

Defining the routes of di/tri-peptide uptake in *Staphylococcus aureus*

Imran Khan

PhD

University of York

Department of Biology

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Abstract

Peptides available in biological niches inhabited by the human pathogen *Staphylococcus aureus* serve as a rich source of amino acids required for growth and successful host colonisation. Uptake of peptides by *S. aureus* involves at least two transport systems: the POT family di/tripeptide permease DtpT and the oligopeptide ABC transporter Opp3. Previously, genetic screening approaches revealed that *dtpT* contributes toward bacterial fitness *in vivo*, but further functional characterisation of the DtpT transporter protein (i.e. in regard to substrate range and physiological roles) remains limited.

In this thesis, the individual and combined functions of DtpT and Opp3 in enabling utilisation of 282 di/tripeptides are studied using a high-throughput phenotypic screen. This approach reveals that many peptides can be utilised via either transporter, though DtpT appears to be the primary route of uptake for dipeptides. Intriguingly, a clear preference is observed for Asp/Glu-containing peptides among putative DtpT substrates. To measure DtpT-mediated peptide transport directly, the protein was purified and reconstituted into proteoliposomes. Active transport of diverse di- and tripeptides was demonstrated using this approach, supporting the conclusions of the phenotypic screen. During this *in vitro* analysis, DtpT was also found to transport the biologically prevalent tripeptide metabolite reduced glutathione (GSH). In the absence of the known glutathione transporter – Gis – the *dtpT* gene is essential for growth when GSH is supplied as the sole sulphur source, identifying DtpT as the predicted second GSH uptake system of *S. aureus*. Site-directed mutagenesis of the predicted ligand binding site of DtpT suggests GSH binds the protein vertically, in a similar conformation to that observed for S-Cys-Gly-3M3SH binding in the homologous POT protein PepT_{Sh}. Finally, GSH transport is revealed to be required by *S. aureus* for complete fitness during *in vitro* macrophage infection experiments, revealing a potential new role for GSH uptake in orchestrating host-pathogen interactions in these infections. Together, these data reveal important new functions for DtpT, both in the utilisation of diverse peptides for growth but also providing the first clues toward a distinct role of glutathione transporters during intracellular infection.

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Author's declaration

I declare that all work presented in this thesis (submitted by myself for the degree of Doctor of Philosophy) is entirely my own original work, except where due reference is made to other authors. This work has not previously been presented for a degree or other qualification at this University or elsewhere.

Some of the work described in this thesis has been published as a pre-print article ("Distinct roles for the *Staphylococcus aureus* POT transporter DtpT in di/tripeptide uptake and glutathione utilisation inside human macrophages") and is currently available to view online on BioRxiv (*doi*: <https://doi.org/10.1101/2024.12.02.626324>)

List of abbreviations

ABC	ATP-binding cassette	MIC	Minimum inhibitory concentration
ACME	Arginine Catabolic Mobile Element	MMP	Matrix metalloprotease
ADP	Adenosine Diphosphate	MRSA	Methicillin Resistant <i>S. aureus</i>
agr	Accessory gene regulator	Mtb	<i>Mycobacterium tuberculosis</i>
AMP	Antimicrobial Peptide	NaCit	Trisodium Citrate
amp	Ampicillin	nanoDSF	nano-differential scanning fluorimetry
ATc	Anhydrotetracycline	NBD	Nucleotide-binding domain
ATP	Adenosine Triphosphate	NMF	Natural moisturising factor
Aur	Aureolysin	NTML	Nebraska Transposon Mutant Library
BCAA	Branched-chain amino acid	Opp	Oligopeptide permease
cam	Chloramphenicol	P5C	Pyrroline-5-carboxylate
CA-MRSA	Community Acquired MRSA	PAGE	Polyacrylamide gel electrophoresis
CD	Circular Dichroism	PBS	Phosphate-buffered saline
CDMG	Chemically Defined Medium with Glucose	PCD	Programmed cell death
CF	Cystic fibrosis	PCR	Polymerase chain reaction
CFU	Colony Forming Units	PG	Peptidoglycan
CMC	Critical micelle concentration	PI	Post-infection
CryoEM	Cryo-electron microscopy	PL	Proteoliposome
DAP	Diaminopimelic acid	PM	Phenotype microarray
DDM	n-dodecyl- β -D-maltoside	PMA	Phorbol 12-myristate 13-acetate
DM	n-decyl- β -D-maltoside	PMF	Proton-motive force
DMSO	Dimethyl sulfoxide	POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
Dpp	Dipeptide permease	POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
Dtp	Di/tripeptide permease	POT	Proton-dependent oligopeptide transporters
eAUC	Empirical area under the curve	ROS	Reactive oxygen species
EDTA	Ethylenediaminetetraacetic Acid	Sap	Sensitivity to antimicrobial peptide
ELISA	enzyme-linked immunosorbent assay	SBP	Substrate-binding protein
erm	Erythromycin	ScpA/B	Staphopain A/B
EtaA/B	Exfoliative toxin A/B	SCV	Small colony variant
FDR	False discovery rate	S-Cys-Gly-3M3SH	S-Cys-Gly-3-methyl-3-sulphanylhexan-1-ol
GFP	(super-folded) green fluorescent protein	SDS	Sodium dodecyl sulphate
ggt	γ -glutamyl transferase	SEC	Size exclusion chromatography
Gis	Glutathione import system	SNP	Single nucleotide polymorphism
GSH	Reduced glutathione	SplA-F	Serine protease-like proteins A-F
GSSG	Oxidised glutathione	SspA	V8 serine protease
GST	Glutathione S-transferase	SSTI	Skin and soft tissue infections
HP	Hypothetical protein	TCS	Two component system
HSD	Honestly significant difference	TEV	Tobacco Etch Virus
IMAC	Immobilised metal affinity chromatography	TM	Transmembrane
IPTG	Isopropyl β -D-1-thiogalactopyranoside	T_m	Melting temperature
kan	Kanamycin	TMD	Transmembrane domain
LB	Lysogeny broth	Tn/TN	Transposon
LDH	Lactate dehydrogenase	TSA	Tryptic soy agar
LMW	Low molecular weight	TSB	Tryptic soy broth
LSD	Least significant difference	UMAP	Uniform Manifold Approximation and Projection
MFS	Major facilitator superfamily	$\Delta\psi$	Membrane potential

Chapter 1 - Introduction

All living cells are surrounded by at least one biological membrane, comprising of a lipid bilayer, which provides a hydrophobic barrier between the intracellular and extracellular environments. The selective exchange of soluble molecules across these biological membranes requires integral membrane-spanning transporter proteins ([D. Drew & Boudker, 2016](#)). As a result, all actively growing cells depend upon transporter proteins for the acquisition of essential nutrients from the extracellular environment, including carbohydrates, nitrogen-sources, metals and vitamins. In the case of bacterial pathogens, transporters are also implicated in processes ranging from drug efflux and toxin secretion to environmental sensing and cell signalling. As a result, many transporter proteins have been identified as essential virulence determinants contributing toward bacterial pathogenesis ([Özkan et al., 2024](#)). In spite of this, many of these transporters remain poorly characterised due to the inherent technical challenges associated with working with these proteins (for instance, their insolubility in aqueous solutions, or their general toxicity when overexpressed in cells) ([Carpenter et al., 2008](#); [Tiefenauer & Demarche, 2012](#)).

Transporter proteins are structurally diverse, reflective of the myriad roles of these proteins in transporting distinct physiological substrates with high selectivity ([X. Bai et al., 2018](#)). Though some transporters facilitate passive diffusion across the membrane, the majority of transporters studied to-date rely on active transport processes driven, for instance, by the hydrolysis of ATP (e.g. primary active transport) or coupling with the favourable movement of a second solute across the membrane during the transport cycle (e.g. secondary active transport).

The work presented in this thesis will focus specifically on the roles of peptide transport systems in the prevalent Gram-positive bacterial pathogen *Staphylococcus aureus*. Here, I aim to provide the context needed to understand the significance of our findings. First, I will provide an in-depth analysis of bacterial peptide transport systems; their structures, mechanisms of action and physiological roles (both in nutrient acquisition, and more generally). Next, I will review existing literature describing the nutrient requirements of *S. aureus*, highlighting the observed importance of peptides and amino acids in this bacterium during survival in relevant biological contexts. Finally, I will bring these themes together to introduce the three known peptide transport systems of *S. aureus* – Opp3, DtpT and Gis – and, in doing so, I will introduce the existing gaps in our understanding of these systems which I hope to address later in this thesis.

Peptide transport in bacteria

Nutrient acquisition is a fundamental metabolic process required by bacteria for successful colonisation and growth ([Davies et al., 2021](#)). In the context of an infection, peptides are highly abundant within a variety of growth niches and represent a desirable source of amino acids, which can in-turn serve as anabolic precursors or energy sources for the cell ([Garai et al., 2017](#); [Lehman et al., 2019](#)). Peptide transporters allow cells to take advantage of this rich nutrient source and are therefore widespread across bacterial phyla ([Garai et al., 2017](#)).

Typically, peptide transporters belong to one of two distinct classes: members of the ATP-binding cassette (ABC) primary active transporter family, and secondary active transporters which utilise the inward proton-motive force to drive peptide import ([Detmers et al., 2000](#); [Detmers et al., 2001](#); [Wilkins, 2015](#)). The latter of these can be further divided into a small number of structurally distinct groups, with the most well-characterised of these being the proton-dependent oligopeptide transporters (POT) which are themselves archetypal members of the major facilitator superfamily (MFS) of secondary transport proteins ([Hagting et al., 1994](#); [Lyons et al., 2014](#)).

Here, I will describe the major features of these two transporter archetypes and outline the molecular basis of peptide transport in each. Where possible, I will also provide examples of how these peptide transporters contribute to bacterial physiology – regarding both their roles in nutrient acquisition, and beyond. Note that I will not discuss other peptide transporter superfamilies – such as OPT ([Gomolplitinant & Saier, 2011](#)) or CstA ([Rasmussen et al., 2013](#)) – as these are not present in *S. aureus* and are, therefore, not relevant to the work presented here.

ABC transporters

ABC transporters are a ubiquitous class of membrane protein complexes implicated in the import and export of diverse soluble molecules across biological membranes ([Thomas & Tampé, 2020](#)). Generally, ABC transporters are characterised by a common architecture comprised of two transmembrane domains (TMD) and two cytoplasmic nucleotide-binding domains (NBD), which may exist as individual protein components or as domains within one or more larger composite peptides ([Biemans-Oldehinkel et al., 2006](#); [Biemans-Oldehinkel & Poolman, 2003](#)). In addition, ABC importers (which are mostly exclusive to prokaryotes) require one or more associated substrate binding proteins (SBPs) localised either in the periplasm (Gram-negative) or as membrane-anchored extracellular proteins (Gram-positive) ([Wilkins, 2015](#)). In some cases, the SBPs may be directly fused to the membrane components, as observed in the glycine-betaine transporter OpuA from *Lactococcus lactis* ([Sikkema et al., 2020](#)). SBPs are primarily responsible for the delivery of substrates to the membrane-associated translocon and are generally believed to convey the majority of the substrate specificity of ABC import systems, often demonstrating exquisite specificity for their cognate substrates ([Maqbool et al., 2015](#); [Thomas & Tampé, 2020](#)).

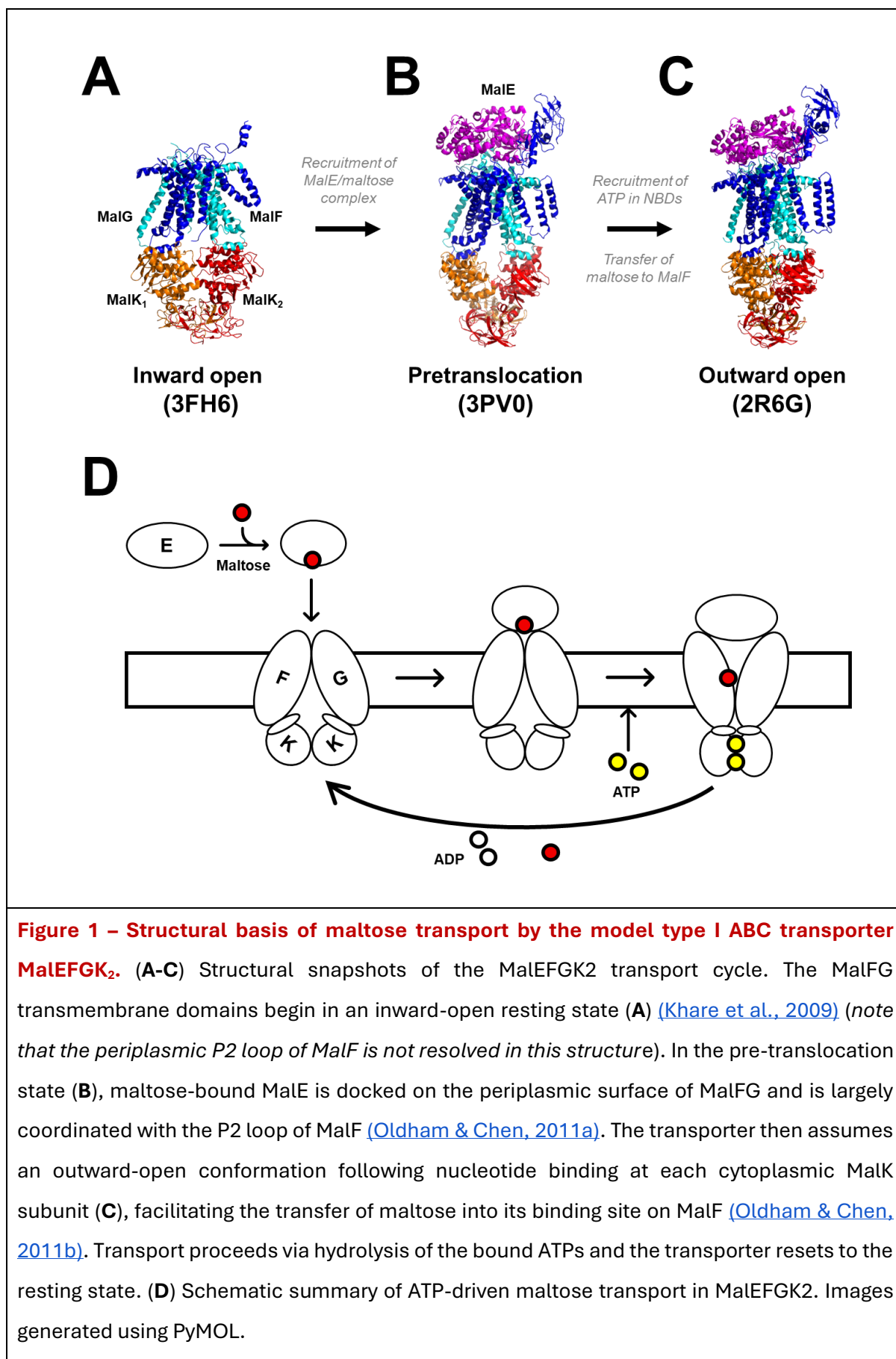
General mechanism and principles of ABC importers

ABC transporters are thought to drive transport via alternating access of the membrane-proximal substrate binding sites of the TMDs between internal and external-exposed states, driven by ATP hydrolysis at the cytoplasmic NBDs ([Procko et al., 2009](#)). Structural studies provide some insight into the conformational changes underpinning the ABC transport cycle, though the exact mechanism of transport is not comprehensively understood and likely differs between individual transporters or subfamilies ([Locher, 2016](#); [Mishra et al., 2014](#)). In the case of type I ABC importers – which includes all currently characterised ABC-type peptide transporters – the *Escherichia coli* maltose transporter MalEFGK₂ is the most well characterised example and serves as a model to understand transport in these systems ([Chen, 2013](#); [Mächtel et al., 2019](#)). Based on structural and biophysical characterisation of MalEFGK₂, the following mechanistic model of type I ABC-mediated import has been proposed (summarised in [Figure 1](#)):

The membrane translocon of type I ABC systems (consisting of the TMDs and NBDs) adopts an inward-facing conformation in the resting state ([Khare et al., 2009](#)) ([Figure 1A](#)). Transport is initiated following docking of a cognate SBP-substrate complex to the TMDs at the extracellular binding interface ([Oldham & Chen, 2011](#)). In the case of MalEFGK₂, these interactions are largely mediated by an extended periplasmic loop (P2) of MalF ([Jacso et al., 2009](#); [Oldham & Chen, 2011](#)).

(Figure 1B). Concurrently, ATP binding to the NBDs facilitates dimerization of these domains on the intracellular side of the complex, which in-turn stabilises the outward-open conformation of the TMDs ([Oldham et al., 2007](#)) (Figure 1C). Site-directed spin labelling spectroscopy and molecular dynamics simulations both suggest that stable homodimerization of MalK is only achieved by concomitant binding of ATP and interactions with the SBP-substrate complex, providing strong evidence that both of these steps precede formation of the outward-open state ([Hsu et al., 2016, 2018; Orelle et al., 2008](#)).

Following adoption of the outward-open state, transfer of the substrate from the SBP to the TMDs is thought to be facilitated by the protruding P3 loop of MalG, which has been observed to invade the binding cleft of the substrate-bound SBP MalE in structures of the complete MalEFGK₂ complex. In this way, the P3 loop acts as a molecular “scoop”, displacing the substrate ([J. Cui et al., 2010; Oldham et al., 2007](#)). Other ABC importer structures also demonstrate the involvement of an orthologous loop structure in this stage of the transport cycle, suggesting that the scoop loop is a conserved feature of all ABC importers ([Hvorup et al., 2007; Liang et al., 2023](#)). In the TMDs, the maltose substrate binds to a single site in MalF formed predominantly by residues in TM helices 5, 6 and 7 ([Oldham et al., 2007](#)). Transposition of the substrate is completed via an outward-to-inward conformational shift, which is coupled to hydrolysis of both bound ATPs and the concomitant release of inorganic phosphate in the cytoplasm ([Oldham & Chen, 2011b; Wilkens, 2015](#)). Thermodynamically speaking, the hydrolysis of ATP at this step provides the energy input which drives the generally unfavourable translocation of the substrate against its concentration gradient.



The nucleotide-binding domains

The mechanism outlined above is often described as the so-called “power-stroke” model, in which alternating dimerization and spatial separation of the NBDs is coupled to an accompanying outward-to-inward rocking-movement in the TMDs to facilitate translocation of the substrate across the membrane ([Stefan et al., 2020](#)). This function of NBDs is apparently essential for ABC-mediated transport, evidenced by the remarkable degree of structural conservation observed for orthologous NBD subunits across diverse ABC systems ([Hopfner et al., 2000](#); [Lewis et al., 2004](#); [Smith et al., 2002](#)). Indeed, several functionally important features are ubiquitous across characterised NBDs, namely: the ATP-coordinating Walker-A/A-loops motifs, the catalytic histidine switch, Walker-B and LSGGQ motifs, and the TMD/coupling helix interface ([Rees et al., 2009](#)).

Dimerization of two NBD subunits is generally only achieved in the “closed” ATP-bound conformation. In this state, the bound ATP molecules of each NBD subunit are brought together at the dimer interface, sandwiched between the Walker A motif of one NBD and the LSGGQ motif of the other ([J. Chen et al., 2003](#)) and are, thereby, co-localised with the catalytic residues essential for ATP hydrolysis ([Zaitseva et al., 2005](#)). In the case of MalFGK₂, stable dimerization and ATP hydrolysis in the MalK subunits is only achieved in the SBP-bound pretranslocation complex, as a result of the consequential positioning of the TMDs in this state ([J. Chen et al., 2003](#); [Khare et al., 2009](#)). This feature of the transport cycle ensures that ATP hydrolysis is exclusively coupled to substrate translocation.

The transmembrane domains

In contrast to the highly conserved NBDs, TMDs are considerably more varied and demonstrate distinct fold-types that are specific to three major ABC transporter families: i.e. type I and type II importers, as well various exporter subtypes ([Locher, 2016](#); [Thomas et al., 2020](#); [Thomas & Tampé, 2020](#)). For type I systems, each TMD is composed of a minimum of 5 transmembrane helices ([Thomas et al., 2020](#)). However, the total number of membrane-spanning helices observed from existing structures varies; from 14 in the case of MalEFGK₂ ([Oldham & Chen, 2011a](#)) to 12 in the molybdate transporter ModAB₂C₂ ([S. Gerber et al., 2008](#)) and 10 in the methionine transporter MetN₂I₂Q ([Kadaba et al., 2008](#)). The only universally conserved features of the TMDs appear to be the presence of an SBP-binding interface and scoop loop on the extracellular face of the translocon (*described above*), and the existence of at least one cytoplasmic coupling helix which forms a ball-and-socket-like interaction with the NBDs and thereby facilitates translational movement of the TMDs during the ATP hydrolysis cycle ([Dawson](#)

[& Locher, 2006; Loo et al., 2013](#)). For type I importers, these coupling helices contain a conserved EAA motif which is seemingly essential for coupling ([Mourez et al., 1997](#)).

The substrate binding protein

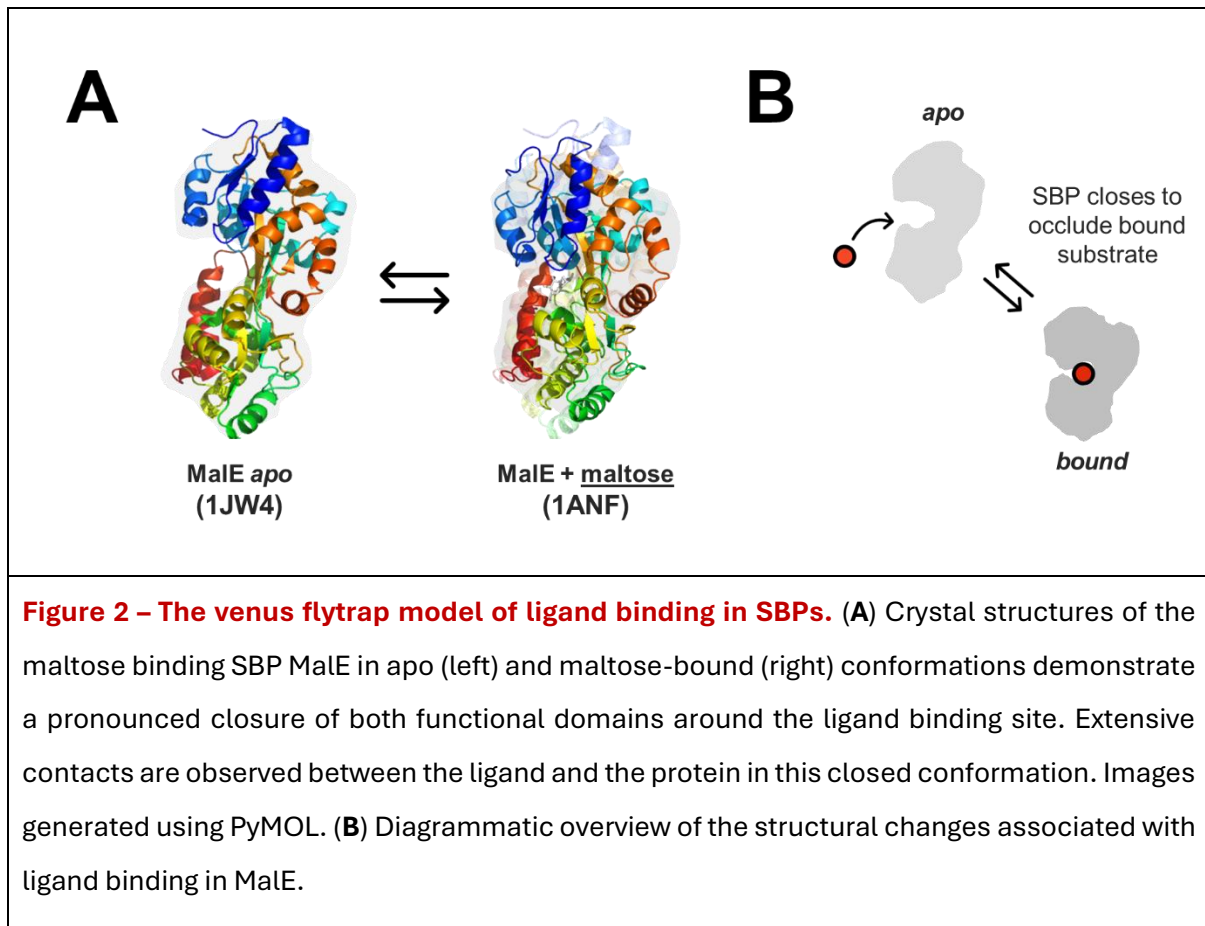
Generally speaking, all SBPs display a conserved architecture comprised of two α/β -domains joined by a flexible hinge-like region ([Berntsson et al., 2010](#)), which allows the two subunits of the protein to come together during substrate binding and thereby form a closed pocket surrounding the bound substrate (*the so-called “venus flytrap” model*; [Figure 2](#)) ([B. Mao et al., 1982](#)). Despite this conserved gross structure, functionally disparate SBPs demonstrate relatively little identity in terms of their primary sequence. This is perhaps unsurprising when considering the requirement for these proteins to recognise and bind structurally diverse substrates with high affinity ([Berntsson et al., 2010; Maqbool et al., 2015](#)).

Historically, there have been multiple attempts to classify SBPs based on structural and phylogenetic analysis ([Berntsson et al., 2010; Chandravanshi et al., 2021; Scheepers et al., 2016](#)). The most recent classification defines eight SBP clusters (A-H) based on their distinct hinge-region architectures and α/β -domain arrangements, with SBPs within these clusters being further subdivided based on substrate profile ([Chandravanshi et al., 2021](#)). Recent structural studies also suggest subtle differences in the conformational changes underpinning substrate binding between these distinct SBP clusters (presumably resulting from structural differences in the hinge region, specifically). For example, *apo*- and *holo*-form structures of the bacterial cluster F SBPs PhnD and GmpC suggest that substrate binding in this cluster is exclusively achieved via a swinging movement of one or both domains around the hinge region ([Alicea et al., 2011; W. A. Williams et al., 2004](#)). In comparison, the metal-binding cluster A SBPs ZnuA, PsaA and YfeA employ a more subtle lateral closing movement in which one domain is seemingly pulled toward the other (often described as a “spring-hammer” motion) to contact the central metal binding site ([Couñago et al., 2014; H. Li & Jogl, 2007; Radka et al., 2019](#)). SBPs belonging to cluster C are structurally distinct due to the presence of a third globular domain, which functions as the hinge region in these proteins and also seemingly contributes to substrate binding ([Tanaka & Pinkett, 2019](#)).

In most systems studied to date, structural and biochemical evidence suggest that substrate binding in the SBP (i.e. adoption of the “closed” substrate-bound conformation) is required for recognition at the membrane translocon interface, and therefore the bound SBP-substrate complex exclusively enables movement through the transport cycle. Such a mechanism is believed to prevent futile cycling of the transporter in the absence of substrate ([Oldham et al.,](#)

[2007](#)). However, experimental evidence suggests that the ligand-free MalE may be capable of binding MalFGK₂ – albeit with a much lower affinity and with a decreased stimulatory effect on the ATPase activity of the transporter compared to the substrate-bound form ([Davidson et al., 1992](#); [Merino et al., 1995](#)). It is also worth noting that some alternative ligands of MalE trigger similar conformational changes in the SBP to those observed for the canonical ligand (maltose) upon binding, though many of these ligands are not viable transport substrates ([de Boer et al., 2019](#)). Hence, much remains to be understood regarding how substrate recognition in the SBP is conveyed to the complete translocon and how this interaction drives progression through the transport cycle.

A notable trait of SBP proteins is their apparent functional versatility. Some SBPs demonstrate exquisite specificity for a single known cognate substrate – for example, the *Streptococcus pneumoniae* manganese-binding SBP PsaA ([Neville et al., 2021](#)) – whilst others recognise a number of structurally related substrates with similarly high affinity ([Fulyani et al., 2016](#); [Russell et al., 1992](#)). Furthermore, though most ABC importers appear to utilise only a single SBP, there are examples in which multiple SBPs are capable of interacting with one ABC translocon to facilitate transport of multiple distinct substrates through a common route ([Alloing et al., 1994](#); [Higgins & Ames, 1981](#)).



Bacterial ABC peptide importers

To date, a number of bacterial ABC peptide importers have been identified and characterised functionally, biochemically and/or structurally to varying degrees ([F. J. M. Detmers et al., 2001](#); [Garai et al., 2017](#)). Based on these existing examples, it is generally accepted that all ABC peptide importers belong to the type I ABC family and utilise cluster C SBPs for peptide recognition ([Fukamizo et al., 2019](#); [Tanaka & Pinkett, 2019](#)). Yet, despite their structural similarities, evolutionarily distant ABC peptide importers can vary considerably in regard to their substrate size and sequence preferences. This is perhaps best exemplified by comparing the *E. coli* dipeptide permease Dpp_{Ec} – which exclusively transports di- and tripeptides – to the *Lactococcus lactis* oligopeptide permease Opp_{Ll}, which is capable of transporting peptides up to 35 residues in length ([F. J. Detmers et al., 2000](#); [Doeven et al., 2004](#); [M. W. Smith et al., 1999](#)).

Oligopeptide permease systems in bacteria

Oligopeptide permease (Opp) systems are a functionally orthologous family of peptide importers found ubiquitously in bacteria and defined by their ability to transport a broad range of oligopeptide substrates. Though first identified in Gammaproteobacteria - specifically *E. coli* and *Salmonella typhimurium* - representatives of this transporter family have since been studied extensively in a range of Gram-positive and Gram-negative species ([Ames et al., 1973](#); [Monnet, 2003](#); [Payne, 1968](#)).

Contemporary studies of Opp transporters have primarily focussed on characterisation of the SBP components (OppA and homologues thereof), as these components are generally thought to determine the substrate specificity for their associated transport systems. Therefore, probing the ligand binding preferences of the SBP exclusively serves as a convenient method to deduce transporter function ([Doeven et al., 2004](#); [Maqbool et al., 2015](#)). In the case of the Gammaproteobacterial Opp systems, the OppA gene forms part of a five-gene *oppABCDF* operon encoding the entire functional transporter and encodes a roughly 60 kDa protein with specificity for peptides between 2 and 5 residues in size, displaying a preference for tri- and tetrapeptides ([Hiles et al., 1987](#); [Klepsch et al., 2011](#); [Tame et al., 1994](#)). Structural studies reveal the presence of a roughly 1000 Å³ peptide binding groove in the protein which is almost entirely conserved between the *S. typhimurium* OppA_{St} and *E. coli* OppA_{Ec} ([Klepsch et al., 2011](#); [Sleigh et al., 1997](#)) (**Figure 3A**). This groove is seemingly rich in negatively charged residues and features extended pockets which accommodate the various possible side-chain structures of the peptide substrate ([Tame et al., 1994](#)). Early characterisation of the *S. typhimurium* protein suggested that substrate recognition in this protein is sequence independent, based on the observation that

various lysine-containing peptides bound to the protein with similar affinities ([Sleigh et al., 1999, 1997; Tame et al., 1994](#)). Later work utilised a mass spectrometry-driven approach to survey the range of peptide associated with OppA_{Ec} purified from cells grown in peptide-rich media. This approach demonstrated that OppA_{Ec} preferentially binds Lys-containing peptides, and the authors suggest that this preference is likely conserved in OppA_{St} ([Klepsch et al., 2011](#)).

While the overall architecture of OppA proteins is largely conserved ([Figure 3A-B](#)), the basis of ligand recognition in these proteins can vary quite dramatically ([Figure 3C-D](#)) and such variance likely explains the distinct functional targets of these different systems. Substrate coordination in gammaproteobacterial OppA proteins is primarily dependent on a pair of salt-bridge interactions with the substrate's N- and C-termini. Specifically, the N-terminus makes extensive polar contacts with the protein and forms a salt-bridge via the sidechain of a conserved Asp residue (Asp419 in OppA_{St} / Asp445 in OppA_{Ec}; [Figure 3C](#)), whilst the C terminal salt-bridge residue varies depending on the substrate size (Arg439 for a tripeptide, as shown in [Figure 3C](#)) ([Klepsch et al., 2011; Tame et al., 1994](#)). Conversely, no equivalent salt-bridge interactions with the substrate's N-/C-termini are apparent in ligand-bound structures of the *L. lactis* OppA_{Li} protein ([Figure XD](#)). Instead, the binding pocket of this protein is rich in charged Ser and Arg residues, which form a number of hydrogen bonds and other polar contacts between the protein and the substrate backbone ([Berntsson et al., 2009](#)). This feature – alongside the presence of an extensive 4900 Å³ binding groove – may explain the ability of this protein to bind larger peptides than the gammaproteobacterial OppA ([Detmers et al., 2000; Doeven et al., 2004](#)). The binding groove of OppA_{Li} also features a distinctive hydrophobic pocket which preferentially accommodates a large, uncharged side chain residue such as isoleucine or phenylalanine ([Berntsson et al., 2009](#)) ([Figure 3D](#)). This pocket provides the protein with a distinct specificity for peptides containing at least one hydrophobic residue ([Berntsson et al., 2011](#)). Such an adaptation may exist to enrich the uptake of peptides containing branched chain amino acids, for which *L. lactis* is auxotrophic ([Godon et al., 1993](#)).

In addition to their canonical role in nutrient acquisition, many Opp systems are also required for the internalization of peptide-based cell-cell signalling and quorum sensing molecules and are, therefore, essential sensory units for various signalling networks ([Slamti & Lereclus, 2019](#)). As such, loss of peptide transporter function can have unexpected pleiotropic effects on bacterial physiology. For instance; Opp homologues are required by various species of Streptococci for the detection of peptide pheromones which induce natural competence and, as a result, loss-of-function of these transporters seemingly inhibits genetic transformation ([Fontaine et al., 2010; Mashburn-Warren et al., 2010; F. Zheng et al., 2018](#)). In Bacilli, Opp facilitates the internalization

of sporulation signalling peptides and thereby plays an important role in coordinated spore formation ([Dubois et al., 2019](#); [Rudner et al., 1991](#)). Moreover, Opp systems have been demonstrated to contribute to virulence traits such as cellular adherence and biofilm formation and have been identified as essential virulence factors in multiple species ([Chang et al., 2011](#); [Moraes et al., 2014](#); [Troy et al., 2016](#); [Verhagen et al., 2014](#); [F. Zheng et al., 2018](#)).

Peptide transport and the Ami system in Streptococcus pneumoniae

The Opp-homologues of the Gram-positive pathogen *Streptococcus pneumoniae* have been the subject of extensive functional characterisation in recent years ([Alloing et al., 1990](#); [Gardan et al., 2009](#); [Kerr et al., 2004](#); [Nasher et al., 2018](#)). Early work demonstrated that most strains of *S. pneumoniae* possess a single membrane-embedded peptide transporter complex (AmiCDEF, or Ami) associated with three seemingly functionally redundant SBPs: AmiA, AliA and AliB ([Alloing et al., 1994](#)). It was later revealed that non-capsular strains of the bacterium often possess additional genes encoding homologues of AliB; AliB-like ORF1 and ORF2 ([Hathaway et al., 2004](#); [Hilty et al., 2014](#)). A number of clinically relevant phenotypes have been noted in *ami* mutant strains of *S. pneumoniae*, namely: inhibition of epithelial adherence and colonisation of the nasopharynx, abolishment of natural competence (as noted in other Streptococci), as well as alterations to the cell membrane potential and branched chain amino acid uptake ([Alloing et al., 1996](#); [Cundell et al., 1995](#); [Kerr et al., 2004](#); [Trombe et al., 1984](#)). Based on these observations, it was proposed that Ami and its associated SBPs may function as important signalling modules involved in environmental sensing, particularly in the context of nasopharyngeal colonisation ([Claverys et al., 2000](#)).

In a number of follow-up studies, *Hathaway* and colleagues isolated the peptide-binding SBPs of *S. pneumoniae* (including AliB-like ORF1/2) and incubated these proteins in the presence of human nasopharyngeal swabs. These proteins were then repurified and bound ligands were investigated via a mass spectrometry-driven approach ([Hathaway et al., 2014](#); [Nasher et al., 2018](#)). In these experiments, it was found that each SBP preferentially bound to one or more ribosomal peptides derived from other bacterial species present in the swab samples, including gammaproteobacterial species which are known colonisers of the nasopharynx ([Hathaway et al., 2014](#); [Nasher et al., 2018](#)). Exposure of cells to these peptide ligands alone was found to be sufficient to trigger a phenotypic response from the bacterium, such as the initiation of programmed cell death in response to the *Klebsiella pneumoniae*-derived peptide SETTFGRDFN ([Nasher et al., 2019](#)). Taken together, these observations demonstrate how Opp-homologues can be utilised by bacteria to sense and respond to environmental changes, including the presence of competitor species during growth. More recently, structural characterisation of the

Ali/Ami SBPs has demonstrated how diverse peptide substrates are accommodated in a conserved spacious binding cavity in each of these proteins. These structures also highlight how differences in substrate preference arise from the characteristics of residues lining the binding pocket in each case ([Alcorlo et al., 2024](#)).

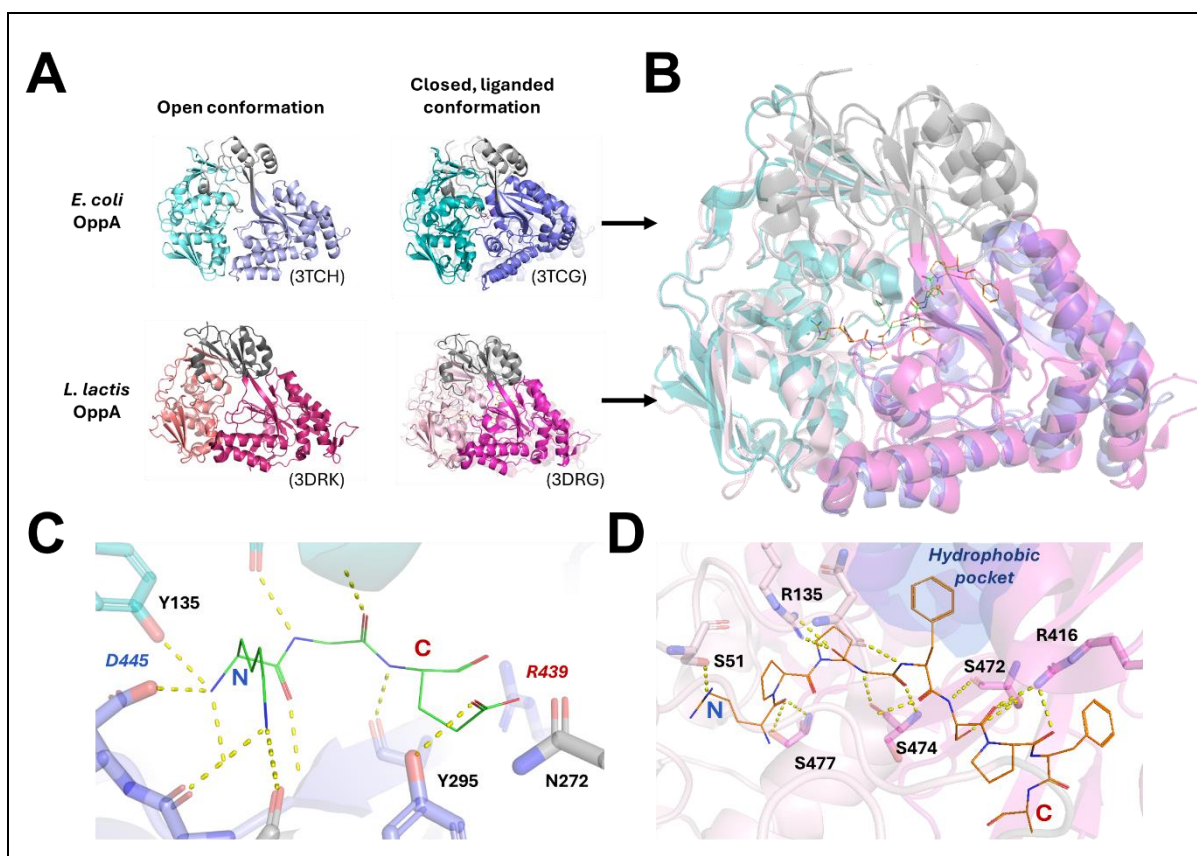


Figure 3 – Structural basis of oligopeptide binding by two functionally distinct OppA family SBPs. (A) Crystal structures of the *E. coli* and *L. lactis* OppA proteins in the open (left) and closed ligand-bound (right) conformations, demonstrating closure of the two functional domains (blue or pink) around the central hinge domain (grey). PDB accession codes are provided in each case. (B) Ligand bound structures are overlaid, demonstrating the conserved architecture of both OppA proteins. Ligands are shown as wires (green = KGE from 3TCG structure; orange = RPPGFSPFA from 3DRG structure). (C) Polar interactions between the *E. coli* OppA protein and bound KGE ligand (yellow, dashed lines) reveals that the N-terminal amino group of the substrate is coordinated through extensive interactions, including a salt-bridge with Asp445. The carboxyl terminus in this structure is coordinated by a salt bridge with Arg439. (D) No such salt-bridge interactions are clearly apparent in the *L. lactis* protein, accounting for its broader ligand promiscuity. A notable hydrophobic pocket (highlighted in blue) in the binding site of the *L. lactis* OppA accommodates the bulky Phe sidechain of the substrate. Images generated using PyMOL.

Other ABC peptide transporters

Generally, Opp systems are categorised based on their ability to transport a broad range of peptide substrates with limited selectivity and are, therefore, primarily implicated in nutrient acquisition (though many such systems have adopted additional roles in other physiological process, as exemplified above) ([Garai et al., 2017](#)). That being said, the functional diversification of peptide ABC importer systems has given rise to a plethora of functionally distinct subfamilies, each with strict specificity to a small number of functional targets. Such specificity confers the ability for these transporters to fulfil more specialised physiological functions.

Here, the alternative ABC peptide transporters of the well-studied model bacterium *E. coli* will be described, in order to exemplify how these specialised transport systems can contribute to bacterial survival beyond their roles in nutrient acquisition (summarised in [Figure 4](#)).

Dipeptide permease

The dipeptide permease (Dpp) is encoded by the *dppABCDF* locus and was amongst the earliest characterised peptide transporters in *E. coli*, alongside Opp ([Abouhamad et al., 1991](#); [Payne, 1968](#)). Early work demonstrated that Dpp, in contrast to Opp, almost exclusively transports dipeptides with high affinity, though the system can also facilitate the transport of some tripeptides with limited affinity ([Manson et al., 1986](#)). Later structural work provided more context for this distinction; the binding groove of the SBP component DppA provides a small volume of only 700 Å³ for ligand accommodation (compared to roughly 1000 Å³ in OppA), and coordinates bound dipeptides tightly through interactions between the substrate C-terminus and Arg355 ([Dunten & Mowbray, 1995](#)). Interestingly, the Dpp system of *E. coli* plays a sensory role in coordinating peptide-directed chemotaxis; a mechanism through which enteric bacteria sense peptide gradients and navigate toward areas of high peptide concentration to facilitate nutrient acquisition ([Abouhamad et al., 1991](#); [Manson et al., 1986](#)).

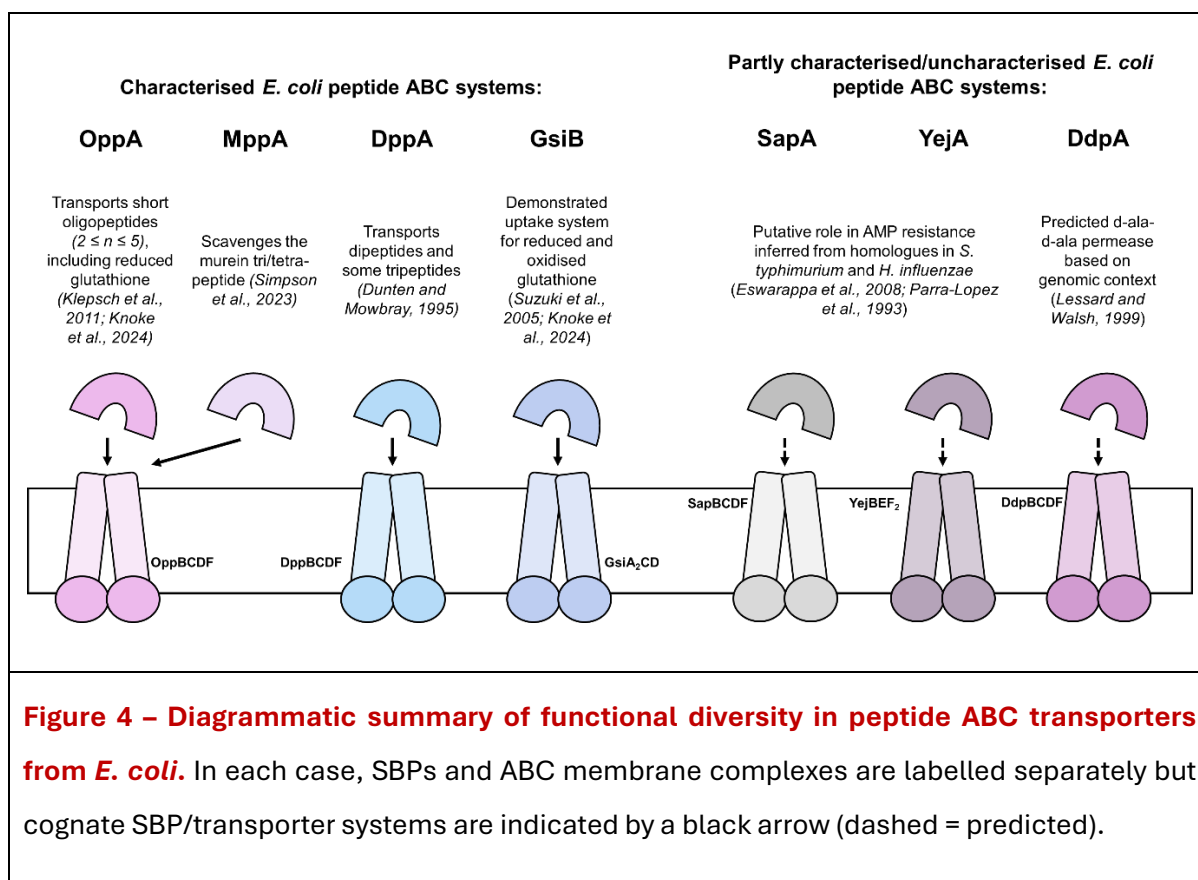
Murein peptide binding protein and the putative D-Ala-D-Ala permease

The bacterial cell wall is a complex structure composed of long polymeric peptidoglycan (PG) chains, structurally supported by oligopeptide cross-links ([Garde et al., 2021](#)). In *E. coli* (and many other Gram-negative bacteria), these linkages are formed between adjacent L-Ala-D-Glu-diaminopimelic acid (DAP)-D-Ala-D-Ala pentapeptide stems, via a covalent linkage between adjacent DAP and D-Ala residues ([Vollmer & Bertsche, 2008](#)). Synthesis and hydrolysis of PG are essential and tightly regulated processes required by bacteria to coordinate the expansion of the cell wall during growth ([Egan et al., 2020](#); [Scheffers & Pinho, 2005](#)). Whilst it is not seemingly essential during growth under rich growth conditions, efficient recycling of PG turnover products (including murein-derived peptides) is one strategy implemented by *E. coli* which is thought to

convey a competitive advantage to the bacterium in polymicrobial communities under nutrient limited conditions ([Goodell & Schwarz, 1985](#); [Simpson et al., 2023](#)).

The first SBP noted for a defined role in peptidoglycan recycling in *E. coli* was the murein tripeptide-binding protein MppA, originally identified based on its essentiality under conditions where growth is dependent on utilisation of the cell wall-derived L-Ala- γ -D-Glu-DAP tripeptide ([Park et al., 1998](#)). In contrast to the other examples already described in this report, the *mppA* gene in *E. coli* does not form part of a complete multigene ABC transporter operon. Instead, early functional studies revealed that MppA delivers bound murein tripeptides to the OppBCDF complex, which facilitates translocation of these ligands across the cytoplasmic membrane ([Park et al., 1998](#)). Later structural work described the basis of muropeptide binding by MppA, demonstrating remarkable similarity between this interaction and the equivalent protein-ligand coordination observed for tripeptide ligands of OppA_{Ec} despite the unconventional D-stereochemistry in the muropeptide substrate ([Maqbool et al., 2011](#)). In this case, accommodation of the muropeptide is enabled by interactions between the sidechain of the MppA Arg402 residue and the α -carboxylate group of D-Glu in the substrate ([Maqbool et al., 2011](#)).

The importance of MppA/Opp-mediated cell wall recycling in the context of bacterial growth and fitness in *E. coli* has been the subject of some debate ([Goodell & Higgins, 1987](#); [Park & Uehara, 2008](#); [Simpson et al., 2023](#)). Mutants of *mppA* do not demonstrate a clear phenotype when grown in nutrient rich conditions, suggesting that any advantage bestowed by the MppA/Opp system may only be relevant during nutrient limitation ([Park & Uehara, 2008](#)). In a recent report by *Simpson* and colleagues, the contribution of MppA/Opp toward murein-derived peptide turnover was determined in relation to the alternative anhydromuropeptide permease AmpG ([Simpson et al., 2023](#)). This study revealed that MppA/Opp was able to effectively scavenge the murein tri- and tetra-peptide from the exogenous environment (as previously suggested by *Maqbool* and colleagues), which the authors suggest may be relevant in mixed bacterial populations ([Maqbool et al., 2011](#); [Simpson et al., 2023](#)).



One aspect of muropeptide recycling in *E. coli* which is not yet comprehensively understood is the fate of the D-Ala-D-Ala moiety of the pentapeptide crosslink liberated during cell wall crosslinking and turnover. Many *E. coli* strains encode a D-Ala-D-Ala dipeptidase (DdpX) which is thought to be cytoplasmically localised, implying the existence of a functional D-Ala-D-Ala permease ([Lessard & Walsh, 1999](#)). Interestingly, the *ddpX* gene is transcriptionally coupled to a predicted peptide ABC transporter of unknown function (*ddpABCDF*) ([Lessard & Walsh, 1999](#)). Given the close genetic association of these two systems, it has been suggested that *ddpABCDF* likely encodes a D-Ala-D-Ala permease which, when combined with DppX, forms a complete D-Ala-D-Ala recycling cluster. However, further functional and biochemical validation for this interaction are still lacking.

Putative antimicrobial peptide susceptibility-associated ABC permeases

Antimicrobial peptides (AMPs) are short (generally < 50 residues) peptide effectors produced by organisms from across the Tree of Life, many of which demonstrate broad antibacterial activity. As such, these molecules are generally implicated in eukaryotic immune systems and contribute toward interspecies competition in polymicrobial communities (reviewed by [Jenssen et al., 2006](#); [Q.-Y. Zhang et al., 2021](#)). AMPs are categorised based on their mode-of-action, with the most widespread of these being cationic AMPs which non-specifically target the negatively-charged cell surfaces of bacteria and directly disrupt lipid membrane ([Sani & Separovic, 2016](#)). Other AMPs must cross the cell envelope in order to target intracellular pathways including DNA replication and protein synthesis ([Scocchi et al., 2016](#)).

Given the ubiquity of these molecules in nature, bacteria have evolved a number of mechanisms to resist antimicrobial peptide activity. The sensitivity to antimicrobial peptide (Sap) locus encodes a complete ABC transporter system and was first identified based on its involvement in resistance to two membrane-targeting AMPs (melittin and protamine) in *S. typhimurium* ([Parra-Lopez et al., 1993](#)). Later studies identified a similar role for the Sap system in a range of bacterial pathogens including *Haemophilus influenzae*, which has since become a model organism for functional characterisation of this transporter system ([Mason et al., 2006](#); [Shelton et al., 2011](#)). In this organism, Sap mutant strains are attenuated in an *in vivo* Chinchilla middle ear infection model, and this attenuation is seemingly dependent on the host-derived AMP Chinchilla β -defensin 1 ([Shelton et al., 2011](#)). The SBP of the *H. influenzae* Sap system – SapA – has been demonstrated to bind various AMPs in surface plasmon resonance experiments ([Mason et al., 2006](#); [K. G. Rivera et al., 2024](#)). In addition, cryo-immunoelectron microscopy has demonstrated that AMPs LL-37 and hBD3 localise to the *H. influenzae* cytoplasm in a Sap-dependant manner ([Shelton et al., 2011](#)). Taken together, these observations suggest a model in which the Sap

system directly recognises and transports membrane-targeting AMPs into the cytoplasm, where these molecules are likely neutralised (for example, by degradation) to confer resistance.

Similarly, the YejABEF (or Yej) transporter is known to contribute to AMP resistance in *S. typhimurium* and *Brucella melitensis*, and is required for full viability of both species in an *in vivo* murine infection model ([Eswarappa et al., 2008](#); [Zhen Wang et al., 2016](#)). Yej is, therefore, postulated to function analogously to Sap in conferring AMP resistance, though robust biochemical evidence of interactions between Yej proteins and AMPs is lacking.

Paradoxically, no role in AMP resistance has been observed for the *E. coli* Sap or Yej transporters, despite the high degree of genetic conservation between these and the aforementioned *Salmonella* systems. Indeed, an *E. coli* Sap mutant strain does not display an altered sensitivity to LL-37. Instead, strains lacking Sap have been found to exhibit decreased export of the organic diamine putrescine ([Sugiyama et al., 2016](#)). In addition, structural studies of the *E. coli* YejA SBP are yet to validate any potential interactions with these aforementioned AMPs. In work by *Ackroyd* and colleagues, it was revealed that the extended N-terminal peptide of YejA occupies the spacious binding groove under the conditions used for structural determination ([Ackroyd et al., 2024](#)). More recent work has revealed how the peptide-nucleotide antibiotic microcin C and other synthetic peptide-conjugated antimicrobials are recognised by YejA in *E. coli*, which thereby exploit the Yej translocon as a means to cross the cytoplasmic membrane ([Luo et al., 2025](#); [Novikova et al., 2007](#)). Despite these studies, the native roles of the Sap and Yej systems in *E. coli* physiology remain elusive, though some functional conservation with the previously described Sap systems is expected.

Glutathione ABC permease

Glutathione is a tripeptide metabolite utilised by prokaryotic and eukaryotic organisms as an antioxidant and redox buffer, with additional roles in cell signalling and virulence observed in some bacterial pathogens (as reviewed by [Ku & Gan, 2021](#); [G. V. Smirnova & Oktyabrsky, 2005](#)). Glutathione exists in two forms – the reduced form (GSH) and the oxidised form (GSSG) – though GSH is generally considered the predominant form in most biological systems. Indeed, measurements of the GSH:GSSG ratio in the cytosol of actively growing *E. coli* cells range anywhere for 200:1 to 66000:1 depending on the methodology utilised to make these measurements ([Knoke et al., 2024](#); [G. V. Smirnova & Oktyabrsky, 2005](#)).

Early work identified an operon in *E. coli* which was essential for glutathione utilisation, consisting of an enzyme of unknown function and a putative ABC transporter system ([Parry & Clark, 2002](#)). Following from this, the ABC transporter described here was functionally

characterised as a specific glutathione importer and labelled GsiA₂BCD ([Suzuki et al., 2005](#)). More recently, the basis of glutathione uptake in *E. coli* was investigated thoroughly by Knoke and colleagues by utilising a fluorescent dye to monitor the cytosolic GSH:GSSG ratio following treatment with either GSH or GSSG ([Knoke et al., 2024](#)). This approach demonstrated that cells lacking GsiA₂BCD are entirely unable to take up GSSG but maintain the ability to transport GSH from the extracellular environment. Further investigation revealed that GSH transport in this case was mediated by Opp. In a double-mutant of both systems, GSH uptake was seemingly abolished entirely ([Knoke et al., 2024](#)). Hence, these two transport systems likely work in-tandem to assimilate glutathione in *E. coli*, though the structural/biochemical basis of glutathione transport in either of these systems remains to be elucidated.

Recent structural studies of peptide ABC importer complexes

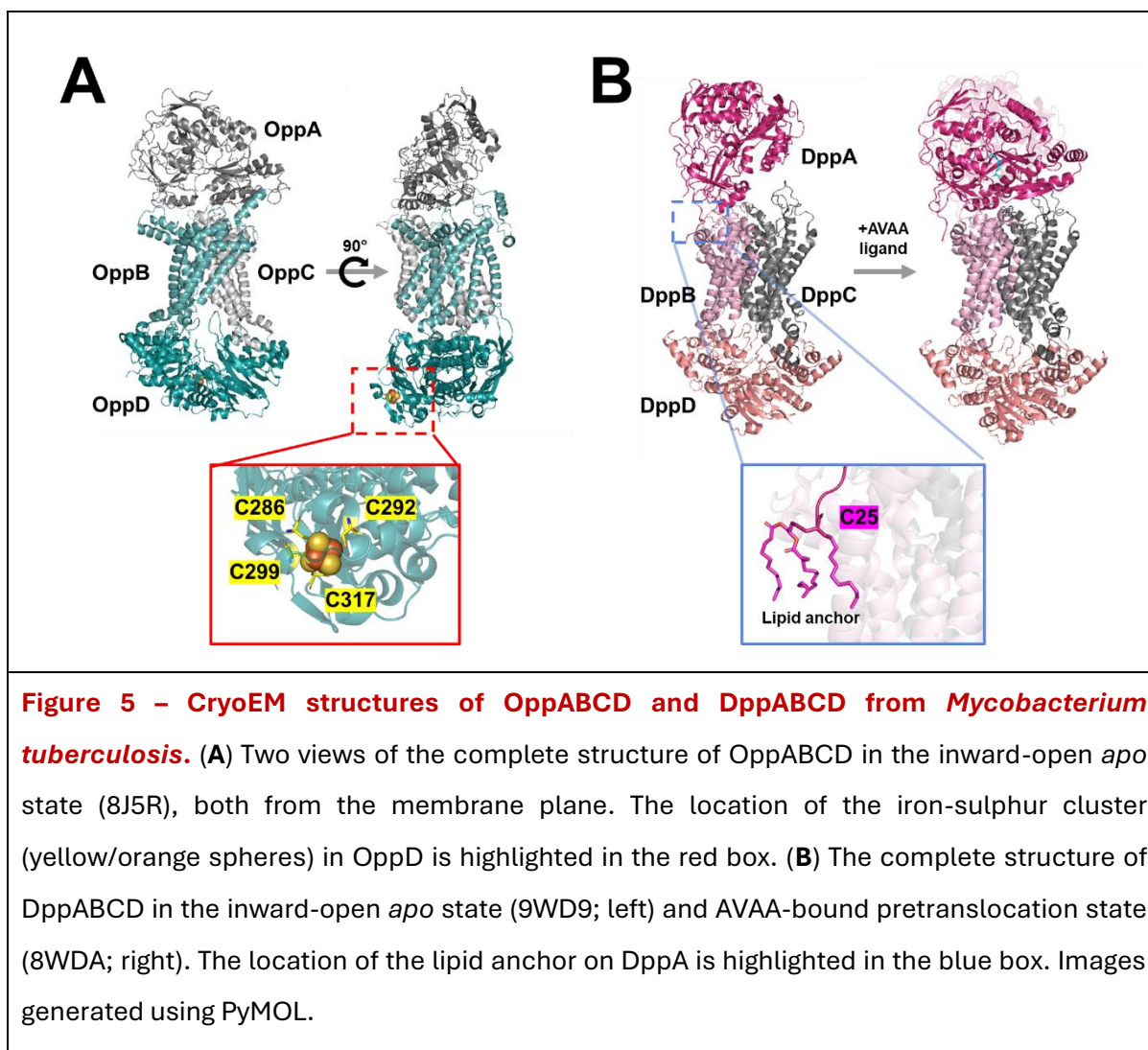
In recent years, technological improvements in the field of cryo-electron microscopy (cryo-EM) have led to revolutionary advances in structural biology and facilitated the determination of many complex macromolecular structures at high resolutions ([A. Cheng & Yu, 2024](#); [E. Y. D. Chua et al., 2022](#)). Specifically, by circumventing the need for crystallisation-based approaches, the development of cryo-EM has provided a major breakthrough in membrane protein structural biology and massively expanded the pool of membrane proteins with the potential for structural elucidation ([Piper et al., 2022](#)). Indeed, cryo-EM allows these proteins to be studied either in detergent micelles or in native-like lipidic environments, allowing for the structural characterisation of proteins which are fundamentally unstable in detergents and more faithfully conserving the native membrane-embedded conformations of these proteins in the final structure ([Abe & Fujiyoshi, 2016](#); [Piper et al., 2022](#)).

Structural characterisation of ABC transporter complexes has been historically challenging for a number of reasons underpinned by their unique quaternary structure consisting of both membrane- and soluble protein components, as well as the dynamic movement of these proteins relative to one another during the transport cycle ([Lewinson et al., 2020](#)). In two recent publications, *Zhang* and colleagues utilised cryo-EM to determine the structural basis of peptide transport via two *Mycobacterium tuberculosis* (Mtb) ABC importer systems: OppABCD and DppABCD, capturing both complexes in multiple conformations corresponding to different stages of the transport cycle ([Hu et al., 2024](#); [Yang et al., 2024](#)). Collectively, these studies report the first structures of complete ABC peptide transporter systems and therefore represent a major step forward in furthering our understanding of these complexes.

The Opp-homologue OppABCD is a peptide importer implicated in glutathione uptake and immune modulation during Mtb infection ([Dasgupta et al., 2010](#)). The complete transporter is composed of the cluster C SBP OppA, the TMDs OppB and OppC and the intracellular ABC subunit OppD, which contains two NBDs fused within a single protein ([Figure 5A](#)). There are some notable differences observed when comparing the structure of OppABCD to the previously characterised type I ABC importer MalEFGK₂ ([Oldham & Chen, 2011b](#)). For instance, *Yang* and colleagues observed that OppA remains docked non-covalently to the membrane translocon during the entire transport cycle via extensive interactions with the TMDs OppB and OppC ([Yang et al., 2024](#)). Ligand binding and release by OppA are achieved through subtle opening and closing movements at the hinge region, which occur laterally – that is, perpendicularly to the membrane plane – thereby allowing the SBP to remain associated to OppB during the transition between these states ([Yang et al., 2024](#)). Additionally, the TMDs OppB and OppC are each made up of six TM helices but do not demonstrate the typical two-fold pseudosymmetry observed in previously characterised ABC importer TMDs ([Khare et al., 2009](#)). Furthermore, OppD contains an iron-sulphur cluster which sits between the two NBDs and is seemingly important for complex assembly ([Figure 5A](#)). The authors note that the cysteine residues required for forming this iron-sulphur cluster are conserved in several homologous Opp systems from across bacteria and archaea, suggesting that this may be a conserved feature in the Opp transporter family ([Yang et al., 2024](#)).

The mycobacterial DppABCD is an alternative peptide transporter predicted to transport short peptides as well as the iron-containing biomolecule haem ([Fernando et al., 2022; Mitra et al., 2019](#)). Similarly to the Opp system described above, this transporter comprises four subunits with the two NBDs being fused within a single protein ([Figure 5B](#)). Intriguingly, the DppA lipoprotein is shown to remain anchored proximally to the TMDs in this structure via a triacyl membrane anchor on Cys25, with this membrane anchor being essential for full assembly of the transporter ([Hu et al., 2024](#)) ([Figure 5B](#)). Similarly to OppABCD, the SBP seemingly remains docked to the DppBCD translocon throughout the transport cycle through interactions with a periplasmic (P1) loop of DppB. This loop is comprised of three small helices and seemingly serves dual roles during transport: stabilising interactions with DppA during the resting state and inserting into the substrate binding pocket of the SBP during translocation (analogous to the P3 “scoop” loop of MalG) ([Hu et al., 2024; Oldham et al., 2007](#)). Unlike OppA, DppA in the *apo* form remains associated to the translocon exclusively via contacts at the N-terminal domain, whilst the substrate-bound conformation makes contacts with both TMDs and more closely resembles the canonical pretranslocation state, as observed in MalEFGK₂ ([Figure 5B](#)). Together, the

structures of both Opp and Dpp indicate that constitutive association of the SBP component to the translocon throughout the entire transport cycle may be conserved in all peptide ABC importers or, more generally, type I ABC transporters associated with a cluster C SBP.



MFS transporters

Major facilitator superfamily (MFS) transporters are widespread across the Tree of Life and represent the most common class of secondary active transporter proteins found in nature ([Pao et al., 1998](#)). Despite all MFS proteins identified to-date possessing a highly conserved core architecture (the so-called “MFS fold”, described below), these proteins demonstrate a remarkable degree of functional heterogeneity and are involved in the transport of diverse substrates, including (though not limited to): sugars, amino acids, peptides, lipids, nucleotides and ions (both organic and inorganic) ([Pao et al., 1998](#)). Historically, classification of MFS transporters has aimed to define distinct subfamilies within this group based on a combination of functional conservation (i.e. those with a common substrate profile) and phylogenetic evidence. One recent classification defines at least 89 such subfamilies and includes importers, exporters and membrane-spanning enzymes such as the bacterial lysyl tRNA-synthetase LysS ([Wang et al., 2020](#)).

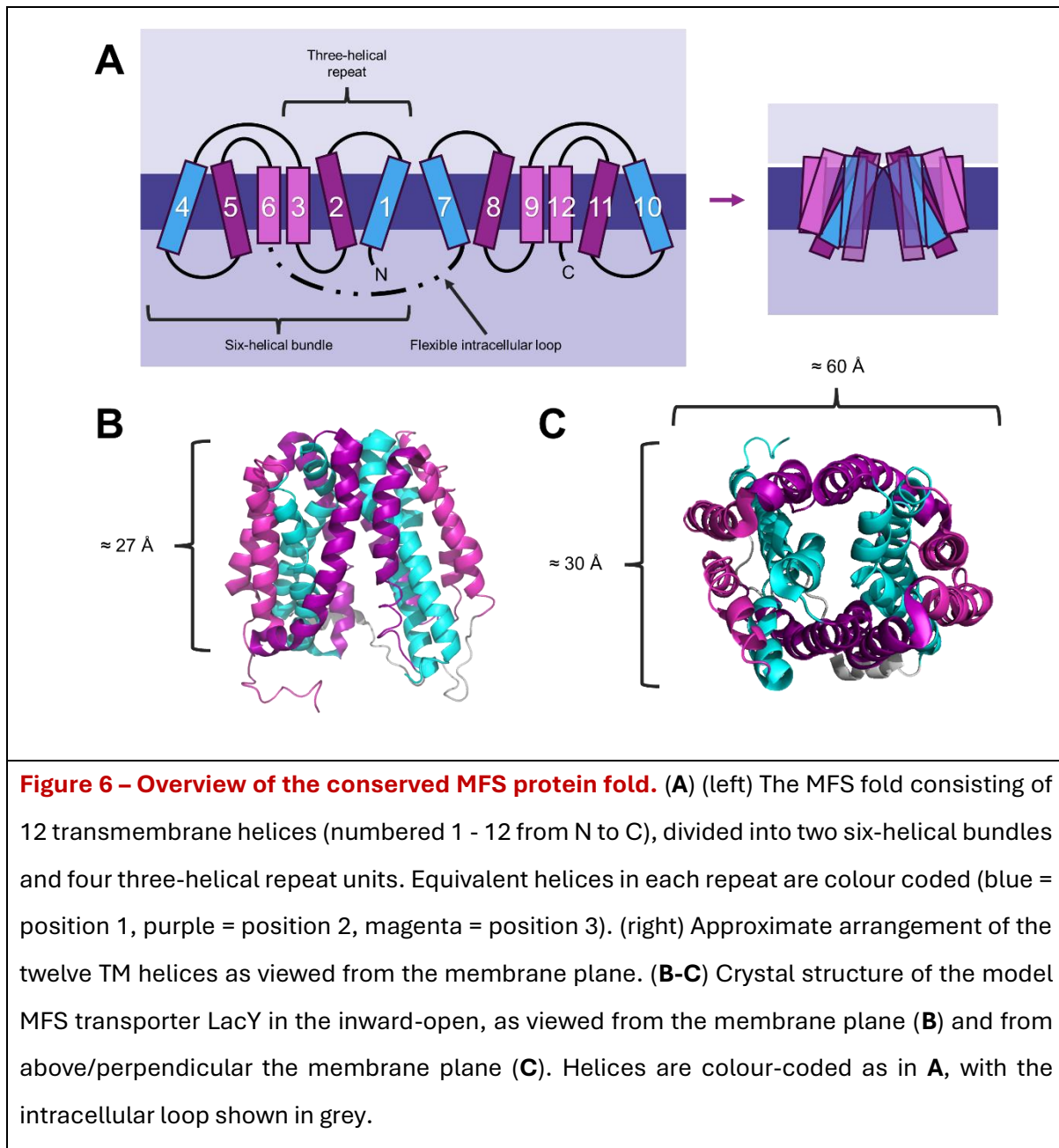
The core MFS fold

In direct contrast to ATP transporter systems (described on **page 14**), archetypal MFS transporters consist of a single membrane-spanning protein composed of two functional domains ([Reddy et al., 2012](#)). In most MFS transporters studied to-date, these domains each consist of a six TM helical bundle (12 total) with both domains flanking the substrate binding site in a pseudo-symmetrical arrangement ([Reddy et al., 2012](#)) (**Figure 6**). This “MFS fold” is highly conserved across structurally characterised MFS transporters, including those with very little homology at the sequence level ([D. Drew et al., 2021](#)).

The two domains of the MFS fold are encoded sequentially as individual units, with helices TM 1-6 making up the N-terminal domain and TM 7-12 constituting the C-terminal domain. These domains are usually connected by a flexible intracellular loop ([Jeff Abramson et al., 2003](#); [D. Drew et al., 2021](#)). For the model MFS transporter LacY, it has been demonstrated that full reconstitution of transporter activity is possible by expressing the two domains as separate polypeptides, providing evidence that they act as distinct functional units during the transport cycle ([Bibi & Kaback, 1990](#)). Each six-helical domain is, itself, composed of two structurally inverted repeats each containing three TM helices (in other words, the complete 12-helical transporter can be imagined as four three-helical repeats, with each domain comprising two such repeats in-tandem) (**Figure 6A**). The complete MFS fold, therefore, likely evolved via repeated gene duplication of a three-helical ancestor protein ([Madej & Kaback, 2013](#)). The first helix in each three-helical repeat (e.g. helices TM1, TM4, TM7 and TM10) is positioned adjacent

to the central cavity and these collectively form the interface for substrate binding, thereby imparting much of the substrate specificity of the transporter. The second helices in each triplet (e.g. helices TM2, TM5, TM8 and TM11) are largely responsible for conveying the conformational changes associated with the transport cycle, whilst the remaining helices provide structural stability and form the majority of the membrane lipid interface ([Yan, 2013](#)).

In addition to this 12-helical core structure, some MFS transporters have been found to contain additional TM helices which are thought to contribute toward protein folding and transporter function, more generally. Indeed, deletion of the two additional TM helices in the 14-helical antibiotic efflux transporter TetL led to abolishment of tetracycline efflux, though cation exchange was seemingly unaffected ([Jin et al., 2001](#)). Conversely, structural characterisation of the human nucleoside transporter hENT1 has revealed that this protein has an 11-helical architecture due to loss of the C-terminal helix TM12 ([Wright & Lee, 2019](#)). Furthermore, many members of the family possess one or more accessory domains with additional functional roles, including those involved in regulating transporter activity or in the mediation of protein-protein interactions ([Harvat et al., 2005](#); [Willson et al., 2019](#)). These examples demonstrate how the highly conserved core structure of MFS proteins provides an adaptable scaffold which can be modified to fine-tune transporter function.



Alternating access in the Major Facilitator Superfamily

In addition to their conserved fold, existing structural evidence suggests that all MFS transporters function through a conserved “rocker-switch” mechanism. In its simplest form, such a mechanism describes the semi-symmetrical movement of both functional domains around a central substrate-binding groove, allowing the protein to alternate between inward- and outward-facing states ([D. Drew & Boudker, 2016](#); [I. Smirnova et al., 2011](#)) (**Figure 7**). Salt-bridge interactions involving the four channel-lining helices (TMs 1/4/7/10) form gates between the N- and C-terminal domains at either face of the membrane, and these interactions serve to both stabilise the inward and/or outward-facing conformations and occlude the substrate from one or both sides of the membrane during the transport cycle ([D. Drew et al., 2021](#); [Parker et al., 2017](#)). In this way, conformational changes during substrate translocation are dictated by the making and breaking of these interdomain salt-bridge (or “gating”) interactions. In addition, the transporter moves through a number of conformations transiently during the transition between the two solvent-facing states, including: partially occluded inward/outward-facing states and a doubly occluded state in which interdomain contacts prevent substrate exposure to either side of the membrane ([Deng et al., 2015](#); [Wisedchaisri et al., 2014](#)) (**Figure 7**). In this way, a bound substrate is conveyed from one side of the membrane to the other without ever being exposed to both sides concurrently (as review by [D. Drew et al., 2021](#); [Quistgaard et al., 2016](#)).

Proton-coupling in MFS transporters

Under standard growth conditions, bacterial cells maintain a negative-inside membrane potential with a magnitude of approximately -150 mV, driven (at least, in part) by the expulsion of protons during ATP synthesis via the electron transport chain ([Lo et al., 2007](#)). The combination of this membrane potential and the resultant proton concentration gradient (i.e. ΔpH) across the cytoplasmic membrane create an inward-facing proton-motive force (PMF), which can be harnessed by the cell as an energy source to drive otherwise energetically unfavourable physiological processes ([Farha et al., 2013](#); [Kashket, 1981](#)). In many cases (though not exclusively), transport of substrates by bacterial MFS systems is actively coupled to proton movement and driven by the PMF, allowing molecules to be transported favourably uphill against a substrate concentration gradient ([Kaback & Guan, 2019](#)). For simplicity, only proton-coupled MFS symporters will be considered here.

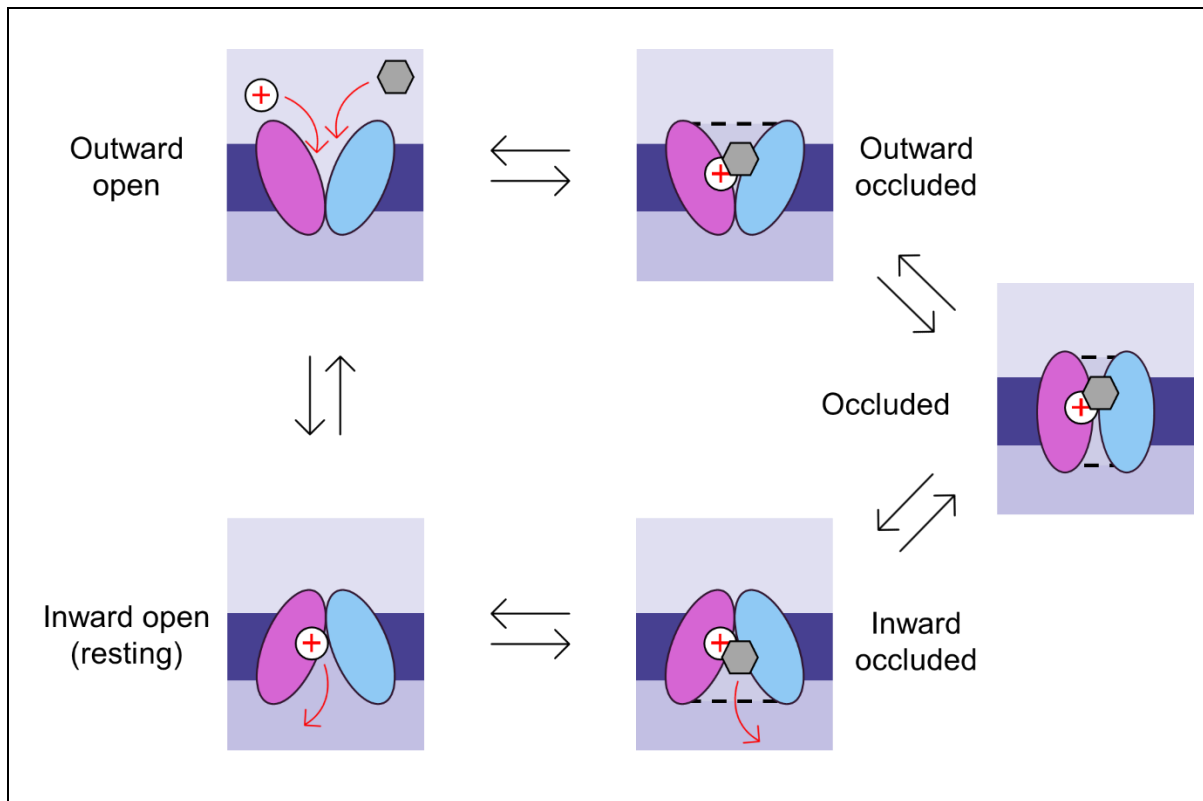


Figure 7 – Diagrammatic overview of alternating access in a proton-driven MFS symporter.

The major conformational states of the complete transport cycle are each depicted in a simplified format. Here, the N- and C-terminal six-helical bundles are depicted as ovals (pink and blue, respectively), the substrate is depicted as a grey hexagon and coupling proton(s) are depicted as a red cross. Figure adapted from [Kaback & Guan, 2019](#).

In proton-coupled systems, completion of the transport cycle requires concomitant binding and release of both the substrate and coupling protons ([Kaback & Guan, 2019](#); [Lolkema & Poolman, 1995](#)). Here, I will use LacY as a model to demonstrate one mechanism by which proton coupling can be achieved in an MFS system ([Figure 8](#)).

In LacY, substrate binding in the outward-open conformation is exclusively permitted by protonation of Glu325, which sits adjacent to the binding groove and interacts directly with residues implicated in substrate coordination including His322 ([H. Kumar et al., 2015](#); [I. N. Smirnova et al., 2008](#)). Substrate binding is believed to trigger the formation of a salt bridge interaction between the adjacent residues Arg144 and Glu269, thereby forming an occluded conformation which drives the transition between outward- and inward-facing states ([Jeff Abramson et al., 2003](#); [I. Smirnova et al., 2011](#)). In the inward-open conformation, the substrate dissociates and Glu325 is deprotonated, with this deprotonation triggering a spontaneous inward- to outward-facing transition ([Andersson et al., 2012](#)). In this way, the complete proton-coupled transport cycle is orchestrated by the protonation of a single glutamate residue ([Kaback, 2015](#)).

Whilst the transport mechanism of LacY provides a clear model for how substrate translocation can be coupled to proton movement in MFS transporters, this system is not reflective of the diversity of proton-coupling mechanisms utilised by distinct MFS transporter subfamilies. For instance, biochemical evidence suggests the existence of three protonation sites in the yeast sucrose transporter Mal11, with any of these three residues being sufficient to enable proton-coupled transport ([Henderson & Poolman, 2017](#)). In some cases, protonation has been implicated in the formation and breakage of salt-bridges between functional domains of the transporter, demonstrating how proton movement itself may be directly responsible for global conformational changes associated with the transport cycle ([Newstead, 2015](#)). For instance: in the xylose transporter XylE, substrate binding is proton-independent and occurs under basic conditions where the essential protonation site Asp27 is predicted to be deprotonated. Protonation of Asp27 in XylE is believed to disrupt interactions between this residue and the nearby Arg133, with this interaction being a key driver of the outward-to-inward conformational switch ([Wisedchaisri et al., 2014](#)). Overall, the high level of diversity observed for proton-coupling mechanisms in MFS systems is likely reflective of the similarly high functional heterogeneity observed for these transporters.

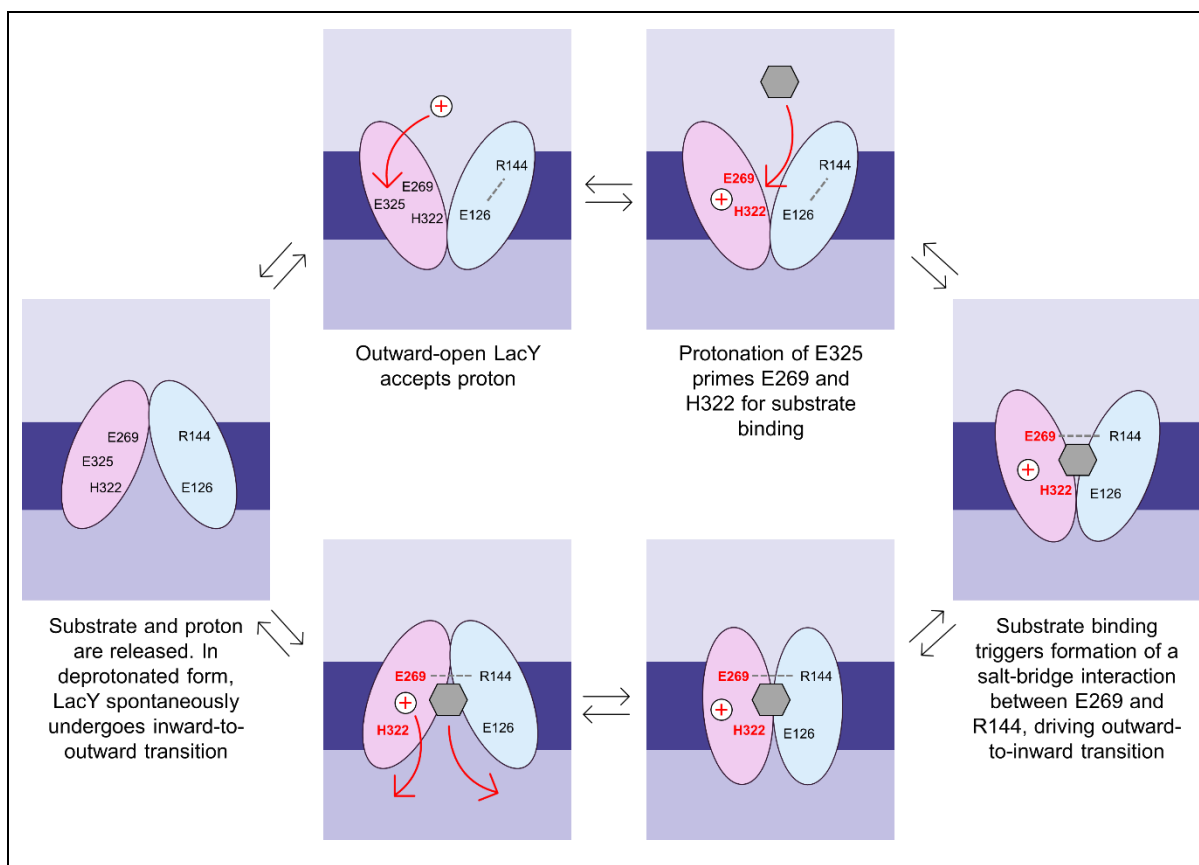


Figure 8 – Proton-coupled transport in the model MFS transporter LacY. The protein, substrate and proton are represented here using conventions established in Figure 7. The approximate positions of important residues implicated in the transport cycle are shown throughout. A grey, dashed line indicates salt-bridge interactions involving Arg144 which facilitate the outward-to-inward conformational transition.

MFS nutrient uptake systems in bacteria

In bacteria, MFS transporters are primarily implicated in two fundamental physiological processes: nutrient uptake (discussed here) and efflux (reviewed extensively by [Lekshmi et al., 2024](#); [Pasqua et al., 2019](#)). In regard to nutrient uptake, these proteins facilitate the acquisition of varied nutrients and thereby allow cells to thrive under diverse growth environments. Well-studied examples of such nutrient uptake systems include: the galactoside permease LacY described above ([Jeff Abramson et al., 2003](#); [Flagg & Wilson, 1976](#)) as well as the muropeptide transporter AmpG ([Cheng & Park, 2002](#); [Li et al., 2016](#); [Simpson et al., 2023](#)), the D-xylose permease XylE ([Davis & Henderson, 1987](#); [L. Sun et al., 2012](#)) and the nitrate/nitrite antiporter NarK ([DeMoss & Hsu, 1991](#); [Noji et al., 1989](#); [H. Zheng et al., 2013](#)), all of which were originally identified and characterised in *E. coli*.

In addition to their roles in nutrient acquisition, individual MFS uptake systems in bacteria have been implicated for their roles in orchestrating the physiological responses to distinct environmental stimuli. For instance, cytoplasmic accumulation of arabinose mediated by the MFS transporter AraE in *E. coli* is known to trigger upregulation of arabinose catabolic genes while suppressing expression of genes involved in the utilisation of less-favourable carbon sources – such as xylose – in a manner dependant on the transcriptional regulator AraC ([Ammar et al., 2018](#); [Desai & Rao, 2010](#)). In this way, AraE allows the bacterium to sense and adapt to changing nutrient availability in the local environment. Another well-studied example is the muropeptide permease AmpG, which is known to contribute toward cell wall turnover in diverse Gram-negative bacteria including *E. coli* ([Cheng & Park, 2002](#); [Sverak et al., 2024](#)). Under conditions where cell wall homeostasis is perturbed (chiefly, during exposure to β -lactam antibiotics) murein breakdown products accumulate in the periplasm and these are rapidly internalised by AmpG. Accumulation of these muropeptide fragments in the cytoplasm is sensed directly by the regulatory protein AmpR in many Gram-negative bacteria, leading to upregulation of the inducible chromosomal β -lactamase gene AmpC ([Jacobs et al., 1997](#)). In the absence of AmpG, this regulatory circuit is disabled and susceptibility to β -lactams is increased ([Dietz et al., 1997](#); [D'Souza et al., 2021](#); [Y. Zhang et al., 2010](#)). Hence, AmpG is an important sensory module required for inducible β -lactamase expression.

The POT family

The POT family of proton-coupled peptide transporter proteins belong to the larger MFS superfamily and were first identified based on their involvement in di/tripeptide utilisation in *L. lactis* ([Hagting et al., 1994](#)). Since then, POTs have been the subject of extensive structural and functional characterisation in a number of organisms ([Newstead, 2017](#)). These proteins are found near-ubiquitously across living systems, being widespread in multicellular eukaryotes (including mammals and plants) as well as in yeasts and bacteria ([Prabhala et al., 2021](#)). In humans, the POTs PepT1 and PepT2 are predominantly expressed in the epithelium of the small intestine and kidneys, respectively, wherein they contribute to the assimilation of important dietary nitrogen sources ([Daniel & Kottra, 2004](#); [Viennois et al., 2018](#)). POT-orthologues in higher plants (generally referred to in these organisms simply as Peptide Transporters, or PTRs) contribute to diverse physiological processes including growth, wound healing and seed defence ([Karim et al., 2007](#); [Komarova et al., 2008](#); [Nour-Eldin et al., 2012](#)). In comparison to these examples, the roles of POTs in bacterial physiology are relatively poorly explored: that is, although numerous bacterial POTs have been implicated generally in the uptake of peptides, there continues to be a poor appreciation for the importance of this process in relevant growth environments. In spite of this, POTs are widespread in bacteria including relevant pathogens such as *E. coli* ([Harder et al., 2008](#); [Jensen et al., 2012](#)), *S. aureus* ([Hiron et al., 2007](#)) and *Yersinia enterocolitica* ([Stauffer et al., 2021](#)). Some bacteria – including *E. coli* – encode multiple POT transporters (in the case of *E. coli*, typically four) with distinct substrate profiles, and this high level of redundancy points toward an important role for these proteins in the bacterial lifestyle ([Harder et al., 2008](#)).

In bacteria, functional POTs are exclusively localised to the cytoplasmic membrane ([Newstead, 2017](#)) and transport peptides present in the extracellular environment (Gram-positives) or those in the periplasm (Gram-negatives). In the case of the latter, these peptides presumably first cross the outer membrane leaflet through passive diffusion processes (i.e mediated by porins) ([Vergalli et al., 2020](#)) or are generated in the periplasm (i.e. through proteolytic activity in this compartment; as exemplified by [Nemoto and Ohara-Nemoto; 2021](#)). The source of nutrient peptides is likely highly organism- and niche-specific. For instance, the Gram-negative periodontal pathogen *Porphyromonas gingivalis* exclusively utilises peptides as a major carbon source ([Mayrand and Holt; 1988](#)), which are generated primarily through the activity of surface-associated proteases called gingipains ([Li and Collyer; 2011](#)). Longer oligopeptides (>10 residues in length) are then specifically translocated across the outer membrane by the RagAB complex ([Madej et al., 2020](#)) before being enzymatically processed in the cytoplasm to produce

dipeptides ([Nemoto and Ohara-Nemoto; 2021](#)), which are then transported into the cytoplasm preferentially by a POT transport system ([Ohara-Nemoto et al., 2020](#)). Other bacteria may similarly utilise peptide transporters to access peptides generated by their own proteolytic activity to fuel growth (as exemplified by [Lehman et al., 2019](#)), or those already present within a given environment.

A notable feature of the POTs is their remarkable substrate promiscuity. In addition to transporting structurally diverse di- and tripeptides (recently demonstrated systematically in the *E. coli* POT DtpB by [Kotov et al., 2023](#)), experimental evidence suggests that some POTs can facilitate the transport of individual amino acids and even tetrapeptides ([Harder et al., 2008](#); [Prabhala et al., 2014](#)). Further functional diversification has been observed in plant POTs, for which transport of nitrate and glucosinolates has also been observed ([Léran et al., 2014](#); [Nour-Eldin et al., 2012](#)). Recent work has also focussed on the ability of these proteins to transport a variety of pharmacologically important peptidomimetic molecules including antibiotics ([Parker et al., 2024](#)) and anti-viral prodrugs ([Minhas & Newstead, 2019](#)), which has ultimately increased scientific interest in POTs as important mediators of dietary drug adsorption and retention in humans and other mammals ([D. E. Smith et al., 2013](#)).

Structurally, POTs are archetypal MFS proteins and universally adopt the conserved 12-helical MFS fold, though most prokaryotic POTs possess two additional TM helices which connect the two functional domains and are believed to contribute toward protein stability ([Minhas et al., 2018](#); [Newstead et al., 2011](#)). These additional helices are absent in eukaryotic POTs including PepT1 and PepT2, which instead possess extracellular C-terminal immunoglobulin domains ([Killer et al., 2021](#); [Parker et al., 2021](#)) that are thought to mediate interactions with the enzyme trypsin, increasing the local concentration of putative oligopeptide substrates outside the cell ([Beale et al., 2015](#)). A characteristic feature common to all POTs is the presence of a conserved ExxERFxyY motif on TM helix 1, which is believed to play a crucial role in proton coupling and substrate coordination in these transporters (described later in this section) ([Aduri et al., 2015](#); [Solcan et al., 2012](#)).

Peptide recognition and binding in POTs

The first crystal structure of a POT transporter (specifically, PepT_{So} from the bacterium *Shewanella oniedensis*) was solved in 2011 by *Newstead* and colleagues ([Newstead et al., 2011](#)). Since then, a number of POTs (see [Appendix 1](#)) have been characterised structurally in complex with diverse ligands, and these structures have provided a wealth of information regarding the molecular basis of substrate recognition in these proteins. Here, we will consider substrate

recognition only in the context of di/tripeptides ([Figure 9](#)), though many recently published structures also demonstrate modes of binding for other peptidomimetic substrates (e.g. [Parker et al., 2024](#); [Ural-Blimke et al., 2019](#)).

Dipeptide binding in POTs

Existing complex structures of POT transporters highlight a number of conserved features implicated in dipeptide binding interactions in these proteins. For instance, all existing dipeptide-bound POT structures demonstrate the existence of a highly conserved triad of residues (Asn/Asn/Glu) required for binding the substrate's amino terminus, and – consistent with this – mutations in these residues are generally detrimental for transport activity ([Doki et al., 2013](#); [Lyons et al., 2014](#)). Recent molecular dynamics simulations predict that peptide binding in POTs is commenced via anchoring of the N-terminus, suggesting that this step is critical for substrate coordination and transport ([Parker et al., 2021](#)). Similarly, the substrate's carboxyl terminus is coordinated through interactions with conserved Arg/Lys residues and a conserved Try residue (part of the ExxERxYY motif), and collectively these interactions stabilise the ligand in a “clamp”-like fashion. As a result, all dipeptides are seemingly bound in a conserved conformation, which is lateral in relation to the membrane plane ([Kotov et al., 2023](#); [Newstead, 2017](#)) ([Figure 9C](#)).

In this lateral conformation, the sidechains of both residues of the bound ligand are accommodated in clearly defined pockets, respectively referred to as P1 and P2 ([Figure 9A-C](#)). In most POTs, P1 is a spacious pocket defined by conserved Asn and Pro residues, while P2 is mostly hydrophobic and comprised predominantly of Trp, Tyr, and Phe residues ([Lyons et al., 2014](#)). Based on the solved structures of the *E. coli* POT DtpB in complex with diverse di/tripeptides, it has been suggested that substrate binding in P1 is mostly consistent between peptides, whilst the structure of P2 is highly plastic and rearrangement of several residues allows the pocket to adopt different conformations and thereby adapt to accommodate diverse ligands ([Kotov et al., 2023](#)). In comparison to the highly conserved N- and C-terminal “clamp” residues, pockets P1 and P2 are less well defined, and variations are observed between structurally characterised POTs, with these variations likely contributing to the distinct substrate preferences observed across representatives of this transporter family ([Figure 10](#)).

In addition to these well-defined contacts, many available structures demonstrate how the peptide bond of a bound peptide ligand can be further stabilised through polar contacts with adjacent residues in the binding groove ([Martinez Molledo, Quistgaard, Flayhan, et al., 2018](#); [Martinez Molledo, Quistgaard, & Löw, 2018](#)). However, these contacts are not universal in POT-

peptide complexes ([Guettou et al., 2014](#); [Lyons et al., 2014](#)). Such an observation strongly suggests that the peptide bond plays a minimal role in substrate recognition.

Tripeptide binding in POTs

In comparison to dipeptide binding, the mode of tripeptide binding in POTs is less well characterised, owing predominantly to the diversity of potential tripeptides and the relative lack of available structures for these complexes. In the *Shewanella oneidensis* POT PepT_{So2} and the *E. coli* POT DtpB, the mode of tripeptide binding is proposed to be similar to that observed for dipeptides (demonstrated in [Figure 9D](#)). In all available tripeptide-bound structures for these two proteins, the position 1 and 2 sidechains of the substrate are accommodated within the previously characterised P1 and P2 pockets, whilst the position 3 sidechain extends into a third, highly spacious P3 pocket comprised of residues in the N-terminal domain of the protein ([Guettou et al., 2014](#); [Kotov et al., 2023](#)) ([Figure 9A-B](#)). In each case, the amino terminus remains anchored in a similar position to that observed for dipeptides, and the same is generally true for the carboxy terminus. The sole exception to this rule was observed in the structure of DtpB bound to Ala-Pro-Phe, in which the proline residue restrains the backbone conformation of the ligand and prevents anchoring of the C-terminus in the expected “clamp” position ([Kotov et al., 2023](#)).

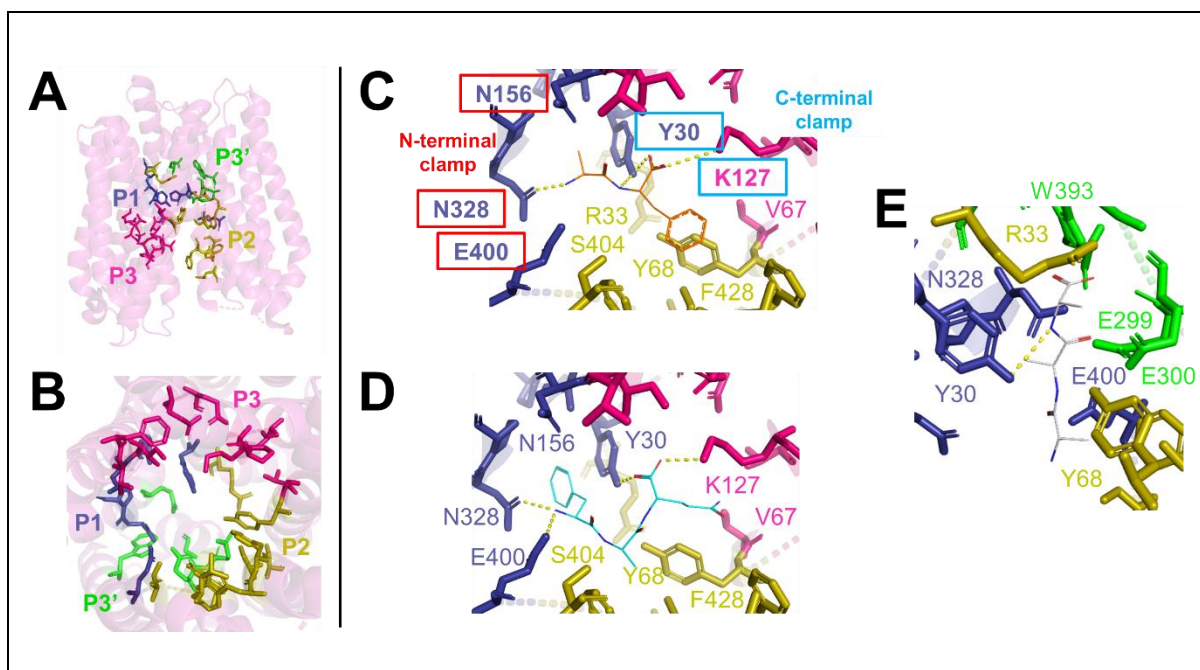
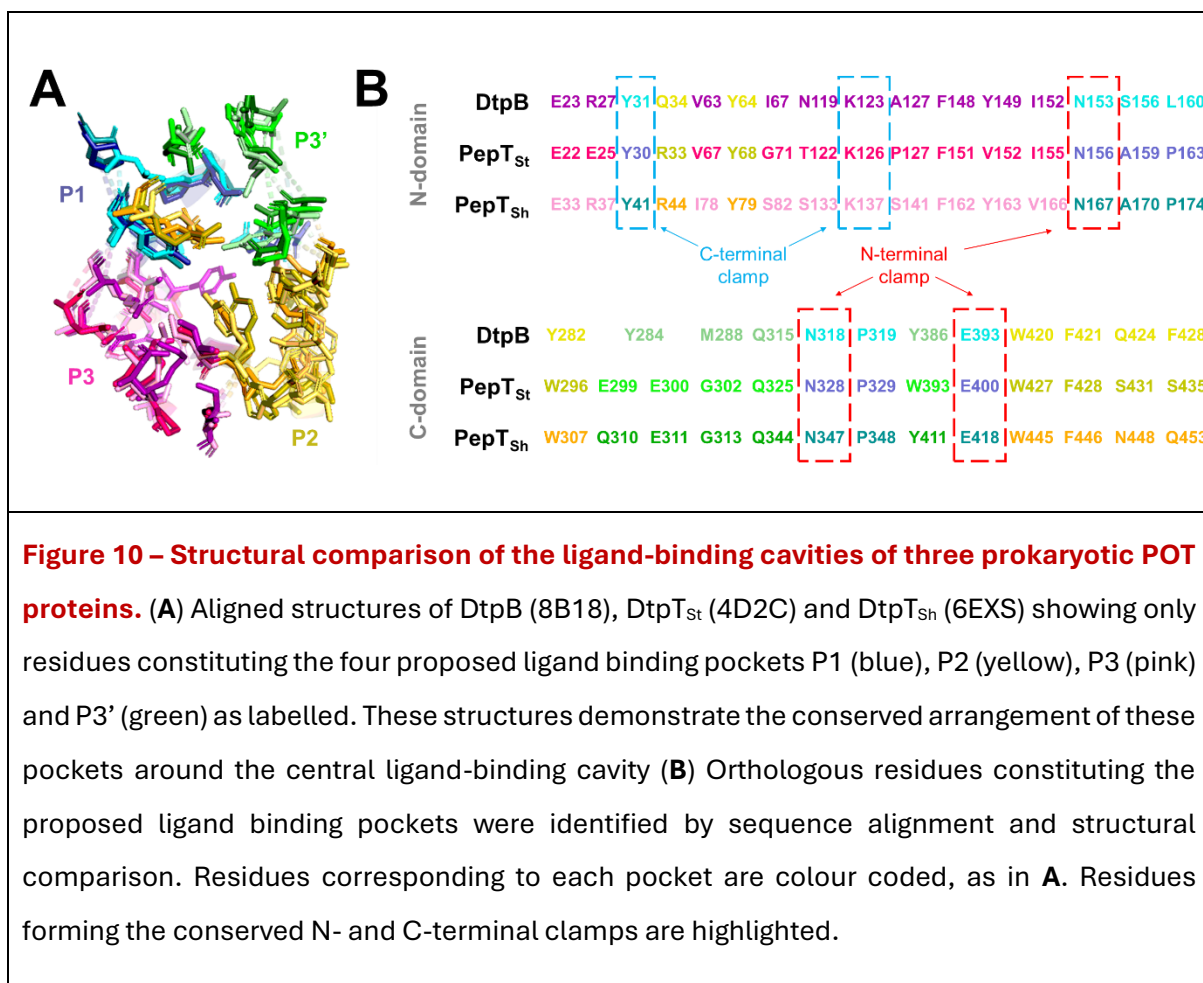


Figure 9 – Peptide binding modes in the model prokaryotic POT transporter PepT_{St}. (A-B)

The inward-open *apo* structure of PepT_{St} (4APS) as viewed from the membrane plane (A) or from below/perpendicular to the membrane plane (B). Side chains comprising the four proposed ligand-binding pockets P1, P2, P3 and P3' are shown as sticks and colour coded, as labelled. (C) Binding mode of the dipeptide ligand Ala-Phe (4D2C; orange) in a lateral confirmation. Conserved residues responsible for coordinating the N- and C-termini of the ligand (so-called “clamps”) are highlighted. (D) Binding mode of the tripeptide ligand Phe-Ala-Gln (6GHJ; cyan) in a lateral confirmation. (E) Proposed binding mode of Ala-Ala-Ala (4D2D; grey) in a vertical confirmation extending into pocket P3'. In each case, nearby residues are labelled and polar contacts between the ligand and protein are indicated by yellow, dashed lines.



In contrast to these examples, a novel binding conformation is observed in the crystal structure of the *Streptococcus thermophilus* POT PepT_{St} bound to Ala-Ala-Ala. Here, the ligand is accommodated vertically in the binding cavity and extends toward the extracellular gate ([Lyons et al., 2014](#)) (Figure 9E). In this conformation, the N-terminus of the ligand is not anchored in the expected position and is instead coordinated primarily through interactions with Glu400 near P1. The position 2 sidechain sits adjacent to P1, while the carboxy terminus and position 3 sidechain are accommodated in a deep polar pocket comprised solely from residues in the C-terminal domain of the protein, hereafter referred to as pocket P3' ([Lyons et al., 2014](#)) (Figure 9A-B). The proposed conformation has been supported by biochemical evidence demonstrating that distinct residues in the protein contribute to transport of Ala-Ala and Ala-Ala-Ala, and that proton stoichiometry differs between these two substrates ([Lyons et al., 2014; Parker et al., 2014](#)). However, it has since been proposed that tripeptide binding in PepT_{St} occurs similarly to that observed in PepT_{So2}, as exemplified by the solved structure of PepT_{St} bound to Phe-Ala-Gln ([Martinez Molledo, Quistgaard, & Löw, 2018](#)) (Figure 9D). Indeed, the authors of this study suggest that the previously observed vertical conformation of Ala-Ala-Ala was, in fact, a structural artefact caused by the vertical binding of HEPES present in the crystallant buffer, which they were able to successfully replicate under similar conditions ([Martinez Molledo, Quistgaard, Flayhan, et al., 2018](#)). Hence, the validity of the originally observed vertical conformation has been called into question, and further structural work is required to confirm the current model.

S-Cys-Gly-3M3SH binding in PepT_{Sh}

In a recent work by *Minhas* and colleagues, it was found that the *Staphylococcus hominis* POT transporter PepT_{Sh} facilitates transport of the peptide conjugate S-Cys-Gly-3-methyl-3-sulphanylhexasan-1-ol (S-Cys-Gly-3M3SH), demonstrating how this activity mediates bacterial uptake and turnover of the compound contributing to malodour generation ([Minhas et al., 2018](#)). In their study, the authors elucidated the structural basis of substrate binding, revealing that S-Cys-Gly-3M3SH adopts a vertical conformation in the central cavity reminiscent of the previous binding mode observed for Ala-Ala-Ala in PepT_{St} ([Lyons et al., 2014](#)). Herein, the bulky thioalcohol moiety of the ligand is bound adjacent to the extracellular gate salt bridge (comprising Arg44 on TM1 and Glu311 on TM7) in the P3' pocket, while the dipeptide component extends out away from the gate and toward P1. Both the N- and C-termini are stabilised by extensive contacts with polar residues lining the binding groove, including Tyr41 of the conserved E₃₃xxERFxYY₄₁ motif ([Minhas et al., 2018](#)). Overall, this structure represents a novel mechanism of ligand binding in POTs and

highlights how different modes of substrate accommodation contribute toward the promiscuity of these transporters.

The proton-coupled transport mechanism of POTs

Based on the wealth of structural information now available for these proteins, a mechanism for the complete proton-coupled transport cycle in POTs has been proposed ([Lichtinger et al., 2024](#); [Newstead, 2015](#)). In this model, alternating access of the bound substrate to either side of the membrane is achieved through sequential breaking and formation of conserved salt-bridge interactions at the intracellular and extracellular ends of the central cavity, driven by proton binding to conserved residues essential for transport ([Figure 11A-B](#)). It is worth noting that the mechanism described here assumes that the substrate binds laterally during the transport cycle, as observed in all dipeptide-bound POT structures solved to-date ([Parker et al., 2021](#)).

In the outward-facing state, an intracellular gate is formed by tight packing of TM helices 4, 5, 10 and 11 ([Figure 11A](#)) and stabilised by a salt-bridge between TM4 and TM11. In the outward-facing structure of the rat PepT2 protein, this salt bridge connects Asp170 on TM4 to Lys542 on TM11 ([Parker et al., 2021](#)), whilst similar contacts connect Arg159 and Glu604 in the outward-facing human PepT1 ([Killer et al., 2021](#)). Structural modelling has previously predicted similar contacts between the Lys136 and Glu400 in the prokaryotic GkPOT, and both of these residues are widely conserved in other prokaryotic POTs ([Solcan et al., 2012](#)). In this outward-open confirmation, the peptide substrate binds first through interactions between the amino terminus and a conserved Glu residue on TM10 (E*) in the binding cavity ([Parker et al., 2021](#)) ([Figure 11C, upper left](#)). The peptide is then reorientated within this cavity, typically such that it is contained laterally in the binding pocket between conserved polar “clamping” residues ([Lyons et al., 2014](#); [Solcan et al., 2012](#)). In this conformation, interactions between the substrate carboxyl terminus and both the Arg and Tyr(2°) residue of the conserved TM1 ExxERxYY motif disrupts an internal salt-bridge interaction within this helix. This interaction allows for subsequent protonation of the first Glu residue of this motif later in the transport cycle ([Aduri et al., 2015](#); [Lichtinger et al., 2024](#)). Substrate binding in the outward-open state is also thought to induce a slight closing motion of the channel-lining TM helices 1, 2, 7 and 8 around the substrate, driving the transition to an occluded conformation ([Figure 11C, upper right](#)) ([Newstead, 2015](#)).

Recent structures and biochemical evidence clearly demonstrate that protonation of residues directly implicated in salt-bridge formation is essential for the transport mechanism of POTs ([Newstead, 2015](#)). Specifically, a conserved Asp/Glu residue on TM7 must be protonated in order to establish a salt bridge with a positively charged residue on TM1 (generally Arg) following

substrate binding. This salt bridge forms an occluded conformation surrounding the bound substrate ([Solcan et al., 2012](#)) (**Figure 11C, lower right**). Concomitantly, protonation of a conserved Glu residue in the ExxERxYY motif of TM1 disrupts interactions between this residue and the adjacent intracellular gate Lys residue on TM4 ([Aduri et al., 2015](#)). As a result, the intracellular gate salt bridge is disrupted and TM helices 4 and 5 swing open to expose the substrate to the cytoplasm (**Figure 11C, lower left**). In this confirmation, tight packing of TM helices 1, 2, 7 and 8 at extracellular gate is stabilised by the newly formed salt bridge interaction. In some structures, a second salt-bridge is observed on the distal side of the extracellular gate, further stabilising the inward-open state ([Lyons et al., 2014](#); [Solcan et al., 2012](#)).

Following the outward-to-inward transition, both the substrate and protons are released into the intracellular milieu. Biochemical evidence suggests that the E^{*} residue responsible for coordinating the substrate N-terminus also contributes toward the proton-coupling mechanism ([Doki et al., 2013](#)). Based on this observation, it has been proposed that extracellular gate proton is transferred to the E^{*} sidechain during the outward-to-inward transition, and this transfer disrupts anchoring interactions with the substrate ([Lichtinger et al., 2024](#)). Following substrate release, both this proton and the ExxERxYY-associated proton are exposure to the solvent and freely dissociate ([Parker et al., 2021](#)). This mechanism therefore proposes that a proton:substrate stoichiometry of 2:1 is sufficient for POT-mediated transport. In spite of this, thermodynamic evidence suggests that peptide transport in PepT_{St} operates with a 3:1 stoichiometry for Ala-Ala-Ala or a 4-5:1 stoichiometry for Ala-Phe ([Parker et al., 2014](#)). Such an observation strongly suggests that one or more additional side chains groups in the central binding cavity must be protonated during transport, and differences in the protonation of these groups may facilitate the accommodation of structurally diverse substrates.

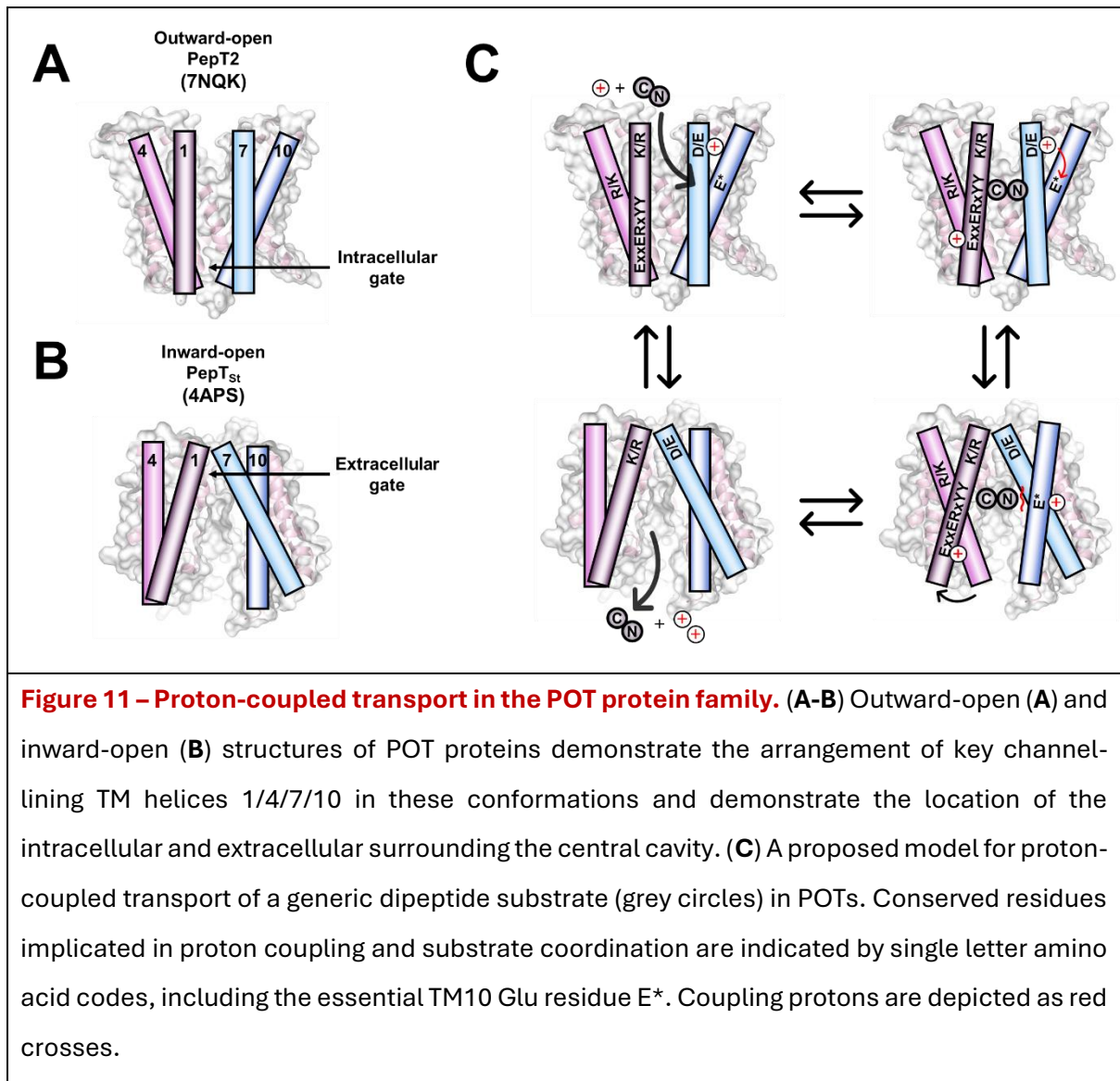


Figure 11 – Proton-coupled transport in the POT protein family. (A-B) Outward-open (A) and inward-open (B) structures of POT proteins demonstrate the arrangement of key channel-lining TM helices 1/4/7/10 in these conformations and demonstrate the location of the intracellular and extracellular surrounding the central cavity. (C) A proposed model for proton-coupled transport of a generic dipeptide substrate (grey circles) in POTs. Conserved residues implicated in proton coupling and substrate coordination are indicated by single letter amino acid codes, including the essential TM10 Glu residue E*. Coupling protons are depicted as red crosses.

Understanding substrate preference in POTs

Elucidating the substrate preferences of individual POT transporters has historically been challenging due, primarily, to the remarkable substrate promiscuity observed in these proteins and their functional redundancy with other peptide transporters in a number of organisms.

Whole cell approaches

Many of the earliest attempts to characterise POT specificity utilised a competitive uptake approach with a radioactively labelled reporter substrate (first utilised by [Hagting et al., 1994](#)) or the fluorescent peptide analogue β -Ala-Lys-AMCA ([Weitz et al., 2007](#)). In this assay, cells overexpressing a given POT are incubated in the presence of a reporter substrate and a putative peptide substrate, and accumulation of the reporter within the cells is quantified over time. Affinity for a given substrate is inferred from the half-maximal inhibitory concentration. Such an approach was utilised in a series of studies by *Daniel* and colleagues to deduce the substrate preferences of the *E. coli* POTs DtpA, DtpB and DtpD ([Casagrande et al., 2009](#); [Harder et al., 2008](#); [Weitz et al., 2007](#)). At around the same time, *Mirza* and colleagues published a similar study elucidating the substrate preferences of DtpC ([Ernst et al., 2009](#)). Briefly, these studies propose that DtpA and DtpB each transport both di- and tripeptides but differ in regard to substrate preferences, with DtpA preferentially targeting cationic dipeptides whilst DtpB demonstrates a preference for anionic peptides ([Harder et al., 2008](#)). In contrast, these studies exclusively demonstrate dipeptide transport in DtpC and DtpD, and both suggest a preference for peptides containing a positively charged residue in the C-terminal position ([Casagrande et al., 2009](#); [Ernst et al., 2009](#)). Collectively, these studies point toward a high level of functional diversification among the POTs of *E. coli*, which likely represents an evolutionary strategy to maximise the variety of peptides accessible from relevant growth environments where these molecules are enriched (i.e. the gastrointestinal tract, wherein *E. coli* is a frequent coloniser) ([Freter et al., 1983](#); [Martinson & Walk, 2020](#)). Indeed, these POTs may function alongside other peptide transport systems including OppABCDF (which can transport peptides up to five residues in length; see [page 21](#)) in these environments to further expand the diversity of potential peptide nutrient sources accessible to the cell.

One major limitation of the studies mentioned above is their use of a limited panel of substrates (<10 in each case), which cannot possibly capture the full extent of structural diversity observed among putative di- and tripeptide substrates. To address this, *Ito* and colleagues constructed a library of 338 dipeptides and applied this library in competitive uptake assays to assess substrate preferences in the *Saccharomyces cerevisiae* POT Ptr2p ([Ito et al., 2013](#)). Overall, this approach highlighted how Trp, Tyr and Phe-containing peptides were generally high-affinity substrates of

P_{tr2p}, while anionic peptides were enriched among low-affinity substrates. Similar affinity patterns have since been deduced for the bacterial POT PepT_{st} by utilising a computational approach ([Samsudin et al., 2016](#)).

Biochemical methods

In recent years, advances in membrane protein biochemistry have allowed for the development of cell-free approaches to facilitate the functional characterisation of POTs. Such approaches generally involve the functional reconstitution of POTs into liposomes, thereby allowing transport to be measured in the absence of potentially convoluting biological factors present in whole-cells ([Solcan et al., 2012](#)).

Since these methods were established, competitive uptake assays have been utilised extensively to understand POT function in liposome-based experiments ([Minhas et al., 2018](#); [Solcan et al., 2012](#); [Ural-Blimke et al., 2019](#)). However, an alternative transport assay has also been developed in which liposomal pH is quantified over time (generally by utilising the ratiometric pH-sensitive fluorescent dye pyranine) to provide an indirect measure of proton-coupled transport in these systems ([Parker et al., 2014](#)). In a recent study, *Kotov* and colleagues utilised the pyranine-based transport assay to compare binding and transport of structurally diverse peptides by DtpB. This thorough study clearly demonstrates how peptides which bind DtpB with high affinity are generally poor substrates in regard to proton-coupled transport, whilst preferred substrates are generally those for which intermediate binding affinities are observed ([Kotov et al., 2023](#)). For instance, the authors highlight how the tight-binding ligand Ala-Phe is an effective competitive inhibitor of transport in DtpB, despite this peptide demonstrating minimal transport in the pyranine-based assay. As a result, the authors propose that competition-based approaches may be entirely unsuitable for inferring transport specificity in POTs ([Kotov et al., 2023](#)).

Amino acids and peptides in *staphylococcus aureus* growth and infection

Staphylococcus aureus is a Gram-positive bacterial pathogen associated with significant morbidity and mortality worldwide, being prevalent in both developed ([A. D. Bai et al., 2022; Cella et al., 2022](#)) and resource-limited countries, and across continents ([E. K. Nickerson et al., 2009; Ruffing et al., 2017](#)). The global burden of Staphylococcal disease has become further exacerbated in recent decades due to the dissemination of highly antibiotic-resistant clones with enhanced virulence properties. Such a paradigm is perhaps best exemplified by the highly virulent community-acquired methicillin resistant *S. aureus* (CA-MRSA) lineage USA300, which emerged in the 1990s and went on to become a major burden to public health in North America in the early 2000s ([Planet, 2017; Thurlow et al., 2013, 2012](#)). A major factor contributing to the severity of *S. aureus* infections is the apparent ability of this bacterium to colonise a variety of body sites and thereby cause a number of invasive and potentially lethal disease manifestations ([Tong et al., 2015](#)). Understanding the molecular factors governing growth and survival in these infection environments is crucial to inform the design of novel therapeutic strategies to combat these severe *S. aureus* infections.

Recent studies highlight how amino acids and peptides serve as important nutrient sources for *S. aureus* in biological environments and may serve as major determinants of successful infection ([Coulter et al., 1998; J. C. Kaiser et al., 2016; Lehman et al., 2019; Lensmire et al., 2020; A. D. Potter et al., 2020; Urso et al., 2024](#)). The bacterium is equipped with an arsenal of secreted proteases which – in addition to playing roles in immune evasion and virulence factor modulation – likely contribute to the generation of oligopeptides and amino acids in the local growth environment ([Kolar et al., 2013; Ohbayashi et al., 2011; Zielinska et al., 2012](#)). A robust metabolic network allows *S. aureus* to catabolise peptides and amino acids to fuel bacterial growth, and it's believed that this process is particularly relevant in glucose-depleted environments where these molecules serve as major carbon sources ([Halsey et al., 2017](#)). In addition, recent evidence suggests that *S. aureus* senses and responds to the availability of these nutrients in the local environment, and these responses may be relevant in the context of infection ([Borezée-Durant et al., 2009; Shibamura-Fujiogi et al., 2022; Urso et al., 2024; Merve S. Zeden et al., 2020](#)). In spite of this, many questions remain regarding the roles of amino acids and peptides in infection environments, as well as the pathways which enable effective utilisation of these nutrients. Specifically, while the routes of amino acid uptake and catabolism are now well characterised,

a complete picture of how peptides are internalised and utilised by this organism remains to be elucidated ([Hiron et al., 2007](#); [Lehman et al., 2019](#)).

Several aspects of *S. aureus* disease and infection – including the epidemiology and disease burden of these infections ([Tong et al., 2015](#)), the prevalence and mechanisms of antibiotic resistance in the bacterium ([Y. Guo et al., 2020](#); [Mlynarczyk-Bonikowska et al., 2022](#)), and the molecular factors contributing toward virulence ([Cheung et al., 2021](#); [Howden et al., 2023](#); [Tam & Torres, 2019](#)) – have all been reviewed extensively elsewhere and are largely outside the scope of this work. Here, I will instead provide a broad overview of the different growth environments encountered by *S. aureus* during infection and the nutrients which contribute toward the success of the bacterium in these niches. Therein, I will introduce the various secreted proteases of *S. aureus* and outline the diverse roles of these enzymes during infection, including the role of proteolysis in nutrient acquisition. Finally, I will describe how this bacterium utilises exogenous amino acids and peptides to fuel growth and introduce the major transporter proteins responsible for their uptake.

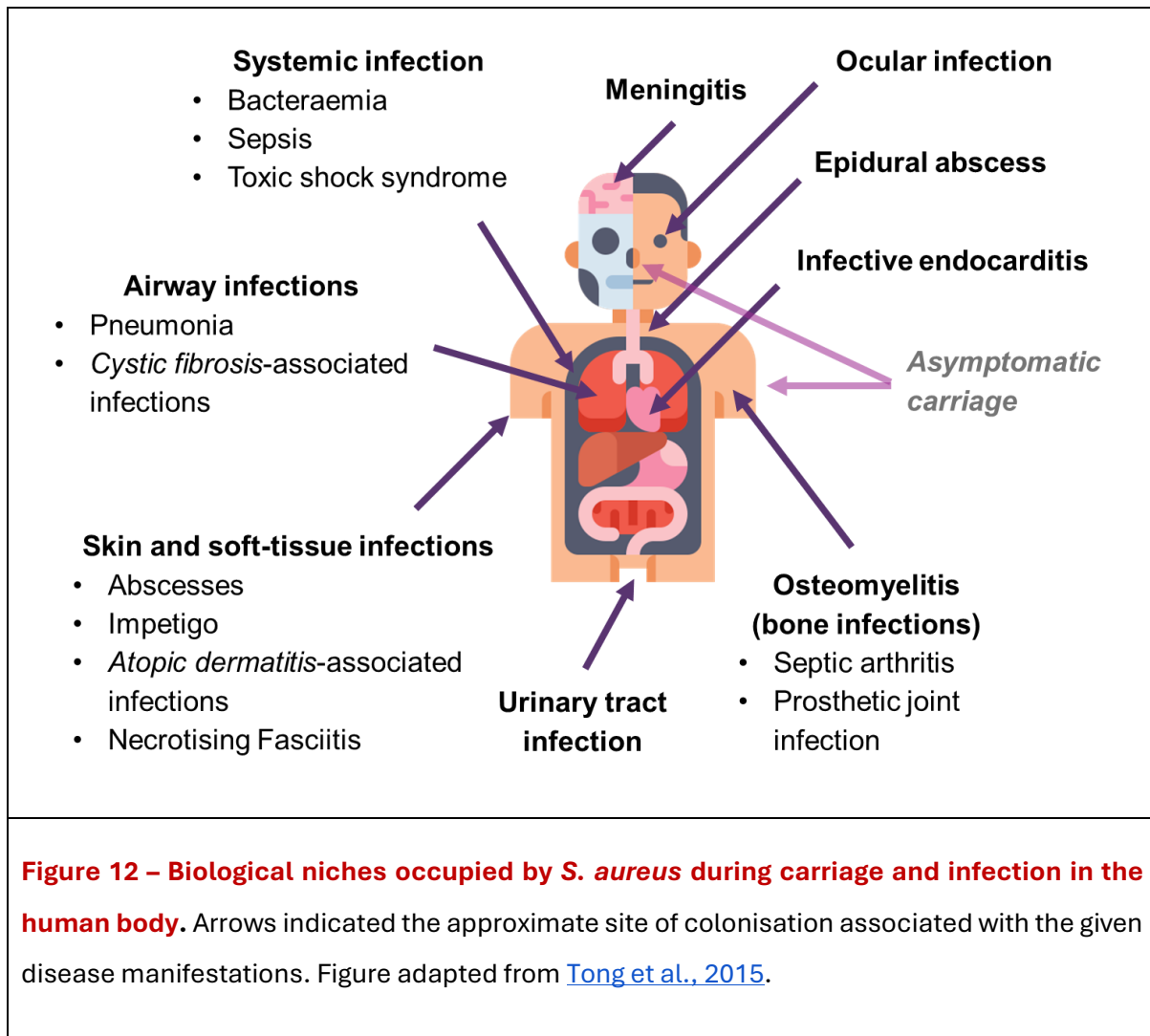
Amino acids, peptides and other nutrients as determinants of *S. aureus* survival in biological environments

The ability of *S. aureus* to survive in a variety of different body sites is likely a major contributor toward its success as a bacterial pathogen (summarised in [Figure 12](#)). In many individuals, *S. aureus* colonises the skin or nasopharynx asymptomatically (as reviewed by [Piewngam & Otto, 2024](#)) and this carriage is believed to serve as a reservoir to drive dissemination of the bacterium ([von Eiff et al., 2001](#)). Regarding infection, *S. aureus* is most commonly associated with topical skin and soft tissue infections (SSTIs) and is a leading cause of these infections in developed countries ([Linz et al., 2023](#); [Ray et al., 2013](#)). Though these cutaneous infections are generally localised and superficial, they may develop into more pervasive forms associated with severe disease and significant mortality ([Tong et al., 2015](#)). *S. aureus* can also colonise the lungs and airways, resulting in pulmonary infections and Staphylococcal pneumonia. Infections of this kind are particularly common among individuals suffering from cystic fibrosis (CF), in which the thick mucus lining the airways provides an ideal niche for long-term colonisation by the bacterium ([Kahl, 2010](#); [Rumpf et al., 2021](#)). If the bacterium is able to disseminate through the bloodstream – for instance, due to intravascular injection ([Palepu et al., 2001](#)), exposure during surgery ([Owens & Stoessel, 2008](#)) or contamination of a prosthetic device ([Lower et al., 2011](#)) – colonisation may occur in sites around the body. These invasive infections can result in a range of life-threatening disease manifestations, including bacteraemia and sepsis (bloodstream), infective endocarditis (heart tissue), osteomyelitis (bone) and meningitis (brain) ([Asgeirsson et al., 2018](#); [Thomer et al., 2016](#); [Tong et al., 2015](#); [Urish & Cassat, 2020](#)).

Notably, the infection environments represented by these various disease states differ greatly in regard to factors such as nutrient availability, and the presence of various chemical and immune stresses. A bacterium colonising the skin, for example, must persist in an acidic and nutrient poor environment, and must interact favourably with other residents of the skin microbiome ([Flowers & Grice, 2020](#)). Recent studies also highlight how the skin barrier forms an active immune response against colonising bacteria, leading to antibody-mediated targeting of cells in this environment ([Bousbaine et al., 2024](#); [Gribonika et al., 2024](#)). In comparison, a wound environment is much more nutritionally rich, but also more accessible to immune cells and other host factors ([Pastar et al., 2013](#); [Zhen Wang et al., 2022](#)). In order to thrive under these diverse conditions, *S. aureus* is equipped with a suite of sensory systems and global gene regulators, and these ultimately coordinate the expression of bacterial virulence factors and fine-tune bacterial metabolism to promote survival ([Dyzenhaus et al., 2023](#); [Jenul & Horswill, 2019](#)). Indeed, a

flexible metabolic network is essential for the bacterium to proliferate in these different environments, in which the availability of primary carbon sources is highly variable.

Here, I will review existing evidence for the importance of nutrient availability during *S. aureus* colonisation and infection in relevant biological environments, highlighting the common role of amino acids and peptides as major carbon and nitrogen sources in each case. This discussion will primarily focus on two niches in particular – that is, the airways and the skin – as these environments are well studied in regard to nutrient availability and the determinants of bacterial survival.



The nasal cavity and airways

Asymptomatic carriage of *S. aureus* in the nasal cavity is observed in about a third of healthy adults, making this the dominant biological niche occupied by the bacterium ([A. M. Edwards et al., 2012](#)). In a study by *Krismer* and colleagues, the metabolome of human nasal secretions was analysed by mass spectrometry ([Krismer et al., 2014](#)). This approach provided compelling evidence that the nasal cavity microenvironment is extremely nutrient limited – particularly in regard to nitrogen sources. For instance, many amino acids were either present at low micromolar concentrations or entirely absent from the environment, with an example of the latter being methionine. When grown in a synthetic medium designed to mimic these conditions, growth of *S. aureus* was limited and genes required for Met biosynthesis were massively upregulated when compared to growth in rich media. Consistent with this, loss-of-function of Met biosynthetic genes significantly reduced the ability of the bacterium to colonise the nasal cavity in cotton rats. Hence, exogenous methionine is limiting during nasal colonisation, and this is reflected in the way *S. aureus* adapts its metabolism to survive this niche ([Krismer et al., 2014](#)). Other genes upregulated during nasal colonisation included high-affinity amino acid and peptide transporter genes, implicating scavenging of these nutrients as a fundamentally important process in this environment ([Krismer et al., 2014](#)).

One way by which bacteria can thrive in nutrient poor environments is through mutualistic or parasitic interactions with other members of the microbial flora. Glycosylated proteins such as mucins are produced by mucosal cells in the nasal cavity, and these macromolecules serve as rich source of carbon, nitrogen and sulphur for bacteria ([Hoffman et al., 2020](#)). Whilst *S. aureus* lacks the secreted carbohydrate-active enzymes required for complete mucin utilisation, the growth of *S. aureus* can be supported by mucin-derived metabolites generated by other members of the nasal flora ([Lucas et al., 2021](#)).

As an example of this phenomenon, *S. aureus* encodes a complete *nan* locus required for the uptake and catabolism of the mucin-derived monosaccharide sialic acid ([Olson et al., 2013](#)). In a study by *Ding* and colleagues, the authors demonstrated how native sialidase-producing bacteria generate free sialic acid in the sputum of CF patients, and this free sialic acid triggers upregulation of *nan* in *S. aureus* ([Ding et al., 2023](#)). Additionally, *S. aureus* isolates adapted to the CF sputum environment (that is, isolates from long-term chronic airway infections) demonstrated a more pronounced transcriptional response to free sialic acid and more rapid consumption of the sugar during growth *in vitro* compared to un-adapted strains. These differences were attributed, at least in part, to dysregulation of the quorum sensing accessory gene regulator (*agr*) locus ([Ding et al., 2023](#)) observed frequently in chronic infection isolates

([Coll et al., 2025](#)). A more recent study by *Shull* and colleagues utilised a Tn-seq approach to identify genetic factors contributing toward the survival of *S. aureus* in CF sputum. Expectedly, genes involved in sialic acid catabolism (specifically *nanE*) were among the most enriched in this data set. However, when grown in monoculture, a *nanE* mutant strain did not exhibit a pronounced phenotype ([Shull et al., 2024](#)). These data are consistent with the model that sialic acid utilisation by *S. aureus* is dependent on the sialidase activity of other members of the microbial community.

Crucially, both this study and the aforementioned study by *Ding* and colleagues demonstrate the importance of metal scavenging in airway environments. In mucosal environment, iron-sequestering proteins such as lactoferrin and calprotectin are secreted by host cells in order to limit the availability of free iron in a process known as nutritional immunity ([Murdoch & Skaar, 2022](#)). Upregulation of the iron-regulated adhesion factor IsdA is observed during *in vivo* nasal colonisation by *S. aureus*, and this is indicative of iron limitation in these environments ([Burian et al., 2010](#); [Krismer et al., 2014](#)). In the presence of free sialic acid, *S. aureus* massively upregulates genes involved in iron scavenging ([Ding et al., 2023](#)), and these systems similarly contributed toward fitness in CF sputum during Tn-seq analysis ([Shull et al., 2024](#)). Hence, metal scavenging seems to be a major determinant of bacterial survival in the airways.

While earlier work suggested that cysteine was heavily depleted in CF sputum ([Krismer et al., 2014](#)), *Shull* and colleagues reported that cysteine was abundant in this niche and demonstrated a key role for the redox-responsive transcriptional regulator CymR in preventing cysteine toxicity at these reported concentrations ([Shull et al., 2024](#)). The authors also highlight the presence of glutathione as an alternative thiol in these environments and demonstrate how this molecule may be present at concentrations ranging from roughly 10 to 300 μ M.

The skin

Despite being a major cause of SSTIs and other cutaneous infections, persistent colonisation of *S. aureus* on healthy human skin is relatively rare as a result of the diverse chemical, immune and microbial barriers which actively inhibit growth in this environment (as reviewed by [Byrd et al., 2018](#); [Swaney & Kalan, 2021](#)). Robust colonisation and infection of the skin, therefore, generally requires these natural defences to be compromised in some way. Exemplifying this, individuals suffering from chronic skin disorders such as atopic dermatitis – an inflammatory condition characterised by barrier dysfunction – demonstrate high rates of *S. aureus* colonisation and reoccurring infections ([Breuer et al., 2002](#); [Ogonowska et al., 2020](#)).

In all cases, colonisation of the skin is commenced by the initial attachment of *S. aureus* cells to corneocytes (forming the outermost layer of the skin barrier) facilitated by diverse cell wall-associated adhesins, such as SasG and ClfB ([Fleury et al., 2017](#); [Mills et al., 2024](#)). If the bacterium is allowed to persist on the skin, various virulence factors facilitate immune evasion and further barrier disruption, ultimately allowing invasion of the underlying tissue ([Foster et al., 2014](#); [Lacey et al., 2016](#)). An acute proinflammatory response at the site of tissue invasion often triggers the formation of a localised abscess; a characteristic feature of *S. aureus* SSTIs ([Kobayashi et al., 2015](#)). Herein, the bacterium is encapsulated by a fibrous proteinaceous capsule comprised of tissue debris, fibrin, collagen and infiltrating phagocytes (primarily neutrophils) ([Cheng et al., 2011](#); [Kennedy et al., 2010](#)) (Summarised in [Figure 13](#)).

Based on the above description, the lifestyle of *S. aureus* in the human skin environment can be described as biphasic; that is, surface-attached growth is strictly limited, while barrier dysfunction facilitates periodic tissue invasion and rapid growth ([Kobayashi et al., 2015](#); [Vitko et al., 2016](#)). Such a dichotomy can be explained, in part, by the availability of essential nutrients in these two scenarios ([Figure 13](#)). Most of the nutrients available to bacteria on the skin surface are derived from secretions of the eccrine (sweat) and the sebaceous (sebum) glands, as well as solutes derived from corneocyte turnover implicated in moisturising the *stratum corneum* (so-called natural moisturising factors, or NMFs) ([Baker, 2019](#); [F. G. Costa et al., 2024](#)). Eccrine sweat is largely nutrient poor and composed primarily of water and inorganic ions ([Baker, 2019](#)), whilst sebum comprises a complex mixture of lipids including glycerides, fatty acids and wax esters ([Picardo et al., 2009](#)). Whilst these sebum components are considered beneficial to some members of the skin flora ([Gribbon et al., 1993](#); [Swaney et al., 2023](#)), their presence inhibits the growth of *S. aureus* and generally contributes toward the antimicrobial properties of the skin barrier ([Angelova-Fischer et al., 2014](#); [S. R. Clarke et al., 2007](#); [Lovász et al., 2017](#); [Swaney et al., 2023](#)). Of the NMFs detectable on human skin, lactate and urea are the most abundant (10-20

mM), though other solutes such as amino acids are also present at low (generally μM) concentrations ([Kutyshenko et al., 2011](#); [Maeno, 2019](#)). Overall, the skin surface is considered a nutrient poor environment for *S. aureus*. In contrast, the underlying epidermis and dermal tissues are rich in carbon sources, including glucose ([Petersen et al., 1992](#)), as well as proteinaceous components of the extracellular matrix (primarily, collagen and elastin), both of which are readily broken down (i.e. into oligopeptides, and then amino acids) and utilised by *S. aureus* for growth ([Lehman et al., 2019](#); [Vitko et al., 2016](#)). These environments are also abundant in essential metal ions including iron and zinc ([Lévêque et al., 2003](#); [Ogawa et al., 2016](#)), and the dermal tissue is considered rich in oxygen as a result of the underlying vasculature ([Evans et al., 2006](#)).

The importance of glucose as a primary carbon source during skin infection is highlighted by the recent observation that glucose fermentation by a commensal skin bacterium (*Cutibacterium acnes*) inhibits the pathogenicity of invading *Pseudomonas aeruginosa* by limiting carbon source availability ([Maslova et al., 2024](#)). Compared to other Staphylococci, *S. aureus* encodes an expanded repertoire of carbohydrate uptake systems, including four glucose transporters ([Vitko et al., 2016](#)). This redundancy points toward an important role for glucose uptake during infection. In two recent studies by Vitko and colleagues, the importance of glucose utilisation was investigated in the context of SSTIs ([Vitko et al., 2016, 2015](#)). The authors demonstrate how, in the presence of antimicrobial factors which inhibit cellular respiration (namely, NO), growth of *S. aureus* is dependent on the glycolytic fermentation of glucose ([Vitko et al., 2015](#)). Both glycolysis and glucose transport are essential for virulence in murine models of SSTI, demonstrating a pivotal role for the sugar in this niche ([Vitko et al., 2016, 2015](#)). Indeed, whilst oxygen is plentiful in the skin environment during the early stages of infection, hypoxia is observed within *S. aureus* abscesses within one-week post-infection in a mouse skin lesion model ([Thurlow et al., 2018](#); [Vitko et al., 2016](#)). It has been proposed that the essentiality of fermentative glucose utilisation during skin infection is driven, at least in part, by the hypoxic environment established within the maturing abscess.

More recent evidence demonstrates that, while glucose is abundant in the early stages of skin infection, mature abscesses are actually depleted of glucose as a result of the acute inflammatory response ([Thurlow et al., 2018](#)). Hence, other carbon sources are likely also required for continued bacterial proliferation over longer infection periods ([Lehman et al., 2019](#)). Indeed, it has been demonstrated that *S. aureus* can grow by utilising lactate – a by-product of glycolytic growth – alongside peptides as a glucogenic substrate in already glucose-depleted environments ([Spahich et al., 2016](#)) ([Figure 13](#)).

A number of studies have utilised genome-wide genetic screening approaches to identify genetic loci implicated in fitness during abscess formation, and these studies help to illuminate the essential nutrients underpinning establishment of this niche ([Coulter et al., 1998](#); [Grosser et al., 2018](#); [Valentino et al., 2014](#)). Whilst the findings of these studies vary substantially as a result of differences in the methodology used, some points of commonality are observed. In all three studies, strains possessing mutations in metal scavenging transporters – namely, the nickel permease Opp2/5 (Nik) ([Hiron et al., 2010](#)) and the nickel/cobalt permease Opp1 (Cnt) ([Remy et al., 2013](#)) – are highly attenuated during abscess formation, presumably due to an inability to accumulate nickel for use as an essential cofactor for intracellular enzymes. One such nickel-dependant enzyme – urease – is required for pH homeostasis in urea-rich environments and has recently been linked to bacterial virulence in these environments ([Sychantha et al., 2024](#); [Zhou et al., 2019](#)). All three studies also identified a key role for carbohydrate metabolism during abscess formation, supporting the proposed essentiality of carbohydrate utilisation in this niche ([Vitko et al., 2016](#)).

In two of the three genome-wide studies, genes implicated in peptide and amino acid uptake/catabolism were also identified as important fitness determinants during abscess formation ([Coulter et al., 1998](#); [Valentino et al., 2014](#)), though the findings of *Grosser* and colleagues suggest that these genes are not significantly associated with fitness ([Grosser et al., 2018](#)). Such a deviation likely stems from the parent strains utilised in these different studies; that is, while the earlier studies utilised transposon libraries in standard laboratory *S. aureus* strains, *Grosser* and colleagues utilised the highly virulent and skin-adapted CA-MRSA strain USA300 LAC. The authors point out that this strain possesses numerous adaptations which both massively influence the infection environment (for instance, increased production of lytic toxins and secreted proteases), as well those which allow the bacterium to efficiently utilise a broader range of different carbon sources during infection ([Grosser et al., 2018](#)). Transport and catabolism of key carbon sources is also often highly redundant, with several compensatory pathways facilitating the utilisation of these nutrients in the absence of individual genetic components ([Halsey et al., 2017](#); [Lehman et al., 2019](#); [Vitko et al., 2016](#)). Hence, the impact of individual nutrients toward bacterial fitness during abscess formation is likely difficult to decipher by genetic screens alone, especially for adapted strains.

There is now a wealth of evidence to suggest that amino acids and peptides are, in fact, major nutrient sources utilised by *S. aureus* during skin infection ([Lehman et al., 2019](#)). Indeed, the success of the USA300 and USA500 lineages as dominant skin-associated pathogens has been attributed to the increased proteolytic activity of these strains ([Min Li et al., 2009](#)). Expression of

protease-encoding genes in USA300 is increased massively in both a skin-like medium ([F. G. Costa et al., 2024](#)) and on *ex vivo* human skin explants ([Burian et al., 2021](#)), while loss of protease-encoding genes leads to attenuation of *S. aureus* in a murine skin abscess model ([Kolar et al., 2013](#)). In addition to roles in immune evasion and skin barrier disruption, these proteases are proposed to contribute toward nutrient acquisition by generating oligopeptides and essential amino acids (e.g. proline) in the local environment, as described below ([Lehman et al., 2019, 2023](#)).

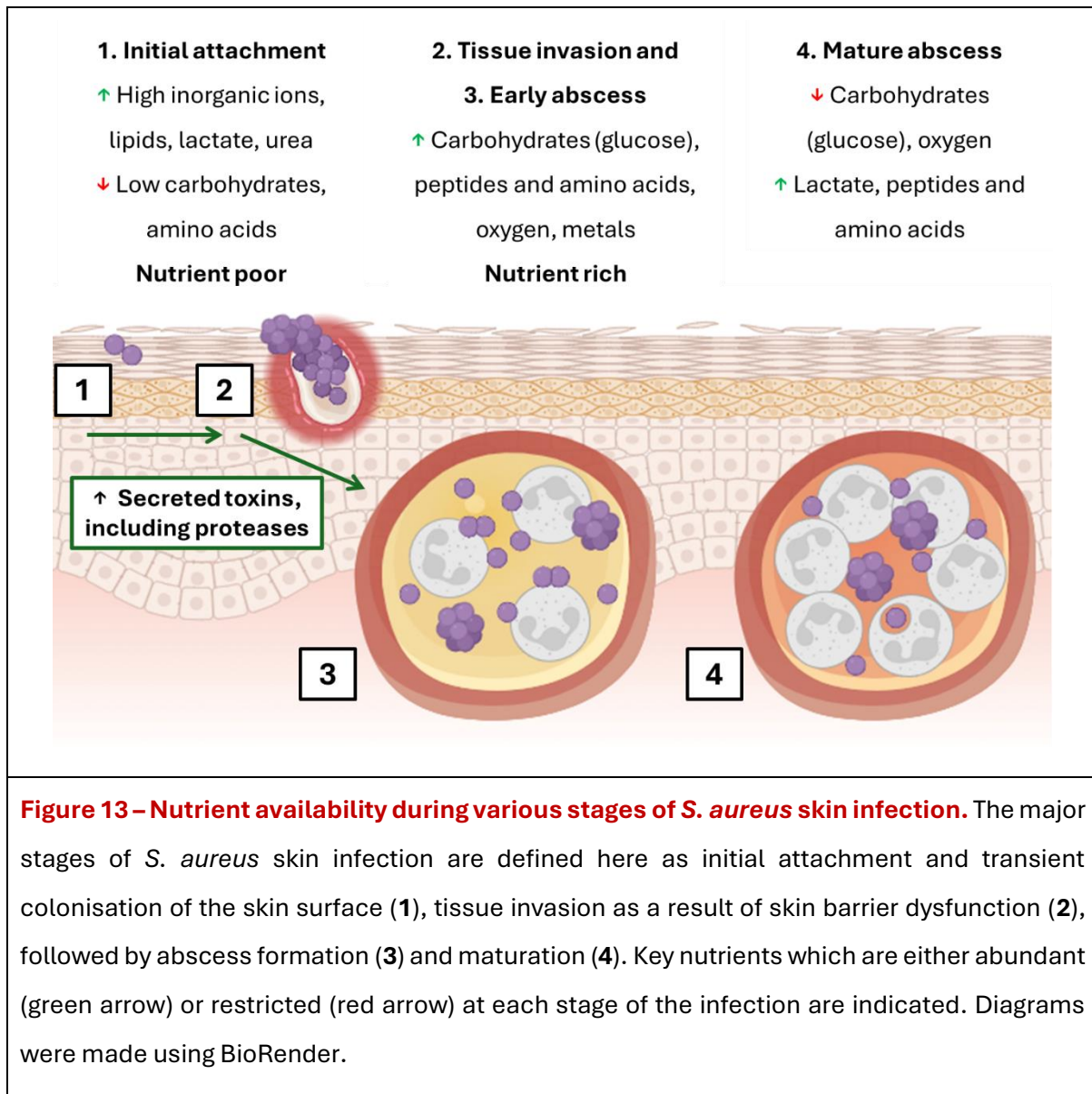


Figure 13 – Nutrient availability during various stages of *S. aureus* skin infection. The major stages of *S. aureus* skin infection are defined here as initial attachment and transient colonisation of the skin surface (1), tissue invasion as a result of skin barrier dysfunction (2), followed by abscess formation (3) and maturation (4). Key nutrients which are either abundant (green arrow) or restricted (red arrow) at each stage of the infection are indicated. Diagrams were made using BioRender.

Secreted proteases of S. aureus

Nutrient acquisition and immune evasion are among the most important physiological processes required by bacterial pathogens for successful infection. In *S. aureus*, secreted proteases are key players in both of these processes and are, therefore, considered important virulence factors in a variety of niches (as reviewed in [Koziel & Potempa, 2013](#); [Tam & Torres, 2019](#)).

S. aureus encodes at least 10 secreted enzymes with proteolytic activity: the metalloprotease aureolysin (*aur*), the papain-like cysteine proteases staphopain A (ScpA) and B (SspB), the V8 serine protease (SspA) and six serine protease-like proteins (SplA-F) ([Shaw et al., 2004](#)). In addition to these, two additional serine proteases – exfoliative toxins A (EtaA) and B (EtaB) – are secreted, but these remain poorly characterised and only have a single known target to-date (Desmoglein 1) ([Hanakawa et al., 2004](#); [Ladhani et al., 1999](#)). The secreted proteases of *S. aureus* differ not only structurally, but also in their molecular targets. For instance, *aur* demonstrates a broad substrate range and cleaves preferentially at peptide bonds immediately before a hydrophobic residue, such as Ala or Val, whilst SspA almost exclusively cleaves after Glu ([Björklind & Jörnvall, 1974](#); [Frey et al., 2021](#)). The Spl proteins are, comparatively, far more limited in regard to substrate profiles and generally demonstrate a consensus target sequence of 4 to 6 residues in length ([Dubin et al., 2008](#); [Stec-Niemczyk et al., 2009](#)).

The major secreted proteases of *S. aureus* are activated following secretion by a well-defined proteolytic cascade ([Figure 14](#)). Briefly, both *aur* and ScpA are secreted as inactive pro-enzymes and undergo autolytic cleavage in the external environment, thereby forming the active proteases ([Nickerson et al., 2010](#); [Nickerson et al., 2008](#)). *Aur* cleaves the inactive pro-SspA to generate active SspA, which goes on to activate SspB in a similar manner ([Massimi et al., 2002](#); [Nickerson et al., 2008](#)). In contrast, the activity of Spl proteins is strictly inhibited by the presence of an N-terminal signal peptide, and cleavage of these peptides during secretion is necessary and sufficient for activation of the protein product ([Dubin et al., 2008](#); [Reed et al., 2001](#)).

The expression of secreted protease genes in *S. aureus* is also tightly coordinated on a transcriptional level in response to environmental conditions through a number of major regulatory circuits. Of these, the global transcriptional regulators CodY and SarA, as well as the quorum sensing *agr* system, are the major factors governing expression of the *aur*, *ssp* and *scp* loci ([Beenken et al., 2014](#); [Campbell et al., 2024](#); [Gimza et al., 2019](#); [Oriol et al., 2021](#)), whilst expression of the Spl proteins is primarily controlled by the SaeRS two-component system ([Cassat et al., 2013](#); [Gimza et al., 2019](#)).

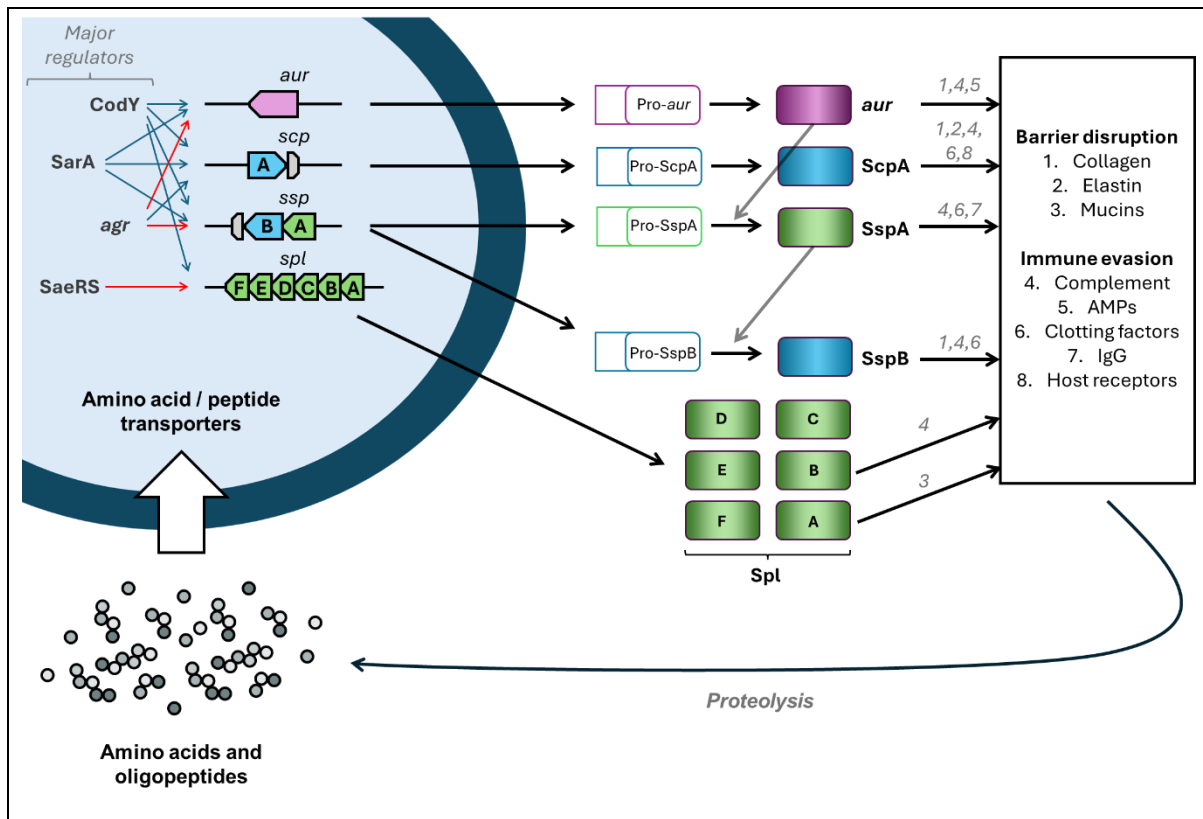


Figure 14 – The major secreted proteases of *S. aureus* and their known functional targets.

(Upper left) The 10 major secreted proteases of *S. aureus* are separately encoded in four distinct genomic loci, as depicted. Regulatory elements primarily responsible for coordinating expression of these loci are also shown (blue arrow = repressor; red arrow = inducer). A number of additional regulatory elements also contribute toward regulating protease expression but are not shown here (described in [Gimza et al., 2019](#)). (Centre) *aur*, ScpA, SspA and SspB are secreted as inactive zymogens and become activated through the proteolytic activity, as described in the text. The Spt proteins are active following secretion without further proteolysis. Purple = zinc metalloprotease; Blue = cysteine protease; Green = serine protease-like. (Upper right) Known functional targets of the *S. aureus* secreted proteases. Numbered arrows denote where a specific protease has been demonstrated to contribute toward degradation of a listed target, as described in the text. (Lower) Proteolytic products including amino acids and oligopeptides are internalised by cognate transporter proteins and serve as a nutrient source for *S. aureus*. Figure adapted from ([W. Chua et al., 2022](#)).

Immune evasion

The secreted proteases of *S. aureus* are, perhaps, most well characterised in relation to their roles in immune evasion during infection. Exemplifying this, there is evidence that *aur* directly cleaves the complement protein C3 during infection to ultimately prevent bacterial killing by the membrane attack complex ([Laarman et al., 2011](#)), and this protein also degrades AMPs including LL-37 ([Sieprawaska-Lupa et al., 2004](#)). In a recent work by Frey and colleagues, the molecular targets of SspA in human serum were systematically elucidated, demonstrating activity against diverse complement proteins, immunoglobulins and clotting factors ([Frey et al., 2021](#)). Both SlpB and the two cysteine proteases of *S. aureus* have also been demonstrated to cleave complement proteins ([Dasari et al., 2022](#); [Jusko et al., 2014](#)), with the latter also demonstrating activity against host receptor proteins and signalling molecules implicated in immune cell function ([Elmwall et al., 2017](#); [Laarman et al., 2012](#); [Smagur et al., 2009](#)).

In addition, there are several examples of *S. aureus* secreted proteases modulating the abundance and stability of the bacteria's own virulence factors directly, including pore-forming toxins, biofilm components and adhesins. Though immediately counterintuitive, this autoinhibitory effect plays an important role in modulating bacterial virulence in order to dampen the severity of the immune response ([Gimza et al., 2021](#); [Kolar et al., 2013](#); [Paharik et al., 2016](#); [Zielinska et al., 2012](#)). The importance of secreted proteases in immune modulation is best illustrated by a protease-null mutant lacking all 10 major secreted proteases, which demonstrates decreased serum survival, increased AMP susceptibility, increased rates of phagocytosis by white blood cells and hypervirulence *in vivo* as a result of virulence factor dysregulation ([Gimza et al., 2021](#); [Kolar et al., 2013](#)).

Nutrient acquisition

Proteolysis is a common mechanism utilised by a number of bacteria to generate amino acids and oligopeptides which serve as nutrients in the local environment ([Cezairliyan & Ausubel, 2017](#); [M. Liu et al., 2010](#); [Nemoto & Ohara Nemoto, 2021](#)). The role of secreted proteases in nutrient acquisition is not yet comprehensively understood for *S. aureus*, though evidence suggests that these enzymes similarly contribute toward growth in relevant biological contexts. Consistent with this, *Kolar* and colleagues have demonstrated how a protease-null strain of *S. aureus* exhibited no strong growth phenotypes in a complex medium containing amino acids and sugars but was inhibited in peptide-rich environments including milk and serum ([Kolar et al., 2013](#)).

The model that secreted proteases may be important players in nutrient acquisition is supported by the observation that their expression is coordinated (at least, in part) by the GTP-dependant

transcriptional repressor CodY ([Gimza et al., 2019](#); [Majerczyk et al., 2010](#); [Rivera et al., 2012](#)). Derepression of the CodY regulon occurs primarily during periods of amino acid limitation, wherein the cellular GTP pool is depleted by the major alarmone synthase *rel* ([Pohl et al., 2009](#)). The majority of genes normally repressed by CodY in *S. aureus* are implicated in nitrogen assimilation via the utilisation of amino acids and peptides, including high-affinity transporters and catabolic enzymes ([Geiger et al., 2012](#); [Majerczyk et al., 2010](#)). CodY also regulates the expression of major virulence determinants, including lytic toxins and secreted proteases ([Geiger et al., 2012](#); [Pohl et al., 2009](#)). Perhaps expectedly, therefore, secreted proteases are among the most upregulated genes in *S. aureus* during growth under nutrient-limited conditions ([F. G. Costa et al., 2024](#)). The observation that these genes are specifically upregulated by a starvation-responsive mechanism provides compelling evidence for their direct involvement in nutrient acquisition.

The proteolytic targets of secreted *S. aureus* proteases include abundant human proteins present in tissues preferentially colonised by the bacterium. Several studies implicate the secreted proteases of *S. aureus* in barrier disruption and tissue invasion ([Amagai et al., 2002](#); [Kolar et al., 2013](#); [Murphy et al., 2018](#); [M. R. Williams et al., 2020](#)), and a major driver of this process is believed to be the ability of these enzymes to degrade major structural components of the extracellular matrix. These include collagen (discussed in more detail below) as well as elastin, which is a major constituent of human connective tissues and comprises at least 30% of the dry mass of major blood vessels ([Cocciolone et al., 2018](#); [Uitto, 1979](#)). The cysteine protease ScpA rapidly degrades elastin ([Potempa et al., 1988](#)), and this activity is likely to be relevant for nutrient acquisition during endothelial colonisation and invasion (both common features of *S. aureus* bloodstream infections) ([Kwiecinski & Horswill, 2020](#)). SplA has been demonstrated to cleave mucins including mucin16, and this activity contributes toward epithelial barrier disruption and virulence in a rabbit pneumonia model ([Paharik et al., 2017, 2016](#); [Stec-Niemczyk et al., 2009](#)). In their study, Frey and colleagues identified 85 substrates of SspA which were abundant in human serum ([Frey et al., 2021](#)), and the breakdown of any of these proteins could feasibly contribute toward the generation of peptides to fuel bacterial growth. Overall, it is likely that these proteases act in tandem to degrade abundant host proteins and generate nutrients (peptides and amino acids) in the local environment (as summarised in [Figure 14](#)).

Collagen utilisation in the skin and airways

Collagen – the primary component of the extracellular matrix – is abundant in many environments associated with *S. aureus* infection, including the dermis of the skin as well as tissues underlying the airways and vasculature ([Bhattacharya & Horswill, 2024](#)). At least three of the *S. aureus* secreted proteases actively degrade collagen: *aur* ([Lehman et al., 2019](#)) and the cysteine proteases ScpA and SspB ([Ohbayashi et al., 2011](#)). Using a fluorescent probe of collagenase activity, *Lehman* and colleagues have demonstrated that *aur* and SspB are responsible for most of the collagenase activity of the bacterium ([Lehman et al., 2019](#)). However, the authors of this study also demonstrated that host proteases – chiefly, the matrix metalloprotease (MMPs) MMP-9 – are overexpressed in response to infection in a murine abscess model and provide evidence that *aur* specifically activates the collagenase activity of MMP-9. MMPs are generally secreted by host cells during acute infection, and their activity remodels collagen to facilitate immune cell migration ([Elkington et al., 2005](#)). Therefore, the secreted proteases of *S. aureus* act synergistically with host MMPs to breakdown collagen during infection ([Lehman et al., 2019](#)).

Several types of collagens are produced in different sites of the human body, which vary based on their structural properties as a result of the presence or absence of various accessory domains. A common feature of all collagens is the presence of Gly-Pro-X repeats (where X is commonly hydroxyproline), which imparts a left-handed helical structure to the protein and facilitates the formation of triple-helical multimeric fibrils ([Ricard-Blum, 2011](#)). As a result of this motif, the breakdown of collagen during *S. aureus* infection theoretically results in the generation of Gly- and Pro-enriched peptide fragments, as well as monomeric Gly and Pro amino acids. In a more recent study, *Lehman* and colleagues demonstrate that uptake of exogenous Pro is essential for *S. aureus* survival in a murine abscess model ([Lehman et al., 2023](#)). In the presence of glucose, *de novo* Pro biosynthesis in *S. aureus* is inhibited by carbon catabolite repression ([Chunling Li et al., 2010](#); as described on **page 80**). Hence, the authors speculate that exogenous collagen-derived Pro (and/or Pro-containing peptides) may be essential in the early stages of abscess formation, where glucose is a dominant carbon source ([Lehman et al., 2023](#)). Indeed, genetic screening approaches have implicated Pro transporters as essential fitness determinants in both abscess and burn wound infection models ([Coulter et al., 1998](#); [Valentino et al., 2014](#)).

To date, the clearest evidence highlighting the importance of collagen utilisation by *S. aureus* during infection was presented in a study by *Urso* and colleagues, focussing specifically on isolates from CF-associated chronic lung infection ([Urso et al., 2024](#)). In a longitudinal study, *S. aureus* strains were isolated from adults with long-term colonisation of the airways. These

adapted strains demonstrated an altered metabolic profile marked by dysregulation of the carbon catabolite repression system, leading to decreased expression of the adenosine synthase *AdsA*. As a result, adapted strains displayed decreased expression of glycolytic genes and upregulation of genes involved in high-affinity Pro transport and Pro catabolism. During growth *in vitro*, these strains demonstrated an increased metabolic output in response to exogenous Pro. In a murine pneumonia model, an *adsA* mutant strain demonstrated increased fitness at 72 h post-infection, but this effect was abolished by loss of the major Pro transporters. Hence, Pro utilisation is an important determinant of survival in the airways and adapted strains are primed to utilise this amino acid by loss of *AdsA* ([Urso et al., 2024](#)).

While the authors of this study did not observe an increase in MMP production in the infection environment (as observed previously by [Lehman et al., 2019](#)), they did note increased occurrence of collagen-producing fibroblasts in the infected tissue, and observed that the expression of collagen biosynthetic genes in this tissue was increased for the adapted strain compared to the wild-type. Inhibiting collagen production nullified the fitness advantage of the adapted strains, demonstrating that these strains were specifically taking advantage of the newly synthesised collagen. Finally, the authors extend their findings to demonstrate that similar adaptations are common among chronic atopic dermatitis isolates, and demonstrate increased collagen production by fibroblasts in these skin infections ([Urso et al., 2024](#)). Hence, the stimulation of collagen production and subsequent utilisation of collagen-derived proline as a major carbon source is common in both skin- and airway-associated chronic *S. aureus* infections. Notably, whilst the authors highlight increased Pro transporter expression as a major fitness determinant in these adapted strains, changes in the expression of peptide transports and the potential for these systems to contribute to Pro assimilation are not explored in this study.

Other infection environments

To date, understanding of the nutrient factors influencing the fitness of *S. aureus* in niches outside of the skin and airways is limited. However, insights from a small number of studies point toward amino acid and peptide utilisation as a common factor affecting bacterial physiology in diverse body sites.

Infective endocarditis

Severe *S. aureus* bacteraemia can lead to infective endocarditis, wherein the bacterium colonises the valves or cardiac tissue of the heart ([Bastien et al., 2023](#)). Following from a genetic screen for factors influencing bacterial survival in number of different infection models, *Coulter* and colleagues demonstrated that the di/tripeptide permease (Dtp) DtpT was essential for establishment of cardiac vegetations following bloodstream infection in a rabbit endocarditis model ([Coulter et al., 1998](#)). In follow-up studies, the same authors also highlighted a role for high-affinity Pro transporters in this model ([Bayer et al., 1999](#); [W. R. Schwan et al., 1998](#)). Analysis of the expression profile of *S. aureus* cells in a rat vegetative endocarditis model demonstrates increased protease expression in this environment, as well as increased expression of genes involved in arginine scavenging and biosynthesis ([Hanses et al., 2014](#)). Collectively, these observations suggest a role for peptide and amino acid utilisation in cardiac infections.

Intriguingly, manganese transporters were also upregulated during vegetative endocarditis ([Hanses et al., 2014](#)). In a study by *Juttukonda* and colleagues, the role of manganese in these infections was explored comprehensively. Herein, the authors demonstrate that increased dietary manganese predisposes mice to *S. aureus* endocarditis by increasing the bioavailability of this metal to the infecting bacterium. Manganese plays a protective role in these environments, allowing the bacterium to overcome reactive oxygen stress and thereby evade immune clearance ([Juttukonda et al., 2017](#)).

Osteomyelitis

The human skeletal system represents a hostile and nutrient-poor environment for infecting bacteria. In spite of this, osteomyelitis is among the most common manifestations of invasive *S. aureus* infection ([Gerber et al., 2009](#)). In this environment, the bacterium liberates essential nutrients from surrounding osteoblast cells through the overproduction of lytic toxins (for instance, phenol soluble modulins), ultimately leading to remodelling and degradation of the bone tissue to benefit bacterial survival ([Cassat et al., 2013](#); [Crémieux et al., 2014](#)). Bone tissue is also rich in collagen and, as a result, one might expect proteolysis to be an essential process for nitrogen assimilation in this nutrient-limited niche. Surprisingly, however, the activity of secreted proteases in this environment is seemingly deleterious to survival ([Campbell et al.,](#)

[2024; Cassat et al., 2013; Ramirez et al., 2020](#)). Indeed, dysregulation of *agr* (associated with decreased protease production) is common in chronic osteomyelitis isolates ([Suligoy et al., 2018](#)), and loss-of-function of the protease-associated repressor SarA attenuates bacterial virulence in a murine osteomyelitis model ([Loughran et al., 2016](#)). Based on proteomics analysis, Ramirez and colleagues proposed that the attenuated virulence of a *sarA* mutant results predominantly from protease expression, leading the degradation of other exotoxins which are more important for survival in this niche ([Ramirez et al., 2020](#)). Furthermore, in a Tn-seq analysis of genetic factors contributing to fitness in an osteomyelitis model, Wilde and colleagues did not identify secreted proteases or amino acid/peptide transporter systems as essential in this niche ([Wilde et al., 2015](#)). Overall, there is poor evidence for the importance of collagen-derived nutrients during colonisation of bone tissue.

A recent study by Potter and colleagues aimed to comprehensively investigate the metabolic pathways required by *S. aureus* for survival in an osteomyelitis model. Therein, the authors identified aspartate biosynthesis as an essential pathway required for survival of the bacterium. However, analysis of the infected bone tissue revealed that exogenous aspartate is not limited in this environment. Instead, the osteomyelitic niche is heavily enriched in glutamate, which competitively inhibits aspartate uptake through the shared Asp/Glu transporter GluT ([A. D. Potter et al., 2020](#)). Overall, this study demonstrates how nutrient availability in the bone microenvironment can determine the bacterial metabolic factors required for survival in this niche.

Intracellular environments

While once considered an exclusively extracellular pathogen, *S. aureus* has now been demonstrated to infect a variety of human cell types including professional phagocytes, such as macrophages and neutrophils ([Flannagan et al., 2016; Rigby & DeLeo, 2012](#)), as well as epithelial, endothelial and bone cells ([Blättner et al., 2016; Krauss et al., 2019; Rollin et al., 2017](#)). During systemic infection in a murine model, Kupffer cells in the liver act as a major reservoir for bacterial proliferation preceding dissemination to other body sites ([Surewaard et al., 2016](#)). In these intracellular niches, the bacterium subverts host cell defences and inhibits programmed cell death pathways such as apoptosis in order to prolong host cell survival (as reviewed by [Hommes & Surewaard, 2022; Vozza et al., 2021](#)). Hence, the intracellular lifestyle is likely an important factor contributing toward survival of the bacterium during invasive infection. The bacterial factors underpinning *S. aureus* survival in these intracellular factors are multifaceted and not yet comprehensively understood. However, the major regulators *agr* and SaeRS have both been implicated as the major determinants of survival within diverse cell types, and this

effect is seemingly conveyed through upregulation of lytic exotoxins including proteases ([M. Grosz et al., 2014](#); [Münzenmayer et al., 2016](#); [Qazi et al., 2001](#); [Schnaith et al., 2007](#)).

A number of conflicting studies propose two major strategies which may be employed by *S. aureus* in the intracellular niche: one in which the bacteria divide within a membrane-bound phagosomal compartment ([Flannagan et al., 2016](#); [Rungelrath et al., 2021](#); [Tranchemontagne et al., 2015](#)), and another in which the bacterium rapidly escapes to the cytosol before replicating ([M. Grosz et al., 2014](#); [Strobel et al., 2016](#)). In reality, internalised bacteria likely exist in both states concurrently during infection and the relative proportions of vacuolar/cytosolic bacteria depends upon strain-specific adaptations and host cell types ([Strobel et al., 2016](#)). Indeed, it's been proposed that phagosomal escape is the predominant survival strategy within non-professional phagocytes, whilst bacteria localise predominantly to the phagosome (and, thereafter, the phagolysosome) in neutrophils and macrophages ([Moldovan & Fraunholz, 2019](#)). In the cytosol, bacteria can utilise diverse nutrients shared with host cell, including rich carbon sources such as glucose and pyruvate, as well as amino acids ([Eisenreich et al., 2010](#)). However, the nutrient factors impacting bacterial fitness inside the phagocytic compartment are less clear, given that this compartment is theoretically depleted of nutrients by the activity of host phagosomal transporter proteins ([Cunrath & Bumann, 2019](#)).

Intracellular bacteria commonly manipulate the metabolism of host cells in order to generate preferable nutrients which drive growth in these environments ([Abu Kwaik & Bumann, 2015](#)). One mechanism by which this is achieved is through the induction and manipulation of autophagy; the process through which cytosolic material is enclosed within a membrane-bound compartment and selectively degraded. Several studies have demonstrated how *S. aureus* modulates host-cell autophagy to enable survival (as reviewed by [Vozza et al., 2021](#); [Wang et al., 2021](#)). In a recent study by *Bravo-Santano* and colleagues, it was demonstrated that autophagy induced by *S. aureus* drastically altered the metabolic profile of these cells during infection, leading to shifts in the levels of intracellular metabolites. The authors speculate that such a response may increase nutrient access to the bacterium, supporting intracellular survival ([Bravo-Santano et al., 2018](#)). In a different study by *Flannagan* and *Heinrichs*, it was demonstrated that nutrients in the extracellular environment are conveyed to intracellular *S. aureus* within macrophages via endocytosis ([Flannagan & Heinrichs, 2020](#)). Overall, there is increasing evidence that intracellular *S. aureus* manipulates the host behaviour in order to parasitise metabolites available to the host cell and thereby thrive within this niche.

Amino acid utilisation in *S. aureus*

As discussed so-far in this chapter, amino acids and peptides derived from host proteins such as collagen serve as an important nutrient source for infecting *S. aureus* in diverse biological contexts, especially in the absence of alternative carbon sources such as glucose. During growth in defined media, some amino acids are preferentially consumed by *S. aureus*, while others are depleted more gradually from the medium ([Halsey et al., 2017](#); [Y.-K. Liu et al., 2020](#)). This phenomenon suggests that a subset of amino acids are more important than others for driving bacterial growth ([Halsey et al., 2017](#)). For some amino acids – namely: arginine, proline and valine – *S. aureus* commonly demonstrates partial or complete auxotrophy under physiologically relevant conditions, making their acquisition essential ([Jeong et al., 2022](#); [Chunling Li et al., 2010](#); [Reslane et al., 2022](#)). In addition to their roles as potential carbon sources and protein synthesis precursors, amino acids are important metabolites contributing to diverse biological processes, including purine and lipid biosynthesis ([Frank et al., 2021](#); [A. D. Potter et al., 2020](#)). As a result, the availability of some amino acids has been observed to have pleiotropic effect on bacterial physiology ([Freiberg et al., 2024](#); [Shibamura-Fujiogi et al., 2022](#); [Merve S. Zeden et al., 2020](#)).

Here, I will provide an overview of amino acid metabolism in *S. aureus*, focussing specifically on how these molecules are transported and catabolised by the bacterium for growth. Where available, I will provide examples of how these processes contribute more broadly to bacterial physiology, highlighting the importance of amino acid utilisation beyond nutrition.

Amino acid catabolism

In a recent study by Halsey and colleagues, the routes of amino acid catabolism in the model *S. aureus* strain JE2 (a derivative of the CA-MRSA strain USA300) were comprehensively investigated by a combination of bioinformatics, microbial growth experiments and NMR metabolomics-based carbon flux analysis (Halsey et al., 2017). From this analysis, a number of key observations were made (summarised in Figure 15A):

1. Glutamate is the preferred carbon source in the absence of glucose, as indicated by the essentiality of the glutamate dehydrogenase GudB under this condition.
2. In the absence of exogenous glutamate, proline is catabolised to produce glutamate via the enzymes PutA and RocA. Both PutA and RocA are therefore essential for robust growth under this condition.
 - 2.a. Arginine and histidine are also converted to glutamate, though these pathways are seemingly less important for supporting growth. Glutamate can also be generated from 2-oxoglutarate or glutamine via the GltB/GltD glutamate synthase, though the importance of this process is poorly explored here.
3. Alanine, threonine, glycine and serine are each catabolised to produce pyruvate, which either enters the TCA cycle directly (via conversion to oxaloacetate by *pyc*) or is catabolised to produce acetate and ATP via the Pta-AckA pathway.
 - 3.a. The essentiality of AckA and observed production of acetate in the absence of glucose strongly suggests that that pyruvate is predominantly converted to acetate under this condition, presumably to maximise ATP yield.
4. Aspartate can also be catabolised directly by the enzyme AspA to yield oxaloacetate, which feeds directly into the TCA to fuel growth.
5. Complete catabolic pathways could not be identified for Cys, Lys, Tyr, Leu, Ile, Phe, Val, Trp or Met, consistent with the more gradual depletion of these amino acids from the medium.

The conclusions of this study provide compelling evidence that amino acids – specifically, glutamate and amino acids which serve as glutamate precursors (i.e. Pro) – act as essential carbon sources for *S. aureus* in the absence of carbohydrates. In other bacteria, glutamate also serves as the major nitrogen donor for biosynthetic processes and is, therefore, maintained at a high cellular concentration (Bennett et al., 2009; Gunka & Commichau, 2012). Consistent with this, carbon flux analysis demonstrates how all amino acids are ultimately utilised for glutamate

biosynthesis in *S. aureus*, particularly in stationary phase where exogenous glutamate may be limited ([Halsey et al., 2017](#)).

Given the central role of glutamate in both carbon and nitrogen metabolism, changes in glutamate availability may result in pleiotropic effects in regard to cell behaviour. In a study by *Shibamura-Fujiogi* and colleagues, the effects of glutamate limitation in *S. aureus* were comprehensively studied in glucose-depleted media, and in strains with disrupted glutamate transport. Intriguingly, these conditions led to a global metabolic rewiring in the cell ultimately resulting in upregulation of the Glu synthase GltD/B and an increased intracellular glutamate pool compared to glutamate-rich conditions ([Shibamura-Fujiogi et al., 2022](#)). Glutamate limitation also triggered enhanced biofilm formation *in vitro* and *in vivo*, implicating availability of this amino acid as an important environmental stimulus impacting relevant virulence-associated behaviour. Whilst the exact basis for this phenomenon is not clearly elucidated in this work, the authors do observe a decrease in the cellular c-di-AMP pool during Glu limitation ([Shibamura-Fujiogi et al., 2022](#)), and more recent work has demonstrated that changes in the concentration of this cyclic nucleotide are a major trigger for *S. aureus* biofilm formation ([Corrigan et al., 2011](#); [Syed et al., 2024](#)).

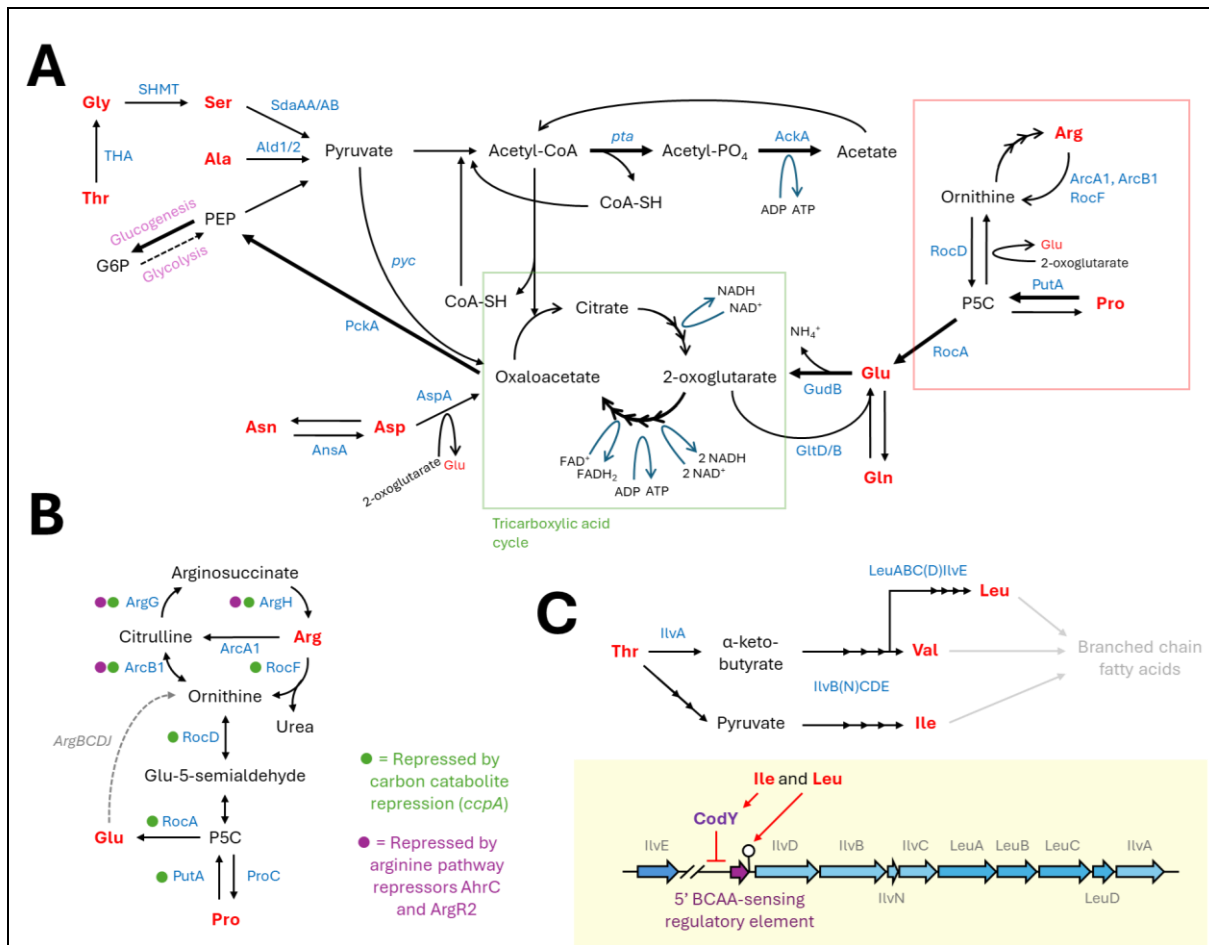


Figure 15 – Amino acid catabolism and auxotrophy in *S. aureus* USA300 JE2. (A) Amino acid catabolic pathways in USA300 JE2. Amino acids are highlighted in red. Known pathways involved in amino acid catabolism are shown as black arrows. Relevant catabolic enzymes involved in various pathways are labelled in blue. Broad arrows denote essential pathways under glucose deprivation (as determined by [Halsey et al., 2017](#)). (B-C) Schematic overview of the metabolic basis for Arg, Pro and Val auxotrophies observed in USA300 JE2. (B) In *S. aureus*, Pro is synthesised from Arg via ornithine and P5C intermediates. In the presence of glucose, essential biosynthetic genes for Pro and Arg are suppressed by *ccpA*. Arg auxotrophy also occurs independently of glucose due to the arginine-specific repressors *AhrC* and *ArgR2*, as well as through depletion of the cellular P5C pool by *ProC*. (C) Branched chain amino acid synthesis is tightly regulated. In the presence of Leu and Ile, synthesis of the entire *Ilv/Leu* operon is repressed via the transcriptional repressor *CodY* and a *cis*-acting Ile/Leu-responsive regulator element. As a result, Val biosynthesis is restricted.

Amino acid auxotrophies

Amino acid auxotrophy occurs when a bacterium is unable to synthesise one or more specific amino acids – for instance, due to the lack of an essential biosynthetic pathway – and therefore obligately requires an exogenous supply of that amino acid to grow ([Ramoneda et al., 2023](#)). Curiously, despite encoding complete biosynthetic pathways for all 20 naturally occurring amino acids, *S. aureus* strains commonly observe amino acid auxotrophies when grown under laboratory conditions ([Lincoln et al., 1995](#)). In the case of the model strain USA300 JE2, three major auxotrophies are well established: proline, arginine and valine ([J. C. Kaiser et al., 2018](#); [Chunling Li et al., 2010](#); [Reslane et al., 2022](#)). In each case, these auxotrophies result from complex regulatory interactions and, therefore, provide keen insights into how nutrient availability can influence central metabolism in this bacterium.

Proline and arginine

In *S. aureus*, both Pro and Arg are synthesised from the metabolic intermediate ornithine. In many bacteria, a canonical ornithine synthesis pathway exists in which glutamate is processed to produce ornithine in a 5-step enzymatic process requiring ArgBCDJ ([Charlier & Glansdorff, 2004](#)). These enzymes exist and are functional when expressed in *S. aureus* ([Jeong et al., 2022](#)), but are generally repressed by the global regulators SarA and SpoVG ([Reslane et al., 2024](#)), as well as by the arginine pathway-specific repressor AhrC ([Reslane et al., 2022](#)). As a result, neither proline nor arginine are synthesised from Glu ([Halsey et al., 2017](#)). Instead, an alternative pathway catalysed by RocD, ProC and PutA allows for the interconversion of Arg and Pro via the intermediate pyrroline-5-carboxylate (P5C) ([David E. Townsend et al., 1996](#)), which can also be utilised to generate glutamate via RocA ([Halsey et al., 2017](#); [Nuxoll et al., 2012](#)). It is believed that this pathway may have evolved as a means of preferably utilising collagen-derived proline for Arg biosynthesis and growth in relevant biological environments ([Reslane et al., 2024](#)) (as discussed in [page 71](#)).

In Gram-positive bacteria, the carbon catabolite repression protein CcpA responds to the availability of preferred carbon sources (e.g. glucose) by repressing pathways involved in the utilisation of alternative nutrients, such as amino acids, as a means of preserving energy ([Hueck & Hillen, 1995](#)). In *S. aureus*, the majority of enzymes involved in Pro/Arg metabolism are repressed by CcpA ([Nuxoll et al., 2012](#)). Crucially, suppression of RocD and RocF prohibit the synthesis of Pro from Arg, resulting in Pro auxotrophy when glucose is present ([Chunling Li et al., 2010](#)) ([Figure 15B](#)).

CcpA also inhibits Arg biosynthesis ([Figure 15B](#)), but Arg auxotrophy is robustly observed even in the absence of glucose ([Nuxoll et al., 2012](#)). Two major factors contribute toward this phenomenon. Firstly, the Arg biosynthesis pathway is specifically repressed by the widely distributed Arg-responsive transcriptional repressors ArgR (specifically ArgR2, part of the Arg Catabolic Mobile Element (ACME)) and AhrC in *S. aureus*, even in the absence of Arg ([Figure 15B](#)). Mutations in either element permit growth in Arg-limited conditions ([Reslane et al., 2022](#)). Secondly, the intracellular P5C pool is restricted by the constitutive activity of the Pro synthase ProC. Mutations in ProC, therefore, permit growth under Arg limitation by increasing the bioavailability of Arg precursors ([Jeong et al., 2022](#)). These observations point toward a model in which Arg biosynthesis in *S. aureus* is stringently and intentionally repressed under most physiological conditions, but the reasons for this remain unclear. In a recent study, *Freiburg* and colleagues demonstrate that Arg is limited within mature *S. aureus* biofilm, and this Arg limitation drives antibiotic tolerance via activation of the starvation-associated stringent signalling response ([Freiberg et al., 2024](#)). Hence – similarly to glutamate – Arg availability may act as an environmental stimulus facilitating the coordinated expression of virulence-associated traits in an infection context.

Valine

In *S. aureus*, the branched chain amino acids (BCAAs; Ile, Leu and Val) are each synthesised from Thr or pyruvate precursors via a complex and resource-intensive multistep enzymatic process. Given the relative inefficiency of these pathways, *S. aureus* preferably utilises exogenous BCAAs for protein synthesis and only expresses BCAA biosynthetic genes under nutrient limitation ([J. C. Kaiser et al., 2016](#); [Pohl et al., 2009](#)). The vast majority of enzymes required for BCAA biosynthesis are encoded by the *Ilv/Leu* operon with the only exception being *IlvE*; an aminotransferase which catalyses the final biosynthetic step for all three BCAAs ([J. C. Kaiser et al., 2018](#)). The orthologous enzyme from *Staphylococcus carnosus* has also been demonstrated to carry out the reverse reaction, catalysing the partial catabolism of BCAAs in a reaction yielding glutamate ([Madsen et al., 2002](#)). Hence, whilst the expression of the *Ilv/Leu* operon is stringently repressed in response to BCAA availability, *IlvE* is differentially regulated ([Lei et al., 2015](#)) owing, presumably, to the bifunctional nature of this enzyme.

Expression of the *Ilv/Leu* operon is generally repressed by the essential transcriptional regulators YeaZ and Gcp ([Lei et al., 2015, 2012](#)), as well as by two BCAA-responsive mechanisms. Firstly, the global transcriptional repressor CodY is specifically activated by Ile (and, to a lesser extent, Val) binding ([Hainzl et al., 2023](#)), and by GTP during periods of high amino acid availability ([J. C. Kaiser et al., 2018](#); [Levdikov et al., 2006](#); [Pohl et al., 2009](#)). Hence, when exogenous Ile is

available, Ilv/Leu is repressed by CodY (Figure 15C). Additionally, a cis acting BCAA-sensing regulator (comprised of a short Leu-rich peptide ORF and terminator/anti-terminator element) sits immediately upstream of the operon and prevents transcription in conditions of high Leu availability ([J. C. Kaiser et al., 2018](#)) (Figure 15C). As a result, biosynthesis of Val is stringently repressed under conditions where either Leu or Ile are present, resulting in an auxotrophic phenotype.

Amino acid transporters

The importance of amino acid utilisation in *S. aureus* is reflected in the extensive arsenal of amino acid transporter proteins possessed by this bacterium. Of the 362 predicted transporter proteins encoded by CA-MRSA strain USA300, at least 33 are predicted to have amino acid transport functions based on homology to characterised systems, and many more remain orphaned in regard to potential substrates (based on analysis by TransportDB2.0; [Elbourne et al., 2017](#)). Of these putative amino acid transporters, only a few have been functionally characterised in the existing literature. These include transporters for: Ala, Cys, Asp, Glu, Gln, BCAAs, Phe and Pro ([Zeden et al., 2021](#)). These proteins, their assigned functions and relevant mutant phenotypes are listed in Table 1.

Several studies demonstrate how amino acid utilisation in *S. aureus* is frequently redundant. Indeed, in their work characterising the alanine transporter CycA, [Gallagher](#) and colleagues demonstrate how alanine consumption occurs in glucose-rich media independently of CycA. Hence, *S. aureus* possesses at least one additional alanine transport system. Intriguingly, the authors also demonstrate that a *cycA* mutant strain possesses an altered cell wall architecture with decreased crosslinking, and this strain is thereby sensitized β -lactam antibiotics ([Gallagher et al., 2020](#)). Similarly, *S. aureus* encodes at least two transporters for Cys (or cystine) – TycP and TycABC ([Lensmire et al., 2020](#)). Even in the absence of these two systems, some growth is observed when 50 μ M Cys is supplied as the sole sulphur source, indicating the existence of at least one additional low-affinity transport system ([Lensmire et al., 2020](#)). Furthermore, the observation that a TycP/ABC double mutant is not significantly attenuated during infection strongly suggests the utilisation of alternative thiol sources present in biological environments, such as the tripeptide metabolite glutathione ([Lensmire et al., 2023](#)).

Redundancy among amino acid transporters is also apparent in regard to Pro uptake, for which *S. aureus* encodes at least two high-affinity transporters – PutP and ProT ([Lehman et al., 2023](#); [D. E. Townsend & Wilkinson, 1992](#)). Three additional transport systems were originally predicted to have proline specificity ([Schwan & Wetzel, 2016](#); [Wetzel et al., 2011](#)), but recent work has

demonstrated how these proteins do not meaningfully contribute to proline uptake in USA300 JE2 ([Lehman et al., 2023](#)). In spite of this, growth of a ProT/PutP double mutant is supported at millimolar proline concentrations, confirming the presence of additional transport systems for proline. This observed redundancy is proposed to contribute toward the utilisation of collagen-derived proline as a major carbon source during infection ([Lehman et al., 2023](#); [Urso et al., 2024](#)).

A number of recent studies highlight the importance of BCAA transporters in influencing *S. aureus* physiology. *S. aureus* encodes at least two high-affinity BCAA transporters – the Leu/Ile/Val transporter BrnQ1 and the Ile-specific system BrnQ2. BrnQ1 contributes toward virulence in a systemic infection model, while a BrnQ2 mutant is, paradoxically, hypervirulent ([J. C. Kaiser et al., 2016](#)). The observation that residual BCAA transport is still observed in a BrnQ1/2 mutant strain led to the identification of a third BCAA transporter – the Leu/Ile/Val permease BcaP ([J. C. Kaiser et al., 2016](#)). In *S. aureus*, BCAAs are precursors required for synthesis of branched-chain fatty acids, which are themselves major components of the cell membrane ([Frank et al., 2021](#)). Anteiso-fatty acids (that is, those derived from Ile) are particularly important for maintaining membrane fluidity under low temperatures ([Zhu et al., 2005](#)). In *S. aureus*, loss of the BCAA transporters (particularly BrnQ1) resulted in changes in membrane composition during growth in defined media, and these changes were associated with growth attenuation under low temperatures ([J. C. Kaiser et al., 2016](#)). These changes in membrane composition may contribute toward the altered virulence phenotypes associated with BCAA transporter mutants *in vivo*.

It is worth noting that *S. aureus* has been demonstrated to rapidly deplete Ser, Gly, Thr and Arg from the environment during growth in glucose-depleted media, and similarly deplete all remaining amino acids more gradually under this condition ([Halsey et al., 2017](#)). Hence, amino acid transporters for all 20 amino acids must exist in *S. aureus*. In some cases, transporter function has been predicted based on genomic context and homology to characterised systems from other organisms. For instance, USA300 possesses two arginine catabolic operons, and each of these encodes a predicted membrane protein with high sequence homology to the characterised *Pseudomonas aeruginosa* ornithine:arginine antiporter protein (ArcD) ([Bourdineaud et al., 1993](#); [Makhlin et al., 2007](#); [Shokrollahi et al., 2022](#)). Hence, whilst arginine transport activity has never been conclusively demonstrated for these proteins in *S. aureus*, their functions can be confidently assigned.

Table 1. Confirmed, predicted and putative amino acid transporters of *S. aureus* USA300

	Gene name	Accession	Known substrates	Biological relevance	Reference
Confirmed transporters	CycA	SAUSA300_1642	Ala	A cycA mutant demonstrates decreased cell wall crosslinking and increased susceptibility to β -lactams	(Gallagher et al., 2020)
	TcyP	SAUSA300_0382	Cys, Cystine	Involved in nutrient sulphur uptake. Contributed to fitness in a competitive systemic infection experiment	(Lensmire et al., 2020)
	TcyABC	SAUSA300_2357 - SAUSA300_2359			
	ProT	SAUSA300_0640	Pro	Either Pro transporter is required for virulence in murine SSTIs. Both transporters contribute to utilisation of collagen-derived Pro in airways. Pro transport protects <i>S. aureus</i> from osmotic stress. PutP was also identified as a fitness determinant in several systemic infection models.	(Coulter et al., 1998; Lehman et al., 2023; D. E. Townsend & Wilkinson, 1992; Urso et al., 2024)
	PutP	SAUSA300_0314			
	GltT	SAUSA300_2329	Asp	Seemingly contributes to general stress response to diverse stimuli, including heat and acetic acid stress.	(A. D. Potter et al., 2020; Yee et al., 2019)
	GltS	SAUSA300_2291	Glu	Loss of GltS alters central metabolism and affects the cellular c-di-AMP pool, resulting in biofilm formation	(Shibamura-Fujiogi et al., 2022; Merve S. Zeden et al., 2020)
	AlsT	SAUSA300_1252	Gln	Loss of Gln transport leads to reduction in the cellular c-di-AMP pool.	(Merve S. Zeden et al., 2020)
	BrnQ2	SAUSA300_0306	Ile	Contribute toward branched chain fatty acid synthesis, thereby	(J. C. Kaiser et al., 2018, 2016)
	BrnQ1	SAUSA300_0188	Ile, Leu, Val		

	BcaP	SAUSA300_2538		affecting membrane composition	
Predicted transporters (from literature)	PheP	SAUSA300_1231	Phe	Contributed to fitness and virulence in a <i>Drosophila melongaster</i> infection model	(Malcolm J. Horsburgh et al., 2004; Needham et al., 2004)
	OpuC	SAUSA300_2390 - SAUSA300_2393	Pro	Contribute to osmolyte stress response	(William R. Schwan & Wetzel, 2016; Wetzel et al., 2011)
	OpuD	SAUSA300_1245			
	ProP	SAUSA300_0558			
	ArcD1	SAUSA300_2568	Arg	N/A	(Bourdineau d et al., 1993)
	ArcD2	SAUSA300_0064			
Other putative amino acid transporters (from TransportDB)	SAUSA300_1613, SAUSA300_0914, SAUSA300_0566, SAUSA300_1329, SAUSA300_1628, SAUSA300_2383, SAUSA300_2385, SAUSA300_2395, SAUSA300_1300, SAUSA300_0021, SAUSA300_1307, SAUSA300_1441, SAUSA300_1638, SAUSA300_1883, SAUSA300_2035, SAUSA300_2145, SAUSA300_2549, SAUSA300_0706 - SAUSA300_0707				(Elbourne et al., 2017)

Peptide transport in *S. aureus*

When considering the role of amino acid utilisation in *S. aureus* physiology and infection, one must also consider oligopeptides as a potential reservoir for these amino acids in biological environments. Indeed, there is wealth of evidence to suggest that peptide transport can compensate for individual amino acid limitations in peptide-rich environments. For instance, the Pro transport-deficient *putP/proT* mutant strain is attenuated in defined media, but shows no phenotype in complex media containing peptides ([Lehman et al., 2023](#)). Similarly, the changes to membrane composition associated with Ile limitation in a *brnQ1* mutant strain are abolished in rich peptide-containing media ([J. C. Kaiser et al., 2016](#)). These examples provide clear evidence that *S. aureus* derives biologically important amino acids from exogenous peptide sources during growth.

In spite of this, the importance of peptide utilisation by *S. aureus* in biological environments is poorly explored. There are many reasons for this. Firstly, the composition of the host milieu is likely highly dynamic, niche-specific and difficult to define as a result of diverse host and bacterial factors contributing to these environments ([Urso et al., 2024](#); [Van Roy et al., 2024](#)). Sufficient analytical methods for determining the gross peptide composition of complex biological samples remains lacking (though such a feat would theoretically be possible through the use of untargeted mass spectrometry-based approaches – for example, MALDI-TOF) ([Tsybin et al., 2019](#)). Secondly, while amino acid transporters are generally highly specific to an individual target, general peptide transporter proteins must be highly promiscuous to facilitate the uptake of diverse substrates. As a result, these proteins are difficult to characterise in regard to their substrate preferences and, ultimately, their physiological roles. Additionally, almost nothing is known regarding how oligopeptides are selectively catabolised following their internalisation, making it difficult to determine the full extent to which these molecules can be utilised for growth ([Carroll et al., 2014](#); [Torres et al., 2023](#)).

Overall, while the routes of amino acid utilisation can be clearly defined, peptide uptake and catabolism are convoluted by the substantial structural diversity observed for these molecules. It is for this reason that attempts to characterise the routes of peptide uptake in *S. aureus* have, so far, utilised limited panels of representative peptide substrates ([Hiron et al., 2007](#); [Lehman et al., 2019](#)). More holistic approaches are needed to better understand how peptides are utilised by *S. aureus* and how these nutrients contribute to growth and infection.

To date, there are two known promiscuous peptide transport systems which are widely distributed in Staphylococci: the ABC importer-type system Opp3ABCDF (or Opp3) and the di/tri-

peptide POT transporter DtpT ([D. Yu et al., 2014](#)). The presence of at least one additional peptide transporter system is predicted, based on the observation that a *dtpT/opp3* double-mutant strain maintains the ability to utilise the peptides Pro-Arg and Ser-Pro-Arg for growth under Arg-limitation ([Lehman et al., 2019](#)). In addition to the aforementioned systems, different *S. aureus* strains can possess up to four additional Opp3-homologues. Of these, Opp1 and Opp2 have been demonstrated to transport nickel and cobalt or exclusively nickel, respectively ([Hebbeln & Eitinger, 2004](#); [Hiron et al., 2010](#); [Remy et al., 2013](#)). However, Opp4 plus one additional Opp system – ACME Opp, encoded as part of the widely disseminated arginine catabolic mobile element – are mostly uncharacterised and could function as additional peptide transporters ([D. Yu et al., 2014](#)). *S. aureus* also possesses an ABC importer system which is specific for the biologically prevalent tripeptide glutathione (GisABCD, or Gis) ([Lensmire et al., 2023](#)). Here, I will outline what is currently known about the peptide transporters of *S. aureus*, as well as highlighting the current gaps in our understanding of these systems.

Oligopeptide permease 3 (Opp3ABCDF/Opp3)

The *opp3BCDFA* operon (SAUSA300_0887 - 0891) of *S. aureus* encodes an archetypal type 1 ABC importer system comprising of two intracellular NBD proteins (Opp3D/F), two TMD proteins (Opp3B/C) and an extracellular lipid-anchored SBP (Opp3A) ([Hiron et al., 2007](#); [D. Yu et al., 2014](#)). A high-confidence (pTM > 0.8) projected structure of the complete Opp3 transporter (generated in AlphaFold 3) is shown in [Figure 16A](#). Herein, we observe the canonical ABC importer architecture, similarly observed in recent cryoEM structures of the *M. tuberculosis* Opp complex ([Yang et al., 2024](#)). In this projection, the cluster C SBP Opp3A is comprised of three distinct domains (two functional domains, and a central hinge domain) and is docked on the extracellular surface of the translocon through extensive contacts with Opp3B ([Figure 16A-B](#)). Both the NBDs and TMDs demonstrate the expected inward-open conformation. Unlike the Mtb Opp system, the projected NBD structures lacks the requisite Cys residues required for formation of an iron-sulphur cluster, which was previously suggested to be a conserved feature of Opp transport systems ([Yang et al., 2024](#)).

In the first major study examining peptide transporter function in *S. aureus*, *Hiron* and colleagues identified Opp3 and DtpT exclusively as the major oligopeptide transport systems of this bacterium. Using growth experiments in glutamate-depleted media, the authors demonstrated Opp3-specific utilisation of 10 Glu-containing peptides, ranging from tripeptides (PHE) to a nonapeptide (KTIPIEKVL) ([Hiron et al., 2007](#)). These findings were later corroborated by *Lehman* and colleagues, who identified an essential role for Opp3 in the utilisation of tri- (PRE) to octapeptides (SSSPRESS) ([Lehman et al., 2019](#)). Both studies failed to observe any utilisation for oligopeptides larger than n=9 residues, suggesting that this value represents the maximum substrate size for Opp3 ([Hiron et al., 2007](#); [Lehman et al., 2019](#)). A substrate profile of this kind is expected for Opp3, given the close phylogenetic association between Opp3A and other oligopeptide binding SBPs known to recognise similarly large peptides (e.g OppA from *Listeria monocytogenes* and PrgZ from *Enterococcus faecalis*) ([Figure 16C](#)).

To date, Opp3 is the best characterised of the *S. aureus* peptide transporter systems in regard to function in relevant biological environments. Early reports demonstrated that the expression of Opp3 varied in response to amino acid availability ([Hiron et al., 2007](#)). Such a phenomenon may be explained by the fact that Opp3 expression is repressed by CodY under nutrient-rich conditions ([Geiger et al., 2012](#); [Majerczyk et al., 2010](#); [Pohl et al., 2009](#)). In contrast, expression of the operon dramatically increases under nutrient-limitation, as encountered in the airways or in skin-like environments ([F. G. Costa et al., 2024](#); [Krismer et al., 2014](#)). Existing studies

demonstrate that the presence of Opp3 is required for robust growth in protein-rich environments (i.e. milk) and contributes toward the utilisation of degraded collagen ([Borezée-Durant et al., 2009](#); [Lehman et al., 2019](#)). Based on this evidence it has been proposed that Opp3 is the predominant transport system implicated in peptide uptake in most relevant physiological contexts ([Lehman et al., 2019](#)). Furthermore, during growth in peptide-rich environments, Opp3-mediated peptide uptake triggers upregulation of the secreted proteases SspA, SspB and *aur* through a yet-uncharacterised signalling pathway ([Borezée-Durant et al., 2009](#); [Lehman et al., 2019](#)). This response is believed to form a simple sensory circuit in which protease production is coupled to the availability of peptides nutrients ([Borezée-Durant et al., 2009](#)). Overall, experimental evidence points toward Opp3 playing an important role in peptide acquisition and protease regulation in proteinaceous biological environments.

Though peptide transport systems are often noted for their broad substrate promiscuity, studies of bacterial ABC peptide importers have previously identified specific amino acid preferences in these transporters, imparting distinct physiological roles in their respective host organisms ([Berntsson et al., 2011](#); [Klepsch et al., 2011](#)). Comparatively, very little is known regarding the substrate preferences of Opp3, owing to the limited panel of substrates identified for this system to-date. It is also worth noting that homologous Opp systems in other bacteria have been attributed to diverse functions beyond nutrient acquisition, such as cell wall recycling and AMP resistance (as discussed in [page 26](#)), but these functions have not yet been observed for the *S. aureus* transporter.

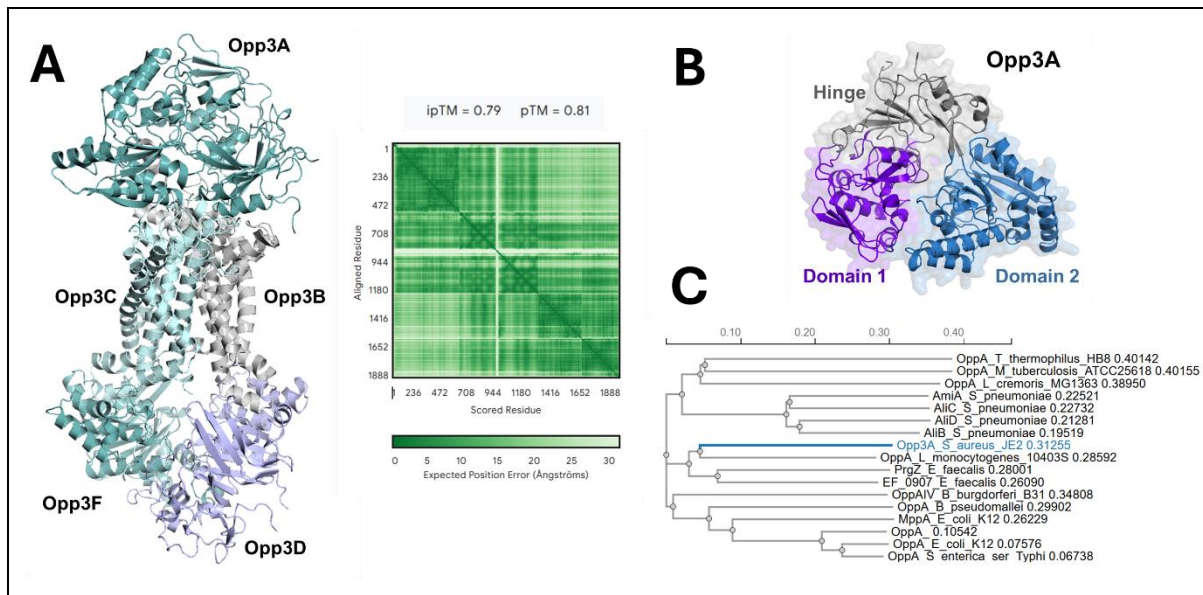


Figure 16 – Predicted structure of the *S. aureus* ABC peptide transporter Opp3. (A) AlphaFold 3 predicts that Opp3ABCDF forms a pentameric complex structure with an archetypal type 1 ABC importer architecture, as viewed from the membrane plane (left). A pTM > 0.8 suggests a high degree of confidence in this projected conformation (right). Areas of low confidence corresponding to disordered signal peptides have been occluded from the presented structure. **(B)** Opp3A is a canonical cluster C peptide-binding SBP, comprised of three clearly distinct domain (as labelled). **(C)** The Opp3A protein sequence from *S. aureus* USA300 was aligned against structurally characterised oligopeptide-binding SBPs and used to construct a simple phylogenetic tree (ClustalOMEGA). The scale bar indicates substitution rate/bp.

Di/tripeptide permease (DtpT)

In *S. aureus*, the *ntpT* gene (SAUSA300_0712) encodes a roughly 55 kDa POT transporter protein with ~87% sequence identity to the recently characterised PepT_{Sh} from *S. hominis* (Minhas et al., 2018). A very high confidence ($\text{pTM} \geq 0.95$) predicted structure of DtpT (generated in AlphaFold 3) is shown in Figure 17. Here, we observe the well-established core MFS fold common to all structurally characterised POTs, comprising of separate N- and C-terminal functional domains flanking a central substrate binding-groove. In this projected structure, DtpT sits in the inward-open resting conformation, as previously observed in many *apo* POT structures. Also visible in this structure are the two additional TM helices conserved in diverse bacterial POTs, which connect the two functional domains (Newstead, 2015).

Notably, all residues implicated in substrate recognition and transport are conserved between DtpT and PepT_{Sh} , providing compelling evidence that these two proteins are structurally and functionally homologous. Hence, the *S. aureus* protein likely transports the human peptide-conjugate metabolite S-Cys-Gly-3M3SH, as previously observed in PepT_{Sh} (Minhas et al., 2018). However, this bacterium lacks the C-S lyase enzyme PatB required for the turnover of this molecule and, therefore, does not contribute to the formation of thioalcohol-based body odour (Rudden et al., 2020). Further sequence comparison between DtpT and other structurally characterised POTs demonstrates how the conserved N- and C-terminal clamping residues first identified in PepT_{St} are also conserved in the *S. aureus* protein, as expected (Figure 18) (Lyons et al., 2014; Solcan et al., 2012). In comparison, several other residues implicated in substrate recognition (i.e. those comprising the four previously identified binding pockets responsible for side-chain accommodation, see page 45) are far more variable among the POTs included here.

In their initial report, Hiron and colleagues demonstrated that DtpT is required for transport of Glu-Glu, Val-Glu and Pro-Gln dipeptides in *S. aureus*, as well as Lys-Glu-Gly and Gly-Gly-Gln tripeptides. Intriguingly, utilisation of a Ser-Glu-Gly tripeptide was facilitated by either Opp3 or DtpT (Hiron et al., 2007). Based on this, it was proposed that DtpT exclusively is responsible for dipeptide uptake in *S. aureus*, whilst tripeptides may be substrates for either peptide transport system. The same authors also demonstrated that an *opp3A/ntpT* double mutant strain was attenuated for growth in milk after 24 hours, as compared to single mutants of either transport system. Hence, it was proposed that peptide utilisation via DtpT may be able to compensate for the loss of Opp3 during prolonged growth in peptide-rich environments (Borezée-Durant et al., 2009). Conversely, Lehman and colleagues failed to observe any phenotypic difference in a *ntpT* mutant strain relative to the wild type in regard to growth supported by collagenase-degraded

collagen ([Lehman et al., 2019](#)). Hence, Opp3 is likely to be the dominant peptide transport system under the conditions tested in this study. Beyond these examples, very little is known regarding the specific transport targets of DtpT in *S. aureus*.

Given their close functional association, one might expect that similar regulatory pathways control the expression of both DtpT and Opp3. In the model Gram-positive organism *Bacillus subtilis*, expression of genes orthologous to *dtpT* and *opp3* are both regulated by a common mechanism dependant on CodY ([Belitsky et al., 2015](#)). A recent study utilising chromatin immunoprecipitation has similarly demonstrated the presence of a conserved CodY binding site immediately upstream of *dtpT* in *S. aureus*, suggesting that *dtpT* is part of the CodY regulon ([Gao et al., 2023](#)). However, CodY-deficient mutant strains of *S. aureus* do not demonstrate a marked change in *dtpT* expression, as is observed for *opp3* ([Gao et al., 2023](#); [Geiger et al., 2012](#); [Majerczyk et al., 2010](#); [Pohl et al., 2009](#)). Such an observation suggests that other yet-uncharacterised factors are involved in the regulation of *dtpT*, specifically.

DtpT is universally conserved in *S. aureus* and other representative Staphylococcal species, including coagulase-negative staphylococci commonly associated with the human skin microbiome ([Rudden et al., 2020](#)). The apparent evolutionary conservation of DtpT suggests that this transporter is fundamentally beneficial to bacterial survival. Consistent with this, a signature-tagged transposon library screen previously identified the *dtpT* locus as an important fitness determinant for *S. aureus* strain RN6390 in a murine burn wound model, though not in an abscess model ([Coulter et al., 1998](#)). These findings are consistent with the later observation that a *dtpT/opp3* double-mutant is not attenuated during abscess formation ([Lehman et al., 2019](#)), presumably due to the presence of glucose as an alternative major carbon source in this environment ([Vitko et al., 2016](#)). In the same study, the authors demonstrate that a *dtpT* mutant strain is strongly attenuated during systemic infection, with this strain being depleted in liver abscesses after 1-3 post-infection and unable to establish infection in a rabbit endocarditis model ([Coulter et al., 1998](#)). In spite of this, the phenotypes associated with *dtpT* mutant strains in peptide-rich environments *in vitro* are generally subtle, particularly when compared to equivalent mutants of Opp3 ([Borezée-Durant et al., 2009](#); [Lehman et al., 2019](#)). Overall, the mechanism by which DtpT contributes toward bacterial fitness during infection remains unclear.

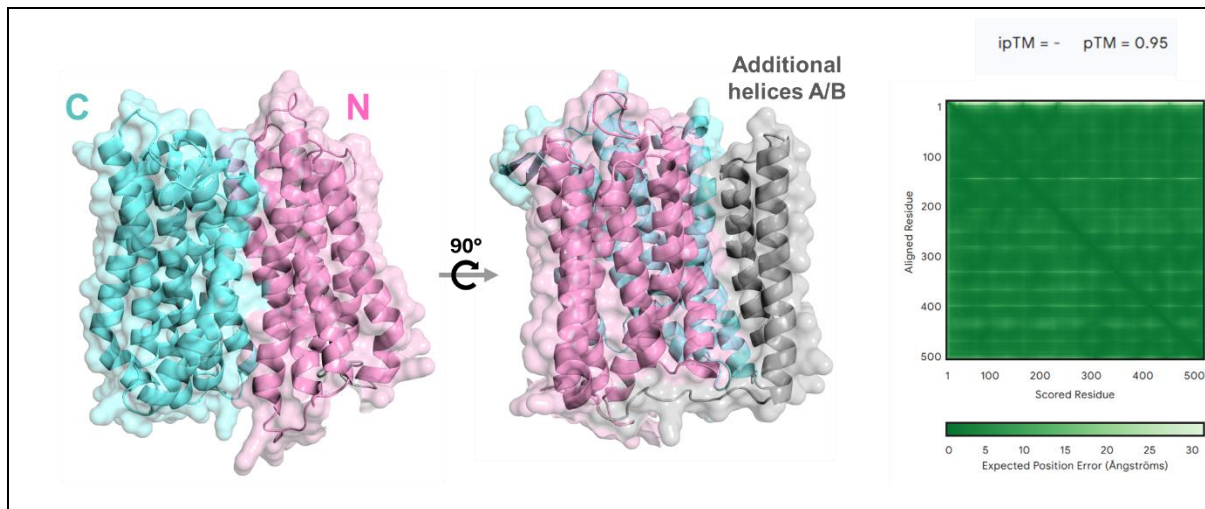


Figure 17 – Predicted structure of the *S. aureus* POT DtpT. (left and centre) AlphaFold 3 confidently predicts that DtpT adopts a canonical MFS transporter fold in which the N- and C-terminal 6-helical bundles (pink and blue, respectively) flank the central binding groove in an inward-open conformation, as viewed from the membrane plane. Two additional TM helices (TM A/B) are also apparent in this structure (grey), as previously observed in the homologous transporter from *S. hominis* (Minhas et al., 2018). (right) A pTM value of 0.95 demonstrates an exceptionally high degree of confidence in the projected structure. The area of low confidence corresponding to the signal peptide has been occluded from the presented structure.

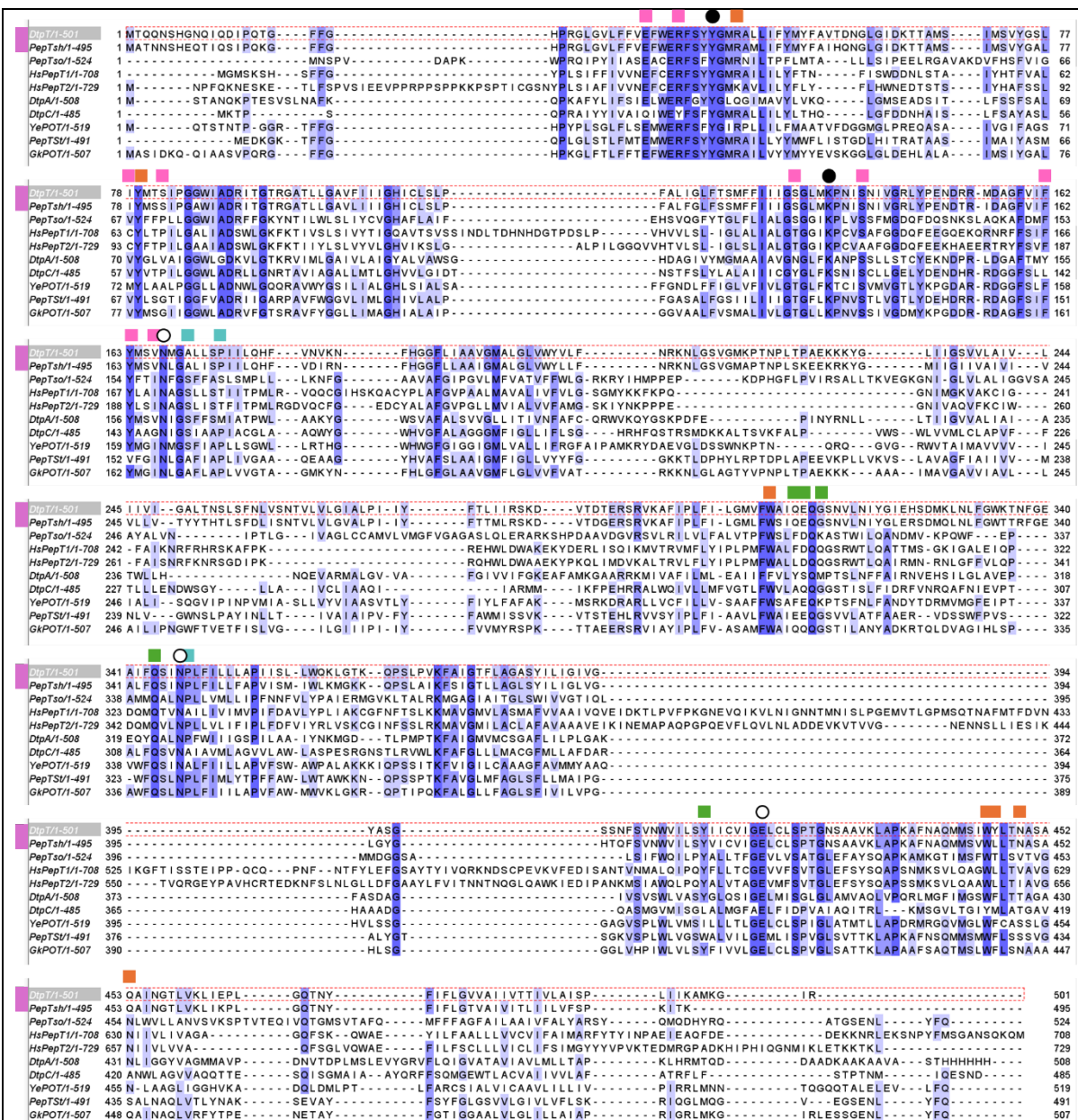


Figure 18 – Multiple sequence alignment of DtpT and structurally characterised POTs.

Multiple protein sequence alignment was carried out by Clustal Omega (Madeira et al., 2024) and visualised in Jalview (A. M. Waterhouse et al., 2009). The sequence of DtpT (from *S. aureus* JE2) is highlighted in the red dashed boundary. Sequences are given in FASTA format and coloured by identity (blue). The highly conserved N- and C-terminal sequence clamping residues are indicated above the aligned sequence by white and black circles, respectively. Coloured squares indicate residues comprising the substrate binding pockets P1 (Cyan), P2 (Orange), P3 (Pink) and P3' (Green) in *PepT_{sh}* (as describe in [page 45](#)).

Glutathione importer system (GisABCD/Gis)

Glutathione is a biologically important tripeptide metabolite with diverse roles in many organisms, primarily serving as an antioxidant in eukaryotes and some prokaryotes ([Lushchak, 2012](#)). In the reduced form (GSH), glutathione consists of a Cys-Gly dipeptide conjugated to a glutamate residue via an unconventional γ -peptide linkage between the glutamyl sidechain and cysteine amino terminus (**Figure 19A**). In the oxidised form of glutathione (GSSG), a disulphide bond links two individual GSH units, forming a non-linear hexapeptide (**Figure 19B**). In most biological contexts, GSH is the most abundant form of glutathione by a significant margin ([Hansen et al., 2009](#)).

GSH biosynthesis typically occurs via a two-step enzymatic process in which glutamate is first conjugated to cysteine (forming γ -glutamyl cysteine) before the addition of glycine via a GSH synthase ([Forman et al., 2009](#)). *S. aureus* lacks the enzymes required to synthesise GSH *de novo*, and there is no existing evidence to suggest that the bacterium utilises exogenous GSH as an antioxidant to resist oxidative stress, as was previously observed in some other Gram-positive pathogens ([Brouwer et al., 2022](#); [A. J. Potter et al., 2012](#)). In spite of this, *Lensmire* and colleagues have recently demonstrated the existence of a high-affinity ABC glutathione importer system (GisABCD, or Gis) in *S. aureus* which allows the bacterium to scavenge GSH and GSSG for use as nutrient sulphur sources ([Lensmire et al., 2023](#)). Notably, *S. aureus* also lacks the enzymatic capability to accumulate sulphur from inorganic sources (e.g. sulphate ions) and, as a result, organic thiols such as cysteine and GSH are believed to serve as essential sources of nutrient sulphur for the bacterium during infection ([Lensmire et al., 2020, 2023](#); [Lithgow et al., 2004](#)). In spite of this, a *gis* mutant strain of *S. aureus* is not attenuated during systemic infection in a murine model when compared to the wild type ([Lensmire et al., 2023](#)). Additionally, while Gis is required during growth in defined medium where GSH is present at low concentrations (< 50 μ M) as the sole sulphur source, growth of a *gis* mutant strain is restored at physiological concentrations of the peptide (> 500 μ M). Together, these observations point toward the existence of at least one additional low-affinity GSH transporter which is active during infection ([Lensmire et al., 2023](#)).

In *S. aureus*, the *gis* locus encodes a tetrameric ABC transporter comprised of the ATP binding cassette protein GisA (containing two predicted NBD domains), the TMD proteins GisB/C and the glutathione-binding SBP GisD. Unlike *opp3BCDFA* – in which all five transporter subunits are encoded as part of a single operon – the *gis* locus consists of two divergent coding sequences which separately encode *gisA* and the remaining subunits *gisBCD*, respectively ([Lensmire et al.,](#)

[2023](#)). The biological significance of this arrangement is unclear, though it is most likely that these divergent coding sequences are differentially regulated in order to stringently control expression and assembly of the complete functional transporter. Indeed, *gisA* – but not *gisBCD* – is repressed by the oxidation-sensing transcriptional regulator CymR, and derepression of this system requires direct thiolation of the protein ([Ji et al., 2012](#); [Soutourina et al., 2009](#)). Hence, it is possible that *gisBCD* is constitutively expressed, whilst *gisA* is specifically upregulated during periods of oxidative stress to rapidly enable GSH transport and increase intracellular thiol availability. Consistent with the proposed role of this transporter in nutrient sulphur acquisition, the *gisBCD* operon also encodes a γ -glutamyl transferase (*ggt*) immediately downstream of *gisD*, and this enzyme catalyses the first step in GSH catabolism. Given the close genetic association between these components, it has been proposed that *ggt* forms a complex with Gis *in vivo*, though experimental evidence for this model is still lacking ([Lensmire et al., 2023](#)).

A high-confidence (pTM > 0.8) predicted structure of the complete Gis complex (generated in AlphaFold 3) is shown in [Figure 19C](#). A few notable distinctions differentiate this predicted structure from the two previously solved peptide ABC importer structures for Opp and Dpp from *M. tuberculosis* ([Hu et al., 2024](#); [Yang et al., 2024](#)). Most notably, six additional short helices at the N-terminal end of GisB are projected to form an unconventional globular-like domain at the cytoplasmic interface of the complex ([Figure 19C, middle](#)). The function of this domain is not immediately obvious, though its position at the intracellular face of the translocon suggests that this region may be implicated in binding *ggt* in the previously proposed Gis-*ggt* complex structure. However, AlphaFold was not able to predict the structure for such a complex with high confidence (*not shown*). In addition, compared to the existing structures, the SBP component GisD occupies an unusual asymmetric position in this proposed structure, and contacts between the SBP and the translocon are overall less extensive (particularly at the interface with GisC). Such a conformation may be influenced by the existence of two additional TM helices on GisC, causing this subunit to extend into the membrane plane ([Figure 19C](#)). Again, the biological significance of these distinctions remain unclear.

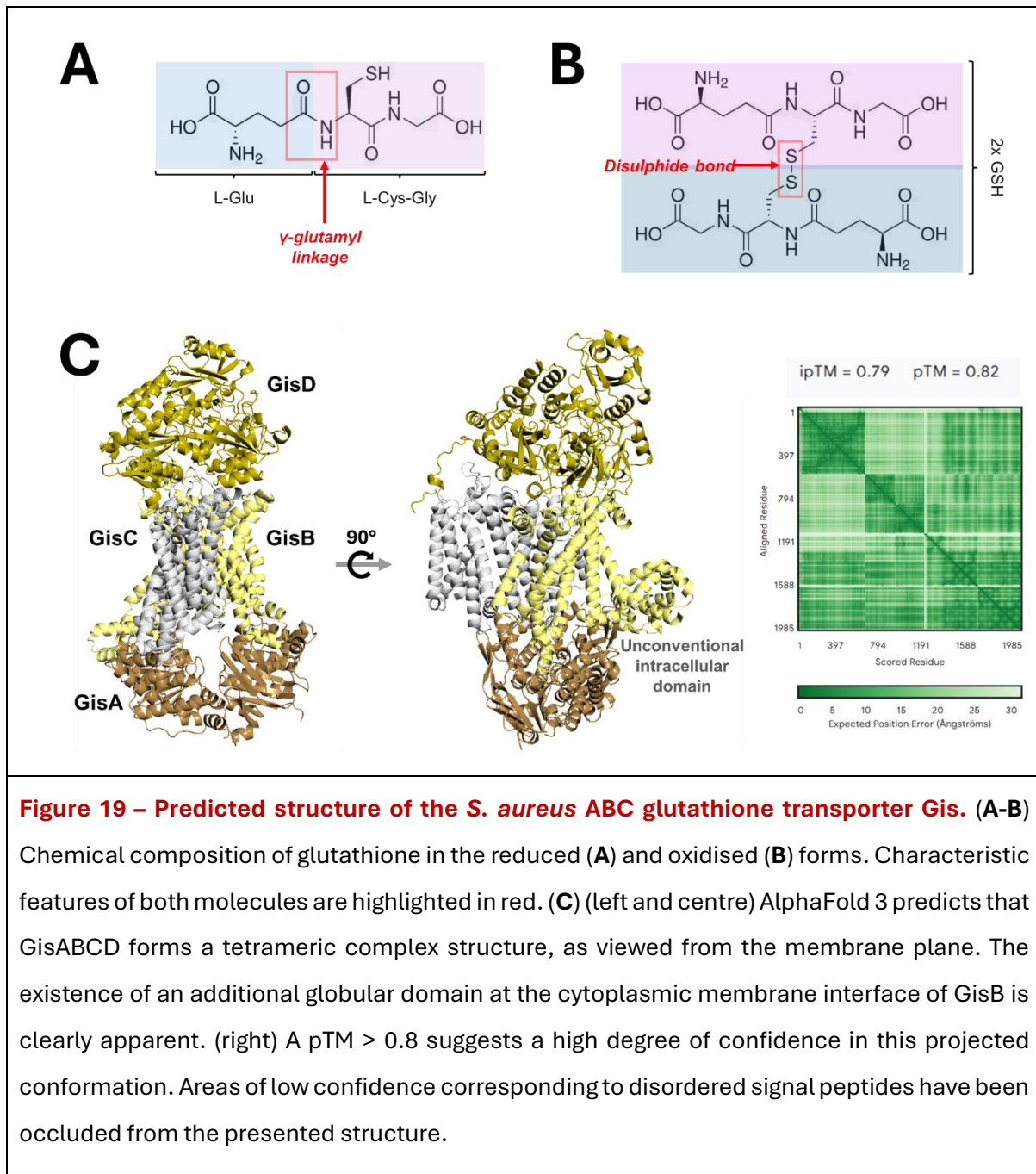


Figure 19 – Predicted structure of the *S. aureus* ABC glutathione transporter Gis. (A-B) Chemical composition of glutathione in the reduced (**A**) and oxidised (**B**) forms. Characteristic features of both molecules are highlighted in red. (**C**) (left and centre) AlphaFold 3 predicts that GisABCD forms a tetrameric complex structure, as viewed from the membrane plane. The existence of an additional globular domain at the cytoplasmic membrane interface of GisB is clearly apparent. (right) A pTM > 0.8 suggests a high degree of confidence in this projected conformation. Areas of low confidence corresponding to disordered signal peptides have been occluded from the presented structure.

Aims of this study

Understanding the physiological factors contributing to the success of *S. aureus* as a bacterial pathogen is essential for identifying novel targets for therapeutic intervention. Highly virulent CA-MRSA strains associated with significant morbidity are known to be highly proteolytic ([Min Li et al., 2009](#)), and a number of recent studies provide compelling evidence that proteolytic products serve as essential nutrients for the bacterium during infection ([Lehman et al., 2019](#); [Urso et al., 2024](#)). Whilst the roles of individual amino acids (and their associated transporters) in these infection environments are gradually becoming more apparent, the importance of oligopeptides as nutrients in relevant biological contexts remains cryptic ([Lehman et al., 2019](#)). Improved understanding of the routes of peptide uptake may provide novel insights into the roles of these molecules during infection.

Existing studies comparing the roles of DtpT and Opp3 in *S. aureus* have utilised a limited panel of peptide substrates as representatives for this diverse class of molecules ([Hiron et al., 2007](#); [Lehman et al., 2019](#)). These studies have also relied exclusively on phenotypic methods to study peptide transport in this bacterium, while other recent examples demonstrate the power of biochemical and structural approaches in understanding peptide transporter function ([Kotov et al., 2023](#)).

Figure 20 provides an overview of our existing knowledge of peptide transporters and their functional targets in *S. aureus*. Here, I aim to apply a multidisciplinary approach to better understand the distinct roles of these peptide transport systems in *S. aureus*, focussing specifically on the often-neglected DtpT. Existing phenotypic assays for assessing peptide transporter function will be combined with high-throughput phenotypic screens and biochemical methods to shed new light on the specific transport targets and substrate preferences of DtpT. In doing so, I aim to shed new light on the biological functions of DtpT and provide the first clues toward the role of this transporter in infection. Finally, I aim to determine the yet-unidentified additional glutathione transporter(s) of *S. aureus* which supports GSH-utilisation in the absence of Gis (see **page 95**), after which I will utilise microbial growth experiments and *in vitro* infection experiments to unpick the roles of glutathione transport in *S. aureus* physiology.

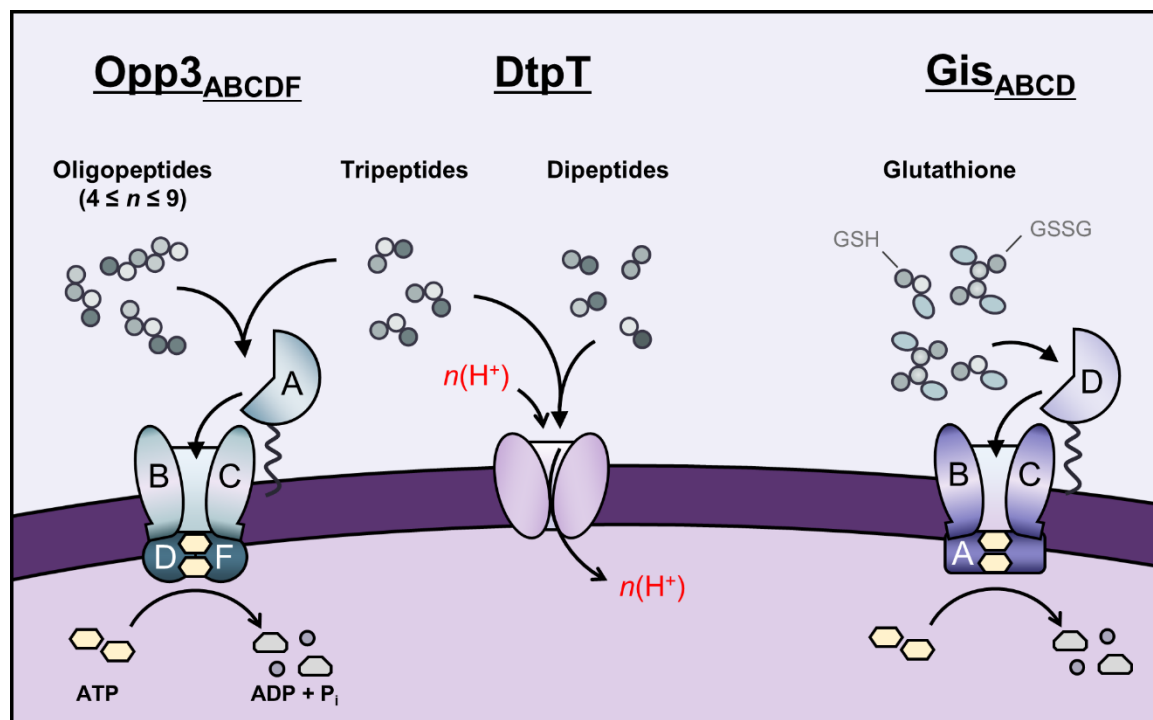


Figure 20 – Diagrammatic summary of existing functions assigned to the major peptide transporters of *S. aureus*.

Chapter 2 – Materials and Methods

1. Materials

Unless otherwise stated, materials utilised in this work were purchased from Sigma Aldrich (Sigma). All materials utilised in this work were reagent-grade products intended for laboratory use.

Some chemicals utilised in this work – namely: antibiotics, peptides (including glutathione), arabinose, Isopropyl β -D-1-thiogalactopyranoside (IPTG; Melford) and anhydrotetracycline (ATc; Cambridge Bioscience) – were prepared as stock solutions at 100x (peptides; gentamicin) or 1000x (all other antibiotics; arabinose, IPTG and ATc) working concentration in water or ethanol, according to solubility. Stocks were filter-sterilised and stored at -20°C for up to 1 year before use.

Peptides

A full list of peptides utilised in this work, along with providers and storage conditions, is provided in [Table 2](#). All peptides were stored as powders and used routinely as aqueous stock solutions, as described above.

Table 2. Di/tripeptides used in this work

Peptide	Provider	Storage
Ala-Ala	Sigma Aldrich	-20
Ala-Ala-Ala	Sigma Aldrich	-20
Ala-Gln	Fluorochem	RT
Ala-Gly	Sigma Aldrich	-20
Ala-Leu	Sigma Aldrich	-20
Ala-Phe	Sigma Aldrich	-20
Arg-Ala	Bachem	-20
Arg-Asp	Santa Cruz Biotechnolog y	-20
Arg-Phe	ALFA Chemistry	-20
Cys-Gly	Bachem	-20
D-Ala-D-Ala	Fluorochem	RT
Gly-Asp	Sigma Aldrich	-20

Peptide	Provider	Storage
Gly-Glu	Sigma Aldrich	-20
Gly-Gly	Sigma Aldrich	-20
Gly-Gly-Gly	Sigma Aldrich	-20
Gly-His	Sigma Aldrich	-20
Gly-Phe	Sigma Aldrich	-20
Gly-Pro	Activate Scientific	-20
Leu-Ala	Sigma Aldrich	-20
Leu-Gly-Gly	Sigma Aldrich	-20
Met-Gly	Fluorochem	-20
Phe-Ala	Sigma Aldrich	-20
Val-Tyr	Sigma Aldrich	-20

2. Microbiological techniques and microbial assays

Bacterial growth conditions

All *S. aureus* strains used in this study were routinely cultured in tryptic soy broth (TSB; VWR) with shaking or on solid tryptic soy agar (TSA; 1.25% agar) medium at 37°C. Selective antibiotics were added during growth when appropriate at the following concentrations: 10 µg/ml erythromycin (*erm*), 10 µg/ml chloramphenicol (*cam*; Melford).

E. coli strains were routinely cultured in lysogeny broth (LB; 10 g/L tryptone; 5 g/L yeast extract; 10 g/L NaCl) with shaking or on LB agar (1.5%) at 37°C. Selective antibiotics were added during growth when appropriate at the following concentrations: 50 µg/ml kanamycin (*kan*; SLS), 100 µg/ml ampicillin (*amp*; Melford).

A full list of bacterial strains and plasmids utilised in this study are available in [Appendices 2-4](#).

Phenotype microarrays and analysis

Peptide utilisation by *S. aureus* strain JE2 and mutant derivatives was assessed on phenotype microarray plates PM 6-8 (Biolog) using a modified version of the manufacturer's protocol. Cells grown on TSA overnight were transferred from solid media into 3 ml IF-0α buffer using a sterile cotton swab and pelleted by centrifugation at 4000 x *g* for 5 minutes. Cells were washed twice and resuspended in IF-0α. This cell suspension was then diluted to a final OD₆₀₀ of approximately 0.004 in 9.975 ml IF-0α and supplemented with 110 µl redox dye H (Biolog) and 915 µl 12X PM additive solution (240 mM tricarballic acid, pH 7.1; 24 mM MgCl₂; 12 mM CaCl₂; 150 µM L-cystine, pH 8.5; 0.06% w/v yeast extract; 0.06% v/v tween 80; 6 mM D-glucose; 12 mM pyruvate). PM plates were inoculated with 100 µl/well of the prepared cell suspension and incubated for 36 hours at 37°C with continuous slow shaking in an Epoch2 microplate spectrophotometer (Agilent). OD₅₉₀ and OD₇₅₀ were measured every 15 minutes.

In preliminary experiments utilising the manufacturer's protocol, the recommended inoculum composition produced a strong metabolic signal in all tested conditions, including the negative control. Hence, the initial OD₆₀₀ and D-glucose/pyruvate concentrations were adjusted through a series of optimisation experiments, until the above conditions were determined to provide a clear phenotypic distinction between the positive and negative control wells of each experiment.

Signal curves (OD₅₉₀ - OD₇₅₀ against time) for all four strains were grouped for each peptide and then analysed by Uniform Manifold Approximation and Projection (UMAP) using the UMAP R

library and a random seed of 888 ([McInnes et al., 2018](#)). K-means clustering was used on the same grouped data with "kmeans" R function and k=7. Empirical area under the curve (eAUC) was calculated using Growthcurver ([Sprouffs & Wagner, 2016](#)).

Arg-peptide utilisation assays

Utilisation of specific peptides was assessed using glucose-supplemented chemically defined medium (CDMG) based on a previously reported methodology ([Hussain et al., 1991](#)). The complete composition of the CDMG used here is provided in [Appendix 5](#). For assessing the utilisation of arginine-containing peptides, L-arginine was omitted from the medium (CDMG - R). Overnight cultures of *S. aureus* strain JE2 and mutant derivatives were diluted 1:100 into fresh TSB and grown to mid-exponential phase. Cells were then pelleted by centrifugation at 4000 x g for 5 minutes and washed twice in CDMG - R before being resuspended and diluted to a final OD₆₀₀ of 0.05 in peptide-supplemented medium. 200 µl of each cell suspension was transferred to a Costar 96 well plate (Corning) and incubated for 24 hours at 37°C with shaking. OD₆₀₀ was measured every 30 minutes in an Epoch2 microplate spectrophotometer.

For assessing the ability of Ala-Phe to inhibit DtpT-mediated Arg-peptide utilisation, growth assays were repeated as described for JE2 in the presence of Ala-Phe at various concentrations.

Glutathione utilisation assays

Utilisation of glutathione by JE2 and mutant derivatives was assessed based on methods previously developed for assessing the utilisation of Arg-containing peptides. CDMG was modified by the omission of L-cysteine and L-methionine, and (NH₄)₂SO₄·FeSO₄·6H₂O was substituted for 15 µM iron (III) citrate to produce a chemically defined medium lacking sulphur (CDMG - s). Overnight cultures of *S. aureus* strain JE2 and mutant derivatives were diluted 1:100 into fresh TSB and grown to mid-exponential phase. Cells were then pelleted by centrifugation at 4000 x g for 5 minutes and washed twice in CDMG - s before being resuspended and diluted to a final OD₆₀₀ of 0.05 in medium supplemented with reduced glutathione (FluoroChem) or oxidised glutathione (SLS). Reduced glutathione solutions were prepared under anaerobic conditions to prevent changes in the oxidation state before commencing assays. 200 µl of each cell suspension was transferred to a Costar 96 well plate (Corning) and incubated for 24 hours at 37°C with shaking. OD₆₀₀ was measured every 30 minutes in an Epoch2 microplate spectrophotometer.

For anaerobic growth assays, cultures were prepared as before being transferred to a Whitley A85 anaerobic workstation (Don Whitley Scientific) and diluted into assay medium which had

been equilibrated under anaerobic conditions for at least 3 hours and supplemented with 100 mM sodium nitrate. Growth was monitored as above in a Stratus 96 well plate reader (Cerillo) for 18 hours at 37°C without shaking.

Antibiotic inhibition curves

For assessing the effect of exogenous glutathione on antibiotic susceptibility, overnight cultures of *S. aureus* strain JE2 (and/or mutant-derivative strains) were each diluted 1:100 into fresh TSB and grown to mid-exponential phase. Cells were then diluted into TSB or TSB supplemented with GSH (0.1 mM, 1 mM or 10 mM) to a final OD₆₀₀ of 0.05. 200 µl of each culture was then transferred to a Costar 96 well plate. Plates were incubated for 1 h at 37°C with shaking and OD₆₀₀ was measured every 30 minutes in an Epoch2 microplate spectrophotometer. Antibiotics were then added at concentrations intended to be inhibitory, based on previous reports ([Vestergaard et al., 2016](#)). For assessing susceptibility to chloramphenicol, chloramphenicol was added to a final concentration of 100 µM, intended to be sub-MIC but still cause moderate growth inhibition (determined separately). Growth was then continued as before for a further 23 h and OD₆₀₀ was measured every 30 minutes.

Hydrogen peroxide survival assay

Hydrogen peroxide susceptibility was assessed after GSH pre-treatment by adapting a previously reported methodology ([Podkowik et al., 2024](#)). Overnight cultures of JE2 or mutant derivatives were diluted 1:100 in TSB and grown for 1 h at 37°C. At this time-point, cultures were split into separate flasks and supplemented with either GSH (1 mM or 10 mM) or water only, before further incubation for 1 h. Cells were then transferred to ice, washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in PBS to a final OD₆₀₀ of 0.1. 20 mM H₂O₂ (VWR) was then added to GSH pre-treated and untreated cells, which were incubated as before for a further 1 h. Excess H₂O₂ was then neutralised by addition of 200 µg catalase followed by further incubation for 10 minutes. Cell survival was determined by serially diluting cell suspensions in PBS and spotting onto TSA before and after H₂O₂ treatment to determine colony-forming units (CFU).

3. Molecular biology techniques, cloning and strain generation

Plasmid purification

Plasmid isolation was routinely carried out using a commercially available Mini prep kit (QIAGEN) following manufacturer's protocols.

Purification of plasmids from S. aureus

For isolation of the low copy-number pSK5630 plasmid and derivative vectors from *S. aureus*, a commercially available Midi prep kit (QIAGEN) was used, following manufacturer's protocols.

For isolation of plasmids from *S. aureus*, Mini and Midi prep protocols were modified by the addition of the following lysis step:

Following resuspension of cells in buffer P1 (step **2**; see manufacturer's protocol), 50 µg recombinant lysostaphin (from *Staphylococcus simulans*) and 1 mg lysozyme (from egg white) were each added, and cell suspensions were then incubated at 37°C with shaking for 1 h (or until visible clearing of the suspension was observed) before proceeding with the remainder of the protocol.

Genomic DNA purification

Genomic DNA was routinely purified using the DNeasy Blood and Tissue DNA purification kit (QIAGEN) following manufacturer's protocols for Gram-negative bacteria.

Purification of genomic DNA from S. aureus

For purification of genomic DNA from *S. aureus*, the manufacturer's protocol was modified by the addition of the following lysis step:

Following resuspension in buffer ATL (pre-treatment step **2**; see manufacturer's protocol), 50 µg recombinant lysostaphin and 1 mg lysozyme were each added. Cells suspensions were then incubated at 37°C with shaking for 1 h (or until visible clearing of the suspension was observed) before proceeding with the remainder of the protocol.

Polymerase chain reaction (PCR)

All PCR reactions described here were carried out using the high-fidelity Phusion DNA polymerase (NEB; with associated 5x Phusion HF buffer) and commercially available 10 mM dNTPs mix (Thermo Fisher). PCR reactions utilised either the T100 (Bio-Rad) or Techne Prime

(VWR) thermo-cyclers. Standard conditions for PCR are listed in Table 3. Annealing temperatures were selected based on the predicted melting temperature associated with each primer pair. A full list of oligonucleotide primers utilised in this work is listed in Table 4. Extension times were generally selected as 30 s/kb of sequence + 15 s. Colony PCRs (see below) were carried out in 20 µl reactions, while all other PCR reactions were carried out in 50 µl.

Cloning PCR

For cloning, linearised inserts and vectors were both prepared by PCR amplification. For vector linearization, pure plasmid vectors were isolated from stocked strains for use as a template (conc ≥ 20 ng/µl). Genomic DNA was utilised as a template for the amplification of DtpT (and the associated promoter sequence) as well as other bacterial POTs (namely DtpA_{Ec}, DtpC_{Ec} and DtpE_{Kp}; as described in the text) (conc ≥ 50 ng/µl).

Diagnostic PCR in *S. aureus*

For preliminary confirmation of plasmid constructs in *S. aureus*, pure plasmids were isolated from successful transformants and used as a template (conc ≥ 20 ng/µl). Following PCR confirmation, all plasmids were verified by DNA sequencing of the insertion site with the same primers utilised here for diagnostic PCR (Eurofins Scientific). For confirmation of *S. aureus* chromosomal mutants, genomic DNA was isolated and utilised as a template for PCR (conc ≥ 50 ng/µl).

Colony PCR

For preliminary confirmation of cloned plasmid constructs in *E. coli*, single colonies were picked into dH₂O and incubated at 98°C for 10 minutes to produce a colony lysate. Lysates were clarified by centrifuging briefly and supernatants were utilised as a template for PCR. Following PCR confirmation, all plasmids were verified by DNA sequencing of the insertion site with the same primers utilised here for diagnostic PCR (Eurofins Scientific).

Agarose gel electrophoresis

All agarose gels were prepared to a final concentration of 1 % agarose in 1x TAE buffer (40 mM Tris HCl; 20 mM acetic acid; 1 mM ethylenediaminetetraacetic acid (EDTA)) and supplemented 1:10,000 with 10,000x SYBR Safe DNA gel stain (Thermo Fisher). Before loading, all samples were mixed with 6x DNA loading dye (NEB). All gels were run with the inclusion of a 1 kb Plus DNA ladder (NEB) for approximation of band sizes. Gels were routinely run for 25-35 minutes at 100 V in 1 x TAE buffer. Gels were visualised using the transilluminator function of a GeneGenius Gel Bioimaging Unit (Syngene) or using the iBright imaging system (Thermo Fisher).

Table 3. Standard conditions used for PCR in this work (per 50 μ l)

Component	Volume (μ l)	Step	Temp ($^{\circ}$ C)	Time (s)	
5x HF buffer	10	Initial denaturation	98	30	
dNTP mix (10 mM)	1	Denaturation	98	10	x 32
Phusion polymerase	0.5	Annealing	<i>variable</i>	30	
Forward primer (10 mM)	2.5	Extension	72	<i>variable</i>	
Reverse primer (10 mM)	2.5	Final extension	72	300	
Template DNA	2	Hold	4	Hold	
DMSO*	1.5				
dH ₂ O	+ to 50 μ l				

* Dimethyl sulfoxide (DMSO) was added to all PCR reactions utilising genomic DNA as a template.

Table 4. Oligonucleotide primers utilised in this work.

#	Target	Sequence (5' > 3')	function	Sources
1	<i>dtpT</i> flanking regions	GGGCAAGTTCACACCACG	diagnostic	
2		GTTAACCGTGCCATCACCG	PCR	
3	<i>opp3A</i> flanking regions	CCTTAGCTCCTGAAAATCTCGTCG	diagnostic	
4		GCGGATTGAACGTTGCGAAG	PCR	
5	<i>gisD</i> flanking regions	GGTGTGGTATTATGTTCTCGTCG	diagnostic	
6		TGGTTCTAACACATTCAATGCC	PCR	
7	SAUSA300_0060 flanking regions	CAAGGTATTGGAAGGAAGTGG	diagnostic	
8		TAGGGGACGCTTTAGACG	PCR	
9	<i>Bursa aurealis</i> Tn	GCTTTTTCTAAATGTTTTTAAGTAAATCAAGTAC	diagnostic PCR	Fey et al., 2013
10	pWaldo	GAAGCAGCCCAGTAGTAGG	diagnostic	
11		GTGTTGGCCATGGAACAGG	PCR / sequencing	
12	pSK5630	CCTTAGCTCCTGAAAATCTCGTCG	diagnostic	
13		GCGGATTGAACGTTGCGAAG	PCR / sequencing	
14	pRab11	TTAAACCTTCGATTCCGACC	diagnostic	
15		CTTGTCTGTAAGCGGATGC	PCR / sequencing	
16	pBADcLIC	ATGCCATAGCATTTTTATCC	diagnostic	
17		TCTGATTTAATCTGTATCAGG	PCR / sequencing	
18	pWaldo	GTCGAGTCTCCTTCTTAAAGTTAAAC	vector	
19	cloning site	GAAAACCTGTACTTCCAGGGTCA	linearisation	
20	pSK5630	GATCCGTCGACCTGCAGC	vector	
21	cloning site	CCCTGGCAGTTTATGGCGG	linearisation	
22	pBADcLIC	CATGGTTAATTCCTCCTGTTAGC	vector	
23	cloning site	CAAGGTCATCATCACCATCATC	linearisation	
24	<i>dtpT</i>	(TAACCTTTAAGAAGGAGACTCG) ACATGACACAACAAAACCTCCCA	Cloning into pWaldo*	
25		(CCTGGAAGTACAGGTTTTTC) ACGTATACCTTTCATCGCTTTGA		
26	<i>dtpA</i>	(TAACCTTTAAGAAGGAGACTCAAA)		

		GTGTCCACTGCAAACCA		
27		(CCTGGAAGTACAGGTTTTC) CGCTACGGCTGCTTTTCG		
28	<i>ctpC</i>	(AACTTTAAGAAGGAGACTC) ATGAAAACACCCTCACAGCCG		
29		(CCTGGAAGTACAGGTTTTC) ATCGTTGCTCTCCTGTATCATA		
30	<i>ctpE</i>	(ACTTTAAGAAGGAGACTCGA) ATGCATTCCTCTGTTAATAAAAACG		
31		(CCTGGAAGTACAGGTTTTC) GGCGTTGCTGACGGATT		
32	<i>ctpT</i>	(CCGCCATAAACTGCCAGGG) ATTAACGTATACCTTTCATCGC	cloning into pSK5630*	
33	<i>ctpT</i> promotor region	(GCTGCAGGTCGACGGATC) GGGTCAAATTGGTGTTACTT		
34		(CCGCCATAAACTGCCAGGG) CATGTATACATCCCATCCTTTC		
35	<i>ctpT</i>	(TATCATTGATAGAGTATG) ATGACACAACAAAACCTCCC	Cloning into pRab11*	
36		(TTCGAGCTCAGATCTGTT) TTAACGTATACCTTTCATCGC		
37	<i>ctpT</i>	(CTAACAGGAGGAATTAACCAT) ATGACACAACAAAACCTCCCATGGA	Cloning into pBADcLIC*	
38		(GATGATGGTGATGATGACCTT) ACGTATACCTTTCATCGCTTTGA		
39	<i>ctpT</i>	CGTACCAATTGCAAATTTTACTGG	diagnostic PCR	
40	<i>ctpT</i>	CTGGGCTATTgcaGAACAAGGGTC	Q310A mutation**	
41		CTGGGCTATTgaaGAACAAGGGTC	Q310E mutation**	
42		AACACCATTCCAAGAATAAATAATG	Q310 reverse	
43		GTTTAGTTATtttGGCATGCGTG	Y41F mutation**	
44		GTTTAGTTATgctGGCATGCGTGC	Y41A mutation**	
45		CTTCCCAGAACTCTACAAAG	Y41 reverse	

46		CTTCTTTGT <u>Agcg</u> TTCTGGGAAAG	E33A mutation**	
47		AGTACGCCTAGTCCTC	E33 reverse	
48		TATGTCAGTT <u>caa</u> ATGGGTGCATTATTATC	N167Q mutation**	
49		TAGAAAATAACAAAACCTGC	N167 reverse	
50	opp3A (codon optimised)	(TCCAGGGACCAGCA) GATGGTATTTATAGCGATAAAGGTCAG	Cloning into pETFPP_ (N)GST* **	
51		(TGAGGAGAAGGCGCG) <u>tta</u> TTTGCCGGTTTCTTTATCGATG		
52	pETFPP_ (N)GST	TGCTGGTCCCTGGAAC	vector	
53		TAACGCGCCTTCTCCTC	linearisation	
54	opp3A (codon optimised)	<u>ga</u> ACCACTCAGGCTTGCTTCAAAG	W438S mutation**	
55		<u>g</u> AGCGCAGATTATCCGGATCCG	W438 reverse**	

**Brackets denote overhang regions used for recombination-based cloning, as described below*

*** Lowercase and underlined sequence denotes non-cognate sequences such as mutations*

*All primers targeting S. aureus genomic sequences (dtpT, opp3A and gisD) were designed using the available whole genome sequence of JE2 (Genbank accession **CP020619.1**)*

Plasmid construction

Complementation vectors

Plasmids were generated using the ClonExpress II One Step Cloning Kit (Vazyme) according to manufacturer's instructions. DtpT complementation in *S. aureus* mutant strains was achieved via introduction of the entire *dtpT* locus into the *E. coli* / *S. aureus* shuttle vectors pRAB11 ([Helle et al., 2011](#)) and pSK5630 ([Grkovic et al., 2003](#)). The latter plasmid was modified by the addition of 1080 bp of sequence amplified from immediately upstream of the *dtpT* open-reading frame in the JE2 genome, comprising the native *dtpT* promoter sequence (herein referred to as pSK56_{pT}). In all cases, plasmids were confirmed by DNA sequencing of the insertion site.

POT-expression vectors

Plasmid vectors for the expression of bacterial POTs in *E. coli* were generated using the ClonExpress II One Step Cloning Kit (Vazyme) according to manufacturer's instructions. The DtpT open reading frame was amplified directly from the *S. aureus* JE2 genome via PCR and cloned into the arabinose-inducible expression vector pBADcLIC ([Geertsma & Poolman, 2007](#)) and the IPTG-inducible super-folded green fluorescent protein (GFP) -tagged fusion vector pWaldo ([D. Drew et al., 2006](#)). Mutant variants of DtpT were generated in the pWaldo vector via site-directed PCR mutagenesis and blunt-end ligation with T4 DNA ligase (NEB). Other bacterial POTs were amplified either from their native genomic contexts (*E. coli* BL21 DE3 for DtpA_{Ec} and DtpC_{Ec}; *K. pneumoniae* ATCC13883 for DtpE_{Kp}) and cloned into pWaldo, or were supplied in previously generated pWaldo expression constructs ([Minhas et al., 2018](#); [Newstead et al., 2011](#)). All expression constructs were verified by sequencing of the insertion site.

Strain generation

Generation of chromosomal mutants in S. aureus by phage transduction

S. aureus JE2 transposon mutants were acquired from the Nebraska Transposon Mutant Library (NTML; [Fey et al., 2013](#)) and transduced into wild-type JE2 via Φ 85 bacteriophage in order to minimise off-target genetic inconsistencies between strains (as outlined by [Olson, 2016](#)).

Phage lysate preparation

Donor strains were grown overnight in phage medium (2:1 LB:TSB; 5 mM CaCl₂) and, the next morning, diluted 1:50 into fresh medium. Cultures were then grown for a further 2.5 h at 37°C. Φ 85 bacteriophage lysates were serially diluted ten-fold in TMG buffer (10 mM Tris, pH 7.5; 10 mM MgSO₄; 0.1% gelatine) and 100 μ l of each dilution was added to 500 μ l aliquots of culture. Cultures were incubated for a further 30 minutes (allowing for one cycle of phage replication)

before being added to 5 ml pre-warmed top agar (0.8% NaCl, 0.8% Bacto-agar, 5mM CaCl₂) and poured over TSA plates with *erm*. Plates were incubated overnight. The next day, plates were inspected and those with a nearly confluent layer of phage plaques were taken forward for lysate preparation. These plates were overlayed with 3 ml TMG buffer at room temperature for 20 minutes and top agar was macerated through continuous scraping with a disposable plastic spreader. Insoluble material was removed via centrifugation at 13,000 x g and supernatant (containing the desired phage lysate) was sterilised through a 0.22 µm PES syringe filter. Lysates were stored at 4°C. Phage titre was assessed in each case by a spotting assay and only lysates with a titre $\geq 10^6$ were stored for further use.

Phage transduction

For transduction, recipient strains were grown overnight in phage medium. The next day, overnight cultures were pelleted by centrifugation at 4000 x g for 15 minutes and concentrated 5x into fresh medium. 250 µl of previously prepared donor lysate was added to 250 µl of concentrated culture and these were incubated 37°C for 20 minutes. Cells were then transferred to ice and washed three times with 1 ml ice-cold 40 mM trisodium citrate (NaCit) to remove exogenous phage. Cells were finally resuspended in 300 µl NaCit and plated on TSA supplemented with 20 mM NaCit and *erm*. Plates were incubated for 48 h. Large colonies (i.e. successful transductions) were restreaked at least twice onto TSA plates supplemented with 20 mM NaCit to prevent phage carry-over. All strains were confirmed by PCR analysis.

Allelic exchange

To generate double mutant strains, the *Erm*^R resistance cassette was exchanged with an unmarked sequence using the allelic exchange vector pTnT (as outlined by [Bose et al., 2013](#)).

pTNT was transduced into marked mutant strains (as described above, but carrying out all growth/incubation steps at 30°C to ensure plasmid maintenance). Successful transductions were streaked extensively over TSA plates supplemented with *cam* and incubated overnight at 44°C. The next morning, large colonies (single-recombinants) were re-streaked onto *cam*-supplemented plates and incubated overnight at 44°C. Strong growing streaks were taken forward for plasmid curing. A single colony isolated from each streak was picked into 3 ml TSB and continuously cultured over 10 days at 30°C, seeding 1 µl of culture into fresh medium every day. On the tenth day, cells were diluted and plated onto TSA supplemented with 100 ng/ml ATc to isolate single colonies. Colonies were then replica patched onto TSA alone, TSA + *cam* and TSA + *erm*. Successful double-recombinants were identified as those with susceptibility to both antibiotics.

S. aureus transformations

Plasmids were transformed into *S. aureus* by electroporation. To prepare competent cells; overnight cultures were diluted 1:100 into 50 ml TSB and grown at 37°C to a final OD₆₀₀ of 0.4 and were then transferred to ice. Cells were pelleted by centrifugation at 4000 x *g* for 15 minutes at 4°C and resuspended in 40 ml wash buffer (ice-cold 0.5 M sucrose). Cells were then successively washed in 30 ml and 20 ml wash buffer and finally resuspended in 0.55 ml wash buffer. Competent cells were snap-frozen and stored at -70°C.

For electroporation, 5 µl of plasmid (prepared in dH₂O; at least 100 ng/µl) was added to 110 µl competent cell suspension on ice. Electroporation was carried out using a Micropulser (Bio-Rad) and standard settings for bacterial transformation (100 Ω; 2.5 kV; 0.1 cm cuvette). Immediately after electroporation, cells were recovered by addition of 0.9 ml pre-warmed Brain-Heart Infusion broth (VWR) supplemented with 0.5 mM sucrose and incubated for 1 h at 37°C. Transformants were plated on *cam*-supplemented TSA and large colonies were picked the next day. All transformants were confirmed by plasmid isolation, followed by agarose gel analysis and PCR.

E. coli transformation

Expression plasmids were shuttled into the *E. coli* protein expression strains BW25113 and BL21 (DE3), as well as BL21-derivative membrane protein overexpression strains C41 (DE3) and C43 (DE3) (generated by [Miroux & Walker, 1996](#)).

To prepare competent cells, overnight cultures were diluted 1:100 into 100 ml fresh LB medium and incubated at 37°C to a final OD₆₀₀ of 0.6 ± 0.1. At this point, cells were incubated on ice for 15 minutes before being pelleted by centrifugation at 4000 x *g* for 15 minutes at 4°C. Pellets were resuspended in 10 ml ice-cold buffer RF1 (100 mM RbCl; 50 mM MnCl₂; 30 mM potassium acetate; 10 mM CaCl₂; 15% glycerol; pH 5.8). Cells were pelleted again as before and resuspended in 9.6 ml ice-cold buffer RF2 (10 mM MOPS; 10 mM RbCl; 75 mM CaCl₂; 15% glycerol; pH 6.8) before being snap-frozen in liquid nitrogen and stored at -70°C.

For transformation, 50 µl of competent cells were thawed on ice. Approximately 200 ng of plasmid DNA was added, and cells were mixed briefly by inverting before a further 30 minutes of incubation on ice. Cells were then heat-shocked at 42°C for 90 seconds and transferred back to ice for 2 minutes. 0.9 ml pre-warmed LB medium was added, and cells were incubated for 1 h at 37°C before being plated on LB agar plates with appropriate selection. Successful transformants were confirmed by colony PCR.

4. Biochemical techniques

SDS PAGE and protein gel analysis

All sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis was carried out using conventional SDS PAGE gels composed of 5 ml resolving gel (375 mM Tris, pH 8.8; 12% polyacrylamide; 0.1 % SDS) and 2 ml stacking gel (125 mM Tris, pH 6.8; 4% polyacrylamide; 0.1 % SDS). All gels were prepared with 29:1 acrylamide/*bis*-acrylamide solution (Bio-Rad) and set by the addition of 0.1% each ammonium persulfate and N,N,N',N'-Tetramethylethylenediamine (Melford).

Before loading, samples were routinely mixed with 4x protein loading buffer (40 mM Tris, pH 7.2; 50% glycerol; 2% SDS; 2.4 g/l bromophenol blue) with addition of 0.3% β -mercaptoethanol and heated at 85°C for 10 minutes. For in-gel fluorescence, samples were mixed with 4x protein loading buffer without β -mercaptoethanol and heated at 55°C for 15 minutes to prevent denaturation of GFP. Samples were routinely run at 160 V for 1 h in running buffer (25 mM Tris HCL; 200 mM glycine; 0.1% SDS). All gels were run with the inclusion of a pre-stained Trident Blue protein ladder (GeneTex) for approximation of band sizes.

Coomassie staining

Total protein content was visualised by overlaying gels with 5 ml Coomassie brilliant blue dye mix (45% methanol; 10% acetic acid; 2.5 g/L Brilliant Blue R) for 15 minutes with shaking. Gels were destained by overlaying with 10% ethanol/10% acetic acid for at least two hours. Stained gels were then imaged using the GeneGenius Gel Bioimaging Unit, or using the iBright imaging system with orange contrast plate.

In-gel fluorescence

Gels were briefly rinsed with deionised water to remove excess salts. In-gel fluorescence of GFP was then visualised using the iBright imaging system or the Amersham Typhoon biomolecular imager (Cytiva) using in-built Cy2 fluorescence setting (ex./emm. = 489/506).

Western blotting

Western blotting was utilised to detect poly-histidine tagged proteins following recombinant expression and purification. Proteins were transferred from SDS PAGE gels onto a nitrocellulose membrane using iBlot dry transfer device (Invitrogen). Uniform transfer of proteins was

confirmed by overlaying membranes with 5 ml Ponceau S staining solution (Thermo Fisher), which was then removed by overlaying membranes with 10 ml dH₂O for 15 minutes with shaking.

Note: For Western blotting, all wash steps were performed by carefully pouring off liquid and overlaying membranes with PBS or PBST (PBS + 0.1% Tween 20) for 5 minutes with shaking at room temperature.

Membranes were blocked by overlaying with 20 ml 5% skimmed milk (Marvel) for 2 h with shaking at room temperature. Membranes were washed twice PBST, before blocking again as before for 30 minutes. Following this, membranes were washed twice with PBST and three times with PBS. Membranes were then overlayed with 5% skimmed milk containing a 1:2000 dilution of 1° murine anti-6His monoclonal antibody (Invitrogen) and incubated overnight at 4°C with shaking. The next day, membranes were washed twice with PBST and three times with PBS. Membranes were then overlayed with 20 ml 5% skimmed milk containing a 1:4000 dilution of 2° rabbit anti-IgG HRP antibody (Invitrogen) and incubated for 1 hour at room temperature with shaking. Membranes were then washed five times with PBST. His-tagged proteins were then detected by briefly rinsing membranes with dH₂O and then overlaying with 1 ml Immobilon Forte Western HRP substrate (Millipore) for 2 minutes and imaging using the iBright imaging system “Chemblots” function. In each case, membranes were exposed for no longer than 2 minutes to detect strong bands corresponding to His-tagged proteins.

Protein expression trials

pBADcLIC

To assess expression of DtpT from pBADcLIC, overnight cultures were diluted to an OD₆₀₀ of 0.05 in LB medium with selective antibiotics and incubated at 37°C with shaking to a final OD₆₀₀ of 0.5 ± 0.1 (*mid-exponential*). Expression was induced by the addition of arabinose (final concentration 0.01%) before incubating for a further 16 hours with shaking at variable temperatures. 1 ml of culture was harvested at set time-points (0/1/2/3/4/16 h) post-induction. Cells were pelleted by centrifugation at 4000 x g for 5 minutes and resuspended in variable volumes of BugBuster lysis solution (Merck) to normalise cell density to t = 0 h. Presence of the target protein was detected via SDS PAGE and Western blot analysis, as described above.

pWaldo

Optimal conditions for the expression of six POT transporters from pWaldo were determined based on previously reported methods ([D. Drew et al., 2006](#)). Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.05 in LB medium with selective antibiotics and incubated at 37°C with

shaking to a final OD₆₀₀ of 0.5 ± 0.1 (*mid-exponential*). Expression was induced by the addition of IPTG before incubating for a further 22 hours with shaking at variable temperatures. At this time-point, cells were pelleted and resuspended in 200 µl BugBuster lysis solution. Presence of GFP-fusion proteins was detected via SDS PAGE and in-gel GFP. In addition, 100µl of each culture was transferred to a NUNC flat-bottomed 96 well plate (Thermo Scientific) immediately after addition of IPTG. Plates were incubated for 22 hours with shaking in an Omega microplate reader (BMG Labtech), measuring OD₆₀₀ and whole cell GFP fluorescence (520 nm emission, 485 nm excitation) every 15 minutes.

Purification of DtpT and mutant variants

Cells carrying DtpT expression vectors were grown overnight in LB with selective antibiotics and diluted 1:100 into fresh media. Cultures were incubated at 37°C until an OD₆₀₀ of 0.5 was reached. Expression was then induced by the addition of 0.01% arabinose (pBADcLIC vectors) or 100 µM IPTG (pWaldo vectors), after which cultures were incubated overnight with shaking under optimal expression conditions (*as described in text*). Cells were then pelleted by centrifugation at 5000 x g for 20 minutes, resuspended in 50 ml resuspension buffer (40 mM Tris, pH 7.5; 10% glycerol) and lysed by sonication. Crude lysates were clarified via centrifugation at 27,000 x g for 30 minutes. Membranes were then pelleted via ultracentrifugation at 164000 x g for 120 minutes. Membrane pellets were pooled and resuspended in 40 ml protein solubilisation buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 20 mM imidazole; 10% glycerol; 0.5% n-dodecyl-B-pyromaltoside (DDM; Avanti)) using a glass homogenizer before being incubated for 60 minutes at 4°C with rolling. The resultant suspension was then ultracentrifuged as before for 60 minutes to pellet leftover membranes and insoluble contaminants. Supernatant containing the solubilised membrane proteins was pooled and applied to a His-TRAP nickel affinity column (GE Healthcare) using an ÄKTA Protein Purification System (Cytiva). This column was washed with 50 ml column wash buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 20 mM imidazole; 10% glycerol; 0.04% DDM) and His-tagged protein was then eluted via application of column elution buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 400 mM imidazole; 10% glycerol; 0.04% DDM).

For purification from pWaldo, the purified GFP-fusion protein was then exchanged into TEV reaction buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 0.5 mM EDTA; 1 mM DTT; 10% glycerol; 0.04% DDM) using a HiTRAP desalting column (SLS). Protein was then mixed with Tobacco Etch Virus (TEV) protease to a final ratio of approximately 3:1. The resultant reaction mixture was incubated overnight at 4°C. Pure DtpT was isolated by applying the reaction mixture to a His-TRAP nickel affinity column as before and washing with column wash buffer, this time collecting the flow-

through fractions. Purified DtpT was exchanged into DDM reconstitution buffer (20 mM Tris pH 7.5; 150 mM NaCl; 0.03% DDM) via size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column (Cytiva). Pure protein was then stored in this buffer at -70°C.

Circular dichroism

Secondary structure elements of the pure DtpT protein were probed by circular dichroism (CD). Purified DtpT was exchanged into circular dichroism buffer (50 mM KPi, pH 7.8; 10 % glycerol; 0.04% DDM) and diluted to a final concentration of 150 µg/ml. For assessing the success of DtpT liposome reconstitution, liposomes were pelleted by ultracentrifugation at 158 000 × g for 30 minutes and resuspended in 50 mM KPi, pH 7.8 to a final protein concentration of 100 µg/ml (assuming 100% reconstitution efficiency). Circular dichroism was repeated five times at 20°C with a J-1500 CD Spectrophotometer (Jasco) using 1mm path-length quartz cuvettes. The resultant spectrum was normalised against the equivalent spectrum for a buffer-only control in each case.

NanoDSF thermal unfolding assay

DtpT-ligand interactions were investigated by nano-differential scanning fluorimetry (nanoDSF) using the Prometheus NT.48 NanoDSF fluorimeter (NanoTemper). Purified DtpT was exchanged into DSF buffer (40 mM Tris, pH 6.8; 200mM NaCl; 0.04% DDM) and diluted to a final concentration of 10 µM. The diluted protein was then mixed 1:1 with 20 mM ligand dissolved in DSF buffer (final concentrations: 5 µM protein, 10 mM ligand) or with DSF buffer only. Melting curves were produced by monitoring intrinsic tryptophan fluorescence as temperature was increased in 1°C increments.

Membrane protein reconstitution

Liposome preparation

All liposomes utilised in this work were composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE; Avanti) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG; Avanti) mixed in a 3:1 ratio. Liposome preparation was carried out in a ventilated cabinet. A large round-bottom flask was first pre-washed successively with dH₂O, 70% ethanol, pentane (VWR) and chloroform. After each wash step, flasks were dried under a stream of nitrogen gas for 30 minutes. Chloroform-solubilised lipids were then each added to the flask (300 mg POPE; 100 mg POPG) and chloroform was evaporated by swirling under a stream of nitrogen gas. Lipids were then successively washed with 5 ml chloroform and 5 ml pentane, drying as before each time. Lipids were then resuspended fully in 50 mM KPi, pH 7.0 to a final

concentration of 20 mg/ml via sonication in an ultrasonic water bath (VWR). Lipids were then subjected to three freeze-thaw cycles before aliquoting into 0.5 ml fractions and storing at -70°C.

Rapid dilution

The pure DtpT protein was thawed and exchanged via SEC (as before) into DM reconstitution buffer (20 mM Tris pH 7.5; 150 mM NaCl; 0.15% n-decyl-B-pyromaltoside (DM; Avanti)). 3POPE:1POPG liposomes were separately prepared and diluted to a final concentration of 5 mg/ml in PBS. Lipids were extruded 11 times through a pre-soaked 0.4 µm track-etched polycarbonate membrane (Whatman) before being slowly added to the protein at room temperature to a final ratio of 50:1. The resultant mixture was incubated on ice for 30 minutes before rapid dilution into 65 ml ice-cold PBS. Resultant proteoliposomes (PLs) were pelleted via ultracentrifugation at $158\,000 \times g$ for two hours. Supernatant was removed and PLs were resuspended in 1 ml PBS. PLs were subjected to three freeze-thaw cycles before dialysing twice against 3 L PBS at 4°C; once for 3 hours, then again overnight. PLs were snap-frozen and stored at -70°C. Variations of the method described here were trialled in an attempt to optimise protein reconstitution, and these are described in the text.

Biobead titration

Purified DtpT in DDM reconstitution buffer was diluted to a final concentration of 0.5 mg/ml. 3POPE:1POPG liposomes were prepared and extruded 11 times each through pre-soaked 0.8 µm and 0.4 µm track-etched polycarbonate membranes (Whatman) before diluting to a final concentration of 10 mg/ml in liposome buffer (50 mM KPi, pH 7). 10 mg lipid mixture was gradually added to 200 µg protein (50:1 ratio) at room temperature before being incubated on ice for 60 minutes. 100 µl SM2 Biobeads (Biorad) suspended in water were added to the lipid-protein mixture, which was then incubated for 1 hour at 4°C while rotating. A further 130 µl Biobeads were added, and the mixture was incubated as before for a further 3 hours. Biobeads were pelleted by centrifugation at $100 \times g$ for 1 minutes and the lipid-protein mixture was transferred to a fresh tube. 160 µl Biobeads were added, and the mixture was incubated as before for a further 16 hours. Biobeads were removed as before and proteoliposomes were pelleted via ultracentrifugation at $158\,000 \times g$ for 30 minutes. Proteoliposomes were then resuspended in liposome buffer to a final protein concentration of 0.5 mg/ml (assuming 100% reconstitution efficiency) and subjected to three freeze-thaw cycles before dialysing extensively against liposome buffer at 4°C. Proteoliposomes were pelleted as before and resuspended in liposome buffer before being stored at -70°C.

Transport assays

Proteoliposomes were pelleted via ultracentrifugation at $158\,000 \times g$ for 30 minutes and resuspended in inside buffer (5 mM HEPES, pH 6.8; 120 mM KCl; 2 mM MgSO_4) spiked with 1 mM pyranine (FluoroChem), then subjected to seven freeze-thaw cycles before being extruded through a pre-soaked $0.4\ \mu\text{m}$ track-etched polycarbonate membrane. Proteoliposomes were then pelleted at 20°C and resuspended in inside buffer. Excess pyranine was removed using a microspin G25 desalting column (Cytiva) according to the manufacturer's protocol. Liposomes were finally pelleted again at 20°C and resuspended to a final protein concentration of 1 mg/ml. For each assay, $6\ \mu\text{g}$ of proteoliposomes were diluted 1:100 into outside buffer (5 mM HEPES, pH 6.8; 120 mM NaCl; 2 mM MgSO_4). Assays were performed in 1 ml clear plastic microcuvettes using a Fluoromax-4 spectrophotometer (Horiba) with a magnetic flea for stirring. Fluorescence of pyranine was measured via excitation at 460 nm and emission at 510 nm. Substrate was added to the reaction at $t = 25\ \text{s}$ and transport was induced by the addition of $1\ \mu\text{M}$ valinomycin (Thermo Scientific) at $t = 50\ \text{s}$.

Purification of Opp3A* and Opp3A*_{W438S}

Optimal expression conditions for Opp3A from the N-terminal glutathione S-transferase (GST) - fusion vector pETFPF_(N)GST were previously determined by project student Steven Kurniawan (*University of York*). BL21 (DE3) cells carrying the truncated Opp3A (Opp3A*) expression vector were grown overnight in LB with selective antibiotics and diluted 1:100 into fresh media. Cultures were incubated at 37°C until an OD_{600} of 0.5 was reached. Expression was then induced by the addition of $500\ \mu\text{M}$ IPTG, after which cultures were incubated overnight with shaking at 20°C . The next day, cells were pelleted by centrifugation at $5000 \times g$ for 20 minutes, resuspended in 50 ml resuspension buffer (see DtpT purification protocol) and lysed by sonication. Crude lysates were clarified via two successive centrifugation steps at $27,000 \times g$ for 30 minutes and 15 minutes, respectively, transferring supernatants to a fresh tube each time. Supernatants were pooled and applied to a His-TRAP nickel affinity column using an ÄKTA Protein Purification System. This column was washed with 50 ml column wash buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 20 mM imidazole; 20% glycerol) and His-tagged protein was then eluted via application of column elution buffer (40 mM Tris, pH 7.5; 200 mM NaCl; 400 mM imidazole; 20% glycerol).

The purified GST-fusion protein was then exchanged into 3C protease reaction buffer (40 mM Tris, pH 7.5; 100 mM NaCl; 1 mM DTT; 20% glycerol) using a HiTRAP desalting column. Protein was then mixed with 3C protease to a final ratio of approximately 50:1. The resultant reaction mixture was incubated overnight at 4°C with rotation. The next morning, insoluble material was pelleted

by centrifugation at 10,000 x g for 10 minutes. Pure Opp3A was isolated by applying the reaction mixture to a His-TRAP nickel affinity column as before and washing with column wash buffer, this time collecting the flow-through fractions. Purified Opp3A was then exchanged into minimal buffer (20 mM Tris pH 7.5; 50 mM NaCl) via SEC using a HiPrep 16/60 Sephacryl S-200 HR column (Cytiva). Pure protein was pooled and stored at -70°C.

Crystallography

For crystallization of Opp3A* (and the mutant variant), protein was first concentrated to produce 10 mg/ml and 20 mg/ml solutions. Crystal trials were performed with both protein solutions using commercially available screening platforms with a Mosquito nanoliter pipetting robot (TTP Labtech): Clear Strategy Screens I and II, JCSG Plus and PEG-ION, each in 2-drop 0.15 µl 96 well plate format. Plates were sealed and incubated for several weeks at 20°C. Conditions yielding protein crystals were taken forward for targeted screening via 2 µl hanging-drop diffusion in a 24 well plate, varying precipitant and salt concentrations to yield desirable (that is, large and regular) crystals. Crystal-containing droplets were mixed 1:1 with a cryoprotectant (20% glycerol, or 35% PEG3350), mounted on a nylon loop and flash-frozen. Crystals which demonstrated clear diffraction in-house (*York Structural Biology Laboratory*) were sent for structural determination at the Diamond Light Source (Oxfordshire) using the i03 beamline.

Structural modelling and blind-docking

A structural model of DtpT was generated using SWISS-MODEL ([A. Waterhouse et al., 2018](#)) using the solved complex structure of the *S. hominis* PepT_{Sh} (6EXS) as a template. For predicting how DtpT recognises glutathione, blind docking was carried out using this projected structure in CB-Dock 2 ([Y. Liu et al., 2022](#)). Projected structural models of various proteins were generated using AlphaFold 3 ([Abramson et al., 2024](#)). Projected and docked structures were visualised in ChimeraX (UCSF) and PyMOL (Schrödinger Inc).

5. Macrophage infection experiments

Macrophage culture and differentiation

(Note: macrophage culture and differentiation was assisted by Dr Dave Boucher, University of York)

THP-1 cells (ATCC) were cultured in macrophages media (RPMI1640 supplemented with 10% Fetal Calf Serum and Glutamine). To obtain a macrophage-like differentiation state, THP1 were differentiated for 48 hrs in macrophage media supplemented with 50 ng/mL Phorbol 12-myristate 13-acetate (PMA; in DMSO). PMA-containing media was replaced with fresh macrophage media and cells were rested overnight prior to infection.

Human primary monocyte-derived macrophages (hMDMs) were differentiated from human peripheral blood mononuclear cells obtained from the NHS (University of York Biology ethic committee project DB202111) and differentiated in complete macrophage media supplemented with 50 ng/mL macrophage colony-stimulating factor (Proteintech) over 7 days.

Macrophage infection

Infection of macrophages and macrophage-like cell lines was carried out based on previously published methods ([Alves et al., 2024](#)). Overnight cultures of *S. aureus* strain JE2 and mutant derivatives were diluted 1:100 into fresh TSB and grown to mid-exponential phase. Cells were then pelleted by centrifugation at $4000 \times g$ for 5 minutes and washed twice in ice-cold PBS before being resuspended to a final cell density of 5×10^7 CFU/mL. For infection, 10 μ l bacterial suspension was added to 50,000 hMDM or THP-1 cells (D.O.I. = 10) in 90 μ l Opti-MEM reduced serum media (Thermo Fisher) and plates were centrifuged for 5 minutes at $500 \times g$ to promote contact. After 30 minutes of incubation at 37°C, the medium was exchanged for Opti-MEM supplemented with 100 μ g/ml gentamicin (Gibco). The addition of gentamicin was designated $t = 0$ h. After a further 30 minutes of incubation this medium was removed, and cells were then maintained in Opti-MEM supplemented with 5 μ g/ml gentamicin. Supernatants were isolated at $t = 4/6/8$ h post-infection for cytotoxicity and enzyme-linked immunosorbent assay (ELISA).

Bacterial survival

To compare bacterial internalisation and survival, cells at $t = 0.5 / 4 / 8$ h were incubated in deionised water for 10 minutes before being lysed by repeated pipetting. Suspensions were

serially diluted in PBS, spotted onto TSA and incubated overnight at 37°C to determine viable CFU.

Cytotoxicity

Cytotoxicity was measured by quantifying the activity of lactate dehydrogenase (LDH) in the extracellular medium using a commercially available kit (Cytotox 96 non-radioactive cytotoxicity kit, Promega). Briefly, culture supernatants were mixed 1:1 with colorimetric LDH substrate in a Corning 96 well plate. Uninfected cells were also included in this analysis, as well as fully lysed cells (by addition of 0.04% Triton X-100, incubated for 10-20 minutes). Plates were sealed and incubated at 4°C overnight, after which A_{490} was measured using an Infinite 200 PRO plate reader (Tecan). Readings were corrected by subtracting the background signal for uninfected cells. Cytotoxicity was determined as a percentage of LDH activity in the lysed cells. All assays were conducted in technical triplicate.

ELISAs

Cytokine levels in cell culture medium were determined at $t = 6$ h by utilising human IL 1- β , IL 6 and TNF- α ELISA assay kits (Invitrogen; all materials listed here).

Coated immunoassay plates were overlayed with 40 μ l/well coating buffer containing a 1:250 dilution of capture antibody and incubated overnight at 4°C. The next day, this buffer was removed, and plates were washed once with 200 μ l PBST. Plates were then blocked with 200 μ l ELISPOT blocking buffer and incubated at room temperature for 1 h. Meanwhile, cell culture supernatants were diluted 1:20 or 1:40 into PBS and standard curves were prepared using cytokine standards. ELISPOT buffer was removed, and plates were overlayed with the prepared samples and standard curves before being incubated overnight at 4°C. The next day, supernatants were removed and plates were washed once with 200 μ l PBST before being overlayed with 40 μ l/well ELISPOT containing a 1:250 dilution of detection antibody and incubated at room temperature for 1 h. Plates were then washed six times with 200 μ l PBST to remove unbound antibodies, after which plates were overlayed with 40 μ l ELISPOT containing a 1:250 dilution of avidin-HRP and incubated for a further 30 minutes at room temperature. Finally, buffer was removed, and plates were washed six times with PBST before overlaying with 40 μ l of TMB substrate. Plates were developed for 30-60 minutes at 37°C until the standard curve was sufficiently resolved, at which point the reaction was stopped by addition of 1 N sulphuric acid. A_{450} was measured using Infinite 200 PRO plate reader. Readings were used to quantify the amount of cytokine present in each sample by comparison to the standard curve in each case. All assays were conducted in technical triplicate.

Glutathione quantification

Total glutathione from differentiated THP-1 macrophage-like cells was quantified at t = 6 h post-infection using a commercially available fluorometric assay kit (Abcam; ab65322; all materials listed here). In each case, fluorescence (Ex./Em. = 360/460 nm.) was detected using the FLUOstar Omega Microplate Reader (BMG Labtech) and glutathione was quantified by comparing to a standard curve.

Chapter 3 – Utilising phenotype microarrays to define the substrate landscapes of DtpT and Opp3 in *S. aureus*

Introduction

Previous attempts to delineate the routes of peptide acquisition in *Staphylococcus aureus* have been limited in scope, largely due to their reliance on a restricted panel of peptide substrates as representatives for this diverse class of molecules ([Hiron et al., 2007](#); [Lehman et al., 2019](#)). Commercially available phenotype microarrays (PM) represent an attractive high-throughput approach for assessing and comparing the ability of bacterial strains to utilise diverse substrates as carbon/nitrogen sources, including di- and tripeptides ([Bochner et al., 2001](#)). PMs were previously utilised to compare the contribution of various dipeptide-binding proteins toward Dpp_{BCDF}-mediated peptide acquisition in *P. aeruginosa* ([Pletzer et al., 2014](#)). Here, we apply a similar methodology to elucidate the distinct roles of DtpT and Opp3 in the utilisation of di/tripeptides by *S. aureus* (it is worth noting that Opp3 has already been implicated in the transport of longer oligopeptides at up to 9 residues in length, but only di- and tri-peptide utilisation will be assessed here due to inclusion of these peptides in existing PM plates).

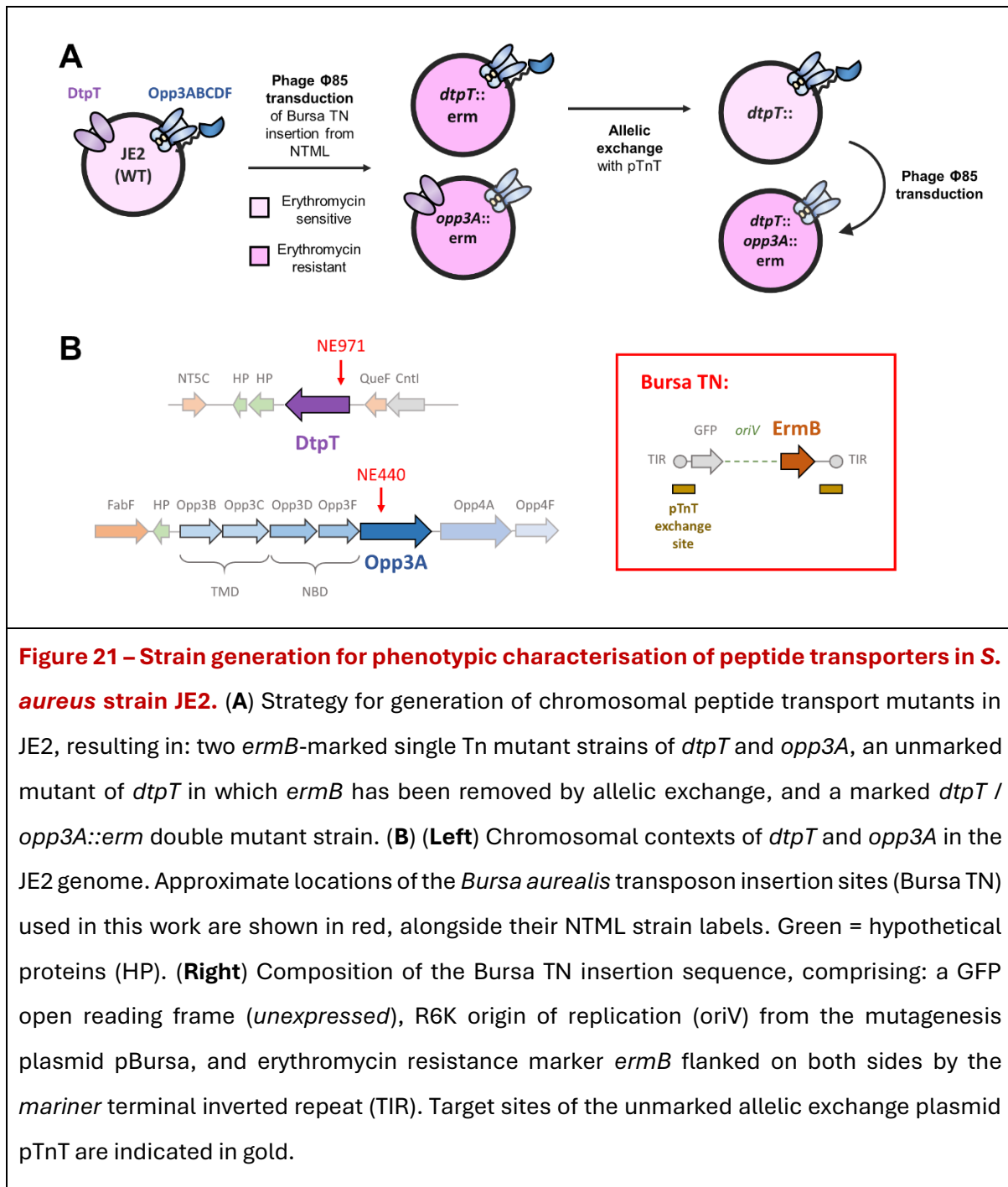
Our data demonstrate that DtpT is the predominant route of utilisation for dipeptides, though some dipeptides can be utilised by either DtpT or Opp3. We also reveal a preference for Asp/Glu-containing peptides among putative DtpT substrates while Arg-containing peptides are commonly utilised via either transport system. Intriguingly, we demonstrate that many Gln-containing peptides are utilised independently of either transport system, suggesting the existence of an alternative transporter(s) specific for these peptides. Finally, we utilise auxotrophy growth assays to confirm the utilisation patterns of three Arg-containing peptides included in our PM assays. Overall, our results provide valuable insights into the substrate preferences of DtpT and Opp3.

Results

Strain design and generation

We first aimed to generate loss-of-function mutants of the two peptide transporters of *S. aureus* – DtpT and Opp3 – in order to assess the contribution of these systems toward peptide utilisation. The strategy by which these strains were generated is outlined in [Figure 21A](#). Transposon-interrupted mutants of *ntpT* and each of the constituents of *opp3BCDFA* were previously generated as part of the Nebraska Transposon Mutant Library (NTML) ([Fey et al., 2013](#)) and these strains were kindly provided by Dr Rebecca Corrigan (*University of Sheffield*). The structure of the *Bursa aurealis* transposon and approximate sites of these insertions in the relevant genes are shown in [Figure 21B](#). *opp3A* is the last gene in the *opp3BCDFA* operon and encodes the substrate binding protein (SBP) component of the complete transporter. An *opp3A* mutant variant was chosen based on the previous observation that loss of the SBP component alone is sufficient to disrupt Opp3-mediated transport ([Hiron et al., 2007](#); [Lehman et al., 2019](#)). Hence, transposon-interrupted variants of *ntpT* and *opp3A* were transduced from the supplied NTML strains into the wild-type strain JE2, which is a derivative of the epidemiologically widespread community-associated MRSA isolate USA300 and the parent strain of the NTML collection ([Fey et al., 2013](#)). JE2 was chosen as the parent strain in these experiments based on the clinical relevance of the USA300 lineage ([Thurlow et al., 2012](#)), as well as the availability of complete genome sequence data and molecular genetic tools specifically for manipulation of this strain ([Bose et al., 2013](#); [Fey et al., 2013](#)).

The allelic exchange vector pTnT ([Bose et al., 2013](#)) was later used to remove the ErmB antibiotic resistance marker from the *ntpT* mutant strain, allowing for the subsequent generation of a *ntpT* / *opp3A* double mutant through phage transduction. All mutants were confirmed by diagnostic PCR ([Appendix 6](#)). In addition, the *ntpT::erm* mutant strain exclusively was analysed by whole genome sequencing to confirm the absence of off-target mutations in relevant genomic loci, and DtpT-dependent peptide utilisation phenotypes were later verified by *in trans* complementation ([page 137](#)). It is worth noting that equivalent analyses were not carried out for the *opp3A::erm* mutant strains utilised here due to time and budget constraints, and hence we cannot rule out the possibility of secondary mutations influencing the observed phenotypes of these strains.



Phenotype microarray analysis of JE2 and *dtpT/opp3A* mutants

JE2 and the transposon mutant-derivative strains were each applied to Biolog phenotype MicroArray plates PM 6-8 in order to assess and compare the utilisation of 282 different peptides, comprising: 246 standard dipeptides (of a possible 400), 1 hydroxyproline-containing dipeptide, 7 β -bonded dipeptides, 2 γ -bonded dipeptides, 12 d-amino acid-containing dipeptides and 14 tripeptides (listed in [Appendix 7](#)).

In this assay, cells are suspended under conditions where a given peptide is supplied as the primary nitrogen source. Efficient internalisation and catabolism of the supplied peptide is therefore necessary to drive cellular metabolism and, ultimately, facilitate respiration ([Bochner et al., 2001](#)). Cellular metabolic output is measured via conversion of a highly redox-sensitive tetrazolium dye, producing a purple colour which is quantified over time to generate a signal curve for each peptide. A positive metabolic signal is indicative of peptide utilisation enabled by a valid route of uptake and downstream catabolic pathways, while intermediate/weak signals are likely to result from poor uptake (or a complete lack thereof) or incomplete catabolism following internalisation. Each plate also includes a negative control well lacking any additional nitrogen source and a positive control well containing L-glutamine. Here, we found it necessary to adjust the manufacturer's protocol to conditions under which there is sufficient distinction between these two conditions for our tested strains (as described in methods). The resultant signal curves for each strain/peptide combination are given in [Appendices 8-10](#).

Cluster analysis and UMAP identify common patterns in PM data

To extract common patterns from our phenotype microarray data, we subjected the raw signal curves for each peptide to unbiased k-means cluster analysis and dimensional reduction using Uniform Manifold Approximation and Projection (UMAP). Briefly, raw signal curves for each peptide were compiled and partitioned into a defined number of clusters via an unbiased clustering algorithm. We found that partitioning the dataset into seven clusters was effective at identifying the most abundant clearly distinct utilisation patterns present within the data ([Figure 22A-B](#)). UMAP, by comparison, allows the dataset to be visualised as a two-dimensional plot wherein each peptide is assigned X/Y coordinates based on the underlying signal curves. As a result, peptides with similar utilisation patterns are spatially close in the final plot. Furthermore, when compared to other dimensional reduction pipelines such as PCA and t-SNE, UMAP attempts to better conserve the global structure of the data in the final plot ([McInnes et al., 2018](#)). Overall, we found that both analytical methods applied here provided complementary outputs, as exemplified by peptides belonging to a common cluster demonstrating close spatial

organisation in the final UMAP plot (Figure 22C). Here, I will outline the distinct peptide-utilisation patterns exemplified within each cluster and briefly speculate on the underlying molecular basis of these patterns (expanded upon in the discussion section of this chapter; page 141).

Cluster 1 – DtpT/Opp3-independent utilisation

Peptides belonging to cluster 1 ($n = 15$) resulted in a strong utilisation signal which was mostly consistent across all four strains tested here – i.e. the wild-type JE2, *dtpT/opp3A* transposon-mutants and the double-mutant strain. Indeed, the mean eAUC across peptides in cluster 1 was comparable to those observed for the positive control well of each plate for each of the four strains, including the *dtpT/opp3A* double mutant ($\text{eAUC} \geq 15$). Such an observation strongly suggests that peptides in this group can be effectively utilised for cellular metabolism independently of either of the two known peptide transporters in *S. aureus*, implying the existence of an alternative route of utilisation (though this does not necessarily exclude the possibility that DtpT or Opp3 also contribute toward internalisation of these peptides).

Cluster 2 – Dependant on either DtpT or Opp3

Utilisation of peptides belonging to cluster 2 ($n = 41$) was maintained in single mutant strains lacking either *dtpT* or *opp3A* but abolished in the double-mutant strain, suggesting that either transport system may facilitate transport of these molecules, and this transport is sufficient to drive cellular metabolism in either case. Notably, our data represent the first clear example of Opp3-mediate utilisation of dipeptides and reveal some redundancy amongst DtpT and Opp3 substrates.

Clusters 3 and 4 – DtpT-dependant utilisation

Two of the clusters identified in our analysis – clusters 3 ($n = 58$) and 4 ($n = 41$) – corresponded to peptides for which a stronger metabolic output was observed in the wild-type and *opp3A* single-mutant strains compared to either of the two *dtpT* mutant strains. These patterns provide compelling evidence that DtpT serves as the primary route of uptake for the almost 100 peptides constituting these two clusters.

The most immediate distinction between these two clusters is that peptides in cluster 3 demonstrated a more pronounced phenotype (that is, a greater difference in signal between “positive” and “negative” utilisation) when compared to those in cluster 4. This difference in signal magnitude indicates that cluster 3 peptides are utilised more effectively than cluster 4 peptides, likely resulting from a higher rate of internalisation by DtpT or more productive downstream catabolism following uptake. For both clusters, we note that the metabolic signal of the *dtpT/opp3A* double mutant strain is slightly reduced on-average when compared to the

ntpT single mutant, suggesting that Opp3 may contribute toward internalisation of some peptides within these clusters (though, if this is the case, the activity of Opp3 is not sufficient to produce a wild-type metabolic output).

Cluster 5 – DtpT-deleterious patterns

For peptides in cluster 5 ($n = 16$), we observed an increased metabolic signal in either *ntpT* mutant strain compared to the wild-type and *opp3A* mutant strains, mirroring the DtpT-dependant pattern observed in clusters 3 and 4 (Figure 22B). The observation that a metabolic signal is restricted in the wild type but produced exclusively in *ntpT* mutant strains suggests that cluster 5 peptides can be utilised by *S. aureus*, but only in the absence of DtpT.

While the exact mechanistic basis for this pattern is unclear, we postulate that DtpT-mediated internalisation of these peptides may be cytotoxic under the conditions tested here. However, a strong signal in the *ntpT* mutant strain suggests an alternate route of internalisation for which toxicity is not observed, perhaps due to a lower overall flux which prevents toxic over-accumulation of the peptide.

Clusters 6 and 7 – Cannot be utilised

The final two clusters in our analysis – clusters 6 ($n = 53$) and 7 ($n = 58$) – correspond to peptides for which a weak signal was observed on average across the tested strains. We therefore conclude that these peptides cannot be effectively utilised as sole nitrogen sources to drive metabolism in *S. aureus*. There are a number of potential factors which may contribute to a lack of utilisation across all four strains in this assay, including: lack of a viable route of cell entry (or an otherwise low rate of internalisation), incomplete pathways for catabolism of the peptide, or peptide toxicity. We do note that the mean eAUC values recorded for cluster 6 are slightly higher than those in cluster 7 across the four strains, perhaps indicating that some peptides in cluster 6 can be utilised to produce an intermediate metabolic signal in the strains tested here. However, if this is the case, utilisation of these peptides by *S. aureus* is not efficient enough to produce a strong signal under the conditions of the PM assay.

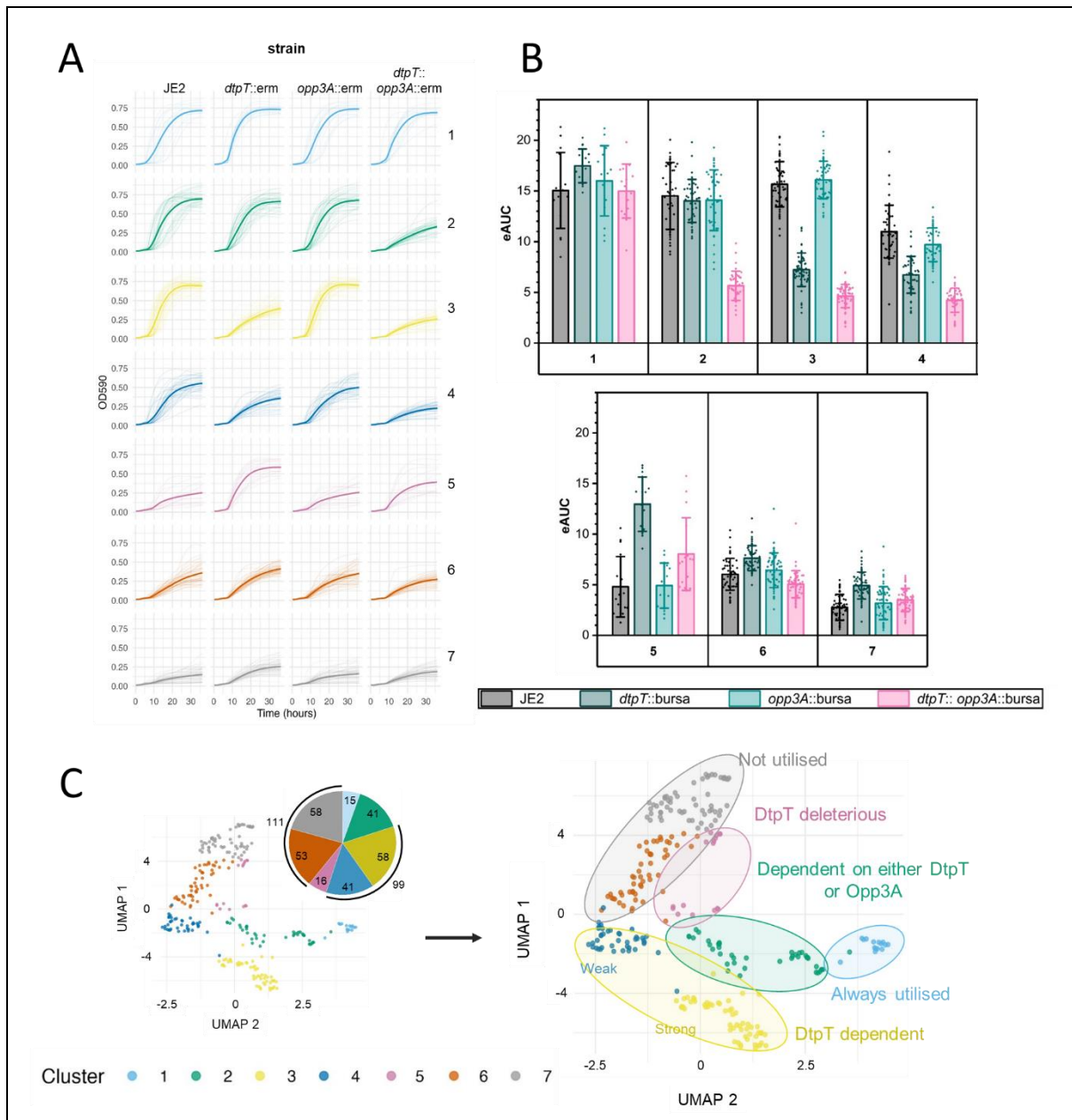


Figure 22 – Cluster and UMAP analysis reveal differential peptide utilisation patterns across *dtpT* and *opp3A* mutant strains. (A) Phenotype microarray signal curves for each peptide/strain combination were subjected to k-means clustering (k-means = 7) to identify strain-specific peptide utilisation patterns. All signal curves for each cluster are overlaid and separated by strain. Mean signal curves for each strain are indicated by the bold line. (B) Empirical area under the curve (eAUC) was calculated for each signal curve and sorted by strain and cluster. Bars show the mean eAUC for each strain within a given cluster. Error bars indicate standard deviation in each case. (C) Signal curves were subjected to UMAP analysis. Peptides belonging to the same cluster are grouped by colour on the UMAP plot and the number of peptides in each cluster is shown in the corresponding pie chart.

DtpT and Opp3 preferentially facilitate the utilisation of di/tri-peptides containing specific amino acids

Considering clusters 2 – 4 in our analysis, our PM dataset implicates DtpT in the utilisation of at least 140 (approximately 50%) of the peptides tested here, with 41 of these substrates being shared with Opp3. On the other hand, the 111 peptide constituting clusters 6 – 7 are not effectively utilised across any of the strains tested here, while the 15 cluster 1 peptides are utilised irrespective of the presence of DtpT or Opp3. The observation that transporter-specific utilisation is limited to a subset of the peptides tested here suggests distinct preferences among the functional targets of these systems in *S. aureus*. To explore this idea further, we next aimed to determine whether specific amino acids were enriched within the peptides of each cluster by applying a Fisher exact test (Figure 23). Given the limited panel of tripeptides assessed here, we focussed our analysis on the 246 standard dipeptides present in the assay (Figure 23A). In doing so, we identified a number statistically significant trends which point toward preferences in the putative substrates of DtpT and Opp3 (Figure 23B). A full list of statistically significant amino acid enrichments and their associated false discovery rate (FDR) q values is provided in Table 5.

Gln-containing peptides are utilised independently of DtpT and Opp3

The strongest enrichment identified in our analysis was for Gln-containing peptides within cluster 1 ($q = 2.89\text{E-}9$). In fact, Gln-containing peptides constituted 60% (9/15) of the peptides in cluster 1 and these peptides represent 75% (9/12) of the Gln-containing dipeptides assessed here, despite cluster 1 comprising only 5.3% (15/282) of the peptides included in the assay. From this, we can conclude that most of the glutamine-containing peptides assessed here can be utilised by *S. aureus* independently of either DtpT or Opp3.

Asp- and Glu-containing peptides are enriched among putative DtpT substrates

Our analysis revealed that peptides containing either aspartate ($q = 6.41\text{E-}5$) or glutamate ($q = 4\text{E-}6$) were enriched within cluster 3. Collectively, these peptides constituted 56.9% (33/58) of the cluster and included 68% (17/25) of aspartate-containing peptides and 80% (20/25) of glutamate-containing peptides tested. Such an observation provides compelling evidence that Asp- and Glu-containing peptides are preferred substrates for DtpT and suggests that utilisation of these peptides is a discrete physiological function of the transporter. For dipeptides, this preference is seemingly independent of amino acid arrangement, with comparable frequencies when aspartate or glutamate are incorporated in the N- or C-terminal position.

Redundant routes of utilisation for Arg-containing peptides

One of the most significant enrichments identified in our analysis was observed for Arg-containing peptides within cluster 2 ($q = 8.68\text{E-}8$). These peptides made up 36.6% (15/41) of peptides in cluster 2 and 62.5% (15/24) of Arg-containing peptides included in the assay. From this, we conclude that both DtpT and Opp3 contribute toward utilisation of arginine-containing dipeptides.

Other trends

In addition to the trends outlined above, we observe a highly significant enrichment of tyrosine-containing dipeptides in cluster 7 ($q = 8.68\text{E-}8$) demonstrating that these peptides are rarely able to support the metabolism of *S. aureus* under the conditions tested here. A number of other statistically significant enrichments were identified from our analysis, though these associations were not as strong as those already discussed ($q \geq 1\text{E-}3$). This includes an enrichment of leucine-containing peptides in cluster 2 ($q = 2.59\text{E-}2$), suggesting that both DtpT and Opp3 may contribute to utilisation of many of these peptides. Furthermore, we observe that serine-containing peptides are enriched in cluster 5 ($q = 1.03\text{E-}2$), suggesting that many serine-containing peptides can only drive metabolism of *S. aureus* when DtpT is absent.

Table 5. Significantly enriched amino acids within each cluster with associated FDR q-values

Amino acid	Enriched cluster	FDR q value
gln	1	2.89E-09
arg	2	8.68E-07
tyr	7	8.68E-07
glu	3	4.00E-06
asp	3	6.41E-05
leu	2	2.59E-02
met	6	1.22E-02
ser	5	1.03E-02

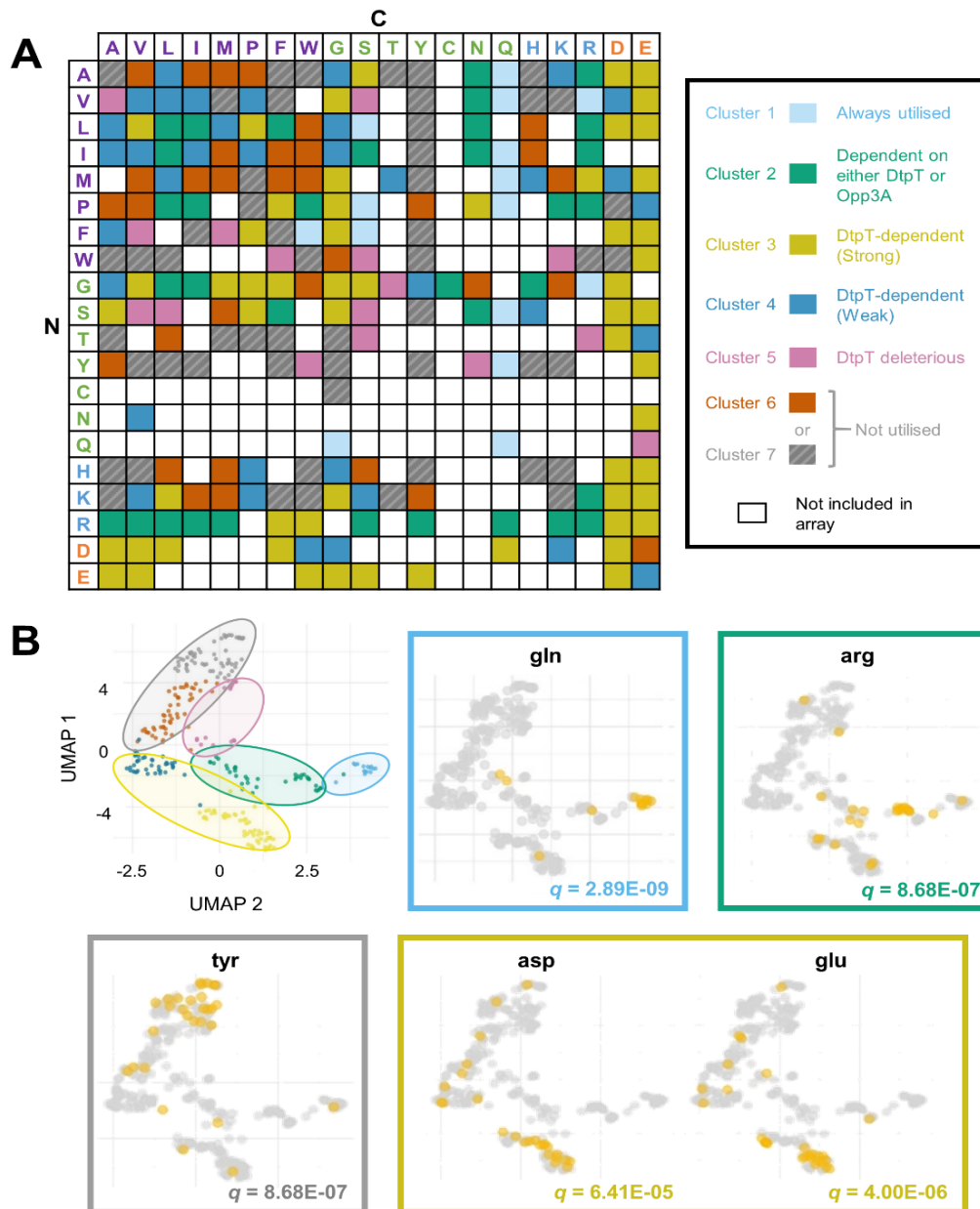


Figure 23 – Specific amino acids are enriched in peptides belonging to a common utilisation pattern. (A) Grid representation of all possible dipeptide structures arranged with N-terminal amino acids on the Y axis and C-terminal amino acids on the X axis. Amino acids are displayed as single-letter symbols and colour-coded according to their chemical properties (purple = hydrophobic/non-polar, green = polar/unique, blue = basic, orange = acidic). Coloured cells correspond to peptides included in the phenotype microarray analysis and their corresponding clusters. (B) Enriched amino acids within each cluster were identified via a Fisher exact test. Positions of peptides containing the five most significantly enriched amino acids are shown overlayed on the original UMAP plot and separated by cluster.

Using bacterial growth assays to verify Arg-peptide utilisation phenotypes

Our phenotypic screens implicate DtpT in the transport of diverse peptide structures, but further validation is required to confirm this DtpT-dependant peptide utilisation under relevant growth conditions. A widely utilised strategy for studying bacterial nutrient transporters is to compare the growth kinetics of wild-type and transporter-deficient mutant strains under conditions where a given nutrient is supplied as the sole source of carbon or nitrogen (exemplified in [Jolkver et al., 2009](#); [Severi et al., 2010](#)). However, these approaches are complicated by the requirement to supplement growth media with a number of essential amino acids for which *S. aureus* is auxotrophic, including arginine (see [page 80](#)) ([Emmett & Kloos, 1979](#); [Reslane et al., 2024](#)). Here, we instead capitalise on the arginine auxotrophy of *S. aureus* in order to verify the DtpT/Opp3A-dependence patterns of three Arg-containing peptides included in our PM assays – Arg-Ala, Arg-Asp and Arg-Phe. It is worth noting that all three of these peptides demonstrated a positive utilisation pattern by the wild-type JE2 under PM conditions, confirming that they are each catabolised following uptake and can thereby, theoretically, serve as arginine sources to drive growth under arginine limitation.

JE2 is auxotrophic for arginine in CDMG

Arginine auxotrophy in *S. aureus* USA300 was previously observed in a chemically defined medium containing glucose (CDMG) and lacking arginine (CDMG - R) ([Jeong et al., 2022](#); [Reslane et al., 2022](#)). To confirm this phenotype, JE2 and the derivative *ntpT* and *opp3A* single/double mutant strains were each grown over 24 hours in CDMG or CDMG - R ([Figure 24A/C](#)). We observed robust growth across all strains in CDMG, while growth in CDMG - R was strictly limited as exemplified by a dramatic reduction in the end-point OD₆₀₀ for each strain ([Figure 24C](#)). Together, these data demonstrate that all four strains are dependent on exogenous arginine for growth under the conditions used here.

Arg-peptides restore growth under arginine limitation only when a viable route of uptake is present

In order to verify the phenotypes identified in our PM data, we supplemented CDMG - R with two cluster 3 peptides (Arg-Asp and Arg-Phe) and one cluster 2 peptide (Arg-Ala) and separately inoculated these media with JE2 and the transposon mutant-derivative strains ([Figure 24B-C](#)). Based on the utilisation patterns observed under PM conditions, we predicted that Arg-Asp and Arg-Phe would restore growth only in strains with an intact *ntpT* gene, whilst Arg-Ala would restore growth in all but the double-mutant strain.

As expected, supplementing with Arg-Asp was able to restore growth in a DtpT-dependant manner, while Arg-Ala restored robust growth in the wild type and single mutant strains but not in the double-mutant strain. These patterns match the transporter-specific utilisation patterns observed in our phenotypic screens. In contrast, supplementing with Arg-Phe restored growth in all but the double-mutant strain (similarly to the cluster 2 peptide Arg-Ala), despite this peptide belonging to cluster 3. We interpret this finding as proof that Opp3 can facilitate the transport of Arg-Phe to suppress arginine auxotrophy, though this transport is not sufficient to drive cellular metabolism under PM conditions (perhaps due to a low transport rate which limits intracellular accumulation and may thereby prevent effective utilisation of the peptide as a nitrogen source). Such a discrepancy highlights a potential limitation in the sensitivity of the PM assay utilised here.

It is worth noting that Arg-Asp and Arg-Phe were each supplemented to a final concentration of 1 mM in this assay, mimicking the concentration of arginine in complete CDMG. However, inclusion of Arg-Ala at this concentration was found to facilitate high levels of background growth in the otherwise deficient *dtpT opp3A* double-mutant strain (as demonstrated in [Appendix 11](#)). Hence, a lower concentration of Arg-Ala (0.5 mM) was used here in order to more clearly distinguish the phenotype of the transport-deficient strain. The observation that growth of this strain is permitted at high Arg-Ala concentrations may suggest the existence of an alternative route of uptake for this peptide, and this may be relevant in environments where the peptide is present at millimolar (or greater) concentrations.

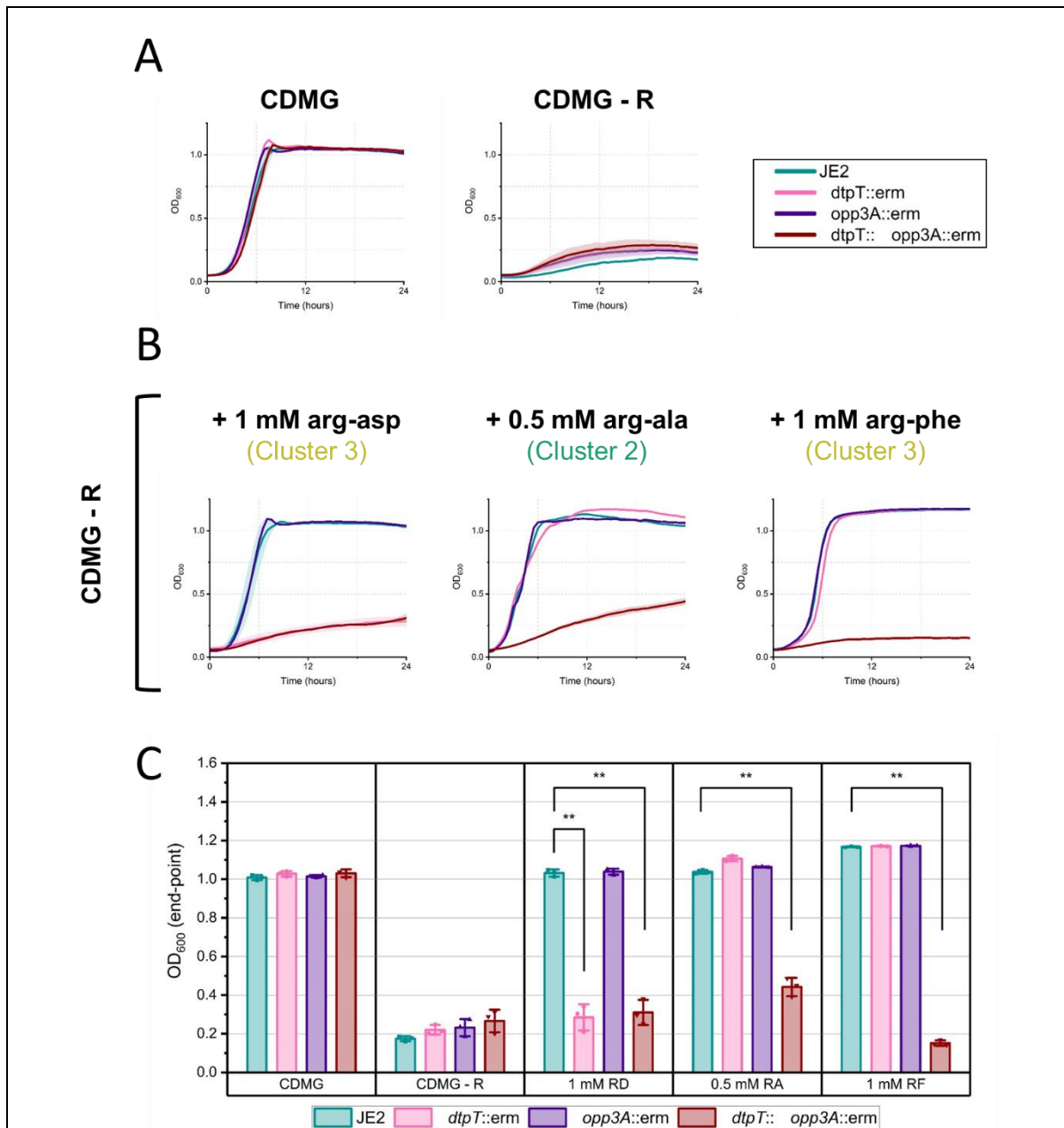


Figure 24 – Arginine-containing dipeptides restore growth under Arg limitation only when a viable route of uptake is available. In each case, strains were grown over 24 hours and OD₆₀₀ was measured every 30 minutes for three biological replicates. Curves indicate the mean values for each reading and the standard deviation in each case is indicated by the shaded area. **(A)** Growth of *S. aureus* strain JE2 and mutant derivatives in CDMG and CDMG – R. Growth of all strains is dependent on an exogenous source of arginine. **(B)** Growth of *S. aureus* strain JE2 and mutant derivatives in CDMG – R supplemented with three dipeptides, as labelled. Corresponding phenotype microarray clusters are also provided. **(C)** End-point OD₆₀₀ values for growth experiments in A and B. Brackets indicate significance as calculated by an unpaired student's t test (** = $p < 0.01$).

Expressing *ntpT* in-trans restores Arg-peptide utilisation in deficient strains

The phenotypes observed in our Arg-peptide growth experiments provide compelling evidence for transporter-dependent utilisation of the chosen peptides. However, *in-trans* complementation is required to verify that the observed phenotypes are entirely dependent on the presence of the transposon-interrupted genes of interest. Given that all three of the tested peptides are seemingly utilised via *DtpT*, we decided to focus our efforts on the design on a suitable complementation vector for *in-trans* expression of *ntpT* only. To achieve functional complementation of *ntpT* in *S. aureus*, expression vectors were constructed by adapting two existing *E. coli* / *S. aureus* shuttle vector architectures.

The first expression plasmid was prepared by adapting the *S. aureus* expression vector pRAB11, which utilises an anhydrotetracycline (ATc)-inducible $P_{xyl/tet}$ promoter in tandem with a TetR repressor to enable stringent control of protein expression levels in response to varying inducer concentrations ([Helle et al., 2011](#)). In this case, the native USA300 *ntpT* gene was inserted immediately downstream of the $P_{xyl/tet}$ promoter to produce the expression plasmid pRAB11-*DtpT* ([Figure 25A](#)). Both this and the parent vector were transformed into the *ntpT* mutant strain and utilisation of the cluster 3 peptide Arg-Asp was assessed, as before. In the absence of inducer, the pRAB11-*DtpT* vector was able to restore growth of the *ntpT* mutant strain after an extended lag period ([Figure 25B](#)). Unexpectedly, addition of the inducer ATc inhibited growth in the presence of Arg-Asp ([Figure 25C](#)). This inhibition was seemingly concentration dependant, with 0.4 μ M ATc leading to near complete inhibition of growth over the 24-hour period tested. The addition of equivalent concentrations of ATc did not have a significant effect on growth for a wild-type JE2 strain carrying the empty pRAB11 vector ([Figure 25C](#)). In comparison, 0.05 μ M ATc did permit some growth at later timepoints, albeit to a drastically lower OD_{max} when compared to uninduced cultures. From this, we can conclude that expression of *ntpT* under the ATc-inducible system used here is detrimental in *S. aureus*, perhaps resulting from toxicity when the protein is overexpressed exogenously. As a result, this vector is not suitable for complementation of *ntpT*-dependant phenotypes.

A second vector was constructed in the low copy-number shuttle plasmid pSK5630, incorporating the replication and partition elements of the *S. aureus* multidrug resistance plasmid pSK1 ([Figure 26A](#)) ([Grkovic et al., 2003](#)). In this case, the entire *ntpT* gene was inserted alongside 1080 bp of upstream sequence from the USA300 genome, in an attempt to reconstitute the native *ntpT* promoter sequence and thereby faithfully recapitulate wild-type gene expression levels. This resulted in the pSK56pT-*DtpT* complementation vector ([Figure 26A](#)). A control plasmid was also constructed containing only the 1080 bp upstream sequence, resulting in the

pSK56pT empty vector (Figure 26A). Both constructs were transformed into the *dtpT* single mutant and *dtpT/opp3A* double mutant strains and the resultant transformants were assessed for their ability to utilise the three arginine-containing peptides assessed above. In each case, *in-trans* complementation with pSK56pT-DtpT restored growth in the deficient strains to near wild-type levels (Figure 26B-C). As a result, we can conclude that DtpT is sufficient to facilitate utilisation of all three peptides.

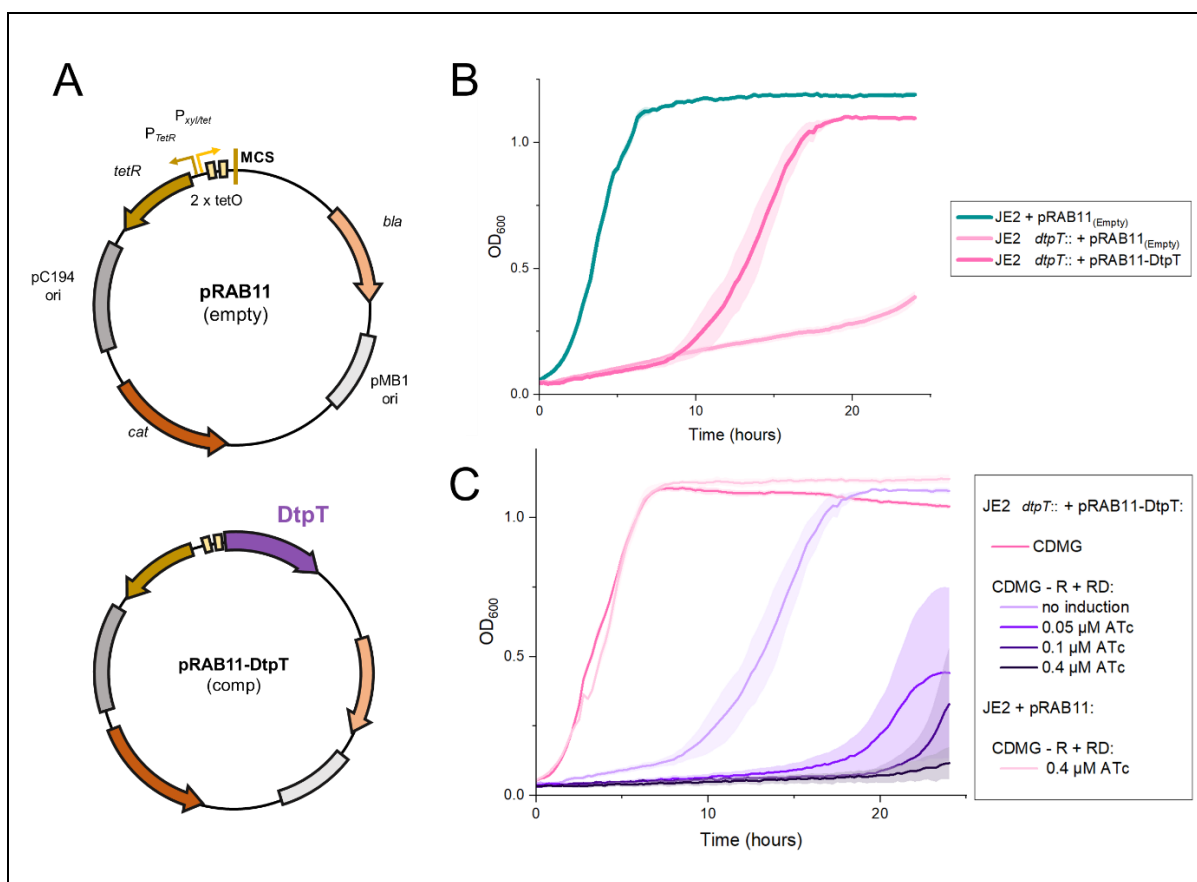


Figure 25 – pRAB11 partially complements a *dtpT* mutant without induction. (A) Overview of a *dtpT* complementation vector based on the *S. aureus* / *E. coli* shuttle vector pRAB11 (upper). Features implicated in plasmid function and replication are coloured in grey. Antibiotic resistance markers are coloured in orange. Elements comprising the tetracycline-inducible expression system are coloured in gold. (lower) The *dtpT* gene was inserted downstream of the dual tetO operator sequence to generate the complementation vector pRAB11-DtpT.

(B) JE2 and a *dtpT* mutant derivative carrying pRAB11 or the pRAB11-DtpT were grown in CDMG – R supplemented with 1 mM arg-asp in the absence of the inducer anhydrotetracycline (ATc).

(C) The complemented strain *dtpT*:: + pRAB11-DtpT was grown in either CDMG or CDMG – R + RD with increasing concentrations of ATc, as indicated by the key. Growth of the wild-type JE2 strain carrying pRAB11 in the presence of inducer is included for comparison (light pink). In each case, strains were grown over 24 hours and OD₆₀₀ was measured every 30 minutes for three biological replicates. Curves indicate the mean values for each reading and the standard deviation in each case is indicated by the shaded area.

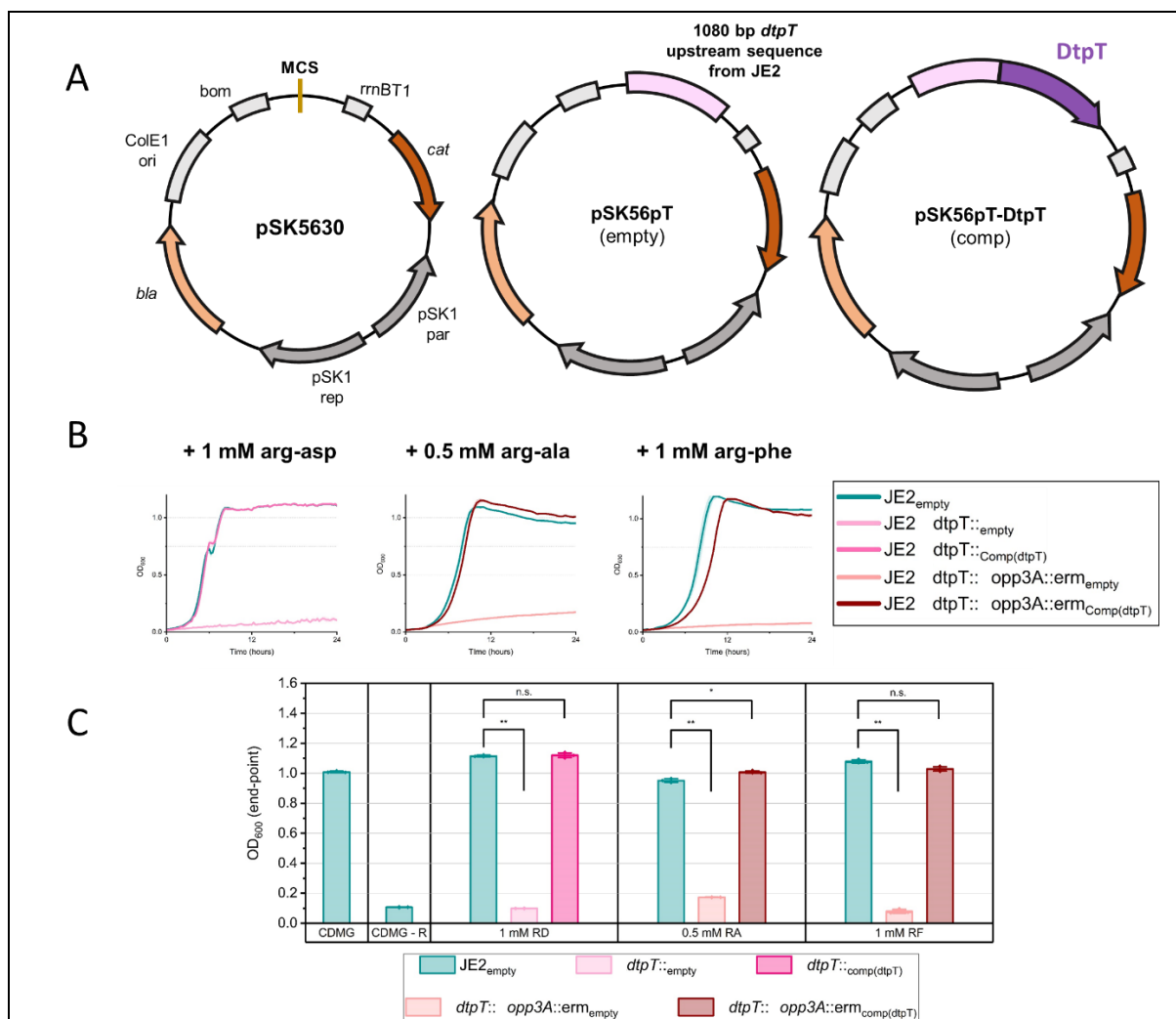


Figure 26 – In trans complementation of *dtpT* restores growth of deficient strains in the presence of Arg-containing peptides. (A) Overview of *dtpT* complementation vectors based on the *E. coli* / *S. aureus* shuttle vector pSK5630 (left). Features implicated in plasmid function and replication are coloured in grey. Antibiotic resistance markers are coloured in orange. (middle) pSK56pT was created by the addition of approximately 1080 bp of sequence immediately upstream of the *dtpT* locus from JE2, encompassing the native *dtpT* promoter. (right) The *dtpT* gene was added downstream of this promoter sequence to generate the complementation vector pSKpT-DtpT. (B) JE2 and mutant derivatives carrying pSK56pT or the complementation vector were grown in CDMG – R supplemented with three dipeptides, as labelled. In each case, strains were grown over 24 hours and OD₆₀₀ was measured every 30 minutes for three biological replicates. Curves indicate the mean values for each reading and the standard deviation in each case is indicated by the shaded area. (C) End-point OD₆₀₀ values for growth experiments in B. Brackets indicate significance as calculated by an unpaired student's t test (* = p < 0.05; ** = p < 0.01; n.s. = not significant).

Discussion

The promiscuous nature of peptide transport proteins and their functional redundancy in many organisms have made these systems historically challenging to characterise regarding substrate range and preference. Molecular dynamics simulations and similar computational methods have been combined with biochemical and structural methods in order predict substrate preferences in POTs, but experimental evidence for the applicability of these models to biological systems is lacking ([Kotov et al., 2023](#); [Chenghan Li et al., 2022](#); [Samsudin et al., 2016](#)). Here, we present an extensive cell-based analysis of peptide transporter function in *S. aureus* utilising a high-throughput phenotypic screen. Therein, we implicate DtpT in the utilisation of 140 peptides (comprising clusters 2, 3 and 4 in our analysis), with utilisation of 41 of these peptides also being enabled by Opp3 (i.e. cluster 2) ([Figure 22](#)). Overall, our findings demonstrate that DtpT is the primary route of uptake for dipeptides in *S. aureus*. Our results also suggest a preference for Asp/Glu containing peptides among putative DtpT substrates identified here, and demonstrate that utilisation of Arg-containing peptides is redundant between DtpT and Opp3 ([Figure 23B](#)). Surprisingly, we also demonstrate that many Gln-containing peptides are utilised by *S. aureus* independently of the two characterised peptide transporters, suggesting alternative uptake mechanisms for these peptides ([Figure 23B](#)).

The observation that peptides incorporating specific amino acids are associated with defined routes of uptake in *S. aureus* may indicate distinct roles for DtpT and Opp3 in nutrient acquisition. Further evidence supporting this hypothesis is presented in later chapters and, therefore, discussion of this topic will be reserved for later (i.e. [Chapter 6](#), at the end of this thesis). Here, I will instead speculate on the molecular basis for some of the utilisation patterns observed in our PM analysis, and compare the data presented in this chapter with the existing literature.

An additional route of peptide uptake specifically targets Gln-containing dipeptides

By applying a phenotypic screening approach, we have demonstrated that a small group of dipeptides comprising cluster 1 in our analysis (15 of the 282 tested here) are robustly utilised by *S. aureus* under PM conditions in the absence of the major peptide transporters DtpT and Opp3 ([Figure 22](#)). We believe that this serves as compelling evidence for the existence of at least one alternative route of peptide utilisation which is independent of both known peptide transporters in *S. aureus*, as was previously suggested by *Lehman* and colleagues ([Lehman et al., 2019](#)). Notably, Gln-containing peptides were strongly enriched in cluster 1 and accounted for more

than half of the peptides in this cluster ([Figure 23B](#)). Hence, our data supports a model in which an additional route of peptide uptake in this bacterium is specific for Gln-containing dipeptides (and, potentially, Gln-containing peptides more generally).

Perhaps the most straightforward explanation for the phenotypic pattern observed in cluster 1 would be the presence of one or more yet-uncharacterised transporters which permit uptake of the peptides in this cluster. *S. aureus* strain JE2 encodes 362 predicted transporter proteins (according to TransportDB 2.0), and the vast majority of these systems remain uncharacterised. Given their evolutionary relationship to Opp3 ([D. Yu et al., 2014](#)), the most obvious candidates for putative peptide transporter systems in this strain are the two yet-uncharacterised Opp-homologues – Opp4 and ACME Opp. The Opp4 transport system is a yet-uncharacterised Opp-homologue which is widespread in *S. aureus* and is likely to have been generated via duplication of the *opp3BCDFA* operon ([D. Yu et al., 2014](#)). Given their close evolutionary relationship, we propose that Opp4 is likely to be a peptide transporter similarly to Opp3 and is, therefore, a strong candidate to fulfil the role of the alternative Gln-peptide transport system in *S. aureus*. An additional Opp-homologue present in USA300 and derivative strains (ACME Opp) may also contribute toward peptide transport, though this system is more closely related to Opp1/Opp2 and is, therefore, more likely to play a role in metal ion transport ([D. Yu et al., 2014](#)). Follow-up experiments should aim to determine whether either of these systems facilitates the utilisation of cluster 1 peptides. This could be achieved by introducing transposon-interrupted variants of *opp4A* and *ACME oppA* into the existing *dtpT/opp3A* mutant strain and repeating the PM assays described, looking for differences in peptide utilisation imparted by these new mutations (as compared to the existing double mutant data set).

Alternatively, strong utilisation of cluster 1 peptides could be explained by extracellular cleavage (or modification), followed by internalisation of the resultant molecular products. *S. aureus* is known to produce at least 10 extracellular proteases with diverse roles in nutrient acquisition and immune modulation (as described in [page 67](#)). Some of these enzymes have well-defined peptide targets or demonstrate exquisite sequence specificity (for instance, the V8 protease SspA almost exclusively cleaves peptide bonds immediately after a Glu residue) ([Frey et al., 2021](#)). However, some secreted proteases – specifically, the staphopains ScpA and SspB, as well as the serine protease-like Spl enzymes) remain poorly characterized in regard to their enzymatic targets and physiological roles ([Tam & Torres, 2019](#)). Hence, these enzymes may cleave Gln-containing peptides in order to liberate free Gln to serve as a nitrogen source for *S. aureus* under PM conditions. More comprehensive biochemical characterisation of the proteases described here is necessary to determine whether any of these enzymes have the capacity to fulfil this role.

Peptides in cluster 5 may be toxic if accumulated by DtpT

Peptides belonging to cluster 5 in our PM assays demonstrated a cryptic utilisation pattern, in which a positive utilisation signal was observed exclusively in strains lacking *dtpT*. Such a pattern may suggest that the DtpT-mediated transport of cluster 5 peptides is toxic under PM conditions. The vast majority of antimicrobial peptides identified to-date are significantly larger than the peptides assessed here (typically 10-100 residues in length), though the existence of di/tripeptides with antimicrobial activity is not unheard of ([Di Somma et al., 2020](#)). Previously, transporter-mediated accumulation of leucine-containing di/tripeptides in *E. coli* was found to inhibit growth, seemingly due to inhibition of isoleucine biosynthesis resulting from over-accumulation of leucine in the cytoplasm ([Tavori et al., 1981](#)). This study exemplifies how peptide transport – while generally considered beneficial to bacterial growth – can become a liability in the context of specific peptides due to the complex metabolic and regulatory consequences of their accumulation. Similar short peptides with activity against *S. aureus* have also been reported, including Ser-Asn-Ala and Gly-His-Lys ([Hong et al., 2015](#); [Liakopoulou-Kyriakides et al., 1997](#)). While the activity of the latter is seemingly conveyed by its interactions with copper ([Bradford et al., 2009](#)), the antimicrobial mechanism of Ser-Asn-Ala is yet to be elucidated.

In our analysis, we note that Ser-containing peptides are significantly enriched within cluster 5, suggesting that Ser-peptides may be generally cytotoxic when accumulated in the cell by DtpT. Such a model could be readily confirmed by carrying out classic antimicrobial susceptibility assays with pure Ser-containing peptides against wild-type and *dtpT* mutant *S. aureus* strains, though we were unable to carry out such experiments in this work due to the relevant peptides being commercially unavailable or prohibitively expensive. It is worth noting that very little is currently known regarding Ser metabolism in *S. aureus*, making it difficult to speculate on a potential mechanism for the apparent toxicity observed for these Ser-peptides in our PM experiments. It is also worth noting that – with the exception of common peptide metabolites or host protein degradation products – *S. aureus* is unlikely to encounter any single di/tripeptide at a high concentration *in vivo*. Hence, if these peptides are indeed toxic to *S. aureus*, this is unlikely to be physiologically relevant under standard infection conditions.

Assuming that the lack of utilisation of cluster 5 peptides by JE2 is a result of toxicity (as outlined above), how could utilisation of these peptides in the *dtpT* mutant strain be permitted? One explanation would be the existence of a secondary transporter for these peptides – perhaps, one with a lower transport rate which prevents toxic accumulation. Close scrutiny of the associated signal curves for peptides in cluster 5 as well as their spatial relationship in the UMAP plot reveals

the existence of two subpopulations within this cluster. Briefly, these subpopulations divide cluster 5 peptides into those for which a strong signal is observed only within the *dtpT* single mutant (cluster 5a, n = 8) and those for which utilisation is observed in both the single and double mutant strains (cluster 5b, n = 8) (Figure 27A-C). Extending the model described above in which DtpT-mediated transport is toxic for cluster 5 peptides, we can infer that Opp3 may serve as an alternative route of utilisation for cluster 5a peptides (as outlined in figure 27D). Such a model would explain why peptide utilisation is permitted in the *dtpT* single mutant strain but suppressed when *opp3A* is also disrupted.

PM assays recontextualise earlier observations regarding peptide utilisation in *S. aureus*

To date, the most comprehensive study comparing routes of peptide utilisation in *S. aureus* was carried out by Hiron and colleagues in 2007. In their study, the authors utilised a minimal medium depleted of Glu and Pro to demonstrate the utilisation of Glu/Gln-containing peptides as a precursor for these essential amino acids (Hiron et al., 2007). A few interesting comparisons can be found between their findings and the PM utilisation patterns observed in our work. Firstly, Hiron and colleagues demonstrated DtpT-specific utilisation for diverse Glu-containing di/tripeptides (Hiron et al., 2007), consistent with the finding that these peptides are specifically enriched within the DtpT-dependent cluster 3 in our analysis (Figure 23B). Specifically, both methods identify DtpT as the major route of uptake for both Glu-Glu and Val-Glu. Intriguingly, however, Hiron and colleagues noted that a *dtpT/opp3A* double mutant was unable to utilise the dipeptide Pro-Gln (Hiron et al., 2007), while our data suggests this peptide is utilised independently of both transporters (i.e. cluster 1) (Figure 23A). Such a discrepancy may be explained by differences in the methodology between these two experiments. Indeed, the utilisation of Gln-containing peptides independently of DtpT and Opp3 may be a specific trait of JE2 (and, potentially, other USA300-derivative strains) which is not shared by the RN6390 strain utilised in the earlier study, perhaps enabled by the presence of distinct genetic factors in JE2 (for instance, ACME and the associated ACME Opp transport system) or differences in transporter expression between these strains.

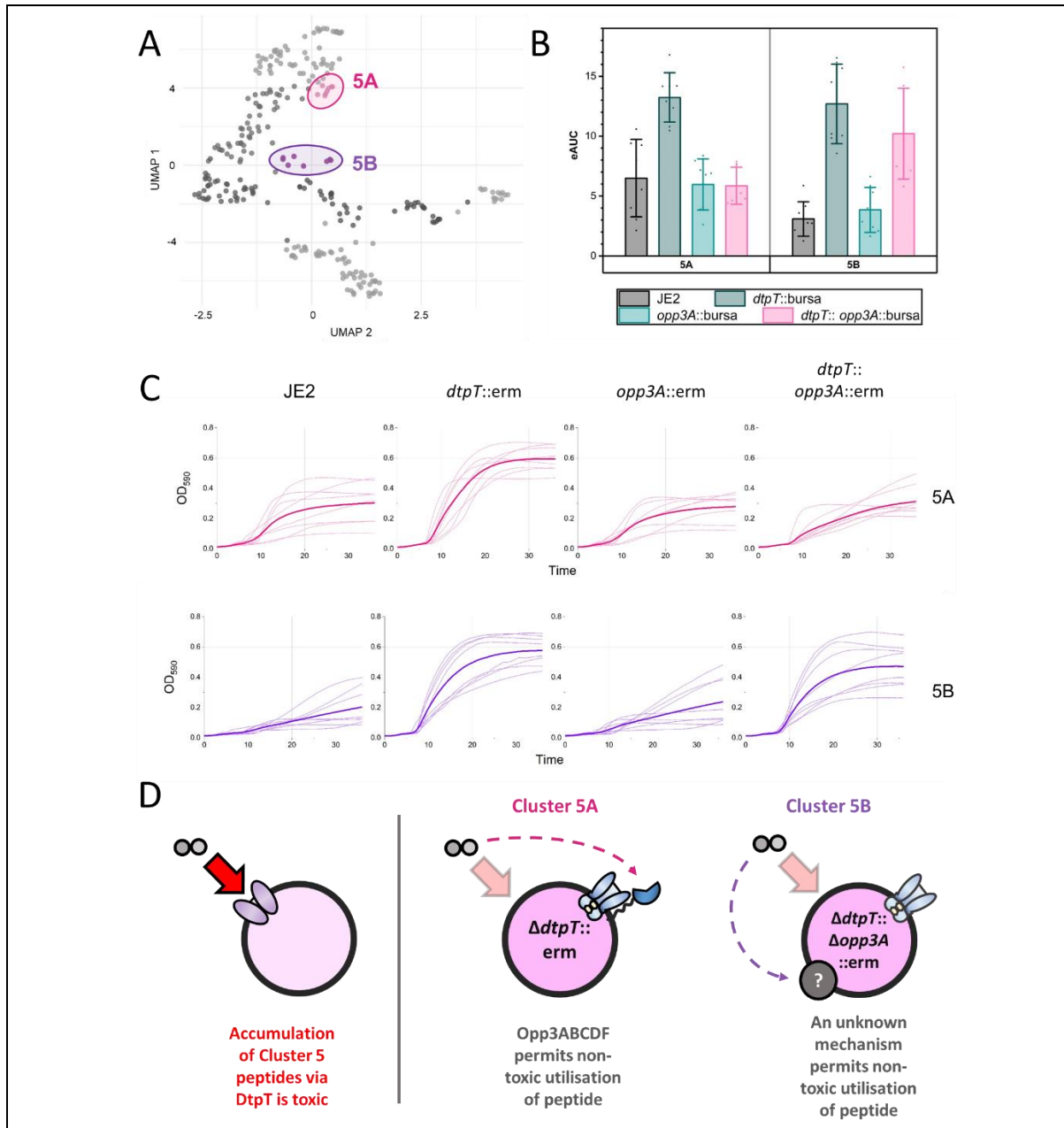


Figure 27 – Cluster 5 encompasses two distinct phenotypic patterns. (A) Peptides belonging to cluster 5 form two distinct spatially clustered groups during UMAP analysis (labelled 5A and 5B), representing the two phenotypic utilisation patterns observed in the data. (B) eAUC was calculated for each signal curve and sorted by strain for each of the two phenotypic patterns observed in cluster 5. Bars show the mean eAUC for each strain. Error bars indicate standard deviation in each case. (C) Overlaid signal curves for the two distinct phenotypic patterns. Mean signal curves for each strain are indicated by the bold line. (D) Proposed routes of utilisation for peptides in each of the cluster 5 subpopulations.

In their more recent study, *Lehman* and colleagues demonstrate that Pro-Arg can be utilised by *S. aureus* JE2 independently of DtpT and Opp3A to facilitate growth under Arg limitation ([Lehman et al., 2019](#)). Comparatively, our PM assays implicate both transport systems in the utilisation of this dipeptide (**Figure 23A**). Hence, we deduce that an alternative transport system facilitates the uptake of Pro-Arg in *S. aureus* and this activity is sufficient to relieve arginine auxotrophy, but is not sufficient to drive cellular metabolism under PM conditions (as similarly observed for the Opp3-dependent utilisation of Arg-Phe in our own growth assays). Notably, this study also demonstrates Opp3-dependent utilisation of diverse Arg-containing peptides ranging from tripeptides to octapeptides ([Lehman et al., 2019](#)); such a finding is consistent with the Arg-peptide specificity observed among putative dipeptide substrates of Opp3 in our PM assays (**Figure 23B**).

Finally, structural analysis of the predicted SBP GmpC (an orthologue of the *E. coli* Met-binding SBP MetQ) from the uncharacterised *S. aureus* ABC transporter GmpABC has demonstrated that this protein purified from *E. coli* bound to the dipeptide Gly-Met ([W. A. Williams et al., 2004](#)). In fact, comparison between the binding cavities of GmpC and MetQ reveals the presence of an expanded pocket in GmpC which specifically enables the binding of X-Met dipeptides ([W. A. Williams et al., 2004](#)). Hence, GmpC has been proposed as a Met-specific dipeptide transporter in *S. aureus*. In spite of this, our PM assays implicate DtpT as the primary transporter required for utilisation of Gly-Met (**Figure 23**). Therefore, while existing evidence demonstrates that GmpC does bind Gly-Met, our results suggest that GmpABC does not mediate robust transport of the dipeptide sufficient to drive cellular metabolism under PM conditions.

It is worth noting, however, that very little is known regarding the expression of GmpABC; hence, it is unclear whether this transporter would even be expressed by *S. aureus* under the conditions tested here. Furthermore, our growth experiments highlight how some peptides (e.g. Arg-Phe) are primarily utilised by DtpT but can also be utilised via a secondary transport system (in the case of Arg-Phe, Opp3) (**Figure 24**), despite no such utilisation being observed in the PM assay (**Figure 23A**). We speculate that this discrepancy results from a limitation in the sensitivity of the PM assay; in other words, low-flux transport systems may fail to meet the cellular requirement for nitrogen, and thereby fail to produce a positive metabolic signal despite facilitating more gradual uptake of a given peptide. Given the broad relevance of methionine in cellular physiology and the limitation of this amino acid in some physiological niches ([Krismer et al., 2014](#)), the existence of an alternative transport system for scavenging of Met-containing peptides is perhaps expected.

Chapter 4 – Understanding substrate recognition and transport in DtpT through biochemical methods

Introduction

In [Chapter 3](#) of this thesis, we utilised a phenotypic screening approach to demonstrate that DtpT is involved in the utilisation of chemically and structurally diverse di/tripeptides as nutrient sources in *S. aureus*. However, peptide utilisation (as determined from PM assays) is a composite phenotype representing the combined consequences of peptide internalisation and catabolism, both of which are required to drive cellular metabolism and produce a positive signal. As a result, peptides with limited uptake may produce a positive PM signal if efficiently catabolised while other peptides may be rapidly internalised (by DtpT or otherwise) without producing a positive PM signal due to incomplete catabolic pathways. Therefore, while the data generated from our phenotypic screen may point toward trends in transporter specificity, targeted functional and biochemical analyses are required to more comprehensively understand the activity and substrate preferences of DtpT on the molecular level.

Here, we describe methods for the exogenous expression and purification of DtpT in *E. coli* cells. We then apply a number of complementary biochemical methods to assess the ability of the protein to recognise and transport a panel of putative peptide substrates. These results confirm the ability of DtpT to transport diverse peptides, as observed in the PM assays. Furthermore, we extend these methods in order to identify novel substrates of DtpT (e.g. the antibiotic chloramphenicol and the tripeptide metabolite GSH) which may point toward novel roles for the transporter in *S. aureus* physiology.

While it is not relevant to the work presented in this chapter, it is also worth noting that Opp3A – that is, the SBP component of the ABC oligopeptide transporter Opp3 – was recombinantly expressed and successfully purified during this project, similarly to DtpT. However, due to time constraints, follow-up work aiming to characterise this protein structurally and biochemically was limited. These efforts are summarised in [Appendix 13](#).

Results

Overexpression and purification of a protein-of-interest is an essential prerequisite step required for functional characterisation of proteins in a cell-free context. Historically, *E. coli* is a preferred bacterial expression system and has been extensively applied for the production of bacterial integral membrane proteins, due in-part to the ability of the bacterium to grow rapidly to a high cell density as well as the vast repertoire of molecular genetic tools available for this organism ([Rosano & Ceccarelli, 2014](#)). However, membrane protein expression is often challenged by high levels of protein toxicity resulting in low yields of the properly folded protein product ([Gubellini et al., 2011](#); [Mathieu et al., 2019](#)). To this end, we constructed two potential *E. coli*-based vectors for the overexpression of DtpT and assessed the ability of these systems to produce high quantities of the protein for purification. The first of these expression systems – constructed in the arabinose-inducible expression vector pBADcLIC – showed promising expression of DtpT in preliminary trials, but ultimately failed to produce high yields of the target protein (as outlined in [Appendix 12](#)). The second system – constructed in the C-terminal GFP-fusion vector pWaldo – resulted in much higher protein yields and was chosen for routine purification of DtpT over the remainder of the project, as described below.

Expression of DtpT and other bacterial POTs in *E. coli* using pWaldo

One limitation of traditional His-tagged protein expression vectors (like pBADcLIC) is that Western blotting (generally required for the detection of membrane proteins) remains a low-throughput and often rate-limiting step required for determining the optimal expression conditions of the target protein. As a result, expression trials utilising such vectors are time consuming unless limited to a restricted pool of tested conditions. This restriction can be detrimental when applied to membrane proteins, for which protein yields are generally low and therefore optimal expression conditions are essential to produce meaningful quantities of protein. A common alternative strategy for optimising the expression of membrane proteins involves the use of C-terminal translational fusions to a fluorescent protein (generally GFP). The basis of this methodology is that GFP does not fluoresce within inclusion bodies, and the fluorescence of membrane protein-GFP fusions is therefore indicative of protein folding and functional insertion into the membrane. The resulting fluorescence is detectable in whole cells and standard SDS PAGE gels, allowing protein abundance to be quantified without the need for Western blotting and thereby facilitating screening of many expression conditions in a short timeframe ([D. E. Drew et al., 2001](#); [D. Drew et al., 2005](#)).

Here, we utilise the low copy number pET28(a+)-derived membrane protein expression vector pWALDO, which incorporates a C-terminal GFP-8xHis fusion downstream of the gene-of-interest and separated by a TEV protease cleavage sequence ([Drew et al., 2005](#); AddGene plasmid #112507) (**Figure 28A**). To capitalise on the high-throughput nature of this approach, DtpT and five alternative bacterial POT proteins were each cloned into pWALDO (*note: these five proteins were intended to serve as alternative protein targets for downstream characterisation in the case that DtpT was not highly expressed under any condition tested*) (**Table 6**). The resulting pWALDO-POT vectors were then transformed into the expression strains BL21 (DE3), C41 (DE3) and C43 (DE3) and expression of all six POTs was trialled under a range of conditions.

Table 6. POT genes cloned into six pWALDO-POT expression constructs

	Gene name	Organism of origin	UniProt Accession
1	PepT _{So}	<i>Shewanella oneidensis</i> ATCC 700550	Q8EKT7
2	DtpA _{Ec}	<i>Escherichia coli</i> K12	P77304
3	DtpC _{Ec}	<i>Escherichia coli</i> K12	P39276
4	DtpE _{Kp}	<i>Klebsiella pneumoniae</i> ATCC13883	A0A377WR44
5	DtpT	<i>Staphylococcus aureus</i> JE2	A0A0H2XIN4
6	PepT _{Sh}	<i>Staphylococcus hominis</i> SK1119	A0AAE5JKN2

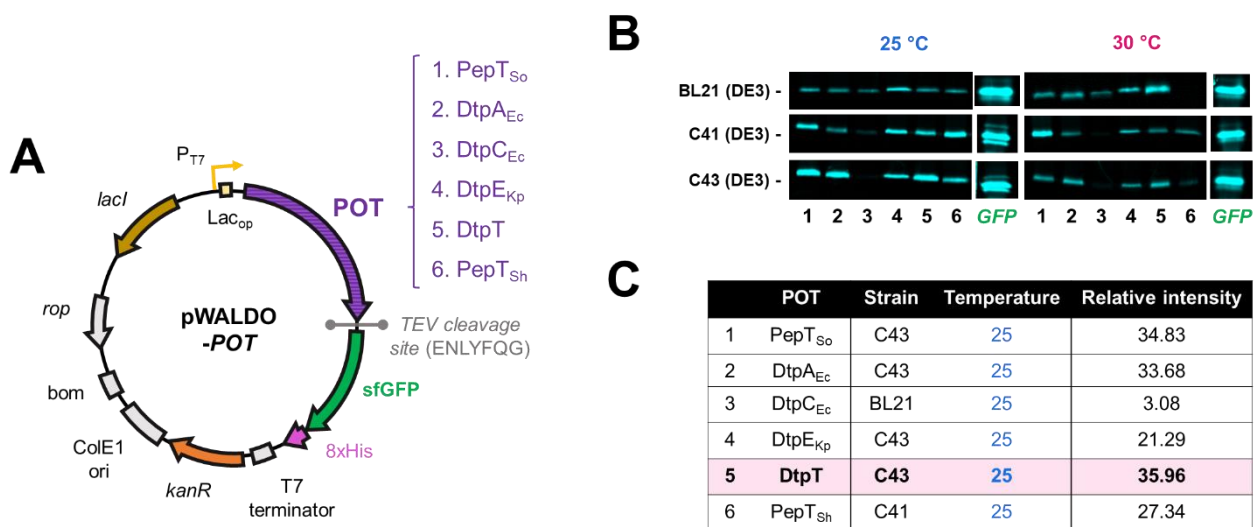


Figure 28 – Expression trials of DtpT and other bacterial POTs in pWALDO. **(A)** Schematic overview of the pWALDO-POT expression vectors. Open reading frames are depicted as arrows. Features implicated in plasmid function and replication are coloured in grey (*rop* protein maintains plasmid at low copy-number; *bom* = *pBR322* basis of motility; *ColE1 ori* = origin of replication). The antibiotic resistance marker *kanR* is coloured in orange. Elements comprising the Lac expression system are coloured in gold. The six POT protein-encoding genes incorporated into this vector are listed in purple. In each case, the entire ORF was introduced into the vector (including the native 5' signal peptide sequence). Elements are roughly to-scale.

(B – C) Expression trials for six POT-GFP fusion proteins expressed overnight at two temperatures in three expression strains. **(B)** In-gel fluorescence of whole cell lysates reveals the presence of a single strong band corresponding to the expressed POT-GFP fusion proteins (numbered 1 – 6, as in **A**). Fluorescence intensity in each case is normalised to a positive control (*GFP* = whole cell lysate of BL21 (DE3) cells expressing GFP only). **(C)** Optimal expression strains and temperatures for each of six POT-GFP fusion proteins. Relative intensity values are given as a percentage of the *GFP* control.

Whole-cell fluorescence

Initially, expression under two IPTG concentrations – 0.1 mM and 0.4 mM – was trialled in BL21 (DE3) cells carrying each of the six pWALDO-POT vectors by measuring whole-cell growth and GFP fluorescence kinetics after induction at 30°C ([Appendix 14](#)). A marked increase in GFP fluorescence was observed for DtpT as well as DtpA_{EC}, PepT_{So} and DtpE_{Kp}, indicative of fusion protein expression. For each of these four proteins, the lower IPTG concentration demonstrated decreased toxicity over four hours and resulted in a stronger fluorescent signal overall. We then compared the expression of each fusion protein over the first 4 hours *post*-induction and after overnight induction in the presence of 0.1 mM IPTG for each of the six POT-GFP fusion proteins ([Appendix 15](#)). In each case, the overall GFP fluorescence was stronger following overnight induction compared to 4 hours. Together, these observations suggest that overnight induction with 0.1 mM IPTG is the optimal expression condition for pWALDO-POT vectors. We also note that fluorescence was higher in both C41 (DE3) and C43 (DE3) for all fusion proteins compared to BL21 (DE3) after overnight induction, with the exception of DtpC_{EC} which expressed poorly in all three strains. For DtpT, GFP fluorescence after overnight induction was slightly greater in C43 (DE3) compared to C41 (DE3).

In-gel fluorescence

While useful for screening a large number of growth conditions *in tandem*, the whole-cell fluorescence experiments described here were carried out in 200 µl culture volumes and may therefore result in growth and expression kinetics which are poorly representative of those attained in the larger expression volumes required for protein purification. Having determined that overnight induction with 0.1 mM was optimal for expression of POT-GFP fusion proteins, these conditions were taken forward for further expression trials in larger flask cultures. Expression of each fusion protein was assessed at 25°C and 30°C in BL21 (DE3), C41 (DE3) and C43 (DE3) by analysing whole-cell lysates via SDS PAGE and in-gel fluorescence ([Figure 28B-C](#)). For all six fusion proteins, expression was strongest at 25°C. In agreement with the whole-cell fluorescence data, expression of DtpT-GFP was strongest in C43 (DE3).

Based on these observations, we chose to move forward with purification of DtpT using the optimal conditions deduced here (overnight induction with 0.1 mM IPTG at 25°C in C43 (DE3)).

Purification of DtpT

We next attempted purification of DtpT from 2 L of bacterial culture in cells carrying pWALDO-DtpT expressed under optimal conditions. First, the complete GFP-8His fusion protein was isolated from purified membrane fractions via immobilised metal affinity chromatography (IMAC) (as described in methods, [page 116](#)). As expected, we observed the elution of a single strong band corresponding to the DtpT-GFP fusion protein, which was confirmed via in-gel fluorescence ([Figure 29A](#)). Elution fractions containing the DtpT-GFP fusion protein were pooled and concentrated. Following treatment with TEV protease (*purified separately*), the cleaved GFP-8His and His-tagged TEV protease were removed via a second round of IMAC to yield pure untagged DtpT protein ([Figure 29B](#)). The cleaved protein was pooled and concentrated as before, and further purified by size exclusion chromatography (SEC). Analysis of the associated $\lambda = 280$ nm absorbance (A280) trace reveals the elution of a single sharp peak corresponding to the pure protein at approximately 13 ml elution volume ([Figure 29C-D](#)).

While our SEC elution pattern suggests that DtpT is mostly monomeric ([Figure 29C](#)), we do note the appearance of at least one larger band seemingly corresponding to DtpT in SDS PAGE gels when the protein is present at high concentrations (this band can be identified as DtpT based on in-gel fluorescence analysis; evident in [Figure 29A](#)). When observed in our SDS PAGE analyses, these bands varied in size from roughly twice that of the expected protein to several times larger (again, evident in [Figure 29A](#)), and could sometimes be observed even after isolation of the pure DtpT protein via size exclusion (as observed in the gels presented later in this chapter in [Figure 39B](#)). Overall, we conclude that DtpT may behave as a dimer or multimer in SDS-PAGE gel analysis, though the biological significance of this phenomenon is not clear.

Following SEC, fractions corresponding to the pure DtpT protein were pooled and quantified by measuring A280. Overall, approximately 2.35 mg of pure DtpT protein was yielded from 2 L of bacterial culture.

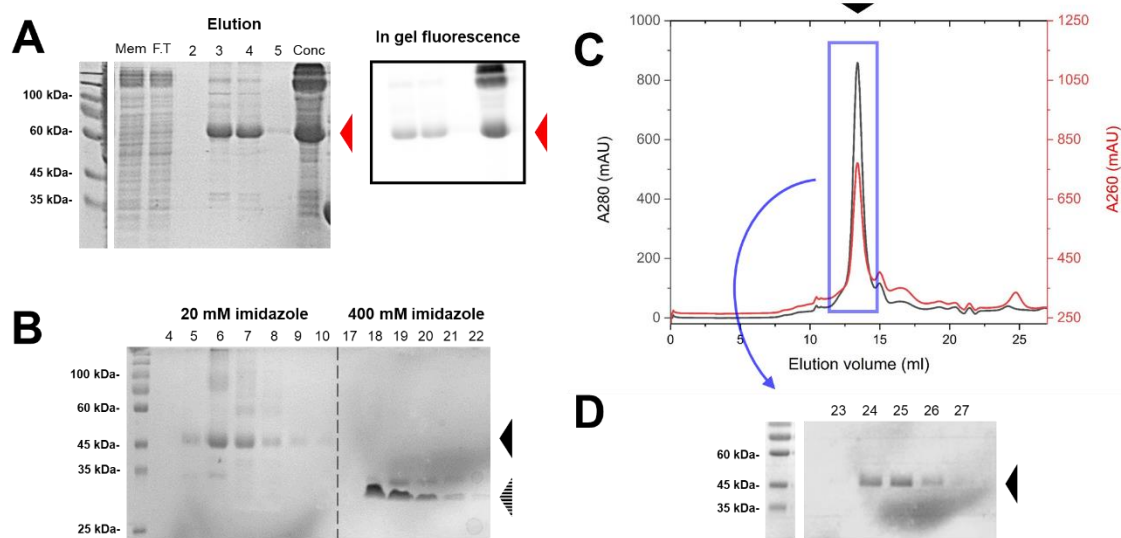


Figure 29 – Purification of DtpT from *E. coli* C43 cells carrying pWALDO-DtpT. (A-D)

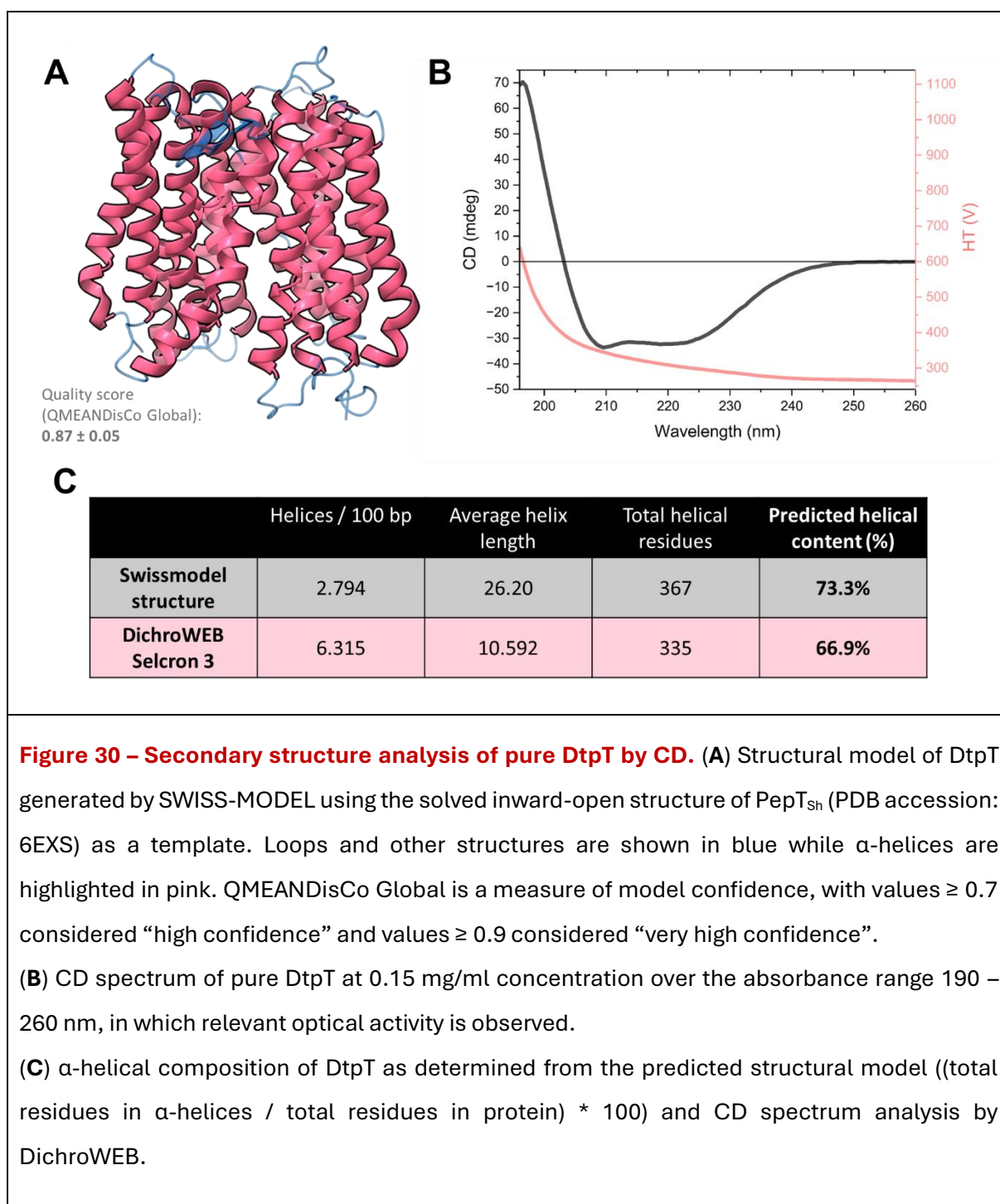
Complete purification of DtpT from C43 (DE3) cells carrying pWALDO-DtpT. **(A)** Nickel affinity purification of the DtpT-GFP fusion protein. Images correspond to total protein (left; Coomassie blue stain) and DtpT-GFP only (right; in-gel GFP fluorescence). **(B)** Representative gel image of a reverse nickel affinity purification of untagged DtpT after TEV cleavage. **(C-D)** SEC purification of pure DtpT protein following TEV cleavage. A single sharp peak is seen in the A280 trace **(C)** corresponding to the pure DtpT protein split across three elution fractions **(D)**. Numbers in **D** correspond to elution fractions (500 μ l) spanning the region of represented by the blue box in **C** (approx. 12 - 14.5 ml).

Mem = Membrane fraction. F.T = Column flow-through. Conc = concentrated fractions. The black arrow in each case indicates the position of a band corresponding to the pure DtpT protein. The red arrow indicates the position of a band corresponding to the expected DtpT-GFP fusion protein. The striped arrow indicates the position of a band corresponding to GFP.

Pure DtpT is largely α -helical

Similarly to other previously characterised bacterial POT proteins, DtpT is predicted to adopt a characteristic MFS fold consisting of 12 membrane-spanning helices as well as two additional TM helices presumed to contribute toward structural stability (AlphaFold 3; [Figure 17](#)). While structural characterisation of DtpT is currently lacking, the structure of a closely related POT protein from *Staphylococcus hominis* (PepT_{Sh}; 80.64% identity to DtpT) in complex with the thioalcohol-peptide conjugate S-Cys-Gly-3M3SH was recently resolved in an inward-open conformation ([Minhas et al., 2018](#)). To verify the AlphaFold projected structure presented here, we also utilised the protein structural homology modelling tool SWISS-MODEL ([A. Waterhouse et al., 2018](#)) to generate a structural model of DtpT using the aforementioned PepT_{Sh} complex structure as a template ([Figure 30A](#)). The two methods produced near-identical projected structures. As expected, DtpT is predominantly α -helical (73.3%) in these projected structures, with the exception of connecting loop regions and a short extracellular β -hairpin motif (previously observed in PepT_{Sh}).

To verify that the DtpT protein remains in a folded state during purification, secondary structure elements in the pure protein were probed using circular dichroism (CD). In this method, the abundance of secondary structure elements (primarily α -helices) is estimated based on the differential absorption of left- and right-handed circularly polarised light over a characteristic wavelength range (approx. 180 – 250 nm) ([Wei et al., 2014](#); [Zavrtanik et al., 2024](#)). We observe that photo-multiplier voltage (HT) remains below 600 V in this relevant absorbance range for our protein sample, allowing confident interpretation of the resultant spectrum with minimal noise. The CD spectrum of DtpT resolved here demonstrates a clear double-dip pattern in the 205 – 235 nm range which is a signature consequence of α -helices ([Figure 30B](#)). The web-based CD spectrum analysis tool DichroWEB ([Miles et al., 2022](#)) predicts an approximate α -helical content of 66.9% in this sample, which is comparable (-6.4%) to the composition of the predicted structural model (assuming that the structural prediction shown here is accurate, variation between these two values may arise from inaccurate determination of the sample protein concentration during CD analysis) ([Figure 30C](#)). Overall, we confirm that the pure DtpT protein is folded in the expected mostly α -helical conformation.



Probing DtpT-peptide interactions via thermostability binding assays

Structural and functional studies of transporter proteins have demonstrated that substrate binding precedes transport and is essential for imparting specificity as well as preventing futile cycling ([Magbool et al., 2015](#); [Quistgaard et al., 2016](#)). Based on these principals, the strength of protein-ligand binding interactions (as inferred by biophysical methods) is generally considered a good indicator of transporter function, i.e. ligands which bind the protein with greater affinity are more likely to be transported. This notion was recently challenged in the case of the *E. coli* POT protein DtpB, for which many high affinities ligands demonstrate poor rates of transport while ligands with middling binding affinities are transported more favourably ([Kotov et al., 2023](#)). Nonetheless, ligand binding screens remain an informative method of probing interactions between a given transporter and a panel of putative substrates to identify likely transport targets.

A common method of quantifying the strength of protein-ligand interactions is through thermal unfolding experiments. These methods typically rely upon the principal that all proteins unfold as they are heated, and this unfolding leads to surface exposure of otherwise sequestered hydrophobic chemical groups. The thermal stability of a given protein under varying conditions can be compared by measuring the mid-point temperature of the transition between folded and unfolded states, referred to as the melting temperatures (T_m). Strong protein-ligand binding interactions are known to stabilise the folded conformation of the protein, and this typically manifests as a change (generally, an increase) in the T_m . Hence, the value of ΔT_m provides an indicator of the strength of the binding interaction for each ligand ([Vivoli et al., 2014](#)), and these values generally correlate to the K_D of binding (although the applicability of irreversible thermal denaturation approaches to determine K_D is the subject of debate) ([Lo et al., 2004](#); [Hall, 2019](#)). It is worth noting that some biologically relevant protein-ligand interactions may also destabilise the protein fold, leading to a decrease in T_m ([Askin et al., 2018](#)).

In a nanoDSF thermal unfolding assay, the intrinsic fluorescence of tryptophan and tyrosine residues at 350 nm and 330 nm is measured while proteins are subjected to a linear temperature ramp. The change in local chemical environment of these residues during protein unfolding can be inferred from the change in the ratio of these two fluorescence values (F350/330 ratio). The T_m of the protein is the temperature at which the rate of change of the F350/330 ratio is maximal (in other words, the greatest positive inflection point of the 1st derivative of the unfolding curve, as exemplified in [Figure 31A-B](#)) ([Magnusson et al., 2019](#); [Wen et al., 2020](#)). Here, interactions between DtpT and a panel of di/tripeptides were screened by a nanoDSF thermal unfolding assay.

DtpT recognises diverse peptide structures

In the absence of ligand, the unfolding of DtpT produces a 1st derivative curve with a slight negative inflection followed by a single positive inflection point (described herein as T_m) at approximately 64.5°C. The addition of some peptides results in a positive shift in this inflection value and therefore a change in the T_m indicative of strong binding interactions (Figure 31A-B). We also note an increase in the magnitude of this inflection for some ligands, seemingly corresponding to a decrease in the initial F350/330 ratio imposed by binding (exemplified in Figure 31A).

ΔT_m was determined for DtpT in the presence of 22 putative ligands. As expected, no change in T_m was observed upon addition of the single amino acid alanine (included here as a negative control). In contrast, our data demonstrates that DtpT interacts with diverse peptide structures – including dipeptides and tripeptides – and these interactions generally induce an increase in the T_m of the protein (Figure 31C). The value of ΔT_m varied from 9.1°C in the case of Ala-Leu to near-zero for Arg-Phe, providing compelling evidence that the strength of DtpT-peptide binding is not uniform and instead depends upon the amino acid composition of the substrate. That being said, any non-zero change in the apparent T_m may be indicative of biologically relevant protein-ligand interactions (Vivoli et al., 2014), and such a change was observed for each tested peptide with the exception of Arg-Phe (Figure 31C).

Our data points toward a number of potential trends in regard to specific substrate characteristics and their effect on protein-ligand interactions, as described below. It is worth noting that these observations are based on a very limited selection of the 8400 possible di/tripeptide structures which could theoretically be accommodated by the transporter. Any potential patterns in the data presented here are, therefore, only speculative and would benefit from further verification via a more comprehensive screen of possible ligand conformations.

N-alanyl peptides bind DtpT more tightly than N-glycyl peptides

In order to identify trends from our thermal unfolding data, we found it useful to compare binding for pairs of ligands which differ only by a single side chain group. In such cases, any deviation in ΔT_m is presumably conferred by changes in ligand binding as a result of the altered group exclusively, allowing the impact of specific amino acid substitutions within the substrate to be assessed. For instance, this approach can be applied to compare binding for two dipeptides which each contain a C-terminal phenylalanine residue: Gly-Phe and Ala-Phe. In this case, we observe that substitution of the N-terminal glycine group for alanine increases the ΔT_m associated with binding from 1.4°C to 7.2°C. A similar difference is observed when comparing

Gly-Gly ($\Delta T_m = 0.3^\circ\text{C}$) with the N-alanyl dipeptide Ala-Gly ($\Delta T_m = 3.0^\circ\text{C}$). In fact, the weakest-binding N-alanyl dipeptide assessed here demonstrated a higher ΔT_m than all N-glycyl dipeptides tested, providing compelling evidence that the presence of an alanyl side chain in this position is favourable for ligand binding.

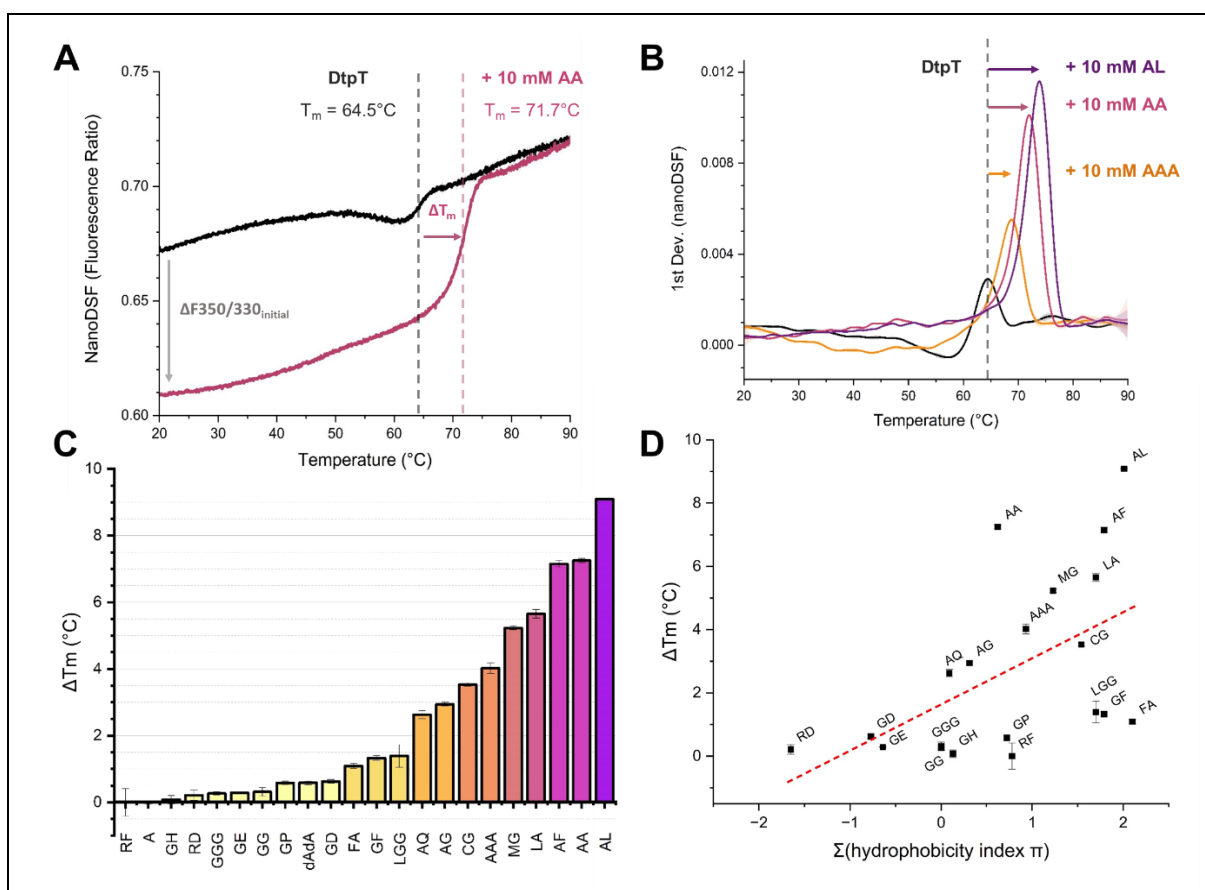


Figure 31 – Screening DtpT-peptide binding interactions by a nanoDSF thermal unfolding assay.

(A) Example nanoDSF unfolding curves of 5 μM DtpT alone or in the presence of 10 mM ala-ala. The T_m is estimated as the temperature at which the rate of change of the fluorescence ratio is maximal for each curve, indicated by the dashed lines.

(B) Example 1st derivative unfolding curves for DtpT alone or in the presence of three peptides, each at 10 mM. In this case, the T_m is estimated as the temperature of the greatest positive inflection point of the curve, as indicated by the dashed line for DtpT.

(C) Estimated values of ΔT_m for DtpT in the presence of 22 putative ligands as determined by nanoDSF. Ligand was added to a final concentration of 10 mM in each case. Bars show the mean of three technical replicates $\pm$ the standard deviation of the mean.

(D) Estimated ΔT_m values from **C** were plotted against the sum of the hydrophobicity index associated with the amino acids in each peptide ligand (as determined by [Fauchere & Pliska, 1983](#)). The dashed red trendline indicates the best linear fit of the data ($r = 0.5432$, $p = 0.0133$; Pearson's correlation coefficient). In each case, peptides are labelled by their single-letter amino acid codes (e.g. AA = Ala-Ala). dAdA = D-Ala-D-Ala.

DtpT binds preferentially to hydrophobic peptides

Inspection of our nanoDSF unfolding data demonstrates how dipeptides containing acidic side chain groups – such as Arg-Asp, Gly-Asp and Gly-Glu – are generally poor binders ($\Delta T_m < 1^\circ\text{C}$). In contrast, many of the tightest binders identified here were comprised of hydrophobic amino acids including leucine and phenylalanine (Figure 31C). Previously, Fauchere and Pliska quantified the hydrophobicity index for each naturally occurring amino acid based upon the partitioning of their N-acetyl amide derivatives in octanol/water (Fauchere & Pliska, 1983). Here, we compare the value of ΔT_m for each peptide ligand against the sum of hydrophobicity indices for amino acids comprising those peptides, to serve as an approximation of total peptide hydrophobicity (Figure 31D). From this, we observe a statistically significant positive association between overall hydrophobicity and the value of ΔT_m ($r = 0.5432$, $p = 0.0133$), suggesting that DtpT preferentially binds to peptide containing hydrophobic side chain groups.

Preparation of DtpT liposomes for measuring and comparing peptide transport

Liposome reconstitution-based methods are generally considered the gold-standard for characterising membrane transporter function. In these methods, a purified transporter protein is functionally inserted into lipid vesicles and transport of a given substrate is tracked (either directly or indirectly) as the substrate is translocated ([D. Drew et al., 2021](#)). As opposed to traditional cell-based transport assays, liposome-based approaches allow the activity of an individual protein to be assessed independently of convoluting biological factors, such as alternate transporters, native inhibitors and the potential for substrate efflux.

Direct methods of assessing transporter activity typically involve the use of radiolabelled substrate analogues, the accumulation of which can be measured over time via scintillation ([Nimigean, 2006](#)). For highly promiscuous transport systems like POTs, this approach would require the use of varied radiolabelled peptide substrates which would be financially unfeasible. Previous studies have instead utilised a competition-based approach in which substrate transport is inferred from the ability of a given peptide to competitively inhibit transport of a model radiolabelled substrate (typically ala-ala, which is a common substrate for all POTs characterised to-date) ([Lyons et al., 2014](#); [Stauffer et al., 2021](#)). However, recent evidence suggests that many peptides with tight binding affinities (which are likely to be effective inhibitors of transport) are themselves poorly transported, challenging the use of competitive inhibition as a proxy for transport in POTs ([Kotov et al., 2023](#)).

An alternative transport assay was previously developed by *Parker* and colleagues to investigate peptide transport in the bacterial POT PepT_{St}, and has since been applied to diverse POT proteins (outlined in [figure 32](#)) ([Parker et al., 2014](#)). Briefly, this assay utilises the pH-sensitive dye pyranine to track the acidification of the liposome lumen resulting from proton-driven transport. Transport of each substrate is driven by an invariable negative-inside membrane potential ($\Delta\Psi$; established by loading liposomes with K⁺, followed by addition of the K⁺ ionophore valinomycin). The initial rate of acidification following the establishment of a negative-inside $\Delta\Psi$ provides an indirect measure of substrate transport. It is worth noting that the rate of acidification depends not only on substrate transport rate, but also on the proton-stoichiometry of transport for each substrate; existing thermodynamic evidence suggests that PepT_{St} may utilise different proton stoichiometries for transport of di-peptide and tri-peptide substrates, though it's currently unclear whether this is the case for other bacterial POTs ([Parker et al., 2014](#)).

In order to carry out the pyranine transport assay described here, we first developed a protocol by which DtpT could be robustly reconstituted into DtpT-proteoliposomes with readily detectable transporter activity. To achieve this, we trialled two well-established methods of membrane protein reconstitution and found that one of these methods – utilising polystyrene bio-beads and extensive dialysis – was effective for reconstitution, producing liposomes with desirable qualities for assessing DtpT-mediated transport.

A “rapid dilution” method results in low reconstitution efficiency and leaky liposomes

Previously, the *S. hominis* POT protein PepT_{Sh} was stably reconstituted into lipid vesicles by a “rapid dilution” method (outlined in [Figure 33A](#)) ([Minhas et al., 2018](#)). Succinctly, this method involves solubilising the pure protein in detergent with a high critical micellar concentration (CMC) such as n-decyl-β-D-maltoside (DM), which is mixed with pre-formed lipid vesicles. In the presence of detergent, the lipid vesicles are destabilised and DtpT can be transiently incorporated into the lipid bilayer. The mixture is then rapidly diluted below the CMC of the detergent, leading to dissolution of detergent micelles. Lipid vesicles containing the reconstituted protein can then be recovered and any residual detergent can be removed by dialysis.

Here, we attempted to reconstitute DtpT via rapid dilution into lipid vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) mixed in a 3:1 ratio. This lipid composition provides an approximation of the *E. coli* membrane composition; that is, roughly 75% PE and 20% PG ([Raetz & Dowhan, 1990](#)). The same lipid mixture was previously used in the reconstitution of PepT_{Sh} and has been demonstrated to produce tight liposomes with the capacity to maintain a strong pH gradient across the membrane ([Minhas et al., 2018](#); [Tsai & Miller, 2013](#)). Before dilution, DtpT was premixed with the lipid mixture in a 1:50 mass ratio. The resultant liposomes were analysed by SEC, revealing that a substantial proportion of the protein (approx. 89%) had moved from the soluble fraction to the insoluble fraction (i.e. eluted in the void volume) ([Figure 33B](#)). After further optimisation of the detergent concentration and initial lipid vesicle size, efficiency was improved slightly from 11.6% to 20.2% ([Figure 33C](#)). However, the resultant liposomes were found to be highly leaky during pyranine transport assays as indicated by an apparent strong acidification of the lumen in a substrate-free control ([Figure 33D](#)). Ultimately, it was decided that this method was not suitable for reconstitution of DtpT.

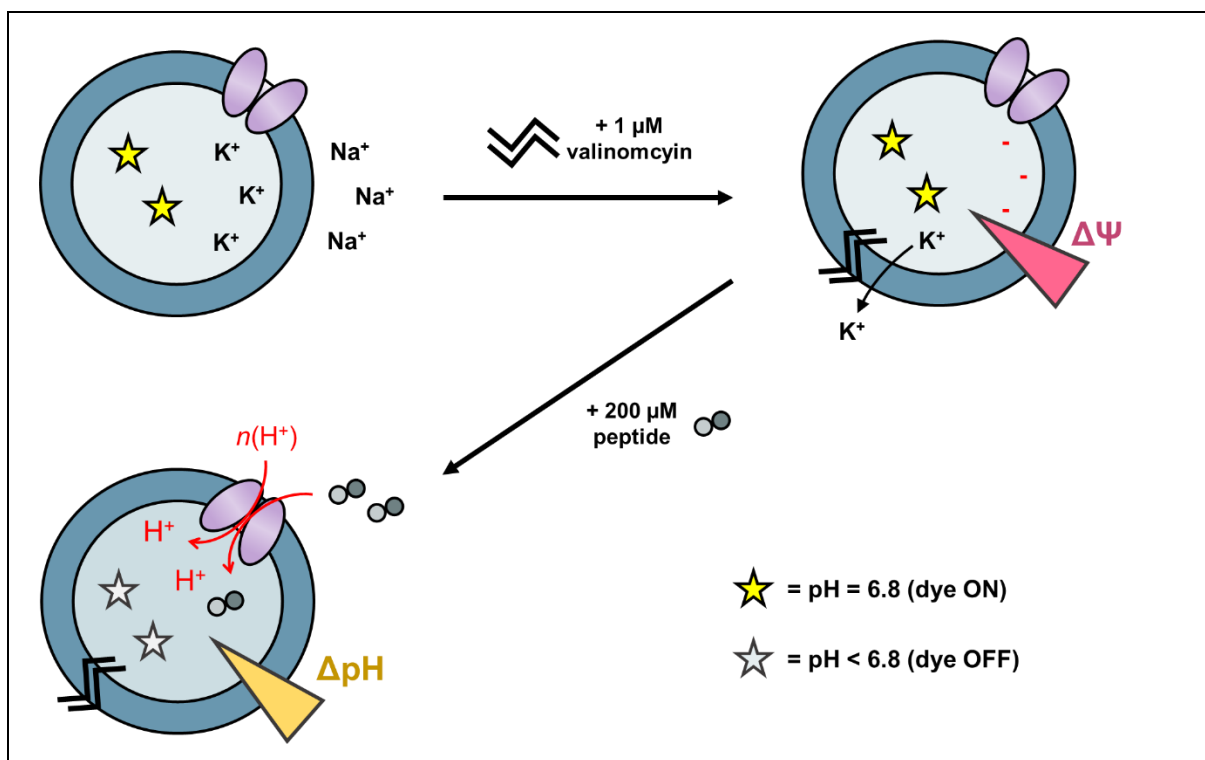
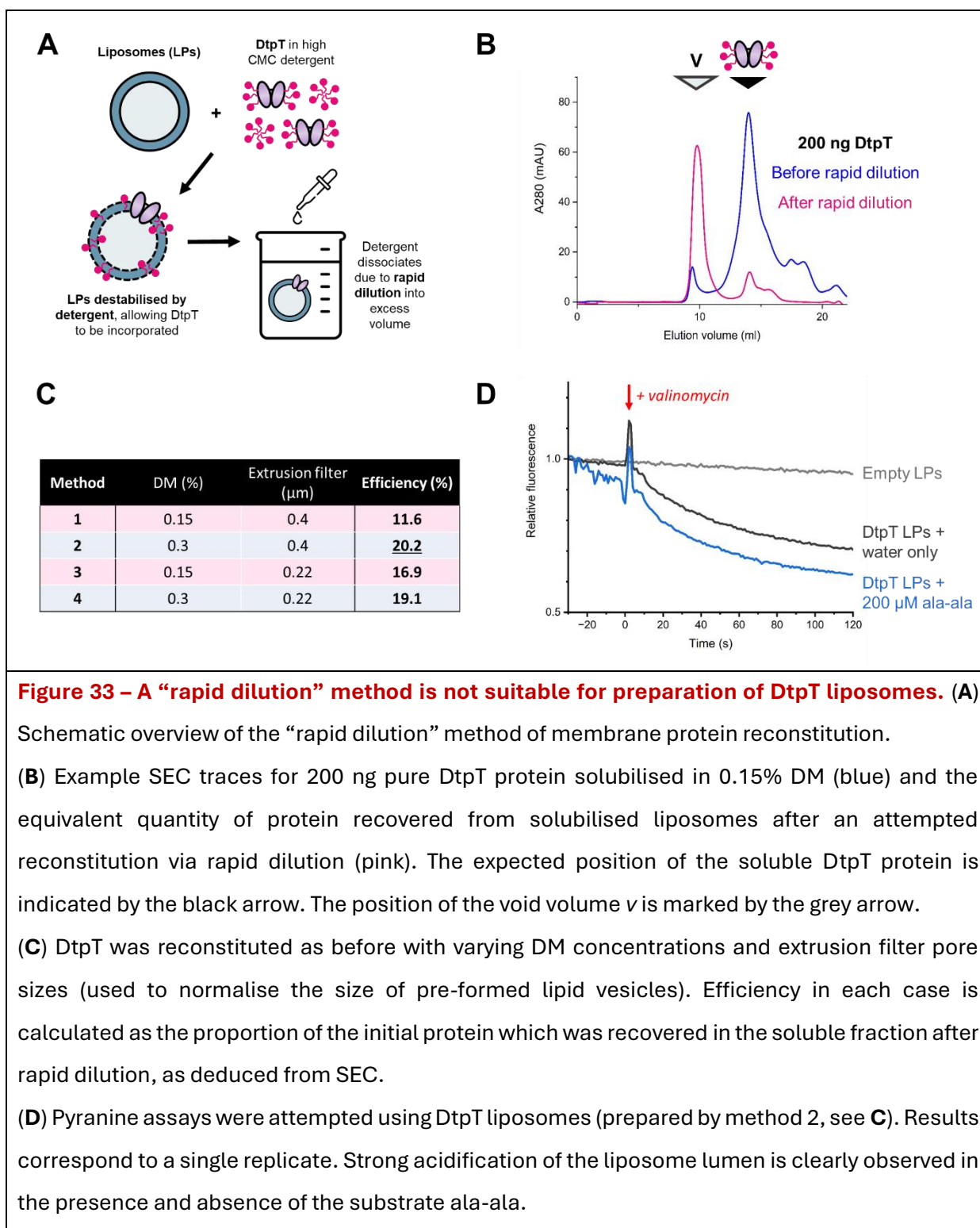


Figure 32 – Molecular basis of the planned DtpT pyranine transport assay. (upper left) In order to study the transport activity of DtpT in a cell-free system, the pure DtpT protein is first reconstituted into artificial lipid vesicles. These vesicles are loaded with the pH-sensitive fluorescent dye pyranine. In addition, a K^+ gradient is established by loading liposomes with a high concentration of KCl before suspending them in a buffer lacking KCl (instead containing an equal concentration of NaCl). (upper right) Upon addition of the potassium ionophore valinomycin, the membrane becomes permeable to K^+ which moves out of the liposome lumen down its concentration gradient. As a result, a negative-inside membrane potential ($\Delta\Psi$) is established. (lower left) The negative-inside $\Delta\Psi$ drives proton-driven transport of peptides by DtpT. When a valid substrate is present, transport occurs, and the liposome lumen becomes acidified leading to a change in the emission intensity of the pyranine dye. The rate of acidification is proportional to the rate of transport for each substrate, assuming that the proton stoichiometry for each substrate is the same.



Reconstitution of DtpT using bio-bead adsorption

One limitation of the “rapid dilution” method applied here is the requirement to utilise high concentrations of detergent to achieve efficient reconstitution. As a result, detergent is likely to remain associated with the liposomes after reconstitution, and this detergent may destabilise the liposomes and cause leakiness. An alternative approach for reconstitution of membrane proteins involves the use of polystyrene bio-beads (outlined in [Figure 34A](#)). This method permits the use of a low-CMC detergent, which can be utilised at much lower working concentrations and is therefore less likely to be maintained in the final liposome product. The macroporous and hydrophobic qualities of bio-beads allow them to adsorb and thereby sequester low-CMC detergents, making them ideal for the preparation of proteoliposomes ([Rigaud et al., 1998](#)). In this method, bio-beads are added to the lipid-protein mixture in a stepwise manner, allowing for the gradual removal of detergent. Liposomes are then extensively dialysed over several days to remove any residual detergent.

Here, we attempted to reconstitute DtpT using the bio-bead adsorption method with the same lipid vesicle composition and protein:lipid ratios described above. In this case, we observed reconstitution efficiencies in excess of 50% (exemplified in [Figure 34B](#)) and the solubilised protein could be readily detected by SDS PAGE analysis ([Figure 34C](#)). To confirm that the reconstituted protein remained in a functional folded state, liposomal DtpT was analysed by nanoDSF in the presence and absence of the tight-binding ligand ala-ala ([Figure 34D](#)). We observe that the membrane-embedded protein is more thermostable relative to the DDM-solubilised form, demonstrating an increase in T_m from 64.5°C (see [page 157](#)) to 73.4°C. In addition, the liposomal protein seemingly interacts with ala-ala resulting in a thermal shift of around 3.2°C in the presence of the ligand. The folded state of DtpT within liposomes was also validated by CD analysis, demonstrating an overall similar spectrum profile to the detergent-solubilised protein in the relevant range ([Figure 34E](#)). However, we note that the signature double-dip motif associated with α -helices is less well defined in the reconstituted protein which we interpret as alterations in the protein fold as a consequence of protein-lipid interactions (observed for other membrane proteins, as in [D. Mao & Wallace, 1984](#)).

Critically, DtpT liposomes produced by bio-bead adsorption demonstrated minimal leakage in a pyranine transport assay in the absence of either substrate or a negative-inside $\Delta\Psi$ ([Figure 34F](#)). In contrast, inclusion of both of these factors resulted in strong acidification of the liposome lumen over the first 100 seconds after the addition of valinomycin ([Figure 34F](#)). From this, we can conclude that the liposomes produced here are functional and suitable for assessing DtpT-mediated peptide transport.

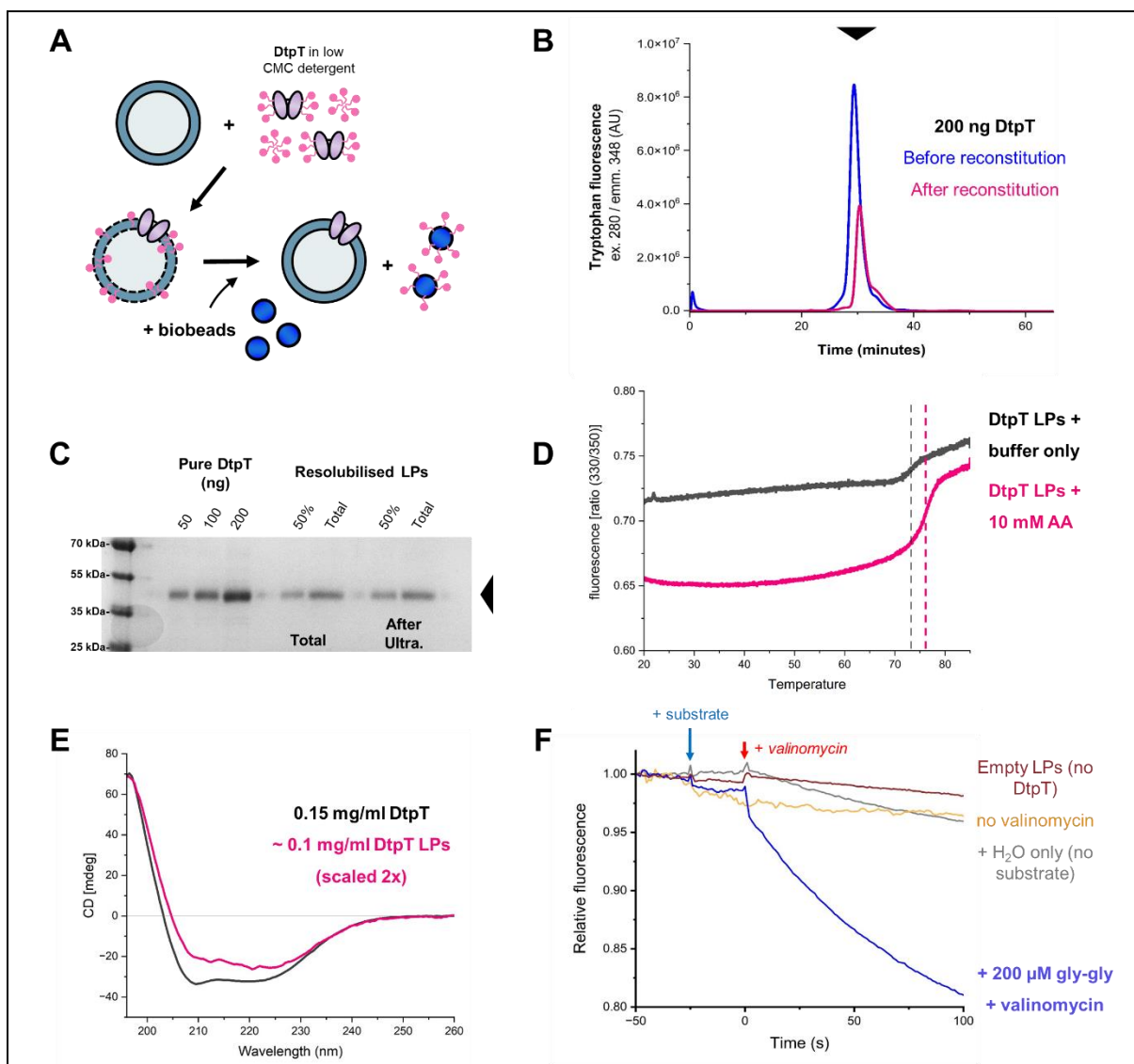


Figure 34 – Reconstitution of DtpT by bio-bead adsorption. (A) Schematic overview of the bio-bead adsorption method of membrane protein reconstitution.

(B) Example SEC traces for 200 ng pure DtpT protein solubilised in 0.03% DDM (blue) and the equivalent quantity of protein recovered from solubilised liposomes after bio-bead (pink). The expected position of the soluble DtpT protein is indicated by the black arrow. No void peak is observed as samples were clarified via centrifugation prior to loading on the SEC column.

(C) Comparison of pure DtpT and DtpT recovered from solubilised liposomes. Following solubilisation, insoluble proteins were removed by ultracentrifugation. Total recovered protein and protein after ultracentrifugation were analysed (labelled below corresponding bands).

(D) Mean nanoDSF unfolding curves of DtpT proteoliposomes in buffer or in the presence of 10 mM ala-ala. Mean T_m values are indicated by the dashed lines.

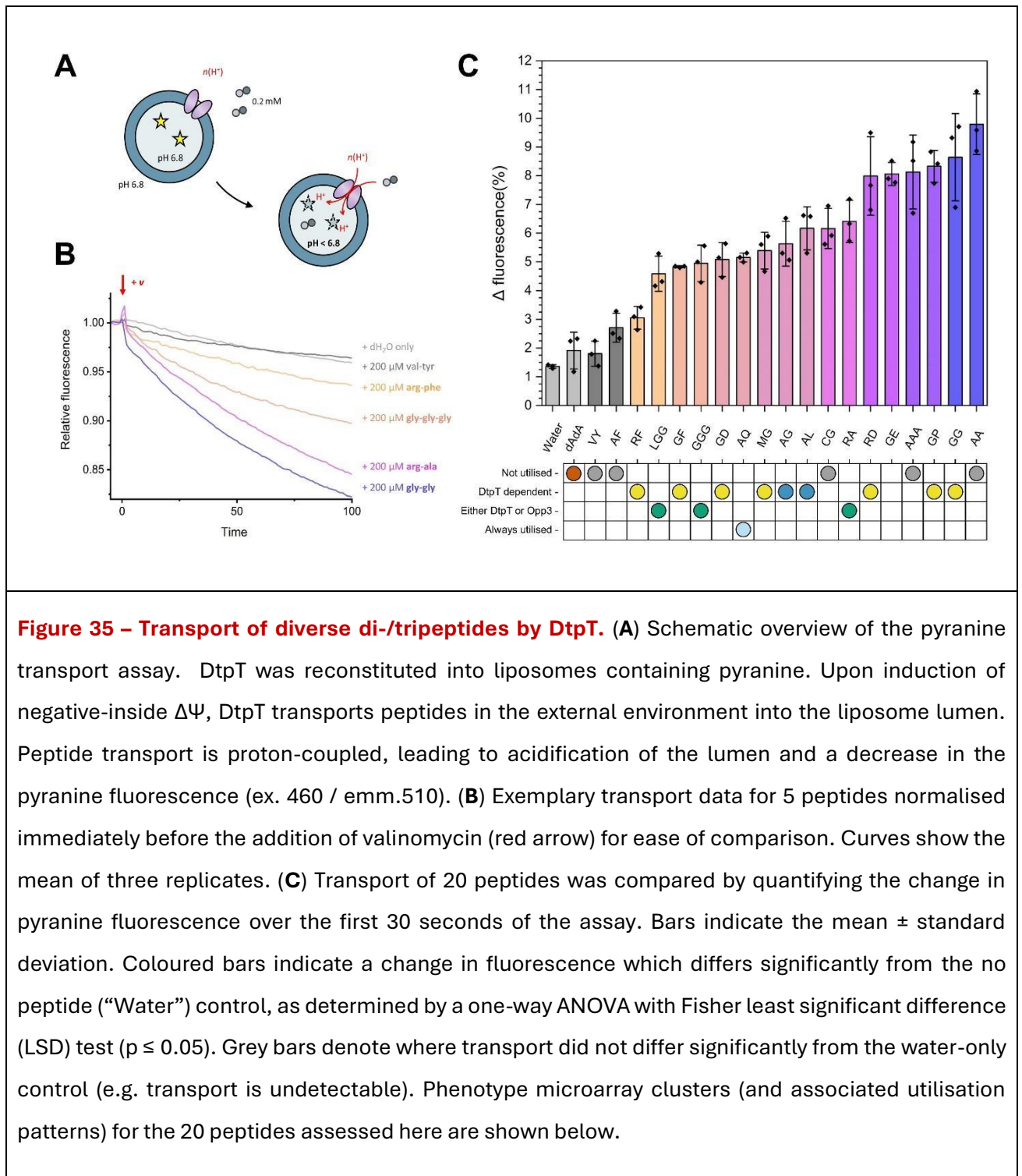
(E) CD spectra of pure DtpT and DtpT liposomes. A scaling factor of 2x has been applied to the DtpT-liposome spectrum to facilitate comparison with the detergent-soluble protein.

(F) Validation of pyranine assays in DtpT liposomes. Curves show the mean of three replicates normalised to the initial fluorescence signal ($t = -50$ s). In each case, Gly-Gly (or H_2O) is added at $t = -25$ s (blue arrow) and a negative-inside $\Delta\Psi$ is established by the addition of valinomycin at $t = 0$ s (red arrow). Strong acidification of the liposome lumen requires both a valid substrate and $\Delta\Psi$ (blue line).

DtpT transports peptides with diverse chemical properties

After verifying that DtpT was successfully reconstituted into liposomes with observable transport activity, transport was assessed for a panel of 20 putative peptide substrates each at a concentration of 200 μ M (Figure 35). In order to compare the initial rate of transport upon establishment of $\Delta\Psi$ for each substrate, we quantified the total % change in fluorescence over the first 30 seconds after the addition of valinomycin in each case (Figure 35C). D-Ala-D-Ala was included as a negative control, as this molecule lacks a standard L-peptide linkage which is thought to be essential for substrate coordination during the POT transport cycle (Parker et al., 2021). Transport was observed for the majority of peptides tested, with only D-Ala-D-Ala, Val-Tyr and Ala-Phe demonstrating a negligible change in fluorescence over the first 30 seconds of the assay when compared to a water-only control. Our data demonstrate that DtpT is highly promiscuous and is capable of transporting substrates with a range of chemical structures and properties, which is similarly noted for other POT transporters studied previously (Figure 35B-C) (Kotov et al., 2023; Parker et al., 2014).

Of those substrates which were transported, we observed a high degree of heterogeneity in the apparent rate of transport over the first 30 seconds of the assay (Figure 35C). Ala-Ala demonstrated the highest rate of transport over this period, with similarly strong acidification observed for Gly-Gly, Gly-Pro, Ala-Ala-Ala, Gly-Glu and Arg-Asp. Of those peptides which were transported, the majority also demonstrated DtpT-mediated transport during whole-cell phenotypic screening (Figure 35C). The only exceptions to this were Ala-Gln – for which utilisation was observed even in the absence of either DtpT or Opp3A – as well as Cys-Gly, Ala-Ala and Ala-Ala-Ala which were not utilised by any strains in the phenotype microarray. Based on this, we can conclude that utilisation of Ala-Gln is facilitated by DtpT and at least one additional route in *S. aureus*, while internalisation of the other three peptides listed here is not the limiting factor for their utilisation. The three peptides tested here which were not transported by DtpT were similarly not utilised by wild type *S. aureus* in the phenotype microarray. All three of the Asp/Glu-containing peptides tested here demonstrated transport by DtpT, consistent with the observation that these amino acids are enriched among DtpT substrates identified in the phenotype microarrays (Figure 35C). We also note that DtpT-mediated utilisation of Arg-Asp, Arg-Ala and Arg-Phe was previously confirmed in bacterial growth experiments (Figure 24), and our transport assays here verify these findings by directly demonstrating DtpT-mediated transport for each of these peptides. Overall, there is good agreement between our liposome-based transport assays and whole-cell peptide utilisation methods.

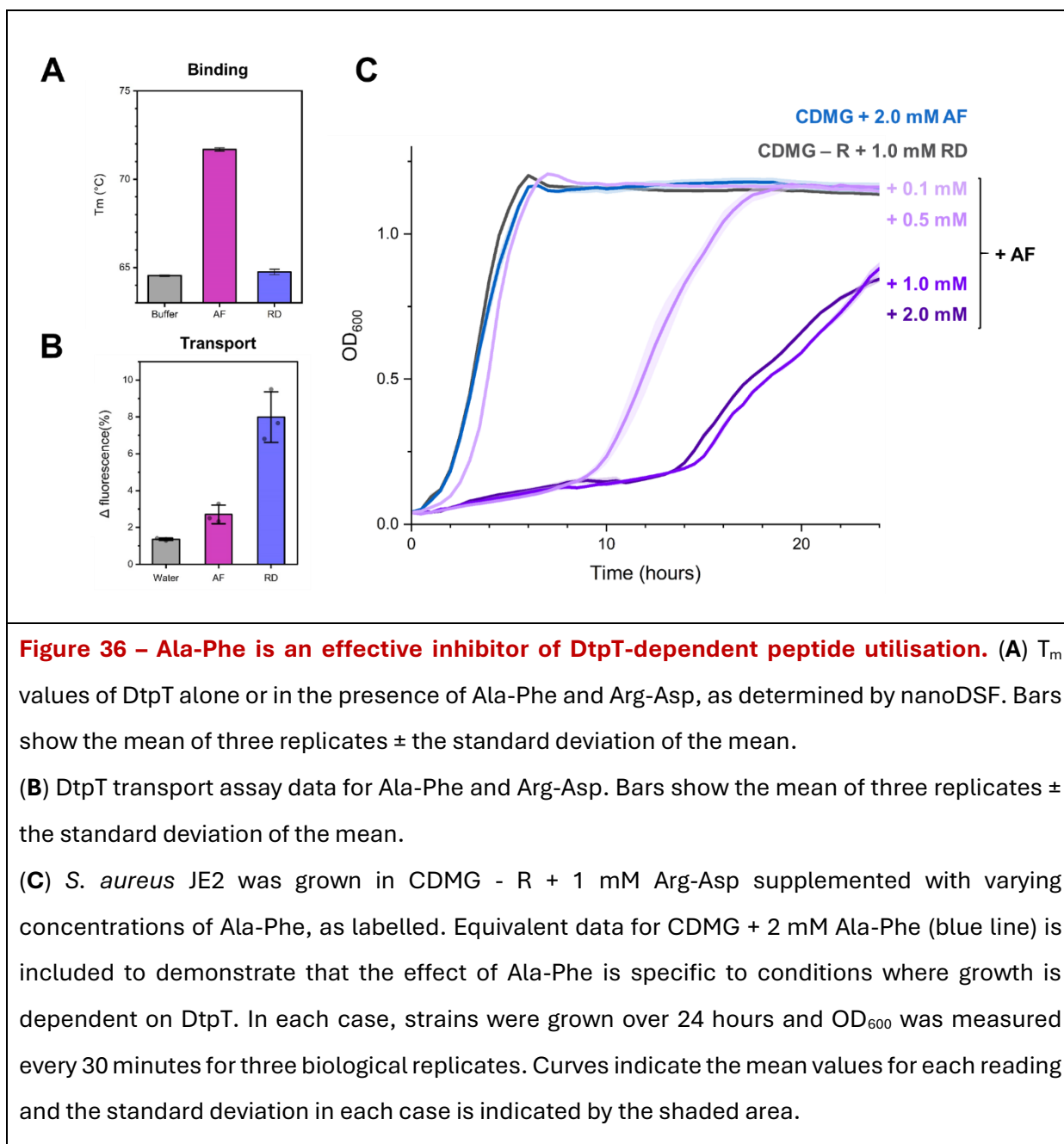


Ligand binding is a poor indicator of transport in DtpT

Unexpectedly, we note a number of inconsistencies when comparing data from our thermostability binding assays and transport assays. Many of the ligands with seemingly poor binding (e.g. Gly-Gly and Gly-Glu) were favourable substrates for transport, which is supported by the DtpT-specific utilisation of these peptides observed from phenotype microarrays. D-Ala-D-Ala demonstrated a stabilising interaction with DtpT and was responsible for a T_m shift comparable to Gly-Pro (Figure 31), but the former peptide was not transported while the latter was transported favourably (Figure 35). Furthermore, DtpT-specific uptake of Arg-Asp and Arg-Phe here has been confirmed in transport assays (Figure 35) and whole-cell growth experiments (Figure 24), despite their seemingly poor binding interactions with DtpT (Figure 31). When comparing our binding and transport assay data, we found no significant correlation between ΔT_m and transport rate across the 17 peptides assessed in both assays (Appendix 17). Overall, these examples illustrate that protein-ligand binding interactions as inferred from a nanoDSF thermostability screen are, in-fact, poor indicators of transport for any given peptide by DtpT.

Ala-phe effectively inhibits DtpT-dependant Arg-peptide utilisation

As described above, we have demonstrated that some peptides bind tightly to DtpT while demonstrating little-to-no DtpT-mediated transport. In the presence of a mixed peptide population, we predict that these peptides would preferentially bind to DtpT without being translocated and would thereby prevent transport of alternative peptide substrates. As such, we predicted that peptides demonstrating this quality may function as effective competitive inhibitors of DtpT. Ala-Phe was among the tightest DtpT-binding peptides assessed in our nanoDSF screens, but demonstrated negligible transport in a pyranine transport assay, making this peptide a promising candidate for competitive inhibition (Figure 36A-B). In contrast, Arg-Asp was among the weakest-binding peptides whilst demonstrating a high rate of transport. Utilisation of Arg-Asp by wild-type *S. aureus* JE2 was previously demonstrated to be entirely DtpT-dependant (Figure 24). To determine whether Ala-Phe could effectively inhibit DtpT-dependant peptide utilisation, JE2 was grown under conditions where growth was dependant on the utilisation of Arg-Asp as a sole arginine source (CDMG – R + 1 mM RD) in the presence of increasing concentrations of Ala-Phe (Figure 36C). Addition of 0.1 mM Ala-Phe caused a subtle increase in the lag phase, but permitted strong growth of the bacterium after approximately 2 hours.

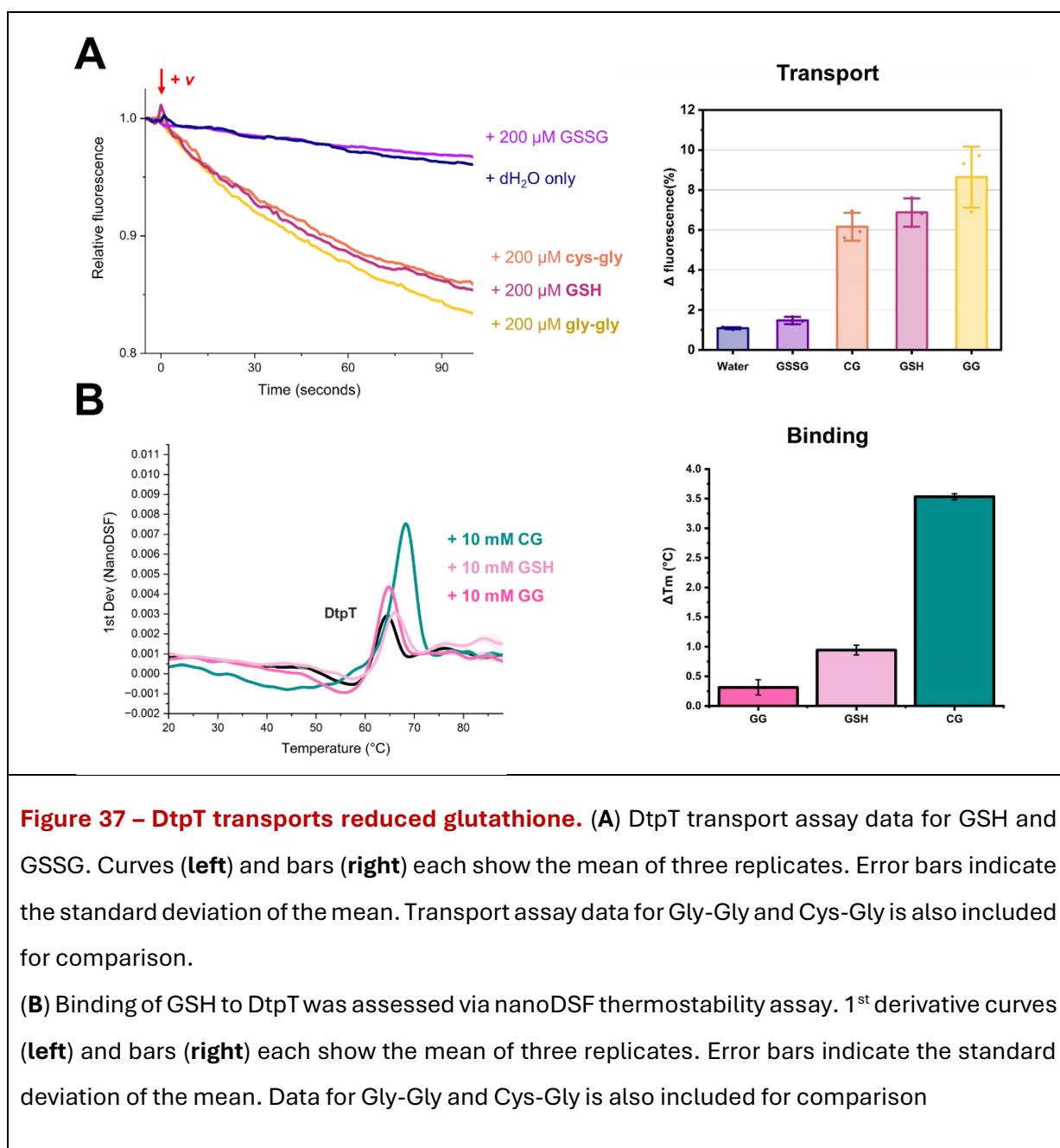


In contrast, 0.5 mM Ala-Phe led to a significantly longer lag phase of 10 hours and slower maximal growth rate. Lag phase and growth rate were most impacted when Ala-Phe and Arg-Asp were included at equimolar concentrations, or when Ala-Phe was added in excess. When growth was not DtpT-dependent (e.g. in the presence of free Arg), equally high concentrations of Ala-Phe did not inhibit growth. Overall, these data demonstrate that Ala-Phe does inhibit DtpT-mediated transport of Arg-containing peptides in a dose-dependent manner.

DtpT transports reduced glutathione (GSH)

Here, we demonstrate that DtpT is able to transport the cysteine-containing dipeptide Cys-Gly (Figure 35), implicating this system in the acquisition of thiol-containing peptides which may serve as a potential source of nutrient sulphur for *S. aureus* in biological environments. With this in mind, we considered whether other more biologically prevalent thiol-containing peptides may also be substrates of DtpT. Reduced glutathione (GSH) is a biologically ubiquitous tripeptide metabolite composed of Cys-Gly conjugated to glutamate via an unconventional γ -linkage (Figure 19A-B). Compared to other putative peptide substrates of DtpT, GSH is known to be abundant in human tissues and enriched in relevant infection contexts (specifically, intracellular environments) (Lensmire et al., 2023; Meister, 1988). Given that glutamate-containing peptides were enriched among DtpT substrates identified in our phenotype microarrays (Figure 23B), we predicted that DtpT may also transport GSH, and this activity may be relevant in biological environments where glutathione is enriched.

To test this, we carried out DtpT transport assays with both GSH and the oxidised form of glutathione (GSSG) (Figure 37A). We observed strong acidification of the liposome lumen upon induction of transport in the presence of GSH, roughly equivalent in magnitude to that observed for Cys-Gly. No such acidification is observed for GSSG, suggesting that DtpT can transport the reduced form of glutathione but not the oxidised form (Figure 37A). This is expected, given that GSSG is effectively a hexapeptide and likely too large to be accommodated within the DtpT binding groove. To better understand the interactions between DtpT and GSH, protein-ligand interactions were probed by nanoDSF (Figure 37B). We found that the addition of GSH had a mild stabilising effect on DtpT causing a T_m shift of approximately 1°C, indicative of binding. Binding of GSH was notably weaker than that observed for Cys-Gly ($\Delta T_m \approx 3.5^\circ\text{C}$), suggesting that the addition of a γ -glutamyl group in this tripeptide results in less favourable binding interactions despite a negligible effect on the transport rate.



Functional characterisation of DtpT residues implicated in the binding and transport of dipeptides and GSH

To the best of our knowledge, our findings provide the first biochemical evidence of glutathione transport by a bacterial POT transporter. In recent years, a number of studies have aimed to elucidate the structural basis of substrate recognition and transport in POTs, and these efforts have resulted in a number of highly informative POT-peptide complex structures ([Appendix 1](#)). These structures allow us to predict how substrates including GSH may be recognised by DtpT, providing valuable insights into the protein-ligand interactions required for transport of this novel substrate.

Blind docking projects two possible binding conformations of GSH

Previously, the structure of the *S. hominis* POT PepT_{Sh} was solved in complex with the human metabolite S-Cys-Gly-3M3SH in an inward-open conformation ([Minhas et al., 2018](#); PDB accession 6EXS). The forementioned structure was used to generate a structural model of DtpT ([Figure 30A](#)) and GSH was docked into this projected structure using CB-Dock2 ([Y. Liu et al., 2022](#)). This blind-docking approach projects two possible structures in which GSH is bound entirely within the central binding groove of the protein with respective docking scores of -5.0 and -5.3 (a more negative docking score denotes a tighter binding affinity) ([Figure 38A](#)).

The first of the two docked structures (labelled “Dock 1”) utilises the aforementioned PepT_{Sh} complex structure as a template, projecting a conformation in which GSH is positioned similarly to S-Cys-Gly-3M3SH within the binding groove. In this projection, GSH adopts a vertical conformation with the Cys-Gly group occupying a nearly identical position to the equivalent groups in the S-Cys-Gly-3M3SH structure, albeit shifted slightly ([Figure 38B](#)). Herein, the substrate C-terminus is stabilised primarily by a hydrogen bond interaction with Tyr41. The γ -glutamyl group of GSH in this projection is accommodated within a deep cleft adjacent to the extracellular gate. As a result, the α -carboxyl-terminus of the glutamate is coordinated by a hydrogen bond with Gln310. The nearby residue Asn347 also forms part of the deep binding cleft and could, potentially, move to within hydrogen bonding distance of the γ -glutamyl carboxyl-terminus.

The second structure (labelled “Dock 2”) predicts that GSH is bound in a position closer to the intracellular gate of the protein in a lateral conformation ([Figure 38C](#)). In this projection, the Cys group of GSH occupies a similar position to the substrate C-terminal Gly residue in Dock 1. The γ -glutamyl group in this conformation sits in a spacious hydrophobic pocket which is mostly

conserved in bacterial POTs and was previously observed to accommodate bulky position 2 side-chain groups of di/tripeptides in both DtpB_{Ec} and PepT_{St} (e.g. pocket P2, as described in [Figure 9](#)) ([Kotov et al., 2023](#); [Lyons et al., 2014](#); [Martinez Molledo, Quistgaard, & Löw, 2018](#)).

It is worth noting that many of the interactions previously observed between S-Cys-Gly-3M3SH and PepT_{Sh} in the solved crystal structure were mediated by water molecules within the protein binding groove. These solvent molecules are absent in our docked structures but may similarly mediate contacts with GSH and thereby influence the binding conformation of this substrate.

Mutagenesis rationale and purification of mutants

To delineate which predicted conformation is more likely to represent the true binding mode of GSH during transport, mutant variants of DtpT were generated with alterations in residues implicated in binding or transport, as deduced from sequence alignment with a number of structurally characterised POT family members ([Figure 18](#); [38A/39](#)). We designed seven mutant variants, each with a single alteration affecting one residue of the protein:

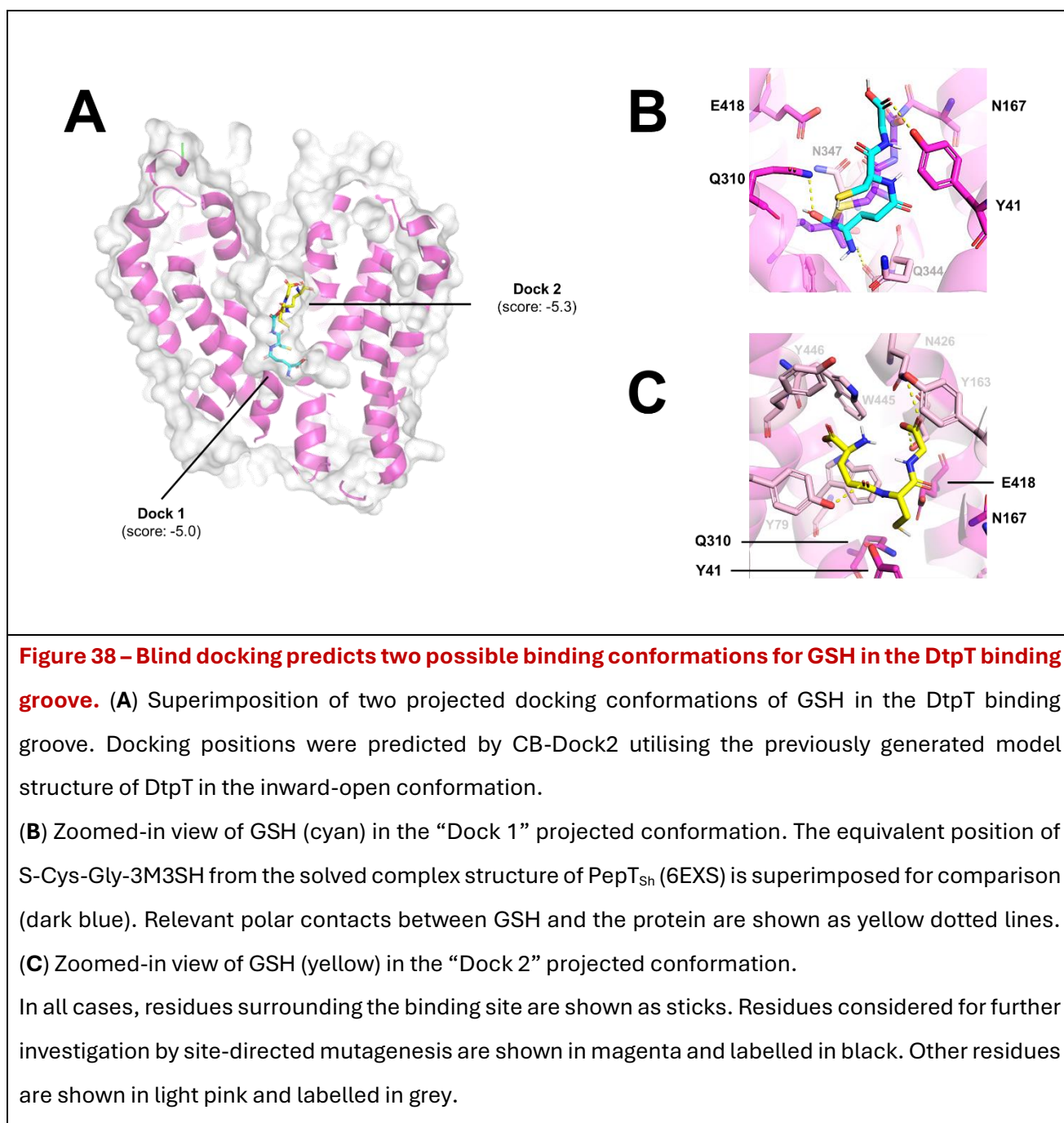
E33A, Y41A and Y41F – Glu33 and Tyr41 form part of the conserved E₃₃xxERFxYY motif found ubiquitously in POT transporters and known to contribute toward proton coupling during the transport cycle ([Lichtinger et al., 2024](#); [Solcan et al., 2012](#)). We therefore expect that alteration of either of these residues will result in a reduced rate of transport for all peptide substrates. Tyr41 was also previously demonstrated to coordinate the carboxy-terminus of S-Cys-Gly-3M3SH in PepT_{Sh} via hydrogen bonding, and the equivalent residue has been shown to interact directly with peptide substrates in other bacterial POTs ([Lyons et al., 2014](#); [Minhas et al., 2018](#)). In the predicted “Dock 2” conformation, no such direct contacts exist between GSH and Tyr41. Hence, in this conformation, transport of GSH may not be affected as dramatically by alterations to Tyr41 as a traditional dipeptide substrate, such as Gly-Gly.

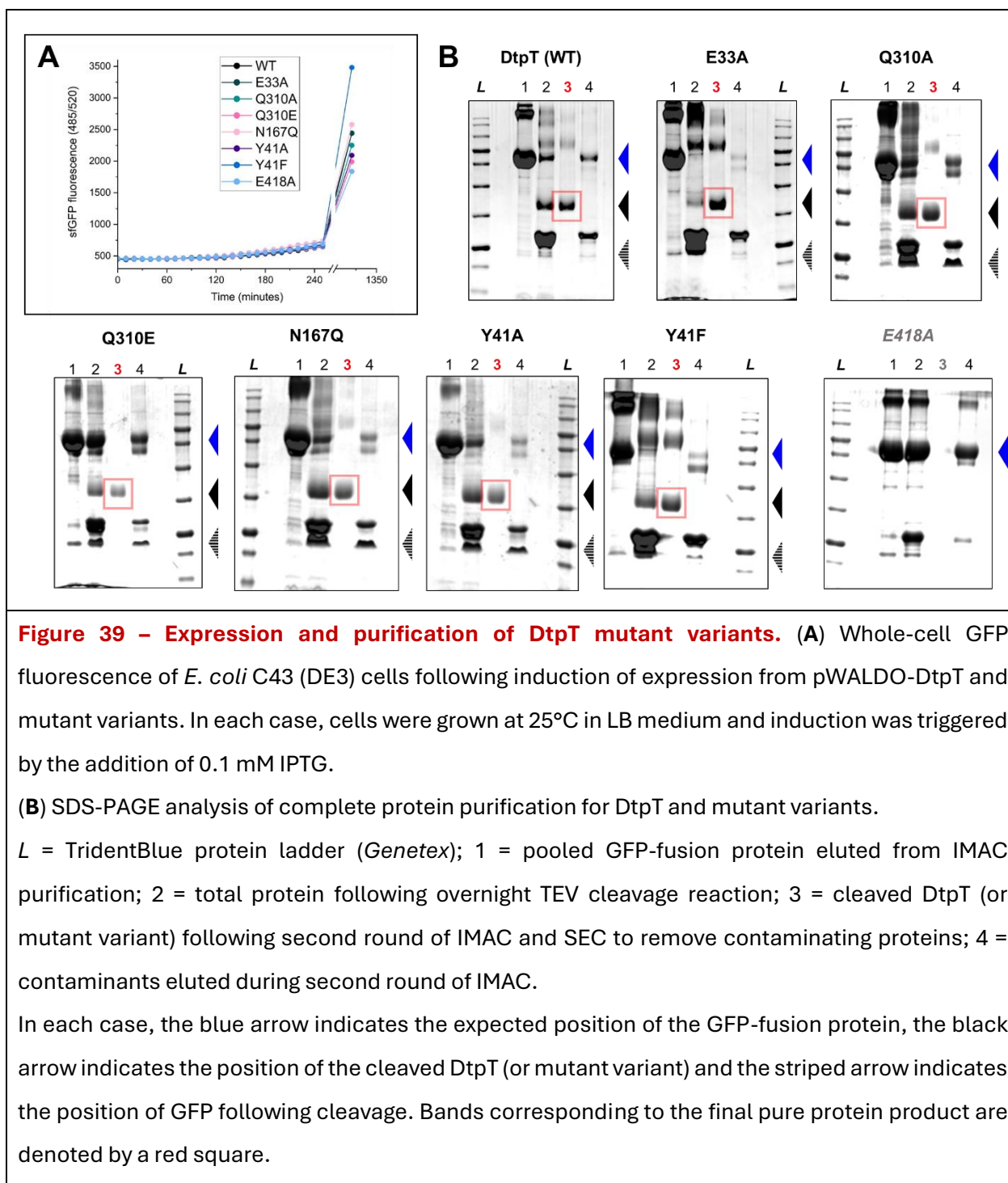
E418A – Glu418 is universally conserved in POTs and is thought to be essential for substrate coordination and proton coupling. Alteration of Glu418 was shown to abolish transport of dipeptides in PepT_{Sh} ([Minhas et al., 2018](#)). We therefore expect that proton-coupled transport will be abolished in an E418A mutant of DtpT.

N167Q – Asn167 is also universally conserved across POT proteins and is thought to be required for anchoring the position 1 amino terminus of dipeptide substrates ([Kotov et al., 2023](#)). As such, modification of Asn167 to a larger glutamine residue is predicted to occlude binding of traditional peptide substrate and thereby abolish transport. In both predicted docking conformations of GSH, the carboxamide sidechain of Asn167 is positioned adjacent to the substrate at a distance

of around 4.2 Å and does not form direct contacts. As such, it is unclear whether modification of this residue will have a significant effect on binding or transport for GSH.

Q310E and **Q310A** – In the solved structure of PepT_{Sh}, Gln310 is positioned close to the extracellular gate and forms part of the deep pocket involved in accommodating the thioalcohol moiety of S-Cys-Gly-3M3SH ([Minhas et al., 2018](#)). In the projected DtpT-GSH “Dock 1” complex structure, the carboxamide sidechain of this residue is positioned at a distance of 2.6 Å from the α-carboxyl group of the GSH substrate, facilitating the formation of a hydrogen bond. In the “Dock 2” conformation, no such contacts exist between Gln310 and the substrate. We therefore expect that GSH transport will be strongly reduced in a Q310A mutant only if GSH is coordinated in a vertical “Dock 1” conformation. Conversely, transport may not be significantly affected in a Q310E mutant, as a Glu residue in this position would still be capable of forming a hydrogen bond with the substrate in this conformation if protonated during the transport cycle.





All seven mutant DtpT variants listed above were generated in the established pWALDO expression plasmid via PCR mutagenesis and blunt-end ligation. Expression of each variant under the previously determined optimal expression conditions for DtpT (Figure 28) was confirmed by whole-cell GFP fluorescence (Figure 39A), and all seven variants were taken forward for purification. From this, six of the mutant DtpT variants were successfully purified and taken forward for further characterisation. Purification was not successful for the E418A variant, seemingly due to failure of the TEV protease cleavage reaction (Figure 39B). Indeed, DNA sequencing subsequently identified a missense single nucleotide polymorphism (SNP) within the TEV cleavage site in the pWALDO-DtpT_{E418A} plasmid which would be expected to abolish the activity of the protease (resulting in a substitution of ENLYFQG > VNLYFQG in the peptide product). Several of the mutant proteins eluted as a single band at the expected size for DtpT, though some variants demonstrated at least one additional band of larger size in the pure product (for instance, E33A; Figure 39B). Similar bands were previously observed for the wild type protein and these were identified as DtpT based on in-gel fluorescence analysis (see page 152).

NanoDSF suggests an important structural role for Glu33 in protein unfolding

To compare thermostability and ligand binding, the six mutant variants of DtpT were subjected to a nanoDSF binding screen against a panel of four peptide ligands: Gly-Gly, Cys-Gly, GSH and Ala-Ala (Figure 40A-D; Appendix 16). In the absence of peptides, each of the mutant DtpT variants demonstrated a T_m decrease relative to the wild type protein, indicative of an overall decrease in stability (Figure 40A-B). All variants also demonstrated a similar 1st derivative unfolding curve with a single negative inflection followed by a positive inflection point, with the exception of the E33A mutant. Protein unfolding was not associated with a strong positive inflection in this variant, but instead caused a single negative inflection point within the expected temperature range corresponding to the T_m (61.1°C) (Figure 40C). If we interpret this negative inflection point as the value of T_m , this would be the lowest value for any of the six DtpT mutants tested here. Overall, these observations suggest a critical role for Glu33 in conformational stability and protein unfolding dynamics. Intriguingly, a positive inflection point is restored in the E33A mutant upon addition of the tight-binding peptide ligands Cys-Gly and Ala-Ala, but this was not observed for Gly-Gly or GSH (Figure 40D; Appendix 16). The observation that a T_m shift and positive inflection maximum is observed upon the addition of known ligands provides compelling evidence that the E33A variant maintains a functional peptide binding site despite the apparent changes to protein unfolding.

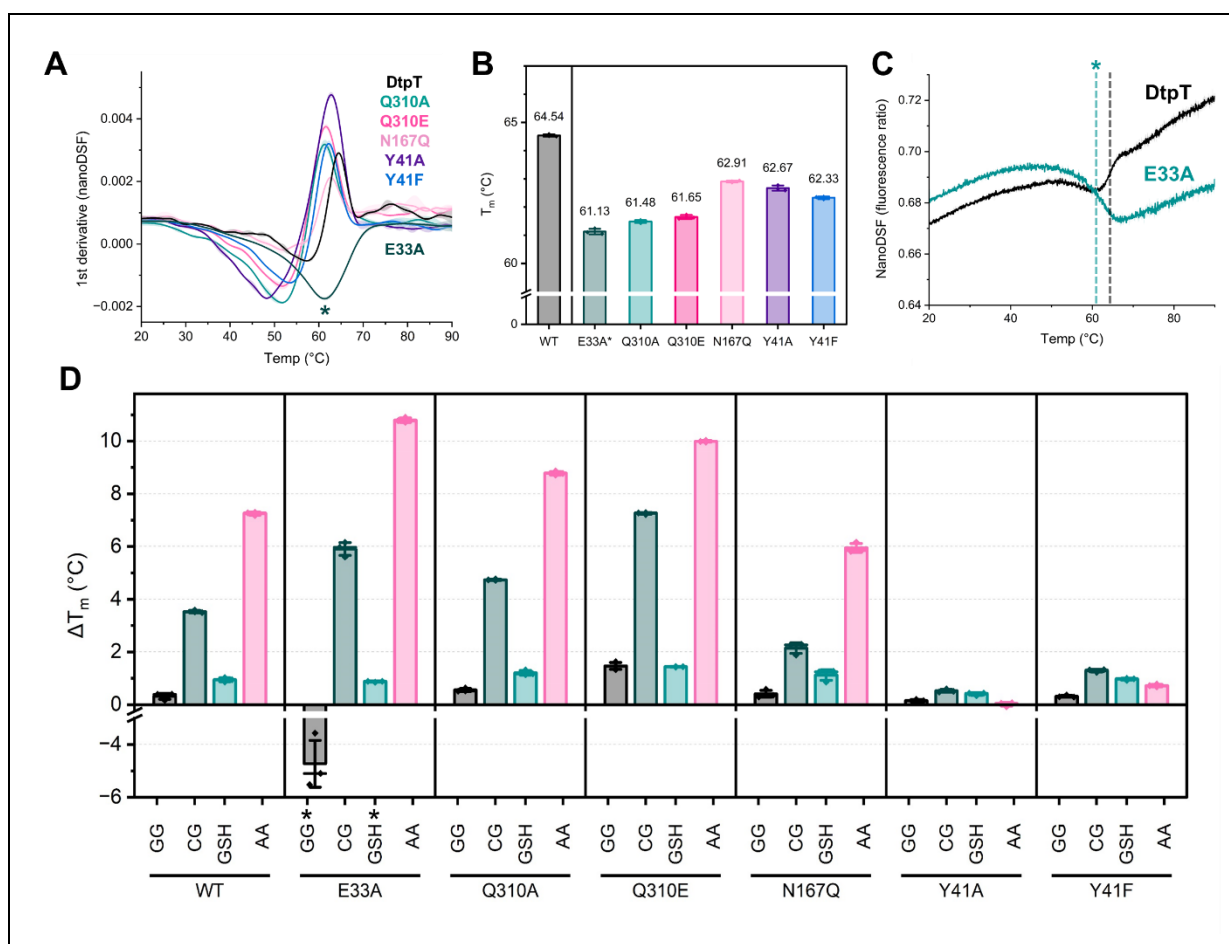


Figure 40 – Site-directed mutagenesis reveals residues contributing toward peptide binding and stability in DtpT. (A) NanoDSF 1st derivative unfolding curves of DtpT and six mutant variants.

Curves indicate the mean for each reading and the standard deviation in each case is indicated by the shaded area.

(B) T_m values of all seven DtpT variants determined by nanoDSF. Bars indicate the mean ± standard deviation.

(C) NanoDSF unfolding curves of wild-type DtpT and the E33A mutant variant. Curves indicate the mean for each reading and the standard deviation in each case is indicated by the shaded area.

(D) Summarised nanoDSF thermal shift data for seven DtpT variants against a panel of four peptide ligands. Bars indicate the mean ± standard deviation.

For some experiments, there was no positive inflection point observed for the 1st derivative curve. In these cases, T_m was interpreted as the single negative inflection point and values derived this way are denoted by an asterisk, as exemplified in A for the E33A variant.

Tyr41 is required for tight ligand-binding interactions

Both Tyr41 and Asn167 are universally conserved residues implicated in substrate anchoring for di- and tripeptides in diverse POT-complex structures ([Figure 9/10](#)). Modification of Asn167 to a glutamine residue led to a decrease in ΔT_m in the presence of the tight-binding ligands Cys-Gly and Ala-Ala, consistent with a partial disruption of substrate binding interactions. On the other hand, binding was almost entirely abolished for all ligands in the Y41A mutant variant, and was severely restricted in the Y41F variant. Hence, we can conclude that Tyr41 is essential for formation of strong protein-ligand interactions in DtpT.

Alterations to Gln310 are neutral or advantageous in regard to peptide binding

In the absence of ligands, both the Q310A and Q310E DtpT variants demonstrate a decrease in T_m relative to the wild-type DtpT protein. In spite of this, both Gln310 mutants also demonstrate a positive thermal shift in the presence of all tested ligands, and in some cases the value of ΔT_m is notably increased relative to the wild type. This is particularly apparent in the Q310E variant, for which binding of Gly-Gly, Cys-Gly and Ala-Ala were all enhanced compared to the wild-type. Altogether, these observations suggest that Gln310 contributes to protein thermostability but is not essential for substrate recognition.

Glu33 is required for proton-coupled transport

In order to assess the contribution of the selected residues toward DtpT-mediated peptide transport, the six mutant variants were each reconstituted into liposomes ([Figure 41](#)). To facilitate comparison between the different DtpT variants, the volume of each liposome suspension was adjusted such that protein concentration was 0.5 mg/ml in each case. Transport activity was then assessed for each DtpT variant for three substrates: Gly-Gly, Cys-Gly and GSH.

The conserved ExxERFxYY motif comprises part of helix 1 and plays an essential role in proton coupling and substrate coordination. Alteration of the first Glu residue in this motif (i.e. the equivalent residue to Glu33 in DtpT) was previously found to abolish transport of the model substrate Ala-Ala in other characterised bacterial POT systems ([Doki et al., 2013](#); [Parker et al., 2014](#)). Consistent with this, we demonstrate that transport across all three tested substrates was abolished for the E33A mutant variant of DtpT by demonstrating that acidification of the liposome lumen occurred at levels equivalent to wild type DtpT liposomes in the absence of substrate ([Figure 41B](#)). These data reinforce the essential role of Glu33 in proton-coupled transport.

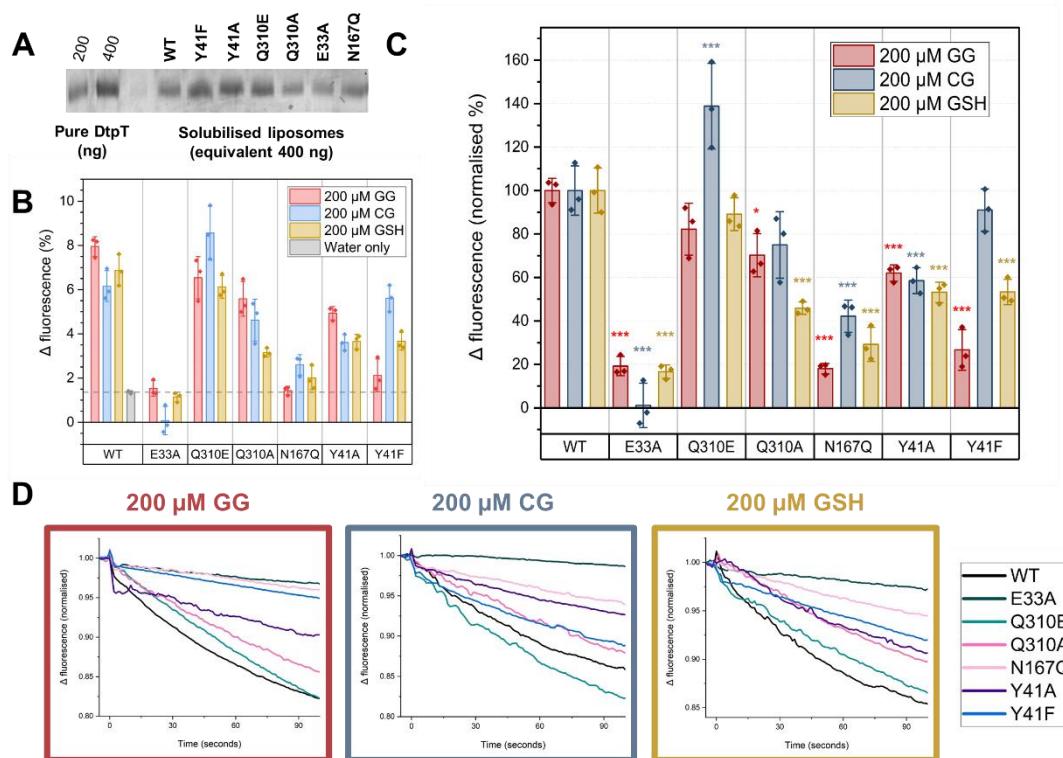


Figure 41 – Site-directed mutagenesis reveals residues contributing toward transport in

DtpT. (A) SDS PAGE comparison of pure DtpT against solubilised liposomes containing wild-type or mutant variants of DtpT, as labelled. Band intensities were used to normalise the final liposomal protein concentration to 0.5 mg/ml.

(B) Summarised transport data for DtpT and mutant variants against Gly-Gly, Cys-Gly and GSH. Transport activity is compared by quantifying the change in pyranine fluorescence over the first 30 seconds of the assay. A grey dashed line indicates the Δ fluorescence (%) recorded for DtpT liposomes in the absence of substrate (“water only”). Bars indicate the mean $\pm$ standard deviation.

(C) Transport data for DtpT mutant variants, expressed as a percentage of the activity for the wild-type protein. Bars indicate the mean $\pm$ standard deviation. * $p < 0.05$, *** $p < 0.005$; one-way ANOVA with Tukey honestly significant difference (HSD) test.

(D) Complete transport assay curves for each of three peptide substrates, normalised immediately before the addition of valinomycin for ease of comparison. Curves show the mean of three replicates.

GSH transport is disproportionately affected in a Q310A mutant

Here, we observe that the initial rate of transport of GSH is reduced by at least 50% for DtpT variants with alterations to either Tyr41 or Asn167 (**Figure 41C**), which is consistent with the expected importance of these residues in proton coupling and substrate coordination. Transport of GSH was similarly reduced in the Q310A mutant variant of DtpT, whilst transport of the dipeptides Gly-Gly and Cys-Gly was decreased only marginally in this variant. Such an observation suggests that Gln310 may play an important role in the transport of GSH specifically, and thereby provides compelling evidence that GSH binds in vertical conformation in which the γ -glutamate group is coordinated specifically by interactions with Gln310 (as in Dock 1). Such a model is further supported by the observation that GSH transport is not significantly affected in the Q310E variant, given that the proposed hydrogen bond interactions between this residue and GSH projected in the Dock 1 conformation would be largely maintained in this variant.

Transport of Cys-Gly is increased in a Q310E mutant

Here, we observe that transport of both Gly-Gly and GSH is not significantly affected in a Q310E mutant, while the initial transport rate of Cys-Gly is increased by roughly 40%. Previously, we observed that the strength of binding interactions between DtpT and Cys-Gly was increased in a Q310E mutant compared to the wild-type (**Figure 40**). Together, these observations suggest that the carboxamide side chain of Gln310 is less favourable than a carboxyl side chain in the equivalent position in regard to both binding and transport of Cys-Gly, perhaps due to difference in the charge/size properties of this side chain which facilitate better accommodation of the substrate.

Transport of Cys-Gly was not significantly reduced in the Y41F mutant despite binding of all tested peptides being almost entirely abolished in this variant. In contrast, transport of both Gly-Gly and GSH was limited in the Y41F mutant. These observations suggest that relevance of Tyr41 may differ for transport of Cys-Gly compared to the other substrates tested here.

DtpT transports the broad-spectrum antibiotic chloramphenicol

In addition to their roles as peptide transporters, several POTs have been implicated in the transport of pharmacologically relevant peptidomimetic compounds including antibiotics ([Parker et al., 2024](#); [Prabhala et al., 2021](#)). In humans, POT-mediated transport is a well-established mechanism by which these compounds are adsorbed into the bloodstream from dietary sources ([Parker et al., 2024](#); [D. E. Smith et al., 2013](#)). Some bacterial POTs have also been shown to transport antibiotics including β -lactams and the protein-synthesis inhibitor chloramphenicol ([Prabhala et al., 2018](#); [Stauffer et al., 2021](#); [Weitz et al., 2007](#)). To date, the impact of POT-mediated transport on antibiotic efficacy and susceptibility in bacteria remains poorly characterised. Here, we assessed the ability of DtpT to transport a small panel of putative antibiotic substrates: three β -lactams (including the first-generation cephalosporin cefalexin), and the protein synthesis-inhibitors linezolid and chloramphenicol ([Figure 42A-B](#)). These antibiotics were selected based on the inclusion of a peptide linkage, which may facilitate recognition and transport by DtpT. Of the antibiotics selected for investigation here, only linezolid is routinely utilised for the treatment of *S. aureus* infections in clinical settings ([Stevens et al., 2002](#)); the remaining antibiotics were used historically, but their continued applicability is challenged by widespread antibiotic resistance in circulating strains of the bacterium ([Lyon & Skurray, 1987](#)).

Due to solubility constraints for some of these compounds, transport was assessed at a concentration of 100 μ M (decreased from 200 μ M, utilised for peptide transport assays) and compared to the known DtpT-substrate GSH. Two of the tested antibiotics demonstrated non-negligible acidification of the liposome lumen, indicative of transport; cefalexin and chloramphenicol ([Figure 42A-B](#)). For both antibiotics, the transport assay curves demonstrated an unusual shape indicative of initially rapid acidification of the liposome lumen, which rapidly transitioned to a more gradual acidification after several seconds (i.e. a sharp “L” shaped curve, as opposed to the more gradual curve observed for GSH) ([Figure 42A](#)). The molecular basis of this pattern is unclear, but may relate to the distinct chemical properties of these substrates.

DtpT-mediated transport of chloramphenicol affects growth inhibition kinetics in S. aureus

Of the two antibiotics with demonstrable transport activity, chloramphenicol was responsible for a stronger fluorescence change over the first 30 seconds of the assay. Based on this, we decided to assess whether the presence or absence of DtpT affected the efficacy of chloramphenicol against *S. aureus* cells ([Figure 42C-D](#)). Cells were grown to early log phase in rich medium before being spiked with chloramphenicol to a final concentration of 100 μ M ([Figure 42D](#)). In the

absence of antibiotic, both wild type and *dtpT* mutant cells grew similarly (Figure 42D). After addition of chloramphenicol, both strains demonstrated a decrease in growth rate. The growth of the *dtpT* mutant under this condition was initially higher than that of the wild type, perhaps as a result of decreased accumulation of the antibiotic in the cytoplasm (Figure 42D). However, upon reaching a maximal OD₆₀₀ value (after ~7 hours of growth), the *dtpT* mutant strain began to decline in optical density, recovering again only after roughly 15 hours of growth. A similar pattern of growth and decline was observed in the wild type, albeit at a later time point (perhaps as a result of the slower initial growth rate) (Figure 42D). Recent evidence suggests that efflux systems are exclusively expressed during rapid bacterial growth in *S. aureus* (Whittle et al., 2021). Hence, the bacterial killing effect of observed at later stages of growth in our assays may correspond to the decreased expression of efflux systems at these cell densities (Figure 42D), potentially explaining why this phenomenon occurs independently of changes to influx.

Overall, our results indicate that DtpT-mediated transport of chloramphenicol may influence intracellular accumulation of the drug and thereby affect the kinetics of growth and inhibition, though this effect is seemingly not very pronounced at the concentration tested here. We also confirm the presence of at least one alternate route of cell entry for chloramphenicol, given that the *dtpT* mutant strain remained susceptible to the drug. Our findings would benefit from confirmation via *in-trans* complementation of *dtpT*, though we were not able to construct suitable expression vectors lacking a chloramphenicol-resistance cassette for this purpose.

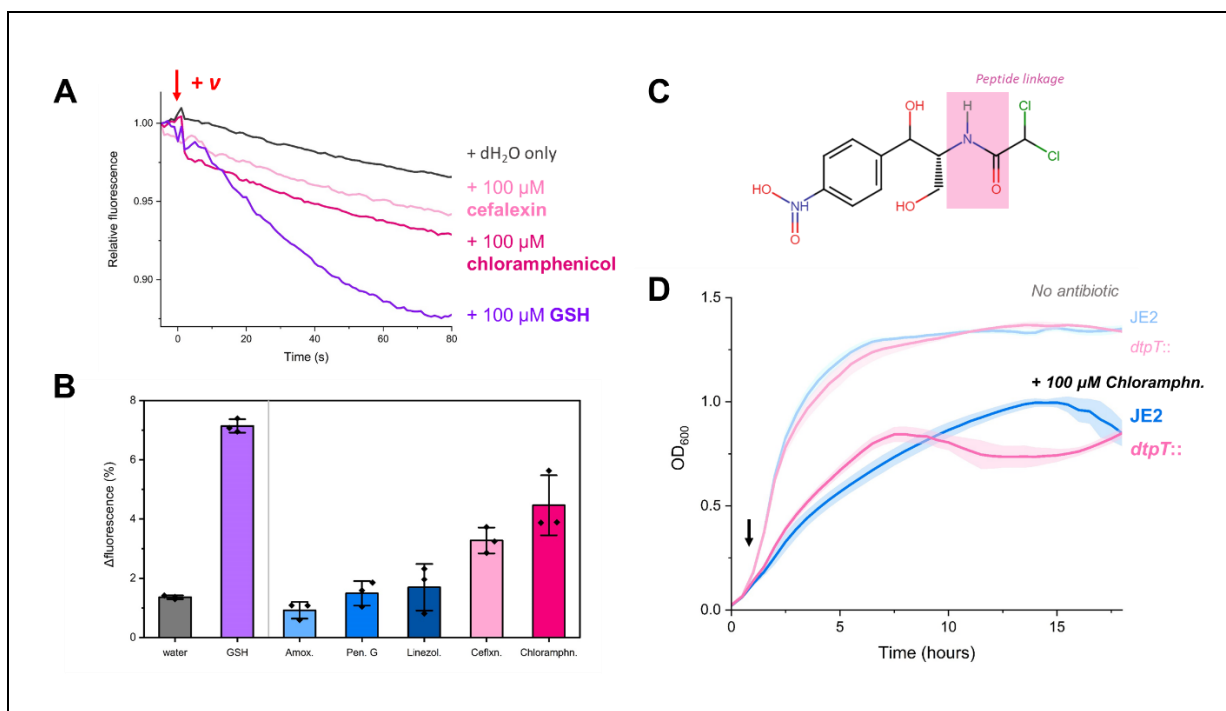


Figure 42 – DtpT transports the protein synthesis inhibitor chloramphenicol. (A) Example transport assay curves for cefalexin and chloramphenicol normalised immediately before the addition of valinomycin (red arrow) for ease of comparison. Curves show the mean of three replicates. The known DtpT-substrate GSH was included as a positive control.

(B) Transport was assessed for 5 peptidomimetic antibiotics by comparing the change in fluorescence over the first 30 seconds after the addition of valinomycin. Bars show the mean of three replicates $\pm$ the standard deviation of the mean. Blue bars indicate a change in fluorescence which did not significantly differ from the water-only control, as determined by a one-way ANOVA with Fisher LSD test ($p \geq 0.05$).

(C) The chemical structure of chloramphenicol. The position of the peptide bond is highlighted in pink.

(D) Wild-type *S. aureus* JE2 and *dtpT::* were grown in TSB for one hour (approx. OD₆₀₀ = 0.15), at which point chloramphenicol was added to a final concentration of 100 μ M (black arrow). In each case, strains were grown over 18 hours and OD₆₀₀ was measured every 15 minutes for three biological replicates. Curves indicate the mean values for each reading and the standard deviation in each case is indicated by the shaded area.

Discussion

Historically, attempts to understand the molecular basis of peptide transport in *S. aureus* have relied entirely on whole-cell growth experiments ([Hiron et al., 2007](#)). While these approaches can demonstrate transporter-specific utilisation of individual peptides, they provide little-to-no information regarding the distinct biochemical preferences for individual peptide substrates, nor can they be readily applied to probe the molecular factors influencing substrate recognition and transport in these proteins.

Here, we present the first biochemical analysis of DtpT in regard to both substrate binding and transport. These data nicely complement the whole-cell phenotypic screening approaches described in [Chapter 3](#), demonstrating that DtpT preferentially transports a subset of peptides with distinct chemical qualities. Specifically, we observed preferential transport of peptides incorporating acidic amino acids (e.g. Arg-Asp and Gly-Glu) or amino acids with small, uncharged side chains (Ala-Ala, Gly-Gly and Gly-Pro) ([Figure 35](#)). In contrast, we demonstrate that dipeptides containing aromatic residues in position 2 (e.g. Val-Tyr and Ala-Phe) generally demonstrated poorer transport when compared to those with less bulky side chains in the equivalent position ([Figure 35](#)). These observed preferences are mostly consistent with patterns observed in our phenotypic analysis ([Chapter 3](#)) and point toward a novel degree of selectivity in DtpT, allowing for the accumulation of specific amino acids during nutrient acquisition (as will be discussed in [Chapter 6](#), at the end of this thesis).

Taken together, the data presented in this chapter provide novel insights into the relationship between substrate binding and transport in DtpT, and these findings may be relevant in understanding protein-substrate interactions in POTs more generally. Briefly, multiple experiments carried out here indicate that transport in DtpT cannot be predicted based on the strength of ligand binding interactions. Here, I will outline the reasoning behind this conclusion and describe how our findings relate to earlier observations regarding POT transporter biology. As part of this discussion, I will also discuss the potential limitations of the methodology utilised in this study and outline how these limitations may be addressed in future works.

Understanding the relationship between peptide binding and transport in POTs

To date, two substrate binding modes have been observed in POT-peptide complex structures; the laterally bound conformation observed for dipeptide-bound structures and some tripeptides ([Kotov et al., 2023](#); [Newstead, 2017](#)), as well as a vertical conformation first proposed for Ala-

Ala-Ala in PepT_{St} ([Lyons et al., 2014](#)) and later demonstrated for S-Cys-Gly-3M3SH in the *S. hominis* POT PepT_{Sh} ([Minhas et al., 2018](#)). Both of these binding modes are enabled by direct interactions between the substrate C-terminus and residues comprising the conserved ExxERxYY motif on TM helix 1, and it is these interactions which are thought to be a major driver of proton-coupled substrate transport in these proteins ([Lichtinger et al., 2024](#)). Meanwhile, in both lateral and vertical conformations, the substrate sidechain groups are selectively accommodated within defined pockets lining the binding groove ([Newstead, 2017](#)) and interactions between the protein and peptide sidechains are largely responsible for differences in the binding affinity of putative peptide substrates ([Kotov et al., 2023](#)). Hence, we can define substrate recognition and transport in POTs as two separate but mutually dependent phenomena, with the former primarily driven by interactions with the substrate sidechains and the latter driven almost exclusively by interactions with the backbone termini. In other words, selectivity in POTs is dependent on interactions which are distinct from those required for the transport cycle.

Logically, therefore, sidechain accommodation is an essential prerequisite step required for selective transport of peptides in POTs, but these interactions play a minimal role in facilitating transport. Accordingly, our data demonstrate that the strength of ligand binding interactions shows no apparent relationship to transport rate in DtpT. Indeed, several peptides which bound poorly to DtpT – for instance, Arg-Asp – were transported at comparable rates to the tightest binding ligands Ala-Ala and Ala-Leu ([Figure 31/35](#)). One of the earliest structural studies investigating ligand binding in POTs demonstrated that the dipeptide Ala-Phe is stabilised by extensive hydrophobic contacts between the Phe sidechain and the conserved hydrophobic pocket P2 in the binding groove ([Lyons et al., 2014](#)). Consistent with this report, we found that Ala-Phe had a strong stabilising effect on DtpT, indicative of tight binding ([Figure 31](#)). In spite of this, DtpT-mediated transport of Ala-Phe was negligible ([Figure 35/36](#)). Gly-Gly – which has no sidechain groups – did not demonstrate strong binding to DtpT, but was transported preferably ([Figure 31/36](#)). Overall, there is no clear correlation between the strength of substrate binding for individual peptides and transport rate in DtpT.

On the other hand, our site-directed mutagenesis experiments provide some indication that binding interactions involving the substrate N- and C- termini do contribute toward transport rate in DtpT. Asn167 and Tyr41 are predicted to coordinate binding of the N- and C-termini of dipeptide substrates in DtpT, respectively ([Figure 9/10](#)). As expected, ligand binding was abolished for all putative dipeptide ligands in the Y41A mutant variant ([Figure 40](#)), and this variant similarly demonstrated a uniform decrease in transport activity across the tested substrates

(Figure 41). The effect of ligand binding in a N167Q mutant was less dramatic (Figure 40), but this variant similarly demonstrated a decrease in transport rate across the tested substrates (Figure 41). Together, these observations point toward a key role for anchoring interactions at the substrate termini rather than interactions involving the sidechains (see Figures 9-10) in determining transport rate in POTs.

Altogether, our data support a model in which tight binding interactions involving substrate sidechains are not a major determinant of transport for DtpT. Considering this raises an important question: why, then, are some peptides transported more favourably than others by DtpT?

In their recent work, Kotov and colleagues proposed a “Goldilocks” hypothesis in which optimal transport substrates of the *E. coli* POT DtpB are, generally speaking, peptides with median binding affinities. These conclusions are based on a combination of estimated binding affinities for a panel of 81 peptides (as determined by nanoDSF) as well as pyranine transport assays for 26 peptides and, subsequently, computational modelling of the remaining possible di/tripeptide structures (Kotov et al., 2023). Based on their evidence, the authors conclude that peptides with poor binding are generally poor substrates (perhaps due to a lack of initial binding interactions required for transport), whilst peptides with high affinities may bind too tightly in the binding groove and thereby fail to dissociate upon binding, effectively inhibiting transport (Kotov et al., 2023). Our data do not clearly indicate a similar relationship between binding and transport in DtpT, though it is important to note that our work has utilised a more limited peptide panel than the aforementioned study (particularly in regard to binding). Expanding the pool of putative ligands assessed may shed new light on the molecular factors governing substrate selectivity in DtpT.

Evidence of differential binding modes for two dipeptides in DtpT

All POT-dipeptide complex structures to-date demonstrate a conserved binding mode in which the substrate is bound laterally via anchoring interactions at the N- and C-termini (as outlined in page 45). Whilst alternate binding conformations have been demonstrated for tripeptides and unconventional POT substrates (i.e. S-Cys-Gly-3M3SH), the existence of alternative binding modes for dipeptides has not yet been thoroughly considered (Lyons et al., 2014; Minhas et al., 2018).

In our mutagenesis experiments, we note a number of distinctions between the behaviour of two dipeptide substrates: Gly-Gly and Cys-Gly. In the case of the E33A mutant variant, binding of Cys-

Gly stabilised the protein and restored the appearance of a positive inflection point in the 1st derivative unfolding curve. Conversely, the addition of Gly-Gly caused an apparent destabilisation of the E33A variant (Figure 40). Binding of both peptides was improved in a Q310E mutant variant (that is, T_m was increased relative to the wild type protein) (Figure 40), but transport rate was exclusively increased for Cys-Gly in this variant (Figure 41). Similarly, transport of Gly-Gly was almost entirely abolished in a Y41F mutant, whilst transport of Cys-Gly was comparable to the wild type. Transport of Cys-Gly (but not Gly-Gly) was also permitted in the N167Q mutant variant (Figure 41). Overall, these observations demonstrate that different residues influence the binding and transport of the two dipeptides Gly-Gly and Cys-Gly, and this serves as compelling evidence for the existence of distinct interactions between the protein and substrate in each case.

A conserved binding mode for Cys-Gly conjugated peptide analogues

The apparent binding strength of GSH was almost entirely unaffected across the mutant DtpT variants assessed here, with the exception of the Y41A variant for which binding was abolished (Figure 40). In spite of this, transport of GSH was significantly decreased in N167Q, Y41A, Y41F and Q310A mutant variants (Figure 41). Tyr41 and Gln310 were previously implicated in binding of the Cys-Gly-conjugate human metabolite S-Cys-Gly-3M3SH in PepT_{Sh} (Minhas et al., 2018), and these residues were similarly predicted to coordinate GSH in DtpT in the first of two blind-docking conformations presented here (i.e. “Dock 1”), which closely resembled the binding mode of the previously described structure (Figure 38). In particular, this docked conformation predicts that coordination of the glutamyl terminal carboxyl group is primarily achieved through a hydrogen bond with the sidechain amide of Gln310. Similar interactions with Gln310 would not be expected for laterally bound dipeptide substrates. We confirm that transport of GSH was disproportionately attenuated in a Q310A mutant variant relative to the dipeptide substrates tested here (Figure 41), supporting the proposed binding conformation. Overall, our findings provide evidence that Cys-Gly-conjugated metabolites such as GSH and S-Cys-Gly-3M3SH are recognised and transported by DtpT via a conserved binding mode.

Limitations of our methodology

While the experiments outlined in this chapter provide valuable insights into the molecular factors influencing binding and transport in DtpT, it is important to note some potential limitations of the approaches utilised here.

Assessing protein-ligand interactions by nanoDSF

Here, we apply a nanoDSF unfolding screen as a medium/high-throughput approach to detect binding of diverse peptide ligands to DtpT. While informative, these assays assume that the concentration of ligand added (10 mM) is in excess of the binding K_D for biologically relevant protein-ligand interactions and is, therefore, sufficient to detect any such potential binding interactions. In reality, it is impossible to determine how the T_m values recorded here relate to absolute metrics of substrate binding (e.g. K_D), and our binding data should be considered as a qualitative indicator of binding strength. Similar thermal shift assays have been utilised to estimate K_D for POT-peptide interactions (e.g. by [Kotov et al., 2023](#)) by utilising variable ligand concentrations in each case, and this method could be readily adapted in future work to estimate ligand binding affinities in DtpT.

In biological systems, membrane proteins exist in a lipid bilayer and interactions with these membrane lipids influence protein conformation and function significantly ([Lee, 2004](#); [Mitchell, 2012](#); [Stieger et al., 2021](#)). Here, we screened DtpT-peptide binding interactions utilising DDM-solubilised protein in the absence of lipids ([Figure 31](#)). After conducting these experiments, circular dichroism and thermal unfolding assays were used to probe the behaviour of DtpT following reconstitution into lipid vesicles ([Figure 34](#)). These approaches suggest that the fold of DtpT in lipid bilayers is modified (and, as a result, more thermostable) when compared to the detergent-solubilised form ([Figure 34D-E](#)). Hence, it is unclear whether ligand binding in the native membrane-spanning conformation is faithfully conserved in the detergent-solubilised form. Future studies would benefit from a comparison between the effects of ligand binding in these two distinct environments.

Pyranine transport assays

Here, we applied a pyranine-based transport assay to compare transport for diverse peptide substrates by DtpT ([Figure 35](#)). One major caveat of this methodology is the assumption that diverse peptide substrates are translocated with the same proton stoichiometry, i.e. that the change in pH (as indicated by quenching of the pyranine dye) relates directly to substrate transport in each case. In reality, it is unclear how many protons are co-transported with each substrate during the transport cycle of DtpT. Thermodynamic evidence has demonstrated that structurally distinct peptides – specifically, dipeptide and tripeptides – may be transported with different proton stoichiometries within the same POT protein ([Parker et al., 2014](#)). Contrarily, recent molecular dynamics simulations and structural evidence point toward a conserved transport cycle in all POTs in which transport of a single substrate is exclusively coupled to the

movement of two protons across the membrane ([Lichtinger et al., 2024](#); [Parker et al., 2021](#)). Further investigation is required to determine whether proton stoichiometry is consistent between substrates of DtpT, and more generally to understand the factors governing proton stoichiometry in POTs as a whole.

Finally, as mentioned previously, the conformation and function of membrane proteins is often influenced by their native lipid environment ([Stieger et al., 2021](#)). Here, we assess transport in lipid vesicles designed to mimic the *E. coli* inner membrane, but these conditions may not faithfully replicate the behaviour of the transporter in native Staphylococcal membrane environments. The cytoplasmic membrane of *S. aureus* differs substantially from that of *E. coli*, lacking both PE and PC and instead being comprised primarily of phosphatidylglycerol and cardiolipin ([DeMars et al., 2020](#)). Hence, we assume that the activity of the protein is unaffected by the different lipid environment used here. Furthermore, by assessing the activity of DtpT in a cell-free context, we also assume that the activity of DtpT in *S. aureus* is not influenced by the presence of other interacting partners which may be present in the native cellular context.

Chapter 5 – Investigating the roles of glutathione transporters in *S. aureus* physiology and infection

Introduction

In [Chapter 4](#) of this thesis, I showed that the *S. aureus* POT transporter DtpT is capable of transporting the prevalent biological metabolite reduced glutathione (GSH) at micromolar concentrations ([Figure 37](#)). In Gram-negative bacteria (and some Gram-positive species, including *Listeria monocytogenes*), glutathione is synthesised *de novo* by the cell and plays diverse roles in the oxidative stress response and bacterial virulence (as reviewed recently by [Ku & Gan, 2021](#)). Other Gram-positive bacterial pathogens (specifically Streptococci) are thought to internalise host-derived glutathione to similarly provide a protective effect against exogenous oxidative stress encountered during infection ([Brouwer et al., 2022](#); [A. J. Potter et al., 2012](#)). Compared to these examples, the roles of glutathione in *S. aureus* remain poorly characterised beyond its potential as an exogenous nutrient sulphur source via the ABC-type glutathione import system (Gis) ([Lensmire et al., 2023](#)). However, utilisation of GSH at physiological concentrations (generally recorded as 0.5 - 10 mM in human tissue ([Cui et al., 2023](#); [N. Li et al., 2023](#))) occurs independently of Gis, suggesting additional routes of uptake for this metabolite in *S. aureus*.

Here, we utilise microbial growth experiments to confirm that DtpT is a functional GSH transporter in *S. aureus*. Based on this observation, we go on to explore the impact of exogenous GSH on *S. aureus* physiology, specifically in regard to oxidative stress responses and immune evasion. Our data sheds new light on the effects of exogenous glutathione on the oxidative stress response of *S. aureus* during growth *in vitro*, and provides the first indications that glutathione transport is an important fitness determinant during intracellular growth inside human macrophages. Collectively, our findings implicate glutathione as an important environmental signal and nutrient source for *S. aureus* in relevant biological contexts.

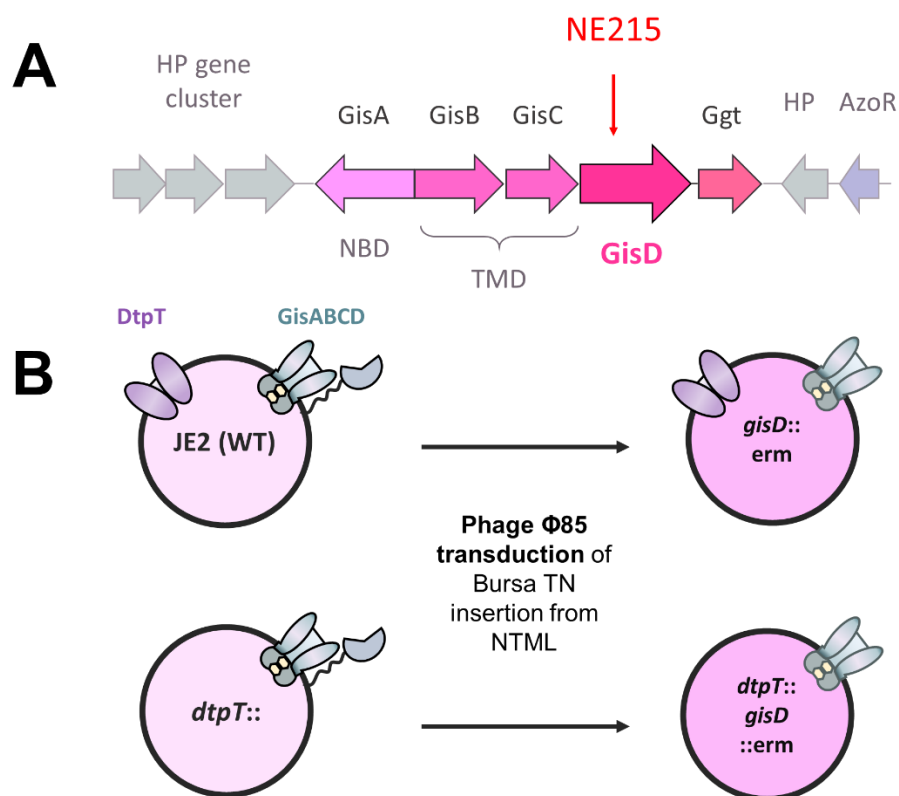


Figure 43 – Generation of *gisD* mutant strains for characterising the routes of glutathione uptake in *S. aureus*. (A) Chromosomal context of *gisD* in the JE2 genome. The approximate location of the Bursa TN insertion site is shown in red alongside the associated NTML strain label. Grey arrows = hypothetical proteins (HP). (B) Strategy for the generation of glutathione transporter-deficient strains utilised in this work.

Results

DtpT and GisABCD are the two glutathione transporters of *S. aureus*

In addition to DtpT – which we have demonstrated as a GSH transporter in our cell-free transport assays (Figure 37) – *S. aureus* encodes at least one other glutathione uptake system; the ABC transporter GisABCD (Lensmire et al., 2023). To investigate the respective roles of these two systems in glutathione utilisation, we generated a transposon-interrupted mutant of the SBP component of the Gis system (*gisD*) as well as a double-mutant of *dtpT* and *gisD* in the wild type strain JE2 (utilising a similar methodology as described on page 125; Figure 43) and both strains were confirmed by diagnostic PCR (Appendix 6). These strains – as well as the wild type and previously-generated *dtpT* single-mutant – were grown in chemically defined medium depleted of sulphur (CDMG – s) and supplemented with GSH or oxidised glutathione (GSSG) at various concentrations.

As expected, growth of the wild type strain was heavily restricted in media lacking an exogenous nutrient sulphur source (Figure 44A). At 1 mM GSH, single-mutants of either transport system demonstrate growth-defects relative to the wild type. Specifically, the *dtpT* mutant demonstrates an extended lag phase, but grows to a similar final cell-density as the wild type. In contrast, a *gisD* mutant behaves identically to the wild type over the first few hours of growth but ultimately plateaus at a lower final OD₆₀₀ after 24 hours (Figure 44A). At this concentration of GSH, growth of the double-mutant is strictly limited in a manner comparable to the wild type strain when no sulphur source is supplied (Figure 44A). When expressed *in-trans* from the complementation vector pSK56pT-DtpT (Figure 26A), *dtpT* restored wild type growth in the *dtpT* deficient mutant and partially restored growth of the double-mutant strain after an extended lag phase of ~ 6 hours (we hypothesise that this increased lag phase may result from competitive inhibition of DtpT by chloramphenicol, which we previously identified as a substrate of the transporter (Figure 42)) (Figure 44B). In addition, given that GSH oxidises spontaneously over time in aerated environments, we repeated the growth experiment with 1 mM GSH under anaerobic conditions to limit changes in the oxidation state of GSH during growth (Figure 44C). Again, we observe phenotypes which are consistent with those observed during aerobic growth. Collectively, these observations confirm that DtpT is a functional GSH transporter in *S. aureus* and provide compelling evidence that DtpT and GisABCD are entirely responsible for GSH uptake in this organism at physiologically relevant concentrations.

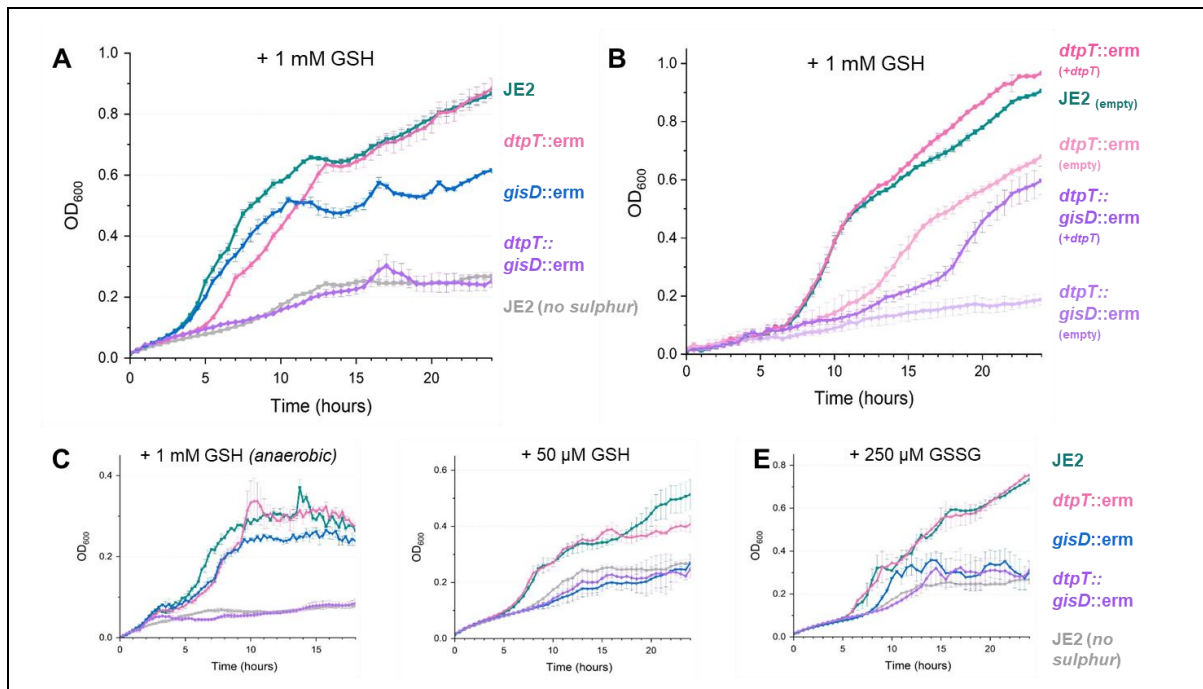


Figure 44 – DtpT and GisABCD contribute to glutathione utilisation in *S. aureus*. Growth of *S. aureus* strain JE2 (cyan), single-mutants of *dtpT* (pink) and *gisD* (blue) as well as a *dtpT gisD* double-mutant strain (purple) was assessed in CDMG – s supplemented with either GSH or GSSG. For **A-B** and **D-E**, strains were grown over 24 hours and OD₆₀₀ was measured every 30 minutes. For **C**, strains were grown over 18 hours and OD₆₀₀ was measured every 15 minutes. Curves indicate the mean values for three biological replicates ± standard deviation in each case.

(A) Growth of JE2 and mutant derivative in CDMG – s ± 1 mM GSH.

(B) Growth of a *dtpT* mutant and *dtpT gisD* double mutant carrying either pSK56pT (empty or pSK56pT-DtpT (+*dtpT*) in CDMG – s + 1 mM GSH supplemented with *cam*.

(C) Cells were grown as in **A**, but under anaerobic conditions in media supplemented with 100 mM sodium nitrate.

(D) Growth of JE2 and mutant derivative in CDMG – s ± 50 µM GSH.

(E) Growth of JE2 and mutant derivative in CDMG – s ± 250 µM GSSG.

GisABCD facilitates the utilisation of GSH at low concentrations

Generally, binding-protein dependant transport systems (including ABC transporters) operate with high affinities (e.g. nanomolar) whilst POTs and similar MFS systems have been reported as having substrate affinities in the high-micromolar to low millimolar range ([Kotov et al., 2023](#); [Samsudin et al., 2016](#)). Previous work has demonstrated how GisABCD exclusively facilitates the utilisation of GSH at low micromolar concentrations ([Lensmire et al., 2023](#)). The observation that GSH-dependant growth of a *gisD* mutant strain is limited at later timepoints suggests that *ctpT* alone is not sufficient to drive glutathione uptake when the tripeptide is depleted from the surrounding medium. Here, we assessed the ability of our mutant strains to utilise GSH at a concentration of 50 μ M. Growth of the wild type and *ctpT* mutant strains was observed under this condition, albeit restricted to a final OD₆₀₀ of 0.5 after 24 hours ([figure 44D](#)). In comparison, growth of both *gisD* mutant strains mimicked that of wild type in the absence of exogenous sulphur. Hence, we confirm that utilisation of GSH at low concentrations is entirely dependent on GisABCD.

GisABCD is solely responsible for GSSG utilisation

Our transport assay data implicates CtpT in the transport of GSH but not GSSG, likely due to the comparatively larger structure of GSSG compared to GSH (see [Figure 19A-B](#)). In-line with this, previous evidence suggests that GisABCD is entirely responsible for utilisation of GSSG as a nutrient source in *S. aureus* ([Lensmire et al., 2023](#)). Here, we confirm that growth of the *ctpT* single-mutant strain is unaffected relative to the wild type in conditions where growth is dependent on GSSG ([Figure 44E](#)). As expected, growth of both the *gisD* mutant and *ctpT gisD* double-mutant strains was heavily restricted under this condition. Overall, these observations confirm that GisABCD is exclusively responsible for GSSG utilisation in *S. aureus*.

Exposure to GSH protects *S. aureus* from oxidative stress independently of transport

In eukaryotes and some prokaryotes, GSH is utilised as a cellular redox buffer with diverse functions linked to the oxidative stress response ([Forman et al., 2009](#)). To date, no such role for GSH has been identified in *S. aureus*. This bacterium does not seemingly possess the enzymatic pathways required to synthesise GSH *de novo*, and instead synthesises a functionally homologous low molecular weight thiol – bacillithiol – which imparts increased survival under oxidative stress imposed by fosfomycin treatment and hydrogen peroxide (H_2O_2) ([Posada et al., 2014](#)). In spite of this, *S. aureus* encodes a homologue of the glutathione peroxidase enzyme BsaA and this gene is known to contribute toward survival during oxidative stress ([F. Sun et al., 2012](#)). Such an observation suggests that exogenous GSH may play a yet-unappreciated role in the *S. aureus* oxidative stress response. Indeed, a similar role for exogenous glutathione has already been demonstrated in other Gram-positive pathogens ([Brouwer et al., 2022](#); [A. J. Potter et al., 2012](#)). Here, we aim to determine whether exogenous GSH (and its associated transporters) contribute toward the oxidative stress response of *S. aureus*.

In order to determine whether GSH provides protection against oxidative stress in *S. aureus*, we devised a robust H_2O_2 killing assay (described on [page 104](#)) based on a reported methodology ([Podkowik et al., 2024](#)) and applied this against JE2 and the previously generated glutathione transporter-deficient mutant strains. Briefly, overnight cultures were diluted to a low cell density and grown in the presence of varying concentrations of GSH (0/1/10 mM). Cells were grown for a total of two hours to reach early log phase (approx. $\text{OD}_{600} = 0.2 - 0.3$), representing a growth stage at which peroxide susceptibility is maximal (due to minimal expression of the *agr* regulatory system and other genes required to detoxify oxide radicals, including the superoxide dismutase SodA, as reported by [Podkowik et al., 2024](#)). Cells were washed twice in ice-cold PBS to remove excess GSH and finally resuspended in PBS at a final OD_{600} of 0.15. The resultant cell suspensions were then treated with 20 mM H_2O_2 for one hour. At this point, H_2O_2 was neutralised by the addition of catalase and survival was determined by spot plating (summarised in [Table 7](#)).

For the wild type JE2, treatment with H_2O_2 alone was effective at killing more than 99.99% of cells. Growth in the presence of either 1 mM or 10 mM GSH before addition of H_2O_2 significantly improved bacterial survival, demonstrating a protective role for this metabolite against oxidative stress ([Figure 45](#)). Given that cells were thoroughly washed prior to H_2O_2 treatment to remove exogenous GSH, we first hypothesised that the protective effect observed here was a result of GSH internalisation during the initial growth period. However, the protective effect of exogenous

GSH prior to H₂O₂ treatment was maintained across strains with transposon mutations in the identified GSH transporter genes (Figure 45), suggesting that GSH-mediated protection from oxidative stress occurs independently of transport. In fact, bacterial survival following H₂O₂ treatment was significantly increased for *dtgT gisD* double-mutant cells grown in the presence of exogenous GSH when compared to the wild type at both concentrations tested (Figure 45). This observation suggests that internalisation of GSH may, in fact, be deleterious under the conditions tested. Altogether, these data demonstrate that growth in the presence of GSH promotes bacterial survival under subsequent oxidative stress.

We also note that survival was increased on-average for the *gisD* mutant and *dtgT gisD* double-mutant strains relative to the wild type in the absence of GSH pre-treatment, though this difference was not statistically significant (Figure 45; grey bars). Such a subtle difference might be explained by the *GisD*-dependant sequestration of the trace levels of GSH (concentration ≤ 0.5 µM) present within the TSB medium utilised here (Newton et al., 1996).

Table 7. Percentage survival of JE2 and mutant derivatives following 60-minute treatment with 20 mM H₂O₂

Strain	% survival	S.D. (±)	Fold-change (vs wild-type untreated)
No pre-treatment:			
JE2	0.0019	0.0011	N/A
JE2 <i>dtgT::erm</i>	0.0026	0.0009	1.39
JE2 <i>gisD::erm</i>	0.0573	0.0605	30.52
JE2 <i>dtgT:: gisD::erm</i>	0.1623	0.1851	86.44
1 mM GSH:			
JE2	7.5482	1.7030	4020.60
JE2 <i>dtgT::erm</i>	10.0441	1.3129	5350.02
JE2 <i>gisD::erm</i>	75.3880	4.5527	40155.82
JE2 <i>dtgT:: gisD::erm</i>	139.7254	48.0196	74425.53
10 mM GSH:			
JE2	2.7804	0.9716	1480.99
JE2 <i>dtgT::erm</i>	20.5917	9.0285	10968.28
JE2 <i>gisD::erm</i>	29.3188	12.3028	15616.84
JE2 <i>dtgT:: gisD::erm</i>	107.1359	24.2903	57066.54

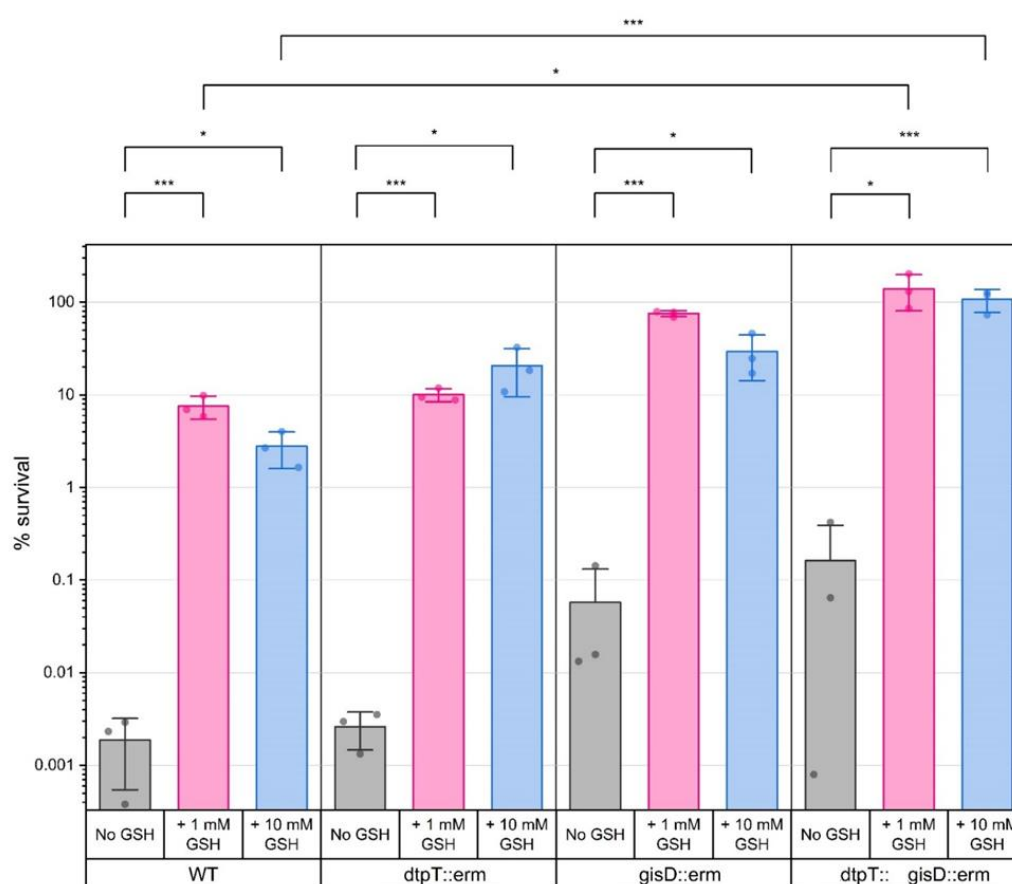


Figure 45 – Pre-treatment with GSH protects *S. aureus* from exogenous oxidative stress.

Overnight cultures of JE2 and transposon-interrupted mutants of *dtpT* and/or *gisD* were diluted 1:100 and grown for 1 hour before addition of either GSH (1 mM or 10 mM) or water (no GSH). After a further hour of incubation, cells were transferred to ice, washed twice in ice-cold PBS and resuspended in PBS to a final OD₆₀₀ of 0.15. Bacterial counts were determined before and after treatment with 20 mM H₂O₂ for 60 minutes and this was used to calculate the percentage survival. Bars show the mean of three replicates ± standard deviation. *p<0.05, **p<0.01, ***p<0.001; Unpaired two-sample student's t test.

Exogenous GSH permits growth under inhibitory concentrations of gentamicin independently of GSH transport

Several classes of antibiotics are known to induce the generation of reactive oxygen species (ROS) in bacteria, but the extent to which this endogenous oxidative stress contributes to antibiotic-mediated bacterial killing is the subject of ongoing debate ([Belenky et al., 2015](#); [Dwyer et al., 2014](#); [Gray et al., 2024](#); [Imlay, 2015](#)). Indeed, previous work has highlighted how several classes of antibiotics induce ROS-dependant DNA damage in *S. aureus* ([R. S. Clarke et al., 2021](#)). In a similar vein, induction of superoxide stress by the redox-cycling agent menadione triggers a decrease in the overall metabolic activity of *S. aureus* and thereby causes antibiotic tolerance, providing further evidence for the interconnectedness of antibiotic action and oxidative stress in this organism ([Rowe et al., 2020](#)). Given that GSH treatment had a protective effect against exogenous oxidative stress generated by H₂O₂ in *S. aureus*, we hypothesised that GSH may similarly permit growth under inhibitory concentrations of bactericidal antibiotics linked to the endogenous generation of ROS.

The aminoglycoside antibiotic gentamicin is a potent protein-synthesis inhibitor which has been utilised extensively for the treatment of *S. aureus* infections in clinical settings ([Chambers & Pallagrosi, 1977](#)). Laboratory evolution experiments reveal how *de novo* evolution of gentamicin resistance in *S. aureus* gives rise to strains with an altered metabolic profile and reduced ROS production ([Pan et al., 2023](#)). Similarly, exogenous peroxide stress drives the generation of small colony variants (SCVs) of *S. aureus* with enhanced survival under oxidative stress, and these SCVs are generally highly gentamicin resistant ([Edwards, 2012](#); [Painter et al., 2015](#)). Collectively, these studies highlight a potential link between gentamicin resistance and the oxidative stress response in *S. aureus*.

Here, *S. aureus* strain JE2 was grown in TSB supplemented with 10 mM GSH, representing the upper bound of GSH concentrations likely to be encountered in relevant biological environments ([Meister, 1988](#)). After one hour of growth, cultures were spiked with gentamicin to a final concentration of 80 µg/ml (roughly 50x the recorded MIC; [Vestergaard et al., 2016](#)). In the absence of GSH, the addition of gentamicin led to growth arrest followed by a gradual decline in optical density consistent with bacterial killing ([Figure 46](#)). Unexpectedly, the inclusion of GSH at this concentration led to a decrease in growth rate compared to cells growing in TSB alone, even in the absence of antibiotic ([Figure 46A](#)). On the other hand, robust growth was permitted in the presence of gentamicin when GSH was also present in the medium ([Figure 46B](#)). To our surprise, the GSH-mediated gentamicin resistance phenotype observed here was conserved in

the *dtgT gisD* double-mutant strain, demonstrating that this effect occurs independently of GSH transport similarly to the H₂O₂ protection effect observed previously (Figure 46B).

To investigate this phenomenon further, the concentration of GSH in the medium was varied over three orders of magnitude (0.1/1/10 mM) and the experiment was repeated as before. Here, we observed that the protective effect of GSH against gentamicin was seemingly concentration-dependent (Figure 47A-C). In the presence of 0.1 mM GSH, growth is severely inhibited upon addition of the antibiotic but OD₆₀₀ continues to increase steadily over the first 10 hours, before eventually declining. A similar phenotype is observed at 1 mM GSH, but here growth is overall stronger when compared to 0.1 mM GSH (Figure 47C). The protective effect of GSH is most apparent at 10 mM GSH, where growth is comparable to the antibiotic-free control.

Neither 0.1 mM nor 1 mM GSH caused a pronounced decrease in growth rate in the absence of antibiotic, as was previously observed in the presence of 10 mM GSH (Figure 47A). Given that glutathione is mildly acidic, we wondered if the decreased growth rate observed at 10 mM GSH may be a pH-dependant effect. To this end, we determined the pH of TSB after supplementation with GSH using a pH probe. Notably, supplementing TSB with 10 mM GSH caused a decrease in pH from 7.2 to 5.6, while pH was maintained around a neutral value with both 0.1 mM and 1 mM GSH (Figure 47C). Adjusting the pH of growth medium to 5.6 using dilute HCl attenuated growth similarly to 10 mM GSH, suggesting that this phenotype is pH-dependant (Figure 47A). Nonetheless, this acidic medium did not provide a protective effect upon addition of gentamicin (Figure 47B-C), confirming that gentamicin protection is dependent on the presence of exogenous GSH rather than pH. It is also worth mentioning that gentamicin does not contain any thiol groups and, therefore, the protective effect of GSH is unlikely to result from a direct chemical modification of the antibiotic by GSH (indeed, this could be readily verified by incubating GSH with gentamicin and analysing the products via mass spectrometry, though we do not apply such an approach here).

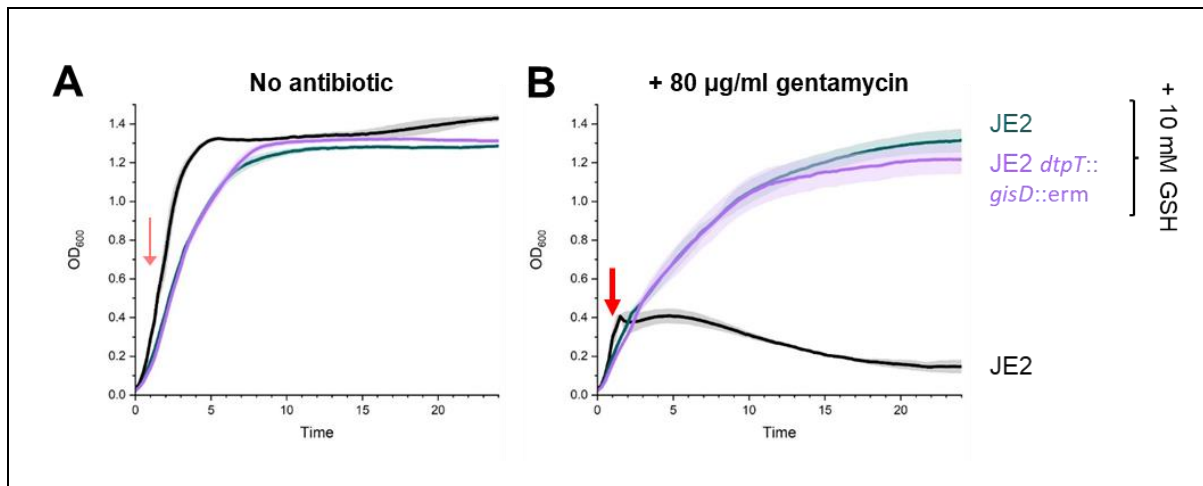
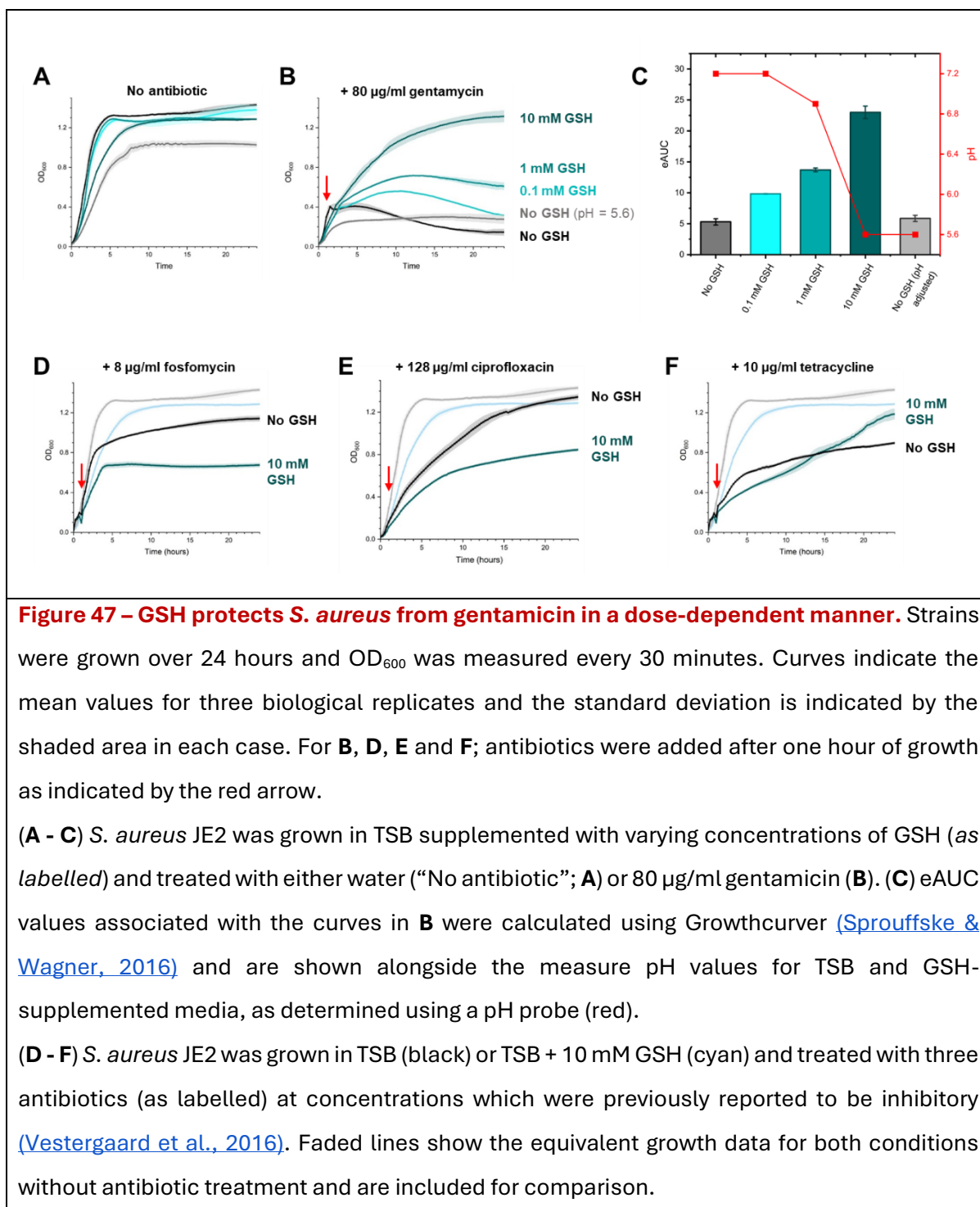


Figure 46 – 10 mM GSH allows *S. aureus* to grow under inhibitory concentrations of gentamicin. *S. aureus* JE2 (cyan) and a *dtpT gisD* double-mutant (purple) were grown in TSB + 10 mM GSH. Growth of JE2 in TSB lacking GSH (black) was included for comparison. After one hour, cells were treated with water (**A**; red arrow) or 80 µg/ml gentamycin (**B**; red arrow). Strains were incubated over 24 hours and OD₆₀₀ was measured every 30 minutes. Curves indicate the mean values for three biological replicates and the standard deviation is indicated by the shaded area in each case.



Surprisingly, work by *Clarke* and colleagues suggests that subinhibitory concentrations of gentamicin do not induce DNA damage (an indicator of ROS generation) in *S. aureus*. Conversely, DNA damage was observed for diverse antibiotics in JE2, including other potent protein synthesis inhibitors ([R. S. Clarke et al., 2021](#)). These findings suggest that the antimicrobial activity of gentamicin may have unique cellular consequences in *S. aureus* when compared to other commonly used antibiotics. Here, we aimed to assess whether the protective effect of GSH was similarly unique for gentamicin, or whether this phenomenon is conserved across antibiotics belonging to different classes and modes-of-action. To this end we repeated the experiment described previously, but instead treated cells with inhibitory concentrations of the cell wall synthesis inhibitor fosfomycin and the DNA gyrase inhibitor ciprofloxacin (**Figure 47D-E**). We also treated cells with tetracycline; a potent protein synthesis inhibitor with a similar mode-of-action to gentamicin (**Figure 47F**). In each case, antibiotic concentrations were chosen based on previously reported minimum inhibitory concentration (MIC) values for JE2 ([Vestergaard et al., 2016](#)).

Upon addition of inhibitory concentrations of gentamicin, growth of JE2 in TSB was almost immediately arrested (**Figure 47B**). In comparison, addition of the other antibiotics tested here attenuated growth to varying degrees but did not inhibit growth entirely, despite these agents being added at concentrations exceeding the recorded MIC (**Figure 47D-F**). Notably, the inclusion of 10 mM GSH in the medium did not demonstrate a protective effect for either fosfomycin or ciprofloxacin at the antibiotic concentrations utilised here, instead exaggerating the phenotypes observed in TSB alone (**Figure 47D-E**). In the case of tetracycline, the inclusion of 10 mM GSH led to a decrease in the initial growth rate but ultimately resulted in an increased cell density at 24 hours compared to TSB alone, indicative of a protective effect over prolonged periods of antibiotic exposure (**Figure 47F**). Hence, our results suggest that exogenous GSH specifically permits growth in the presence of antibiotics which inhibit protein synthesis – namely, gentamicin and tetracycline, with the latter demonstrating only a mild protective effect. Further experiments are required to assess whether this trend is consistent when applied to an extended panel of antibiotics and conditions, particularly under growth environments which better replicate relevant infection conditions.

The glutathione transporters of *S. aureus* contribute toward bacterial survival during intracellular THP-1 infection

Whilst generally considered an extracellular pathogen, work in recent years has demonstrated that *S. aureus* can survive within human phagocytes, and it's thought that this intracellular lifestyle contributes toward bacterial dissemination and immune evasion during systemic infections (as discussed in [page 74](#)). Several studies have identified bacterial factors required for survival in these intracellular niches, including key virulence regulators such as the accessory gene regulator (*agr*) locus and alternative sigma-factor *sigB* ([Alves et al., 2024](#); [Blättner et al., 2016](#); [Hachani et al., 2022](#); [Kubica et al., 2008](#)). In spite of this, many aspects of the intracellular lifestyle of *S. aureus* remain poorly understood. For instance, several studies report that *S. aureus* remains predominantly localised to the phagolysosome within professional phagocytes and divides exclusively within these membrane-bound compartments, whilst contradictory studies suggest that bacterial replication is preceded by phagolysosomal escape ([Blättner et al., 2016](#); [Flannagan et al., 2016](#); [M. U. Grosz, 2015](#); [Rungelrath et al., 2021](#)). Ultimately, the fate of intracellular *S. aureus* is seemingly influenced by both strain-specific and host cell factors ([Strobel et al., 2016](#); [Rodrigues Lopes et al., 2022](#)) (discussed further on [pages 74-75](#)). In either scenario, very little is known regarding the relevant nutrients and nutrient acquisition strategies which allow the bacterium to thrive in these distinct intracellular niches.

Our findings demonstrate that *S. aureus* has evolved to possess two redundant routes of uptake for glutathione, providing evidence that glutathione transport imparts a fitness advantage to the bacterium in relevant growth environments. In humans, glutathione is a predominantly intracellular metabolite and concentrations within human cells range from 0.5 mM to 10 mM depending on the cell type ([C.-Y. Cui et al., 2023](#); [Meister, 1988](#)). Hence, glutathione is the most abundant non-protein intracellular thiol. Based on this observation, we hypothesised that glutathione utilisation may contribute toward the fitness of *S. aureus* in intracellular environments.

Loss of both glutathione transporters causes decreased survival inside THP-1 macrophage-like cells

THP-1 is an immortalised human leukaemia-derived monocytic cell line which can be stimulated to differentiate into adherent phagocytic macrophage-like cells ([Chanput et al., 2014](#); [Tsuchiya et al., 1980](#)). To better understand the role of glutathione transporters in the intracellular lifestyle of *S. aureus*, differentiated THP-1 macrophage-like cells were infected with JE2 as well as transposon-interrupted mutant derivatives of *dtgT* and/or *glsD*. To account for any changes in

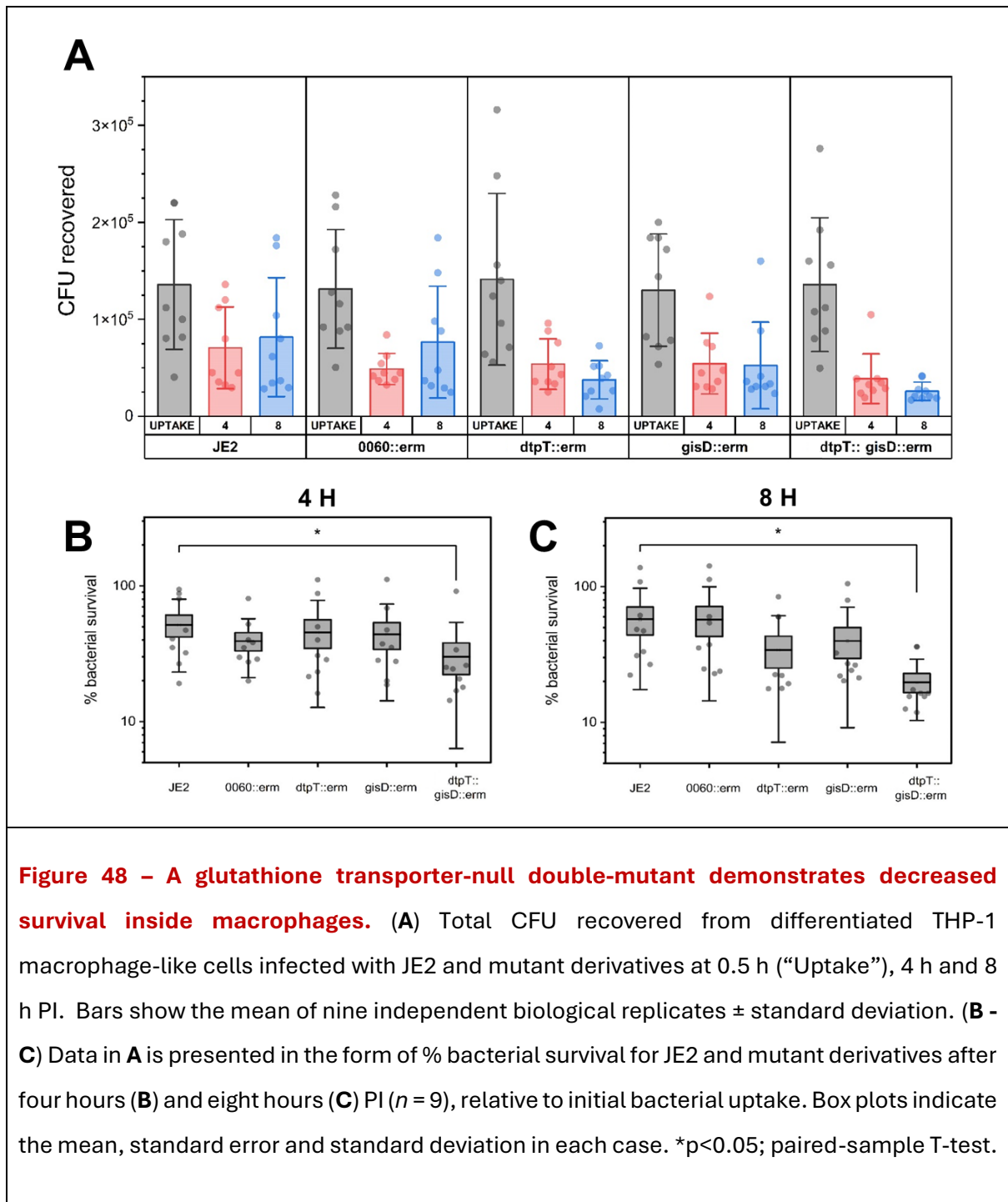
fitness resulting from inclusion of the *bursa aurealis* transposon insertion sequence, we also assessed survival of a mutant strain carrying the transposon in a presumed phenotypically-null locus corresponding to a predicted IS3-family transposase gene (SAUSA300_0060; herein referred to as 0060).

Following infection of THP-1 cells, extracellular bacteria were killed by treatment with 100 µg/ml gentamicin, after which cells were maintained in 5 µg/ml gentamicin to prevent proliferation of any bacteria subsequently released into the culture medium and thereby limit reinfection. It is worth mentioning that prolonged gentamicin exposure has previously been demonstrated to trigger the manifestation of small colony forming variants of *S. aureus* both *in vitro* and *in vivo* ([Massey et al., 2001](#); [Painter et al., 2015](#)), and these variants typically demonstrate altered antibiotic resistance and virulence factor expression ([Painter et al., 2015](#); [Kahl et al., 2016](#)) which may influence their interactions with host cells in the experiments described here. Hence, a more effective strategy for preventing reinfection of cells may have been the addition of alternative bactericidal effectors such as lysostaphin, and such an approach would ideally be applied in any future work of this nature.

To determine total bacterial uptake, THP-1 cells were lysed and bacterial CFU was determined by spot plating after 30 minutes post-infection (PI) (e.g. immediately after exchange into 5 µg/ml gentamicin). CFU was subsequently determined in this way after four hours and eight hours PI for each strain. All five tested strains demonstrated comparable levels of uptake by THP-1 cells with an average of around $1.25 \pm 0.5 \times 10^5$ CFU detectable at 30 minutes post-infection ([Figure 48A](#)). After four hours, the wild type JE2 strain demonstrated roughly 50% survival when compared to initial bacterial uptake. This value was comparable across the tested strains with the exception of the *ntpT gisD* double-mutant, which demonstrated significantly lower survival than the wild type at four hours PI ([Figure 48B](#)). After eight hours, % survival of the wild type and control strain (0060::erm) increased on average compared to the four hour time-point, indicative of bacterial proliferation. In comparison, no such increase was observed for either of the *ntpT/ gisD* single-mutants or *ntpT gisD* double-mutant strain. These strains instead demonstrated decreased survival at eight hours compared to the wild type, though this decrease was only statistically significant in the case of the *ntpT gisD* double-mutant ([Figure 48C](#)). Based on these observations, we can conclude that glutathione transporters are required for complete fitness of *S. aureus* inside THP-1 macrophage-like cells, though these systems are seemingly redundant, and the presence of either transporter is sufficient to restore survival to near wild type levels.

S. aureus glutathione transporters prolong survival of infected THP-1 cells

In eukaryotic systems, glutathione acts as an important cellular antioxidant and contributes to varied physiological processes including metal homeostasis and alleviation of lipid oxidation stress, ultimately contributing to immune cell function and preventing cell death via ferroptosis ([Amaral et al., 2019](#); [Dasgupta et al., 2010](#); [Lushchak, 2012](#)). In *M. tuberculosis* (Mtb), bacterial glutathione transport has been implicated in regulating the activity of Mtb-infected macrophages by sequestering host glutathione, thereby contributing toward intracellular survival of the bacterium ([Dasgupta et al., 2010](#)). Based on this, we hypothesised that bacterial sequestration of glutathione may act as a conserved strategy to disrupt healthy phagocyte function and thereby promote bacterial survival during infection.



To determine whether *S. aureus* glutathione transporters are involved in modulating host cell behaviour during intracellular infection, the activity of lactate dehydrogenase (LDH) in the medium (indicative of lytic mammalian cell death) was determined at four- and eight-hours PI. After four hours, the wild type JE2 and all mutant derivatives demonstrated considerable cytotoxicity against the THP-1 cells (approximately 50%), though there were no differences observed between strains at this time point (Figure 49A). Conversely, cytotoxicity was significantly increased in the *ntpT gisD* double mutant strain exclusively after eight hours PI compared to the wild type (Figure 49B). Such an observation provides compelling evidence that glutathione transporters in *S. aureus* prevent host lytic cell death by a yet-uncharacterised mechanism.

To better understand the relationship between bacterial glutathione transporters and host glutathione homeostasis, we quantified the glutathione content of infected THP-1 cells at six hours PI. This timepoint was chosen to reflect the mid-point between four- and eight-hours PI, wherein we expect the activity of bacterial glutathione transporters to exert their observed effects on host cell behaviour. THP-1 cells infected with wild type *S. aureus* JE2 demonstrated a 30% decrease in glutathione compared to uninfected cells at this time point, most likely resulting from cell lysis or changes in the cellular redox state following infection (Figure 49C). A similar shift in cellular glutathione content following *S. aureus* infection has been observed previously in THP-1 cell infection experiments (Li et al., 2024). In contrast, we demonstrate that THP-1 cells infected with the *ntpT gisD* double-mutant contained significantly higher levels of glutathione at six hours PI when compared to the wild type, consistent with the model that these transporters allow *S. aureus* to modulate host glutathione levels (Figure 49C). However, we note that all glutathione measurements collected in this work were approaching the lower limit of detection for the kit utilised here (as exemplified in Figure 49D), resulting in a high degree of variability and overall low confidence in our readings. Hence, alternative methods are required to accurately measure and compare the glutathione levels of infected THP-1 cells to better understand how host glutathione levels are impacted by *S. aureus* via glutathione transport.

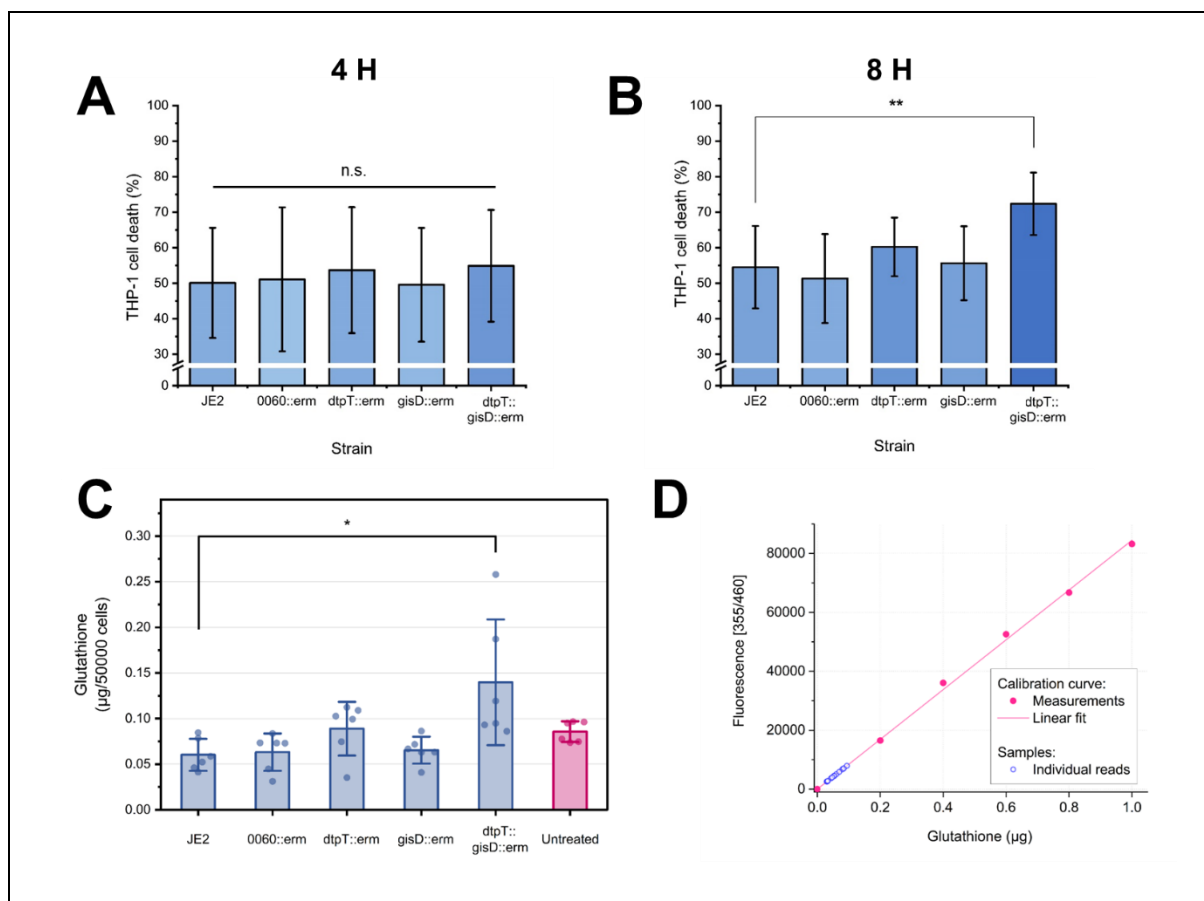


Figure 49 – A glutathione transporter-null double-mutant demonstrates increased cytotoxicity against THP-1 macrophage-like cells. (A-B) Cytotoxicity of JE2 and mutant derivatives inside THP-1 cells at 4 hours (A) and 8 hours (B) PI ($n = 6$ biological replicates). In each case, cell death was quantified via activity of LDH in the cell culture supernatant using a commercially available kit (Promega). Values are given as a percentage of the LDH activity for cells lysed entirely by addition of triton X-100 and corrected for background signal (C) Total glutathione detected from lysates of infected THP-1 cells at 6 hours PI ($n = 6$). Bars indicated the mean of all replicates $\pm$ the standard deviation in each case. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; as determined by a paired-sample t-test. (D) Glutathione was detected from cell lysates using a commercially available kit (blue circles) and quantified via a standard curve (pink line). In each case, recovered glutathione levels were lower than the lowest available standard and approached the lower limit of detection for the kit.

Glutathione transporters may facilitate intracellular survival in THP-1 cells by inhibiting ferroptosis

Generally, programmed cell death (PCD) in compromised mammalian cells can occur through one of four clearly defined pathways: apoptosis, pyroptosis, necroptosis and ferroptosis. These cell death pathways are primarily distinguished by the molecular factors implicated in their activation, and each has been demonstrated to contribute toward the response to intracellular pathogens including *S. aureus*. Likewise, these pathogens employ diverse strategies to manipulate host cells and thereby modulate PCD in a manner beneficial to bacterial survival (reviewed by [Jorgensen et al., 2017](#); [Soe et al., 2021](#)). Glutathione homeostasis in eukaryotic cells is exclusively implicated in the activation of ferroptosis, wherein depletion of cytosolic glutathione leads to the accumulation of oxidised lipids which ultimately results in membrane pore formation and cell death ([Chen et al., 2021](#); [Kang et al., 2021](#)).

Our data suggest that cytotoxicity in the early stages of infection (up to four hours PI) is uniform across the strains tested here, whilst cytotoxicity at later timepoints (eight hours PI) is significantly enhanced in the *dtpT gisD* double-mutant strain ([Figure 49A-B](#)). To determine whether PCD pathways contribute toward the increased cytotoxicity of the *dtpT gisD* double-mutant strain at this time point, we repeated the previous infection experiment in the presence of three well-characterised cell death inhibitors: the pyroptosis inhibitor VX-765 ([Wannamaker et al., 2007](#)), the necroptosis inhibitor GSK872 ([Kaiser et al., 2013](#)) and the ferroptosis inhibitor Ferrostatin-1 ([Zilka et al., 2017](#)) ([Figure 50A-C](#)). Overall, we observed that cell death at eight hours PI is mostly unaltered by the presence of VX-765 ([Figure 50A](#)) when compared to values previously recorded for untreated cells ([Figure 50](#)), suggesting that the cytotoxicity of *S. aureus* in infected THP-1 cells occurs independently of pyroptosis at eight hours PI. In comparison, we observed a general increase in lytic cell death across all tested strains in the presence of either GSK872 or Ferrostatin-1 compared to values previously observed for untreated cells ([Figure 50B-C](#)), though it is worth noting that untreated and treated cells were infected in distinct experiments (that is, utilising separately prepared THP-1 cells) and this is likely to result in inconsistencies in the viability of these cells during infection.

Notably, the increased cytotoxicity of the *dtpT gisD* double-mutant was maintained in the presence of both VX-765 and GSK872 ([Figure 50A-B](#)), suggesting that this phenotype is dependent on neither pyroptosis nor necroptosis. In contrast, the increased cytotoxicity phenotype was abolished in the presence of Ferrostatin-1 ([Figure 50C](#)), implicating host-cell

ferroptosis as a major driver of cytotoxicity for the double-mutant strain. Our data therefore supports a model in which glutathione transport allows *S. aureus* to prolong host survival by circumventing ferroptosis specifically.

S. aureus glutathione transporters modulate the host response to infection in THP-1 cells

So far, the data presented here provide compelling evidence that *S. aureus* glutathione transporters prolong host-cell survival during intracellular infection (Figure 49B), seemingly by modulating ferroptosis signalling responses (Figure 50C). Inflammatory cytokines are direct initiators of programmed cell death pathways, and these proteins are also secreted during pro-inflammatory PCD pathways including ferroptosis (Y. Chen et al., 2023). Based on this, we aimed to determine whether *S. aureus* glutathione transporters also modulate the host inflammatory response during THP-1 infection. To this end, production of inflammatory cytokines IL-1 β , IL-6 and TNF- α was quantified at six hours PI for each strain, representing the mid-point between two time points over which an increase in cytotoxicity was observed (i.e. four- and eight-hours PI). At this time point, we found that THP-1 cells infected with both the *gisD* mutant and *ntpT gisD* double-mutant strains produced significantly less IL-1 β when compared to the wild type (Figure 51A). Similarly, the double-mutant strain resulted in a decrease in IL-6 production (Figure 51B). However, there were no differences observed in the production of TNF α across the tested strains (Figure 51C). Altogether, these findings suggest that glutathione transporters allow *S. aureus* to modulate the inflammatory status of host macrophage cells during infection, which may serve to prolong host cell survival or otherwise favour bacterial survival in the intracellular niche. That being said, the biological relevance of these observed changes in cytokine production is not entirely clear; particularly in the case of IL-1 β , wherein differences in production are seemingly quite subtle and may not be reflective of relevant changes in the physiological response to infection (indeed, such an effect may also be accounted for by the previously observed decrease in macrophage survival in the case of the *ntpT gisD* double-mutant strain).

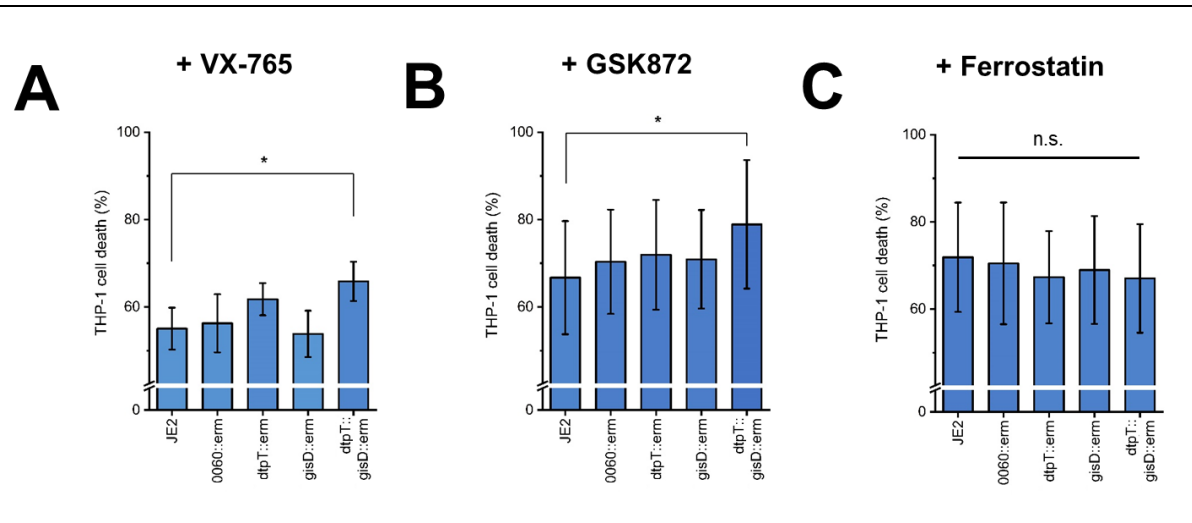


Figure 50 – The increased cytotoxicity of the *dtpT gisD* double-mutant strain is nullified by Ferrostatin-1. (A–C) Cytotoxicity of JE2 or mutant derivatives in THP-1 cells was determined at 8 hours PI in the presence of three PCD inhibitors: VX-765 (A), GSK872 (B) and Ferrostatin-1 (C) ($n = 3$ biological replicates). In each case, cell death was quantified via activity of LDH in the cell culture supernatant using a commercially available kit (Promega). Values are given as a percentage of the LDH activity for cells lysed entirely by addition of triton X-100 and corrected for background signal. Bars indicated the mean of all replicates $\pm$ the standard deviation in each case. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; as determined by a paired-sample t-test.

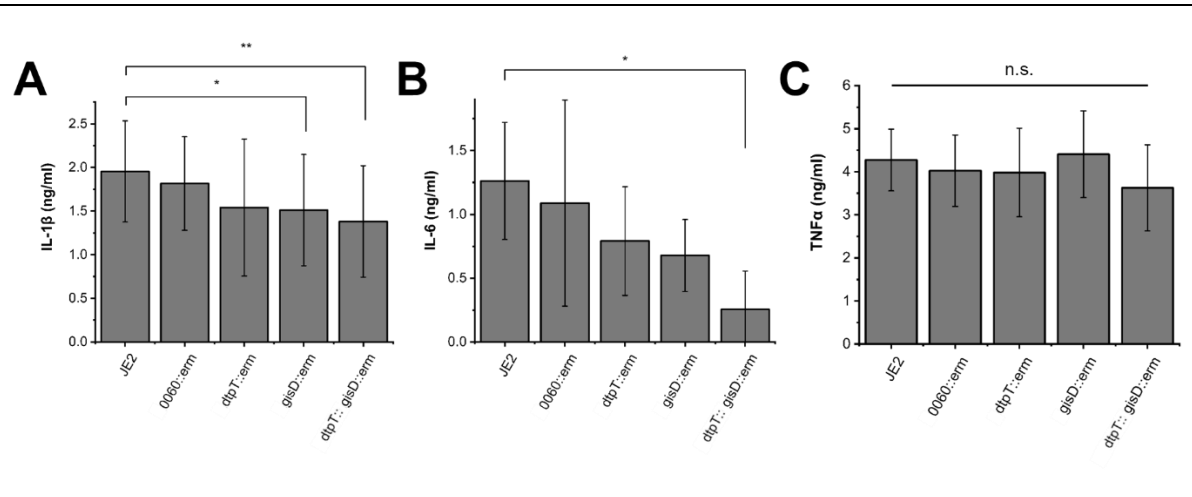


Figure 51 – *S. aureus* glutathione transporters modulate the host-cell inflammatory response during intracellular THP-1 infection. (A-C) Production of inflammatory cytokines IL-1 β (**A**), IL-6 (**B**) and TNF α (**C**) by infected THP-1 cells at 6 h PI, as determined by ELISA (n = 4, each measured in technical triplicate). In each case, bars indicate the mean $\pm$ standard deviation. *p<0.05, **p<0.01; as determined by paired-sample t-test.

S. aureus glutathione transporters are required for complete fitness inside human monocyte-derived macrophages

Differentiated THP-1 macrophage-like cells represent a highly versatile model for intracellular macrophage infection, in part due to their fast doubling-time and ability to be stored and passaged repeatedly as needed ([Chanput et al., 2014](#)). The use of an immortalised cell line also circumvents the high degree of phenotypic versatility often associated with primary human cells. However, these cells differ in their response to pro-inflammatory stimuli when compared to *ex vivo* human primary cells, and these differences are likely to affect the ability of cells to respond to bacterial infection ([Tedesco et al., 2018](#)). Hence, the applicability of these cells as a model for real-world human infection is limited.

In order to validate that *S. aureus* glutathione transporters contribute to bacterial fitness in physiologically-relevant intracellular environments, we repeated our infection experiments in human monocyte-derived macrophages (hMDMs) differentiated from peripheral blood mononuclear cells. As before, bacterial survival was determined at four- and eight-hours PI. We observed a high degree of heterogeneity in bacterial uptake – as was similarly observed for THP-1 cells ([Figure 52A](#)). Generally, bacterial survival within hMDMs was similar to that observed in THP-1 cells at four hours PI, and no significant differences were observed between strains at this time point ([Figure 48B](#); [Figure 52B](#)). In contrast, hMDMs were more effective than THP-1 cells at killing intracellular *S. aureus* at the eight-hour time point. Indeed, bacterial survival was quantified as 57% for the wild type JE2 inside THP-1 cells at eight hours PI, but this value was decreased to approximately 18% in hMDMs ([Figure 54A](#)). Such a distinction highlights the intrinsic differences between these two cell types as a model for intracellular infection. Notably, we observed a significant decrease in survival for both the *gisD* mutant and *dtgT gisD* double-mutant strains compared to the wild type at eight hours PI inside hMDMs ([Figure 52C](#)). Hence, we demonstrate that glutathione transporters are required by *S. aureus* for complete fitness inside human macrophage cells, as was previously observed in THP-1 cells.

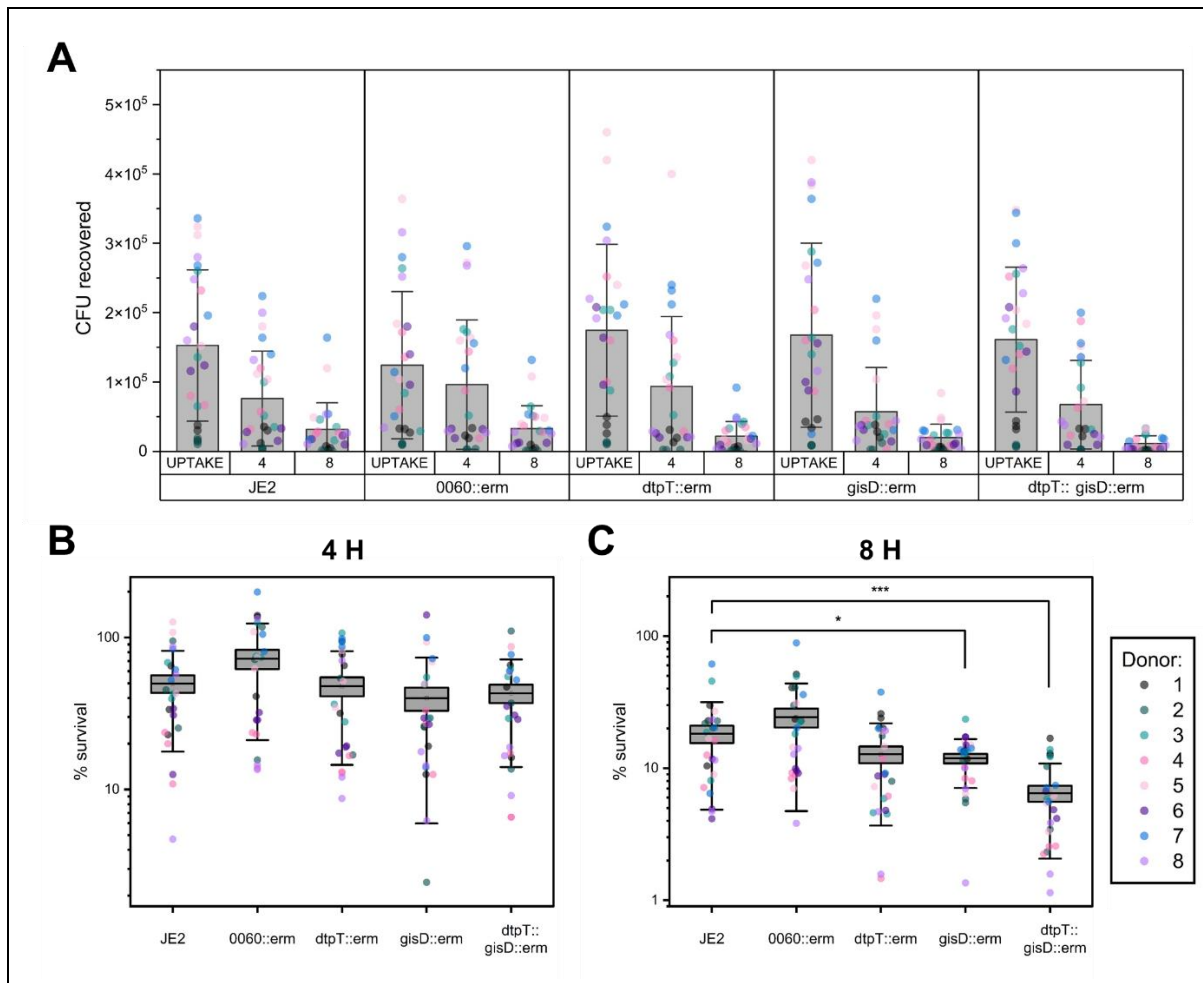
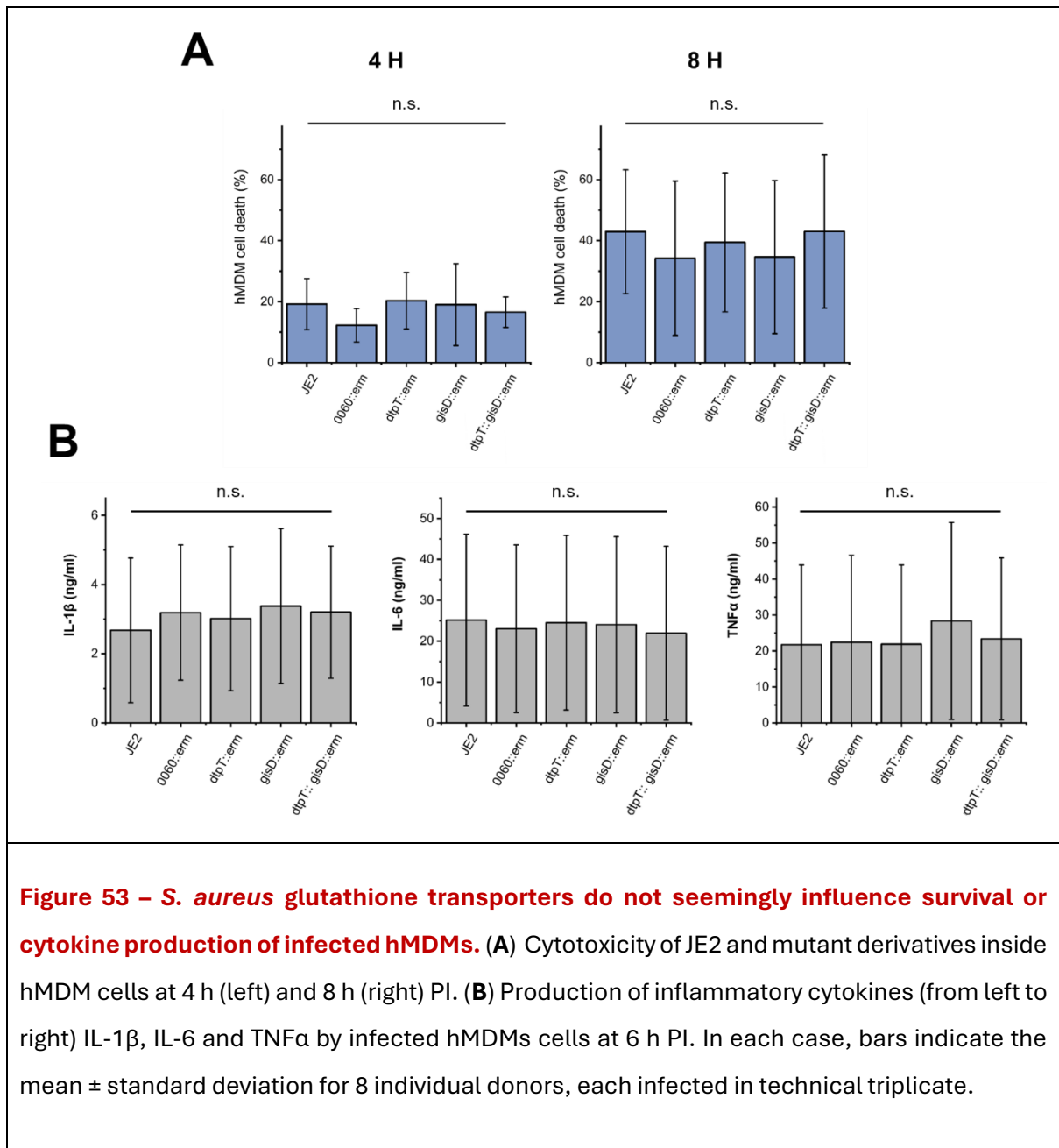


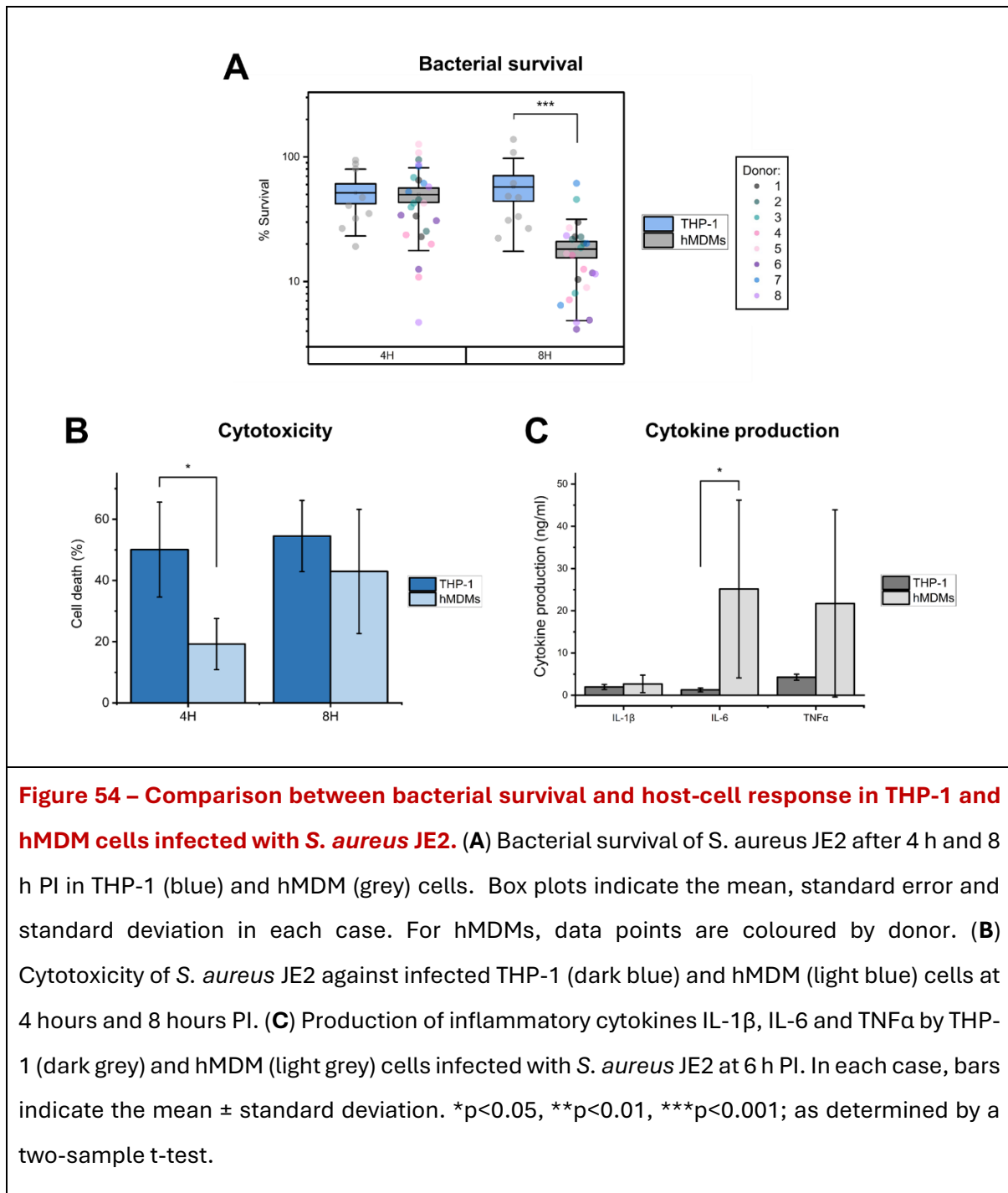
Figure 52 – Glutathione transporters contribute toward the fitness of *S. aureus* inside human MDMs. (A) Total CFU recovered from hMDMs infected with JE2 and mutant derivatives at 0.5 h (“Uptake”), 4 h and 8 h PI. Bars show the mean of 24 replicates (8 donors, each infected in triplicate) $\pm$ standard deviation. (B - C) % bacterial survival of JE2 and mutant derivatives after four hours (B) and eight hours (C) PI in hMDMs. In each case, survival is calculated as a percentage of bacterial uptake. Box plots indicate the mean, standard error and standard deviation in each case. Data points are coloured by donor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; as determined by a paired-sample t-test.

hMDM responses to intracellular infection are highly variable and differ from THP-1 cells

Next, we aimed to determine whether *S. aureus* glutathione transporters modulate the behaviour of host hMDM cells, as previously observed for infected THP-1 cells. To this end, we determined bacterial cytotoxicity and inflammatory cytokine release for hMDMs following infection with JE2 and mutant derivatives. Intriguingly, no significant differences were observed between strains in regard to either cytotoxicity (at four- or eight-hours PI) or inflammatory cytokine production (at six hours PI) in infected hMDMs (Figure 53A-B). However, we did observe a high level of variability in regard to both cytotoxicity (at eight hours PI) and cytokine production in hMDMs, indicative of high donor-to-donor heterogeneity in the infection response (Figure 53A-B). Such variability may mask subtle strain-dependent differences in these aforementioned host cell response phenotypes.

Overall, a lack of increased lytic cell death in hMDM cells during infection with the *dtgT gisD* double-mutant may point toward intrinsic differences between the responses of hMDMs and THP-1 cells to intracellular *S. aureus* infection. To investigate this, we compared bacterial survival and host cell responses during infection with wild type JE2 in both cell types. Bacterial survival at four hours PI was comparable in both cell types, but hMDMs more effectively cleared intracellular bacteria after eight hours (Figure 54A). On the other hand, hMDMs infected with the wild type JE2 demonstrated decreased cell death at four hours PI compared to THP-1 cells (Figure 54B), and produced higher levels of the inflammatory cytokine IL-6 at six hours PI (Figure 54C). These comparisons highlight how *S. aureus* infection dynamics differ greatly between THP-1 cells and hMDMs, at least when differentiated and cultured under the conditions utilised in this work.





Discussion

Versatile bacterial pathogens such as *S. aureus* are equipped with diverse transporter proteins which facilitate utilisation of the nutrients present in distinct biological niches. Nutrients with important physiological roles during infection – e.g. major carbon sources like glucose and proline – are internalised via multiple redundant transport systems, seemingly to maximise the availability of these nutrients to the cell ([Lehman et al., 2023](#); [Vitko et al., 2016](#)). Here, we demonstrate that glutathione can serve as a source of nutrient sulphur for *S. aureus* and determine that the reduced form, GSH, is taken up via two redundant transporter systems: DtpT and Gis ([Figure 44](#)). Furthermore, our phenotypic growth data suggests that these two systems have distinct roles in regard to GSH uptake; specifically, Gis is a high affinity system which enables scavenging of GSH in depleted environments, whilst DtpT allows for rapid utilisation of the tripeptide in GSH-enriched environments ([Figure 44A/D](#)). Despite this distinction, changes to the fitness of *S. aureus* in intracellular environments are exaggerated in the *dtpT gisD* double-mutant compared to the wild type or single mutant strains ([Figure 48/52](#)), suggesting some functional redundancy between these transporters under this condition. Overall, we propose that this observed redundancy of GSH transporters is indicative of an important role for glutathione utilisation in *S. aureus* physiology, though the biological relevance of each system during nutrient acquisition may vary in accordance with nutrient availability in other biological contexts.

Glutathione is a tripeptide comprised of three amino acids: L-Cys, L-Glu and Gly. Earlier work investigating nutrient acquisition in *S. aureus* suggest that this bacterium exclusively assimilates nutrient sulphur in the form of organic thiols, primarily Cys ([Lensmire et al., 2020](#)). However, we and others ([Lensmire et al., 2023](#)) have demonstrated that glutathione at physiologically relevant concentrations is sufficient to meet the nutrient sulphur requirements of *S. aureus* in defined media ([Figure 44](#)). In other words, glutathione likely serves as an alternative source of Cys for *S. aureus* during infection and permits growth in Cys-depleted environments. Of the other amino acids present in glutathione, Glu is particularly notable as a major glucogenic carbon source for *S. aureus* during glucose deprivation ([Halsey et al., 2017](#)).

Further speculation regarding the role of glutathione as a potential nutrient source for *S. aureus* in biological environments will be provided in [Chapter 6](#) at the end of this thesis. Here, I will instead discuss two other major takeaways of the work presented in this chapter. Firstly, I will consider the observation that exogenous GSH protects *S. aureus* from H₂O₂-mediated killing and

gentamicin inhibition during *in vitro* growth. Therein, I will speculate on possible mechanisms through which this effect is conveyed and discuss the potential significance of this finding in real-world contexts. Secondly, I will discuss how our *in vitro* macrophage infection experiments highlight a novel role for GSH transport in intracellular *S. aureus* infection. In both cases, I will outline potential follow-up experiments which would build upon our findings and shed new light on the novel GSH-associated phenotypes observed here.

Reduced glutathione is a novel environment signal influencing the oxidative stress response in *S. aureus* independently of DtpT/Gis

Highly virulent *S. aureus* strains – such as the widespread CA-MRSA isolate USA300 (from which the JE2 strain used here is derived) – possess diverse mechanisms of oxidative stress resistance which are selectively activated under relevant stress conditions ([Linzner et al., 2021](#)). For instance, ROS species generated during H₂O₂ exposure are sensed intracellularly by redox-responsive transcriptional regulators, including PerR and CymR ([M. J. Horsburgh et al., 2001](#); [Ji et al., 2012](#)). These regulators collectively coordinate the expression of enzymes involved in the detoxification of reactive oxygen species, including the Mn-dependent superoxide dismutase enzymes SodA and SodM and major catalase KatA ([Clements et al., 1999](#); [M. J. Horsburgh et al., 2001](#); [Valderas & Hart, 2001](#)). The quorum sensing regulator system *agr* is also redox responsive and coordinates the transition toward fermentative growth during oxidative stress, relieving the oxidative burden associated with respiratory growth ([Podkowik et al., 2024](#)), as well as upregulating the H₂O₂ detoxifying enzyme BsaA ([K. Kumar et al., 2017](#); [F. Sun et al., 2012](#)). Other redox-responsive regulators include the global gene regulators MgrA, SarZ and SarA, which coordinate pleiotropic changes to bacterial behaviour under oxidative stress and thereby promote survival ([P. R. Chen et al., 2006, 2009](#)). The low-molecular weight (LMW) thiol bacillithiol (alongside its associated disulphide reductase YpdA) provides a cytoplasmic buffer for oxidative stress, and also modulates the activity of diverse cellular targets by selective S-bacillithiolation during oxidative stress ([Dickerhof et al., 2020](#); [Linzner et al., 2019](#)). Collectively, these mechanisms provide resistance to H₂O₂ concentrations in excess of 100 mM ([Podkowik et al., 2024](#); [Weber et al., 2004](#)) and enable survival during the oxidative burst employed by macrophages and other innate immune cells during phagocytosis ([Winterbourn & Kettle, 2013](#)).

In this chapter, we have demonstrated that growth in the presence of either 1 mM or 10 mM GSH primes *S. aureus* cells to subsequent oxidative stress in the form of H₂O₂ treatment ([Figure 45](#)). Specifically, we assessed H₂O₂-mediated killing of *S. aureus* JE2 when cells were grown to early-log phase in the absence of oxidative stresses, representing a condition under which the cells

are susceptible to acute H₂O₂ treatment (as determined by [Podkowik et al., 2024](#)). Presumably, this observed H₂O₂ susceptibility results from the intrinsic oxidative burden associated with respiratory growth and, concomitantly, a lack of expression of oxidative stress response genes. As a result, survival of wild type JE2 cells was as low as 0.0019% after one hour of H₂O₂ exposure ([Figure 45](#)). In contrast, the same cells grown in the presence of GSH demonstrated an increase in survival in the order of magnitude of >10³ ([Figure 45](#)). Furthermore, exogenous GSH enabled bacterial proliferation in the presence of inhibitory concentrations of the antibiotic gentamicin ([Figure 46/47](#)); a phenotype previously linked to the oxidative stress response in *S. aureus* ([Painter et al., 2015](#); [Pan et al., 2023](#)). Collectively, these observations point toward a novel role for exogenous GSH in influencing the oxidative stress response of this bacterium.

The response to GSH occurs independently of DtpT and Gis

A similar role for exogenous GSH in conveying resistance to oxidative stress was previously reported in streptococci, though this phenomenon was found to be dependent on the internalisation of GSH by the ABC import system GshT ([Brouwer et al., 2022](#); [A. J. Potter et al., 2012](#)). Here, we demonstrate that growth in the presence of GSH protects *S. aureus* from oxidative stress imposed by H₂O₂ treatment even in the absence of the two glutathione transport systems Gis and DtpT (survival was, in fact, enhanced in the *dtpT gisD* double-mutant relative to the wild type ([Figure 45](#))), suggesting intrinsic differences between the modes of action of GSH between these two bacteria. This is perhaps expected, given that *S. aureus* does not encode an orthologue of the glutathione reductase enzyme required for the GSH-dependent oxidative stress response in streptococci ([A. J. Potter et al., 2012](#)). Additionally, growth of a *dtpT gisD* double-mutant mimicked the wild type JE2 in the presence of gentamicin when GSH was present in the medium ([Figure 46](#)). Overall, our data suggest that exogenous GSH is sensed in the local environment by the bacterium independently of DtpT and GisD and subsequently conveys survival under oxidative stress, presumably through upregulation of other oxidative stress response pathways such as those described above (though we cannot also rule out the possibility that the observed phenotypes are the result of direct physical interactions between GSH and the cell surface).

GSH sensing may be used to detect intracellular environments during infection

In the human body, glutathione is predominantly an intracellular metabolite and, as such, bacterial pathogens utilise GSH as an environmental signal to coordinate the expression of genes required specifically for intracellular survival ([Reniere, 2018](#)). In *Listeria monocytogenes*, GSH is imported through the CtpP/P1/P2 OppD/F ABC transporter system and coordinates global

changes to gene expression through interactions with the master virulence regulator PrfA ([Berude et al., 2024](#); [Hall et al., 2016](#); [Reniere et al., 2015](#)). Conversely, the facultative intracellular pathogen *Burkholderia pseudomallei* senses GSH through the redox-responsive two component system (TCS) VirAG and responds by upregulating expression of the type VI secretion system required for cell-to-cell dissemination ([Wong et al., 2015](#)). Our data support a model in which *S. aureus* similarly responds to the presence of exogenous GSH, potentially serving as an environmental cue signifying the transition from extracellular to intracellular environments during phagocytosis. Indeed, human cells specifically synthesise high quantities of GSH in response to bacterial infection, raising the local concentration of this metabolite encountered by the bacterium within intracellular environments ([Tandon & Tandon, 2024](#)).

Our observations raise several questions regarding how extracellular GSH affects *S. aureus* physiology. For instance, does GSH sensing in *S. aureus* occur directly (e.g. through specific interactions between bacterial receptors and GSH), or indirectly through changes to environmental factors such as redox potential and pH?

S. aureus encodes 16 TCSs implicated in environmental sensing and many of these remain poorly characterised in regard to their environmental triggers and downstream effectors ([Bleul et al., 2021](#)). The bacterium lacks a direct homologue of the *B. pseudomallei* VirAG system, but does possess a functionally orthologous redox-responsive TCS called SrrAB ([Bleul et al., 2021](#); [Yarwood et al., 2001](#)). In a study by [Tiwari](#) and colleagues, it was revealed that the histidine kinase component of this system (SrrA) detects changes in the redox state of the environment through an internal disulphide bond ([Tiwari et al., 2020](#)). Specifically, SrrA activity is enhanced in the reduced form ([Tiwari et al., 2020](#)). It has been proposed that SrrAB coordinates the response to oxidative stress and respiratory dysfunction in *S. aureus*, seemingly through sensing of reduced menaquinones, which accumulate under these conditions ([Kinkel et al., 2013](#); [Mashruwala et al., 2017](#)). Genes upregulated by SrrAB include those required for NO $\cdot$ detoxification and anaerobic metabolism ([Kinkel et al., 2013](#)). Collectively, these properties make SrrAB a potential candidate for the putative GSH sensory system of *S. aureus*; in other words, exogenous GSH may reduce SrrA (directly, or indirectly through the reduction of membrane-associated redox-responsive elements such as menaquinones) and thereby trigger the upregulation of factors permitting survival under oxidative stress.

Further experiments are required to elucidate the molecular basis of GSH sensing in S. aureus

Our data suggests that GSH is sensed by *S. aureus* and influences oxidative stress responses, enabling both survival in the presence of acute oxidative stress ([Figure 45](#)) and growth in the

presence of gentamicin (Figure 46). However, much more work is needed to elucidate the molecular basis of these phenotypes.

To the best of our knowledge, the regulatory response of *S. aureus* in the presence of exogenous GSH has never been investigated, making it difficult to interpret the phenotypic responses to GSH observed here. Transcriptomic or proteomic approaches may provide valuable insights into how GSH influences the oxidative stress response of *S. aureus* and highlight genes/proteins required for this response. Indeed, coordinated changes in protein expression may implicate specific TCSs (for instance, SrrAB) or other known regulatory elements in mediating the cellular response to GSH. Follow-up experiments could then determine whether the GSH-sensing phenotypes observed in this work are maintained in strains lacking the genetic elements identified this way. Furthermore, responses to exogenous GSH could be compared between the wild type JE2 and the *dtgT gisD* double-mutant in order to elucidate why this strain displays an enhanced H₂O₂ resistance phenotype following growth in the presence of GSH. Overall, it is clear that this is an area of *S. aureus* biology where much remains to be elucidated and understood.

GSH may influence gentamicin susceptibility *in vivo*

Several recent studies highlight how antibiotic susceptibility in *S. aureus* is influenced by the infection environment, leading to differences in antibiotic efficacy during real world infection scenarios when compared to laboratory conditions (Ledger et al., 2021; Ranganathan et al., 2024). However, our understanding of the specific environmental stimuli responsible for these *in vivo* phenotypes remains incomplete. Our data implicate GSH as an environmental factor influencing antibiotic susceptibility in *S. aureus*, and this phenomenon may be relevant during infection given that glutathione concentrations in human tissue vastly exceed those found in common microbiological media such as TSB (Forman et al., 2009). Specifically, we demonstrate how physiologically relevant GSH concentrations impart a resistance-like phenotype to gentamicin in *S. aureus*; that is, supplementing the medium with GSH allows cells to proliferate under normally inhibitory concentrations of the antibiotic (Figure 46), indicative of an increase in the MIC (though we do not quantify MIC here). This effect is seemingly independent of changes in pH triggered by the addition of GSH, given that adjusting the pH alone failed to recapitulate the GSH-associated phenotypes (Figure 47B).

Notably, we observed that GSH did not impart a resistance-like phenotype for the antibiotics ciprofloxacin and fosfomycin, instead enhancing the efficacy of these antibiotics in inhibiting growth (similar changes to growth kinetics in the absence of antibiotic suggest that this enhanced inhibition is probably a pH-dependent effect, though this was never confirmed

experimentally) (Figure 47D-E). The inclusion of GSH did have a subtle effect on growth in the presence of tetracycline, though it's unclear whether this effect was GSH dependant or caused instead by changes to the pH of the medium (as the effect of the latter in regard to tetracycline susceptibility was not tested in the work presented here).

Previously, Clarke and colleagues demonstrated how the cellular response to gentamicin in *S. aureus* differed from other clinically relevant anti-Staphylococcal antibiotics. Specifically, of eight antibiotics assessed in this work, gentamicin was the only one which failed to induce the DNA damage-responsive SOS response in *S. aureus* at inhibitory and sub-inhibitory concentrations (R. S. Clarke et al., 2021). Similarly, we demonstrate how the presence of GSH impacts susceptibility to gentamicin specifically in *S. aureus* (Figure 47). Hence, our findings support a model in which the antimicrobial activity of gentamicin is conveyed through distinct molecular mechanisms when compared to other antibiotics in this bacterium. However, our observations are based on a limited panel of antibiotics and excludes some important drugs with ongoing widespread clinical use, such as vancomycin and daptomycin. I propose that the effects of exogenous GSH should be investigated against an expanded panel of antibiotics, in order to determine whether the observed resistance phenotype is a unique trait of gentamicin, and to better understand the potential relevance of this phenomenon in clinically relevant contexts.

GSH transporters contribute toward the fitness of *S. aureus* during intracellular infection

To date, very little is known regarding how *S. aureus* meets its nutritional requirements in intracellular environments, though recent studies suggest that this bacterium is able to access nutrients available to the host cell from within a membrane-bound phagocytic vacuole in human macrophages (Bravo-Santano et al., 2018; Flannagan & Heinrichs, 2020). In order to proliferate, bacteria require exogenous sources of essential chemical elements including carbon, nitrogen and sulphur. Cys (in both the reduced and oxidised forms) has been proposed as the major sulphur source for *S. aureus* in biological environments (Lensmire et al., 2020), but the concentration of free Cys inside mammalian cells is strictly limited ($< 100 \mu\text{M}$) (Abu-Remaileh et al., 2017; Bonifácio et al., 2021). Furthermore, the majority of Cys within the cell is selectively compartmentalised within lysosomes and, therefore, unlikely to be freely available to intracellular bacteria (X. Guo et al., 2022). In comparison, glutathione is abundant within cells (10x to 100x more than Cys) and is distributed throughout cellular compartments (Vázquez-Meza et al., 2023). Hence, we predict that glutathione is the predominant thiol available to *S. aureus* during intracellular infection and thereby contributes toward bacterial proliferation within

intracellular environments. Indeed, glutathione transport is seemingly a common trait of prominent intracellular bacteria, and these transporters have been implicated in the utilisation of host-derived glutathione in *L. monocytogenes*, *Salmonella* and *Mtb* ([Berude et al., 2024](#); [Dasgupta et al., 2010](#); [Zhongshan Wang et al., 2017](#)). In *Salmonella*, the glutathione ABC transport Gsi is required for complete fitness in a murine systemic infection model *in vivo* ([Zhongshan Wang et al., 2017](#)). Our data similarly implicate GSH transport as a determinant of fitness for *S. aureus* inside human macrophages, and provides novel insights into the importance of GSH transport in this environment, as described below.

Firstly, mutations in *ntpT* and/or *gisD* did not have a significant impact on bacterial uptake in either THP-1 or hMDM cells ([Figure 48/52](#)), suggesting that these factors do not influence the initial internalisation process. Secondly, survival of a *ntpT gisD* double-mutant was reduced compared to single mutants of either transporter at eight hours PI in both THP-1 and hMDM cells ([Figure 48/52](#)), suggesting that these mutations have an additive effect on bacterial fitness and, therefore, both transporters may function redundantly to transport GSH in order to enable intracellular survival. Thirdly, bacterial survival in hMDMs differed significantly in the *gisD* and *ntpT gisD* mutant strains at eight hours PI but did not differ at four hours PI ([Figure 52](#)). Similarly, changes in cytotoxicity of the *ntpT gisD* double mutant in THP-1 cells manifested exclusively at the later time point ([Figure 49](#)). Collectively, these observations suggest that glutathione transport is most relevant after several hours post-infection, potentially coinciding with other events such as the initiation of bacterial proliferation or disruption of the phagolysosome ([Flannagan et al., 2016](#); [Rodrigues Lopes et al., 2022](#)). Based on this, we propose that glutathione transport may enable survival or proliferation within macrophages over prolonged time periods, though the effect of glutathione transporters at later time points (for instance, 24 hours PI) was not assessed here.

The role of GSH transport during intracellular infection remains unclear

While our data demonstrate a clear link between glutathione transport and fitness inside macrophages, the precise role of glutathione transport during the intracellular lifestyle is difficult to elucidate. In the simplest model, GSH provides a rich source of nutrient sulphur to *S. aureus* and thereby enables proliferation of the bacterium in the intracellular niche. While our bacterial CFU data certainly supports such a model ([Figure 52](#)), we also note increased cytotoxicity in the *ntpT gisD* double-mutant strain ([Figure 49](#)) which is seemingly dependent on host cell ferroptosis ([Figure 50](#)). Hence, increased survival of the wild type JE2 may instead result from prolonged host-cell survival, perhaps via modulation of host glutathione levels by the bacterium. Whilst some evidence supports such a model, this hypothesis is overall counterintuitive; logically, one

would expect disruption of bacterial glutathione transport to lead to increased concentrations of GSH in the host-cell cytosol, which would prevent ferroptosis ([Y. Yu et al., 2021](#)). Indeed, disruption of glutathione transport in Mtb is reported to lead to a decrease in cytotoxicity, whereas we observe the opposite phenomenon here ([Dasgupta et al., 2010](#)). Furthermore, the expression of IL-6 (previously associated with ferroptosis) was significantly lower during infection with the *dtgT gisD* double mutant in our experiments, while expression of TNF- α (also associated with ferroptosis) was unchanged by the presence of glutathione transporters ([Figure 51](#)) ([Y. Chen et al., 2023](#)). Finally, strain-specific changes to host-cell viability were not clearly observed during infection of hMDMs, though the fitness of glutathione transporter mutants was still significantly decreased in these cells ([Figure 53](#)). Therefore, whilst our findings hint toward a novel interplay between bacterial glutathione transport and host cell ferroptotic regulation, we cannot definitively conclude that changes in cytotoxicity observed in our experiments are dependent on glutathione transport directly.

Another potential explanation for the observed fitness defect of the *dtgT gisD* double-mutant during macrophage infection is that glutathione transport allows *S. aureus* to more effectively survive the diverse oxidative stresses encountered in the intracellular niche. Our data suggests that neither transporter contributes toward survival to acute H₂O₂ stress ([Figure 45](#)), but these *in vitro* experiments may poorly reflect the conditions encountered by *S. aureus* during intracellular infection. Indeed, GSH transport in *S. aureus* may provide a protective effect against other oxidative stresses present in the phagolysosome, such as reactive nitrogen species or copper ([Bogdan, 2014](#); [Samanovic et al., 2012](#)). Conversely, GSH serves as a source of the amino acid Cys in *S. aureus* and may thereby fuel biosynthesis of the LMW thiol bacillithiol: a known contributor toward bacterial fitness inside macrophages ([Posada et al., 2014](#)).

To summarise, glutathione transport in *S. aureus* may contribute toward bacterial fitness inside macrophages by one or more of the following mechanisms: 1. providing nutrients to fuel bacterial proliferation, 2. disrupting host-cell glutathione homeostasis, or 3. providing increased resistance to oxidative killing in the phagolysosome.

A major limitation of the methodology utilised thus far in our infection experiments is the reliance on culture-scale approaches to quantify bacterial survival and macrophage viability. While these methods are informative in regard to overall population dynamics, they lack the resolution required to understand how the success of intracellular *S. aureus* cells is impacted in the *dtgT gisD* double-mutant strain. An attractive alternative is the use of microscopy-based approaches – for instance, fluorescence microscopy – to determine the fate of individual bacterial cells over

the course of infection. These techniques are well established for the study of *S. aureus* inside macrophages and would allow for a clearer picture of how glutathione transport impacts phenomena such as bacterial killing and proliferation over the duration of infection ([Flannagan et al., 2016](#); [Rodrigues Lopes et al., 2022](#)). This methodology would also facilitate the use of GSH-specific probes to assess changes in host-cell GSH content during infection, allowing us to determine whether bacterial glutathione transporters do, in fact, modulate host-cell glutathione levels to influence traits such as viability and inflammatory status ([Mingzhang Li et al., 2024](#)). Overall, I believe these experiments would shed new light on the roles of GSH transport in the intracellular lifestyle of *S. aureus* inside macrophages.

The importance of GSH transport may vary in different host cell types

Here, we have considered the role of glutathione transport in *S. aureus* exclusively in the context of macrophage infection. However, *S. aureus* has been observed to infect diverse cell types, including endothelial cells and other non-phagocytes ([Blättner et al., 2016](#); [Rollin et al., 2017](#)). Furthermore, the dynamics of intracellular infection have previously been found to be both strain-specific and cell-type specific ([Rodrigues Lopes et al., 2022](#)). In regard to the latter, *S. aureus* is most commonly localised within a membrane-bound vacuole in professional phagocytes (i.e. macrophages and neutrophils) ([Rigby et al., 2012](#); [Flannagan et al. 2016](#)) whereas the bacterium rapidly escapes this compartment in non-phagocytic cells and multiplies primarily within the cytosol ([Moldovan & Fraunholz, 2019](#)).

To date, there is a poor understanding of the differences in nutrient availability in these two compartments. Studies of the intracellular pathogen *Salmonella enterica* have demonstrated how this bacterium resides predominantly within a membrane-bound vacuolar compartment inside host-cells and manipulates the endosomal system to access nutrients available to the host ([Liss et al., 2017](#)). As a result, the bacterium has plentiful access to glucose-derived carbon sources and various micronutrients ([Diacovich et al., 2017](#); [Röder et al., 2021](#)). Yet, in spite of this, glutathione transport is required for robust infection of *S. enterica* in a murine systemic infection model ([Zhongshan Wang et al., 2017](#)). Such an observation suggests that host-derived glutathione is both available to *S. enterica* inside the vacuolar compartment and, more importantly, serves as an important nutrient contributing to bacterial fitness in this context. Equally, glutathione serves as an important nutrient source for *L. monocytogenes* even though this bacterium generally resides in the host cytosol during infection ([Berude et al., 2024](#)). Hence, studies of other intracellular pathogens demonstrate that glutathione can serve as an important nutrient source for bacteria in both of the intracellular environments occupied by *S. aureus* during infection.

Overall, further experiments are needed to compare the importance of glutathione transporters on fitness in *S. aureus* within a range of different host-cell types. Such experiments may reveal that the requirement for glutathione is specific for distinct host cell types, or more generally associated with the vacuolar or cytosolic lifestyles.

In vitro experiments point toward an important role for glutathione transporters *in vivo*

Our data provide compelling evidence that GSH transport (mediated by DtpT and Gis) is required for the complete fitness of *S. aureus* during macrophage infection *in vitro*. Given the proposed importance of the intracellular lifestyle in bacterial persistence and dissemination *in vivo*, our observations thereby implicate glutathione transport as a potential determinant of bacterial success during infection. Specifically, while scavenging of glutathione in GSH-depleted environments is likely to be facilitated exclusively by Gis, both DtpT and Gis may support bacterial survival in infection environments where glutathione is enriched or those where innate immune cells are enriched and, therefore, a significant proportion of cells must survive intracellularly.

Studies of systemic *S. aureus* infection in a murine model identified Kupffer cells in the liver as an early niche colonised by the bacterium in the initial stages of infection ([Surewaard et al., 2016](#)), making survival within these cells a major bottleneck for the success of the bacterium ([Pollitt et al., 2018](#)). GSH concentrations in the human body are highest in liver tissue (~ 10 mM), where hepatic cells are specifically implicated in the synthesis and export of GSH for dissemination to other organs around the body ([Lu, 2020](#)). As a result of these factors, the liver represents a niche wherein glutathione transport may play a significant role in bacterial survival, both intracellularly (i.e. within Kupffer cells) and – potentially – extracellularly (i.e. utilising excreted GSH within liver abscesses).

Consistent with this hypothesis, *Coulter* and colleagues identified *dtpT* as a fitness determinant during intravenous *S. aureus* infection and specifically demonstrated decreased bacterial burden of a *dtpT* mutant in the livers of infected mice ([Coulter et al., 1998](#)). However, mutations in *gis* do not meaningfully impact bacterial fitness in the liver ([Lensmire et al., 2023](#)). Hence, either DtpT is the major route of cell entry for GSH for *S. aureus* in the liver, or the fitness cost of a *dtpT* mutant results from disruption to the transport of other peptide nutrients present in this niche. Overall, understanding the relative contributions of DtpT and GisD toward survival *in vivo* requires further investigation. Indeed, analytical techniques such as imaging mass spectrometry ([Buchberger et al., 2018](#)) may allow for the local glutathione concentrations of different infection

environments to be compared to better understand how availability of this nutrient influences survival in different host tissues, and this information may correlate with the importance of glutathione transport in these environments.

Chapter 6 – Concluding remarks

Nutrient acquisition is a fundamentally essential process required to drive cellular metabolism and synthesise new biological material in all living organisms. For bacterial pathogens, effective nutrient uptake is a determinant of successful colonisation, as demonstrated by the importance of nutritional immunity as an innate defence against infection ([Murdoch & Skaar, 2022](#)). Multiple studies have indicated an important role for host-derived proteolytic products (that is, amino acids and oligopeptides) as an essential nutrient source for the prominent bacterial pathogen *S. aureus* in diverse biological environments ([Coulter et al., 1998](#); [Lehman et al., 2019](#); [Urso et al., 2024](#); [Valentino et al., 2014](#)). Specifically, there is evidence that virulent *S. aureus* strains are highly proteolytic ([Min Li et al., 2009](#)), and recent studies highlight how host-adaptation in these strains is driven by utilisation of host collagen-derived nutrients as a major carbon source ([Urso et al., 2024](#)). Hence, amino acid and peptide utilisation represents an attractive target for the development of novel therapeutic interventions to inhibit the growth of virulent, host-adapted *S. aureus* strains. In spite of this, many aspects of bacterial physiology relating to nutrient acquisition and utilisation in *S. aureus* remain poorly understood. In particular, the promiscuous nature of peptide transport proteins and their functional redundancy in many bacteria (including *S. aureus*) have made these systems historically challenging to characterise regarding substrate range and preference. Indeed, existing studies fail to capture the full scope of substrate promiscuity in these systems in *S. aureus*, limiting our understanding of their distinct roles in bacterial physiology ([Borezée-Durant et al., 2009](#); [Hiron et al., 2007](#); [Lehman et al., 2019](#)).

In this report, I have significantly expanded upon these earlier findings by utilising a high-throughput phenotypic screening approach to distinguish the functional di/tripeptide targets of DtpT and Opp3 ([Figure 22/23](#)). Briefly, these experiments demonstrate that DtpT serves as the primary route of dipeptide uptake in *S. aureus*, but also reveals some overlap in substrate specificity between this transporter and Opp3. Specifically, many peptides (in particular, Arg-containing dipeptides and tripeptides) are substrates of both DtpT and Opp3, whilst Asp- and Glu-containing peptides are preferentially utilised via DtpT only ([Figure 23B](#)). Biochemical methods were applied to further interrogate the substrate preferences of DtpT and assign new functional roles to this transporter. Intriguingly, these experiments demonstrate that peptide composition has a profound effect on transport rate in DtpT. Notably, we demonstrate that preferable substrates of DtpT include dipeptides containing acidic amino acids, or those with short/non-polar sidechains such as Gly, Pro and Ala ([Figure 35](#)). During this *in vitro* analysis, we also found that DtpT could transport the biologically prevalent tripeptide GSH ([Figure 37](#)). In the

absence of the known glutathione transporter Gis, *ntpT* is essential for growth under conditions where GSH is supplied as the sole sulphur source (Figure 44), identifying DtpT as the predicted second GSH uptake system of *S. aureus*.

In Figure 20, I gave an overview of how the three known oligopeptide transporters of *S. aureus* – Opp3, DtpT and Gis – each contribute toward nutrient acquisition in this bacterium, based on insights from the existing literature. As a result of our endeavours, we are now able to provide a more detailed picture of how *S. aureus* utilises the diverse peptide nutrients present in biological environments (Summarised in Figure 55).

Peptide transporters allow *S. aureus* to access important amino acids in complex biological environments

Substrate promiscuity in peptide import systems is required to permit the translocation of chemically and structurally diverse oligopeptides across biological membranes (Kotov et al., 2023). Indeed, in both phenotypic and biochemical analyses of DtpT, we demonstrate a remarkable degree of heterogeneity among potential substrates (Figures 23/35). However, our findings also highlight how both DtpT and Opp3 preferentially facilitate transport for peptides containing specific amino acids (Figure 23B). Based on these trends, we propose that peptide transport in *S. aureus* functions to selectively accumulate specific amino acids in the cell, with both DtpT and Opp3 working to maintain a high intracellular concentration of these amino acids. In other words, the emergence of preferred functional targets for these systems may represent an evolutionary strategy to maximise the availability of biologically important amino acid targets required to support the survival of *S. aureus* under physiologically relevant growth conditions.

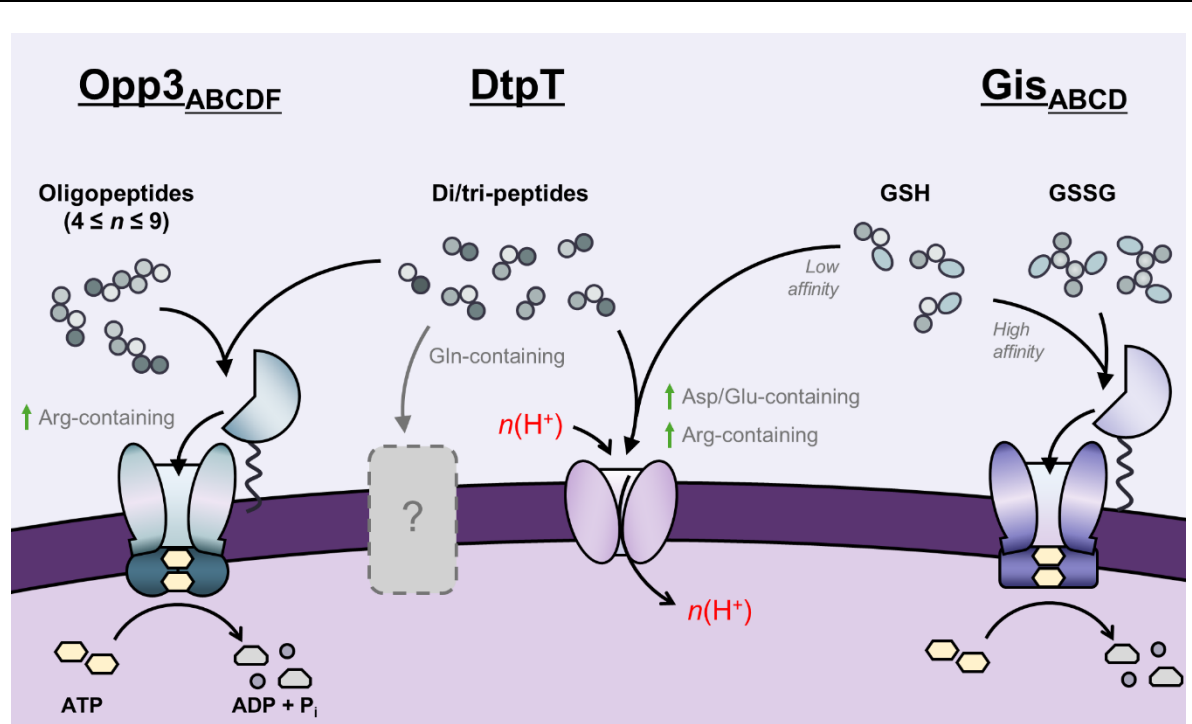


Figure 55 – Diagrammatic summary of the functions of three major *S. aureus* peptide transporters in nutrient acquisition, based on evidence collected in this work and earlier findings. Amino acid preferences observed for substrates of DtpT and Opp3 in our phenotype microarrays are labelled with green arrows. A grey, dashed box indicates the existence of at least one additional route of utilisation for Gln-containing peptides.

For instance, glutamate is known to serve as a major gluconeogenic carbon source for *S. aureus*, as demonstrated by the observation that strains deficient in Glu catabolism are attenuated under glucose-limited conditions ([Halsey et al., 2017](#)). Perhaps as a result of this physiological importance, Glu restriction has pleiotropic effects on cellular physiology, ultimately resulting in increased biofilm formation both *in vitro* and *in vivo* ([Shibamura-Fujiogi et al., 2022](#)). Taken together, these observations implicate exogenous Glu as an important factor contributing to both growth and environmental sensing in *S. aureus*. Acquisition of free Glu in *S. aureus* is primarily facilitated by the amino acid permease GltS ([Zeden et al., 2020](#)), but the availability of Glu in biological environments is likely to vary in different tissues and infection environments. Here, we demonstrate that DtpT is responsible for the utilisation of diverse Glu-containing dipeptides and show that these peptides are specifically enriched among DtpT substrates ([Figure 23](#)). These findings suggest that DtpT-mediated peptide uptake may serve as an additional source of exogenous Glu in peptide-rich (or, conversely, Glu-depleted) growth environments. Excitingly, we also demonstrate for the first time that glutathione transport in *S. aureus* is mediated by both Gis and DtpT ([Figure 44](#)). To date, glutathione uptake in *S. aureus* has only been explored in the context of nutrient sulphur acquisition ([Lensmire et al., 2023](#)). However, given the abundance of glutathione in many mammalian tissues ([Forman et al., 2009](#)), we propose that this tripeptide metabolite may also serve as an additional source of Glu in some growth environments. It is worth noting that we also demonstrate a preference for Asp-containing peptides among putative DtpT substrates in our phenotypic screens ([Figure 23B](#)), though the relevance of aspartate as a nutrient source in *S. aureus* is less well studied and this amino acid is not predicted to be essential for growth, given that *S. aureus* can freely synthesise Asp from oxaloacetate during aspartate limitation ([A. D. Potter et al., 2020](#)). Therefore, it is difficult to predict whether Asp-containing peptides serve as an important nutrient source in any relevant physiological contexts.

During Glu limitation, *S. aureus* maintains a high intracellular pool of Glu primarily via catabolism of the amino acids arginine and proline ([Halsey et al., 2017](#)). Consistent with this, both Arg and Pro are rapidly internalised by the bacterium during growth in a defined medium, and carbon flux analysis demonstrates that these amino acids are predominantly converted to Glu once inside the cell ([Halsey et al., 2017](#)). However, *S. aureus* does not synthesise Arg from either Pro or Glu under physiologically relevant conditions ([Reslane et al., 2022](#)). As a result, the bacterium must consistently source arginine from the environment during growth to maintain an intracellular pool of this amino acid required for protein synthesis and, ultimately, growth. Arg-limitation also triggers the starvation-associated stringent signalling response in *S. aureus*, stimulating

antibiotic tolerance in *S. aureus* biofilms ([Freiberg et al., 2024](#)). Hence, Arg availability is an important environmental stimulus used by *S. aureus* to coordinate the switch between proliferation in nutrient-rich environments and persistence during nutrient limitation. Here, we demonstrate how diverse Arg-containing dipeptides are redundantly utilised via either DtpT or Opp3. We propose that the redundancy observed here for uptake of Arg-containing peptides likely signifies an evolutionary strategy to increase the diversity of potential arginine sources available to the cell, which agrees with the apparent importance of exogenous arginine in *S. aureus* physiology.

In addition to arginine, *S. aureus* is also auxotrophic for the amino acid proline in glucose-rich environments ([Li et al., 2010](#)), and several reports demonstrate the importance of collagen-derived Pro as a nutrient source of *S. aureus* in relevant biological contexts ([Lehman et al., 2023](#); [Urso et al., 2024](#)). However, unlike arginine, we do not observe any clear trends regarding the utilisation of Pro-containing peptides by *S. aureus* in our phenotypic screens. Whilst some Pro-containing peptides could be utilised by the bacterium (via DtpT, DtpT/Opp3 or independently of either transporter), many showed no utilisation across the strains tested here ([Figure 23A](#)). Hence, our data suggest that neither peptide transport system in *S. aureus* demonstrates a clear preference for Pro-containing peptides, perhaps suggesting that *S. aureus* preferentially utilises free collagen-derived proline instead of Pro-containing peptides as a nutrient source in biological environments. Indeed, the attenuation of proline transporter mutants in collagen-rich infection environments is consistent with such a model ([Lehman et al., 2023](#)). We do, however, note that Gly-Pro was preferentially transported by DtpT in our biochemical analyses ([Figure 35C](#)), and this specific dipeptide motif is notably enriched in human collagen ([Ricard-Blum, 2011](#)). Hence, we cannot rule out the possibility that DtpT possesses specific adaptations facilitating the acquisition of common collagen-derived Pro-containing dipeptides. Meanwhile, a role for Opp3 in the utilisation of longer collagen-derived oligopeptides has already been demonstrated ([Lehman et al., 2019](#)).

To date, the importance of exogenous glutamine in *S. aureus* is relatively unexplored. Unlike the other amino acids discussed so far in this section, *S. aureus* can freely synthesise Gln from Glu via the glutamine synthetase GlnA ([Gowrisankar et al., 2021](#)). Work by Zeden and colleagues has demonstrated that exogenous Gln can serve as a nutrient source to promote the growth of *S. aureus* in defined media ([Zeden et al., 2020](#)). The authors highlight how Gln availability modulates the cellular c-di-AMP pool ([Zeden et al., 2020](#)), and is thereby likely to influence phenotypes such as biofilm formation and osmotic stress tolerance (though follow-up work confirming these phenotypes is yet to be published) ([Schuster et al., 2016](#); [Syed et al., 2024](#)). Here, we

demonstrate that DtpT is capable of transporting the glutamine-containing dipeptide Ala-Gln (Figure 35), and our phenotypic screens point toward the existence of an additional yet-unidentified peptide utilisation system in *S. aureus* which is specific for Gln-containing peptides (Figure 23B). We propose that the existence of such a system demonstrates a yet-unappreciated role for Gln-containing peptides in *S. aureus* physiology, though such an observation is surprising given that glutamine is among the most abundant free amino acids present in human tissues and is, therefore, unlikely to be limiting in relevant infection environments (Roth, 2008). Indeed, these peptides may instead serve alternative roles beyond nutrient acquisition (for instance, acting as an environmental stimulus indicating nitrogen-rich environments).

In contrast to the examples listed above, we demonstrate that tyrosine-containing dipeptides are scarcely utilised by *S. aureus* (Figure 23). This is perhaps unsurprising, given that *S. aureus* JE2 lacks a complete catabolic pathway for tyrosine utilisation and does not rapidly consume exogenous tyrosine when grown in defined media (Halsey et al., 2017). Hence, tyrosine is probably not a preferred nutrient source for *S. aureus*. Intriguingly, no DtpT-mediated transport was observed for the sole tyrosine-containing peptide (Val-Tyr) assessed in our biochemical analysis (Figure 35), providing evidence that DtpT possesses a distinct biochemical preference toward peptides which are readily catabolised over those which lack the potential to serve as a nutrient source. That being said, we do observe the utilisation of some dipeptides composed of the BCAAs (i.e. leucine, isoleucine and valine) in our phenotypic screens (Figure 23A), and we have demonstrated that BCAA-containing peptides can be transported by DtpT *in vitro* (Figure 35). Such an observation is surprising, given that a catabolic pathway for these amino acids in JE2 is yet to be elucidated (Halsey et al., 2017). However, in addition to their roles as anabolic precursors for protein synthesis, BCAAs have diverse roles in *S. aureus* physiology and are preferentially acquired from exogenous sources (J. C. Kaiser et al., 2018, 2016; Pohl et al., 2009). Hence, our findings implicate BCAA-containing peptides as a potential reservoir for these important amino acids in peptide-rich environments.

One surprising observation from our phenotype microarrays is the apparent utilisation of peptides comprised exclusively from BCAAs – for instance, Leu-Leu – despite the lack of any known catabolic pathways for these amino acids in *S. aureus*. How does *S. aureus* utilise these peptides as a nitrogen source to drive metabolism? Previously, it was demonstrated that the BCAA aminotransferase IlvE – the final enzyme in the BCAA biosynthesis pathway of Staphylococci – was able to catalyse the biosynthesis of glutamate using BCAAs as a precursor (Madsen et al., 2002). Hence, we propose that these BCAAs (and, hence, BCAA-containing

peptides) may serve as nitrogen sources for *S. aureus* under nitrogen-limited conditions in an IlvE-dependent manner

GSH and other peptides serve as sources of nutrient sulphur for *S. aureus* in biological environments

In earlier works, it was proposed that peptide transport in *S. aureus* (specifically, promiscuous peptide transport mediated by DtpT and Opp3) primarily serves as a means for cells to assimilate carbon and nitrogen from diverse peptide sources present in biological environments ([Borezée-Durant et al., 2009](#); [Hiron et al., 2007](#); [Lehman et al., 2019](#)). However, we demonstrate that DtpT also contributes toward the accumulation of thiol-containing peptides, including the abundant human metabolite GSH ([Figure 35/37](#)). Specifically, we utilise bacterial growth experiments to demonstrate that DtpT-mediated GSH transport is sufficient to meet the bacterium's requirement for nutrient sulphur at physiological GSH concentrations ([Figure 44](#)). In the absence of DtpT and the glutathione ABC transporter Gis, bacterial fitness is attenuated during intracellular infection of human macrophages and macrophage-like cell lines ([Figure 48/52](#)), and we speculate that this phenotype may be linked to nutrient sulphur limitation (discussed further on [pages 226-227](#)). Collectively, our data implicates DtpT as an important route of nutrient sulphur acquisition in *S. aureus*, particularly in GSH-enriched infection environments.

Glutathione levels in human tissue are regulated through a continuous cyclical process called the γ -glutamyl cycle, whereby the metabolite can be either synthesised or catabolised by cells in response to oxidative stress or low cysteine availability, respectively ([Bachhawat & Yadav, 2018](#); [Viña et al., 1989](#)). In addition to GSH, we demonstrate that DtpT can also transport the dipeptide Cys-Gly: the major intermediate thiol formed during glutathione turnover by the γ -glutamyl cycle ([Figure 35](#)). Hence, our data implicate both DtpT and Gis in the uptake of glutathione, and DtpT specifically in the utilisation of Cys-Gly (though we do not exclude the possibility that either Opp3 or Gis may also contribute to Cys-Gly transport). Based on these observations, we propose that *S. aureus* may leverage DtpT to exploit multiple stages of host γ -glutamyl cycle as a source of nutrient sulphur in intracellular environments. That being said, the concentration of Cys-Gly in relevant human tissues has not been thoroughly investigated, so the potential relevance of Cys-Gly transport in DtpT remains uncertain.

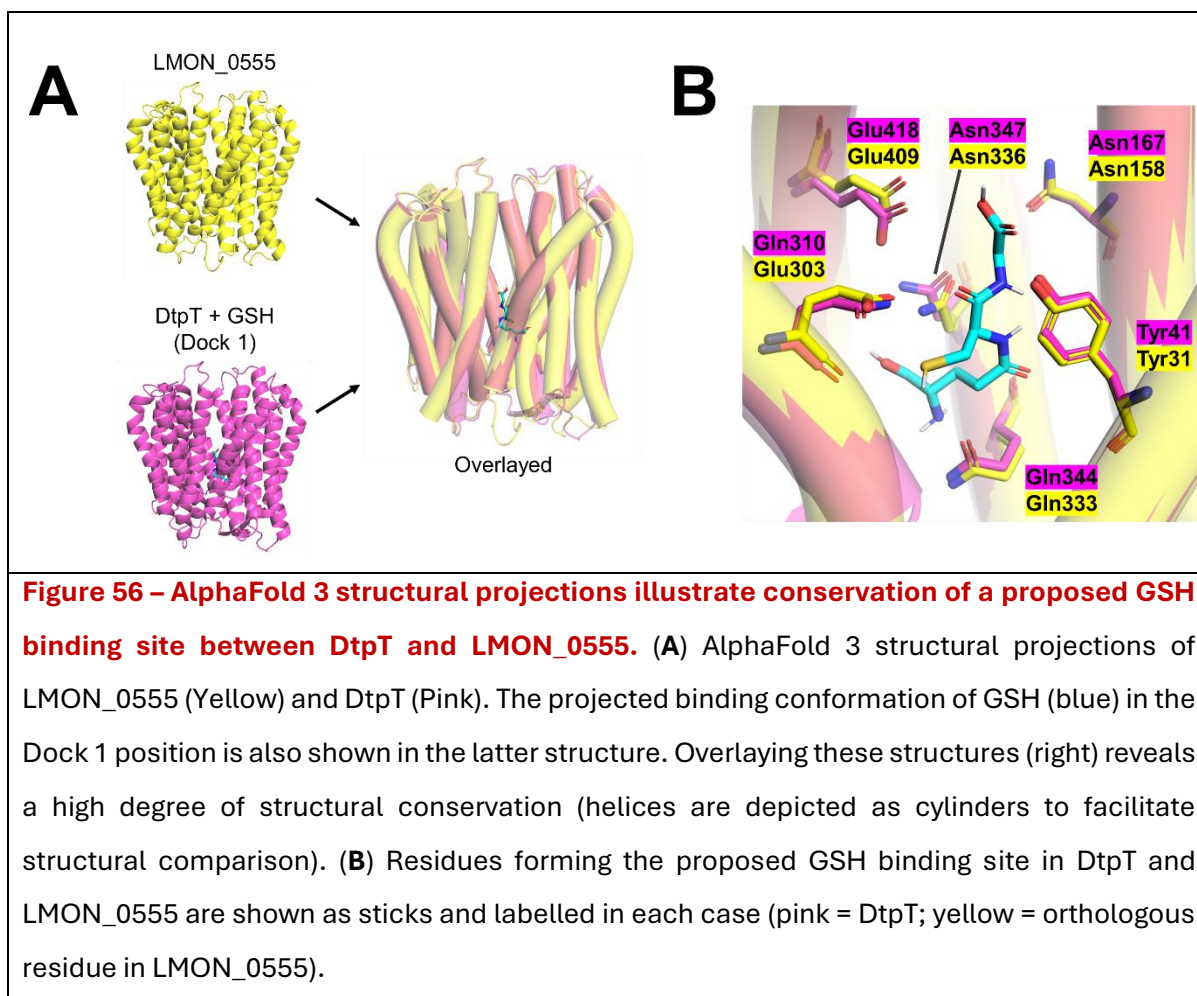
GSH transport is likely to be conserved in closely related bacterial POT transporters

Based on our structural projections and site-directed mutagenesis experiments, we predict that GSH binds DtpT in a vertical conformation during transport, stabilised in-part by two hydrogen bonds: one between the conserved TM1 residue Tyr41 and the substrate C-terminus, as well as one between Gln310 and the terminal carboxyl group of the substrate glutamate residue ([Figure 38](#)). Consistent with this, mutation of either Tyr41 or Gln310 to an alanine residue significantly reduced GSH transport in this protein ([Figure 41](#)). Additionally, transport was attenuated in a N167Q mutant, indicating an essential role for this conserved Asn residue in either substrate coordination or transport ([Figure 41](#)).

To the best of my knowledge, our findings represent the first evidence of POT-mediated GSH transport in a bacterium. With that being said, POTs are widespread across the bacterial domain and GSH transport is unlikely to be a unique trait of DtpT. For instance, all residues implicated in substrate recognition in DtpT are conserved in the closely related *S. hominis* POT PepT_{Sh}, suggesting that this protein similarly transports GSH ([Figure 18](#)). If we consider the conservation of Tyr41, N167 and Gln310 (or Glu in the equivalent position) as essential prerequisites for POT-mediated GSH transport, then we may infer that GSH transport is conserved in at least two other structurally characterised bacterial POTs: GkPOT from *G. kaustophilus* and PepT_{St} from *S. thermophilus* ([Figure 18](#)). In contrast, Gln310 is not conserved in the enterobacterial POTs (DtpA/B/C/D/E) and, as such, these proteins probably lack the ability to transport GSH effectively. This prediction is seemingly confirmed by recent work demonstrating that GSH transport in *E. coli* is entirely dependent on the ABC transporters Opp and Gsi at millimolar concentrations ([Knoke et al., 2024](#)).

One exciting possibility is that DtpT-mediated GSH transport is a conserved trait utilised by Gram-positive bacteria to assimilate nutrient sulphur in intracellular environments. *L. monocytogenes* is perhaps the most well characterised Gram-positive intracellular bacterial pathogen, and glutathione acquisition is a known determinant of fitness in this bacterium ([Berude et al., 2024](#); [Reniere et al., 2015](#)). Similarly to *S. aureus*, *L. monocytogenes* possesses an ABC glutathione import system, but mutants of this system maintain the ability to utilise exogenous GSH as a sulphur source at physiological concentrations ([Berude et al., 2024](#)). *L. monocytogenes* also possesses a yet-uncharacterised POT protein (LMON_0555 in *L. monocytogenes* EGD) with approximately 50% identity to DtpT ([Figure 56A](#)). Notably, all residues implicated in the projected GSH binding pocket of DtpT are conserved in LMON_0555, with the

exception of Gln310 which is substituted for a Glu residue in the equivalent position (Figure 56B). In DtpT, this substitution did not significantly affect GSH transport (Figure 41). Hence, I propose that LMON_0555 is highly likely to serve as a GSH transporter and may contribute to the survival of *L. monocytogenes* within human cells.



Could DtpT serve as a target for antimicrobial intervention?

The observation that nutritional immunity restricts the success of bacterial pathogens during infection ([Murdoch & Skaar, 2022](#)) highlights the potential of targeting nutrient acquisition processes as a means of attenuating bacterial growth and virulence therapeutically. In a recent article by Zeden and colleagues, the authors discuss how amino acid and peptide transport systems may serve as promising therapeutic targets to inhibit growth and virulence in *S. aureus* ([Zeden et al., 2021](#)). Therein, the authors highlight the importance of several amino acids (and their associated transporter proteins) in bacterial physiology, and posit that inhibition of amino acid transport may be an effective strategy to limit bacterial propagation by denying essential environmental nutrients ([Zeden et al., 2021](#)).

However, in our work, we demonstrate that DtpT, Opp3 and Gis allow *S. aureus* to access oligopeptides as a source of important amino acid nutrients present in biological environments (*as discussed above*). For instance, we show that Arg-containing peptides can serve as arginine sources to permit growth during Arg limitation, and that utilisation of these peptides is often redundant ([Figure 24](#)). Similarly, we demonstrate that glutathione can be utilised by *S. aureus* via either DtpT or Gis, and this tripeptide can serve as a source of cysteine in Cys-depleted environments ([Figure 44](#)). Several examples in the available literature similarly demonstrate how individual amino acid transporter mutants do not display strong phenotypes in peptide-rich media *in vitro* ([J. C. Kaiser et al., 2016](#); [Lehman et al., 2023](#); [Lensmire et al., 2020](#)), suggesting that peptides can serve as an alternate source of these nutrients when free amino acids are inaccessible to the cell. It is worth noting, however, that some amino acid transporters have been demonstrated to contribute toward the fitness of *S. aureus in vivo* (for instance, the proline transporters PutP and ProT) ([Lehman et al., 2023](#); [W. R. Schwan et al., 1998](#); [Urso et al., 2024](#)), as well as contributing toward relevant virulence-associated phenotypes including β -lactam resistance ([Gallagher et al., 2020](#)), nucleotide signalling responses ([Zeden et al., 2020](#)) and biofilm formation ([Shibamura-Fujiogi et al., 2022](#)). Overall, based on the evidence collected by ourselves and others, we propose that amino acid transporters may represent promising targets for attenuating *S. aureus* virulence during infection, but the effectiveness of such an approach may be limited in biological environments where peptides are available as an alternative source for these nutrients. Indeed, the expression of both Opp3 and DtpT is controlled by the amino acid starvation-responsive CodY regulon ([Gao et al., 2023](#); [Pohl et al., 2009](#)) and – as a result – one can expect that these systems would be upregulated under conditions where amino acid transport is limited by therapeutic intervention.

Prior to the work presented in this thesis, very little was known regarding the peptide transporters of *S. aureus* and their roles in nutrient uptake (particularly DtpT, which had remained largely uncharacterised outside of a single study by *Hiron* and colleagues) ([Hiron et al., 2007](#)). Here, we demonstrate that DtpT transports diverse peptides in *S. aureus* ([Figure 22](#)), including the biologically prevalent tripeptide GSH ([Figure 44](#)), and our results implicate DtpT (along with Gis) in fitness during intracellular environments where GSH is enriched ([Figure 48/52](#)). Such observations raise an interesting question: could inhibiting peptide transport by DtpT be utilised to attenuate *S. aureus* during infection?

DtpT does not seemingly contribute toward abscess formation in a murine SSTI model, suggesting that targeting DtpT may be ineffective for attenuating virulence in this environment ([Coulter et al., 1998](#); [Lehman et al., 2019](#)). However, in foundational work by *Coulter* and colleagues, DtpT was shown to contribute toward bacterial fitness in multiple other murine infection models, including a burn wound model and a systemic infection model ([Coulter et al., 1998](#)). In regard to the latter, a *dtpT* mutant strain was specifically attenuated during colonisation of the liver, suggesting an important role for DtpT – but not Opp3 or Gis – in this niche ([Coulter et al., 1998](#)). Studies of *S. aureus* systemic infection dynamics demonstrate a key role for liver colonisation in the early stages of systemic infection in mice ([Pollitt et al., 2018](#); [Surewaard et al., 2016](#)), and *S. aureus* liver abscesses are also a common feature of systemic infection in humans ([Brook & Frazier, 1998](#); [Straughan et al., 2018](#)). Hence, we propose that targeting DtpT may be an effective strategy for attenuating *S. aureus* in the liver, and may thereby alleviate the burden of systemic *S. aureus* infections by disrupting colonisation of this important niche.

Ala-Phe and other dipeptides as inhibitors of DtpT for therapeutic applications

Intriguingly, our biochemical analyses demonstrate how some dipeptides bind tightly to DtpT ([Figure 31](#)) but are not detectably transported by the protein ([Figure 35](#)), suggesting that these peptides may inhibit DtpT-mediated transport of other preferred substrates. Extending this, we utilise a simple bacterial growth experiment to demonstrate how one peptide with this quality (Ala-Phe) can inhibit growth of the bacterium under conditions where DtpT is essential ([Figure 36](#)). Collectively, our findings serve as compelling evidence that Ala-Phe and other dipeptides can inhibit the activity of DtpT and, therefore, we propose that these compounds may be effective at attenuating the growth of *S. aureus* *in vivo* in environments where DtpT is important for fitness (i.e. in the liver). However, the applicability of such an approach to real world infection scenarios remains uncertain, especially given that our data implicates DtpT in intracellular survival

specifically (Figure 48/52), and cells in this environment may be generally protected from external interventions. Hence, the applicability of such an approach to target DtpT in the liver requires *in vivo* confirmation.

DtpT-mediated chloramphenicol uptake does not meaningfully contribute toward antimicrobial activity in *S. aureus*

Here, we have demonstrated that DtpT is a highly promiscuous transporter, facilitating the utilisation of roughly half of the di/tripeptides assessed in our phenotypic screens (Figure 22). Perhaps due to this remarkable ability to accommodate structurally diverse substrates during transport, peptide transporters are frequently implicated in the transport of chemically diverse non-peptide substrates including peptidomimetic antibiotics. In humans, the POTs PepT1 and PepT2 transport β -lactam antibiotics including amoxicillin and cefadroxil, and are the primary routes through which these compounds are absorbed and maintained in the bloodstream following oral administration (Parker et al., 2024). In Gram-negative bacteria, POTs have similarly been implicated in the transport of β -lactams (Prabhala et al., 2017; Stauffer et al., 2021) and the protein synthesis inhibitor chloramphenicol (Prabhala et al., 2018), whilst ABC peptide transporters serve as routes of entry for antibiotics including negamycin (Hörömpöli et al., 2021) as well as kasugamycin and blasticidin S (Shiver et al., 2016).

Antibiotics with targets in the bacterial cytoplasm – such as chloramphenicol – must accumulate within the cytoplasm to a high concentration in order to exert their antibacterial effects. In this work, we demonstrate that DtpT transports chloramphenicol in *S. aureus* (Figure 42A-B), as previously demonstrated for the POT DtpA in *E. coli* (Prabhala et al., 2018). In spite of this, changes to the efficacy of chloramphenicol in the *dtpT* mutant strain were subtle compared to the wild type over the first few hours of growth in the presence of subinhibitory antibiotic concentrations. Similarly, in their report, Prabhala and colleagues do not indicate a change to chloramphenicol susceptibility in a *dtpA* mutant strain of *E. coli*, though they do demonstrate increased chloramphenicol susceptibility in strains overexpressing the transporter from an exogenous promoter (Prabhala et al., 2018). Furthermore, we observed a gradual decrease in OD₆₀₀ consistent with antibiotic-mediated killing in both the wild type and the *dtpT* mutant strain at later stages of growth (Figure 42D). Overall, our data suggests that DtpT may influence the accumulation of chloramphenicol in *S. aureus* cells, but alternative routes of cell entry likely compensate for the loss of this transporter and thereby maintain the antimicrobial activity of the antibiotic in the *dtpT* mutant strain.

Antibiotic accumulation within bacterial cells involves two major processes: influx and efflux. Recently, it was proposed that antibiotic influx mechanisms are generally highly redundant in bacteria, limiting the impact of individual import systems toward antibiotic resistance phenotypes ([Mendes et al., 2020](#)). Unlike Gram-negative bacteria, Gram-positives lack the ability to modulate cell permeability via changes to expression of outer-membrane porins ([Pagès et al., 2008](#)). Hence, changes in antibiotic influx are rarely implicated in antibiotic susceptibility in Gram-positive bacteria, while efflux is regarded as the dominant mechanism impacting antibiotic accumulation in these organisms ([Schindler & Kaatz, 2016](#)). Indeed, *S. aureus* possesses several multidrug efflux systems with broad substrate specificity which may influence the accumulation of chloramphenicol in these cells during growth ([S. S. Costa et al., 2013](#)). Overall, inhibition of efflux systems represents a promising approach to increase the intracellular accumulation of chloramphenicol (via DtpT and other routes) to enhance bacterial killing.

DtpT could be hijacked as a route of cell entry for trojan-horse antimicrobials

A promising approach for increasing the efficacy of existing antimicrobials against *S. aureus* and other bacterial pathogens is the introduction of chemical modifications to enhance the ability of these drugs to cross the bacterial cell envelope, increasing their rate of accumulation inside the cell ([Muñoz & Hergenrother, 2021](#)). A related strategy involves the conjugation of existing antimicrobials to membrane-permeable compounds to enable illicit transport into the bacterial cytoplasm, wherein the active antimicrobial is cleaved enzymatically and accumulates ([Kim et al., 2006](#); [Nolan et al., 2007](#)). To-date, these so-called “Trojan horse” approaches have been explored extensively in enteric Gram-negative bacteria as a means of enhancing permeability of the outer membrane barrier, but their applications in Gram-positive bacteria are poorly explored. That being said, conjugation of existing fluoroquinolone antibiotics to iron-binding siderophores has been demonstrated to enhance the activity of these drugs against *S. aureus*, presumably by increasing their membrane permeability ([Wencewicz et al., 2013](#)). Such a finding highlights the potential of other Trojan horse strategies in the design of novel antibiotic variants with enhanced activity against *S. aureus*.

The highly promiscuous nature of peptide transport systems and their observed ubiquity make these systems promising targets for the design of novel Trojan horse antimicrobial variants. In a recent study, *Luo* and colleagues demonstrated that peptide-conjugated antimicrobials could be illicitly transported into *E. coli* by the ABC peptide permease Yej, and this method was applied to enhance the effectiveness of the oxazolidinone antibiotic Eperezolid ([Luo et al., 2025](#)). We

therefore postulate that peptide conjugation may similarly stimulate the accumulation of antimicrobials in *S. aureus* in an Opp3- or DtpT-dependent manner, and this modification may enhance the effectiveness of the antimicrobials. Here, we have demonstrated that Arg-containing dipeptides are frequently substrates of Opp3 (Figure 23B), but our efforts to further structurally and biochemically characterise how substrate selectivity is achieved in Opp3A were strictly limited (Appendix 13), making it difficult to rationally design peptide conjugates which may enable antimicrobial transport in this system. Conversely, our experiments provide a wealth of information regarding potential scaffold structures which could serve as conjugates to enable DtpT-specific transport and accumulation of antimicrobials in *S. aureus*.

Firstly, we demonstrate that DtpT is highly promiscuous (Figure 23) and can serve as a route of uptake for peptidomimetic antimicrobials in *S. aureus* (Figure 42), underscoring the potential of this transporter to facilitate Trojan horse antimicrobial transport. In our biochemical analysis, we identify several peptides which are transported favourably by DtpT, including the dipeptides Ala-Ala, Gly-Gly and Arg-Asp (Figure 35). Given the ability of DtpT to transport tripeptides such as Ala-Ala-Ala (Figure 35), we propose that linear dipeptide conjugates (e.g. Ala-Ala-X, where X is an antimicrobial) may serve as substrates of DtpT. We also identify GSH as a substrate of DtpT (Figure 37A), and previous work has identified S-Cys-Gly-3M3SH as a substrate of the closely related transporter PepT_{Sh} (Minhas et al., 2018). Hence, we can conclude that DtpT accommodates structurally distinct Cys-Gly conjugates as potential transport targets. Finally, given the apparent preference of DtpT for anionic peptide substrates (Figure 23/35), we propose that the presence of a negative charge in a putative peptide-conjugated antimicrobial may enhance DtpT-mediated transport.

When designing Trojan horse antimicrobials in this way, it is also important to consider the properties of the antimicrobial effector. Firstly, effective Trojan horse antimicrobials must target processes in the cytoplasm – those with extracellular targets (for instance, β -lactams) or those which target the membrane (for instance, polymyxins) are unlikely to benefit from cytoplasmic accumulation and are, therefore, unsuitable for this kind of approach. Secondly, suitable effectors must be sufficiently small to be accommodated within the DtpT binding groove during transport, preferably mimicking the size and chemical properties of an amino acid (as would be found in a typical peptide substrate of DtpT). Finally, the chosen effector must be chemically compatible with peptide conjugation; for instance, possessing either a free carboxyl or amino group suitable for the formation of a peptide bond.

Taking these considerations into account, one class of existing antimicrobials which might be suitable for peptide-conjugation are the amino acid analogue D-Ala racemase inhibitors, such as D-cycloserine ([Gallagher et al., 2020](#); [Tabuchi et al., 2017](#)) and L- α -ethynyl glycine ([Meffre & Le Goffic, 1996](#)). Notably, these molecules mimic natural amino acids in terms of size and the approximate arrangement of chemical groups, and could be readily incorporated into a di- or tripeptide substrate via a peptide bond, allowing the active antimicrobial effector to be released in the cytoplasm via the activity of nonspecific bacterial peptidases (as proposed by *Luo* and colleagues for the peptide-based Trojan horse antimicrobial microcin C7 ([Luo et al., 2025](#))). That being said, it's difficult to predict whether the unconventional chemical properties of these antibiotics (for instance, the D-stereochemistry of D-cycloserine) would permit their transport by DtpT. If one were to pursue this approach further, *in silico* approaches such as blind docking could be utilised to screen possible binding conformations of potential Trojan horse antimicrobials in DtpT, and thereby assess the likelihood that these compounds will be favourably transported by the protein.

Appendices

Appendix 1 – Table of currently available POT protein structures (as of Feb 2025):

Protein name	Organism	Ligand	Accession
PepT _{So}	<i>Shewanella oneidensis</i>		2XUT
PepT _{St}	<i>Streptococcus thermophilus</i>		4APS
		Ala-Phe	4D2C
		Ala-Ala-Ala	4D2D
		Ala-Gln	5OXX
		Ala-Leu	5OXL
		Asp-Glu	5OXM
		Phe-Ala	5OXN
		Phe-Ala-Gln	6GHJ
PepT _{So2}	<i>Shewanella oneidensis</i>	Alafosfalin	4LEP
		Ala-Ala-Ala	4TPJ
		Ala-Tyr	4TPG
		Ala-Tyr-Ala	4TPH
PepT _{Gk}	<i>Geobacillus kaustophilus</i>		4IKX
		Alafosfalin	4IKZ
NRT1.1	<i>Arabidopsis thaliana</i>		5A2N
		Nitrate	4OH3
DtpD	<i>Escherichia coli</i>		4Q65
YePEPT (DtpE)	<i>Yersinia enterocolitica subsp. palearctica</i>		4W6V
		LZNV	7Q0M
XCC1406	<i>Xanthomonas campestris</i>		6EI3
PepT _{Sh}		S-Cys-Gly-3M3SH	6EXS

	<i>Staphylococcus hominis</i>	Valacyclovir	6GZ9
		5-aminolevulinic acid	6H7U
DtpA	<i>Escherichia coli</i>		6GS1
		Valganciclovir	6GS4
HsPepT1	<i>Homo sapiens</i>	Ala-Phe	7PMX
			7PN1
HsPepT2	<i>Homo sapiens</i>	Ala-Phe	7PMY
		Cefradoxil	9BIR
		Amoxicillin	9BIS
		Cloxacillin	9BIT
PepT2	<i>Rattus norvegicus</i>		7NQK
DtpC	<i>Escherichia coli</i>		7ZC2
DtpB	<i>Escherichia coli</i>	Ala-Trp-Ala	8B17
		Ala-Phe	8B18
		Ala-Phe-Ala	8B19
		Ala-Ile	8B1A
		Ala-Leu	8B1B
		Ala-Leu-Ala	8B1C
		Ala-Pro-Phe	8B1D
		Ala-Gln	8B1E
		Ala-Val	8B1F

		Ala-Trp	8B1G
		Lys-Val	8B1H
		Met-Ser	8B1I
		Ser-Leu	8B1J
		Asn-Val	8B1K
PHT1	Gallus gallus		8CNI
PHT1	Homo sapiens		8JZR
		Feeblin	8JZX
		Lys-Leu	8WX4
PHT2	Homo sapiens		8WX2

Appendix 2 – *Staphylococcus aureus* strains utilised in this work

Strain	Background	Relevant genotype	Notes	Relevant source
IK5	JE2		Wild type	Fey et al., 2013
IK2		<i>ntpT::ErmR</i>	Generated via Φ 85 phage transduction from NTML strain NE971 into <i>IK5</i>	Fey et al., 2013
IK163		<i>opp3A::ErmR</i>	Generated via Φ 85 phage transduction from NTML strain NE440 into <i>IK5</i>	Fey et al., 2013
IK38		<i>ntpT::</i>	<i>ErmR</i> cassette cleared from <i>bursa aurealis</i> via allelic exchange	Bose et al., 2013
IK165		<i>ntpT:: opp3A:: ErmR</i>	Generated via Φ 85 phage transduction from NTML strain NE440 into <i>IK38</i>	Fey et al., 2013
IK12	RN4220		Cloning intermediate strain	Kreiswirth et al., 1983
IK208	JE2	<i>gisD::ErmR</i>	Generated via Φ 85 phage transduction from NTML strain NE215 into <i>IK5</i>	Fey et al., 2013

IK207		<i>dtpT::gisD::</i> ErmR	Generated via Φ 85 phage transduction from NTML strain NE215 into <i>IK38</i>	Fey et al., 2013
IK206		SAUSA300_006 0::ErmR	Generated via Φ 85 phage transduction from NTML strain NE806 into <i>IK5</i>	Fey et al., 2013

Appendix 3 – *Escherichia coli* and *Klebsiella pneumoniae* strains utilised in this work

Strain	Background	Notes	Relevant source
	DH5α	Cloning intermediate strains	
	XL-1 Blue		
GHT190	BL21(DE3)	Routine protein expression strains	Studier & Moffatt, 1986
GHT397	BW25113		Baba et al., 2006
GHT313	C41	BL21(DE3)-derived expression strains for the expression and purification of toxic/membrane proteins	Miroux and Walker, 1996
GHT314	C43		
IK27	<i>K. pneumoniae</i> ATCC13883		Schroeter, 1886; Trevisan, 1887

Appendix 4 – Plasmids utilised in this work

Plasmid name	Organism	Resistance marker	Source
pRab11	<i>E. coli</i> / <i>S. aureus</i> shuttle vector	Amp100 (<i>E. coli</i>) / Cam10 (<i>S. aureus</i>)	Helle et al., 2011
pRab11-DtpT			This work
pSK5630	<i>E. coli</i> / <i>S. aureus</i> shuttle vector	Amp100 (<i>E. coli</i>) / Cam10 (<i>S. aureus</i>)	Grkovic et al., 2003
pSK56pT			This work
pSK56pT-DtpT			
pTnT	<i>S. aureus</i>	Cam10	Bose et al., 2013
pBADcLIC	<i>E. coli</i>	Amp100	Geertsma and Poolman, 20078
pBADcLIC-DtpT			This work
pBADcLIC-NanT			Roy et al., 2010
pWaldo	<i>E. coli</i>	Kan50	Drew et al., 2005
pWaldo-DtpT			This work
pWaldo-DtpA _{Ec}			
pWaldo-DtpC _{Ec}			
pWaldo-DtpE _{Kp}			
pWaldo-PepT _{Sh}			Minhas et al., 2018
pWaldo-PepT _{So}			Newstead et al., 2011
pWaldo-DtpT _{Y41F}	<i>E. coli</i>	Kan50	This work
pWaldo-DtpT _{Y41A}			
pWaldo-DtpT _{Q310E}			
pWaldo-DtpT _{Q310A}			
pWaldo-DtpT _{E33A}			

pWaldo-DtpT _{N167Q}			
pWaldo-DtpT _{E418A}			
pETFPP_(N)GST	<i>E. coli</i>	Kan50	University of York Technology Facility
pETFPP_(N)GST- Opp3A*			This work
pETFPP_(N)GST- Opp3A* _{W438S}			

Appendix 5 – Composition of CDMG (adapted from [Hussain et al., 1991](#)):

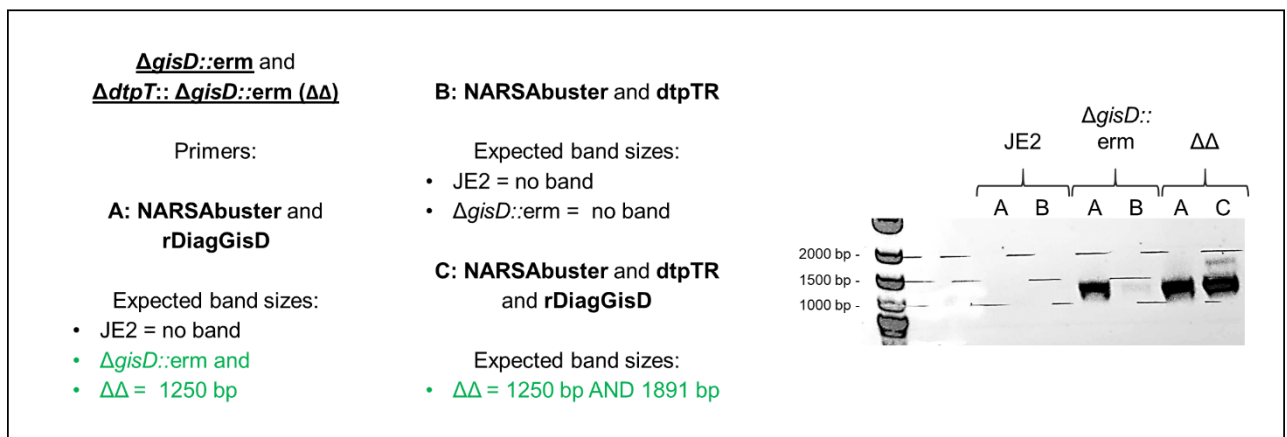
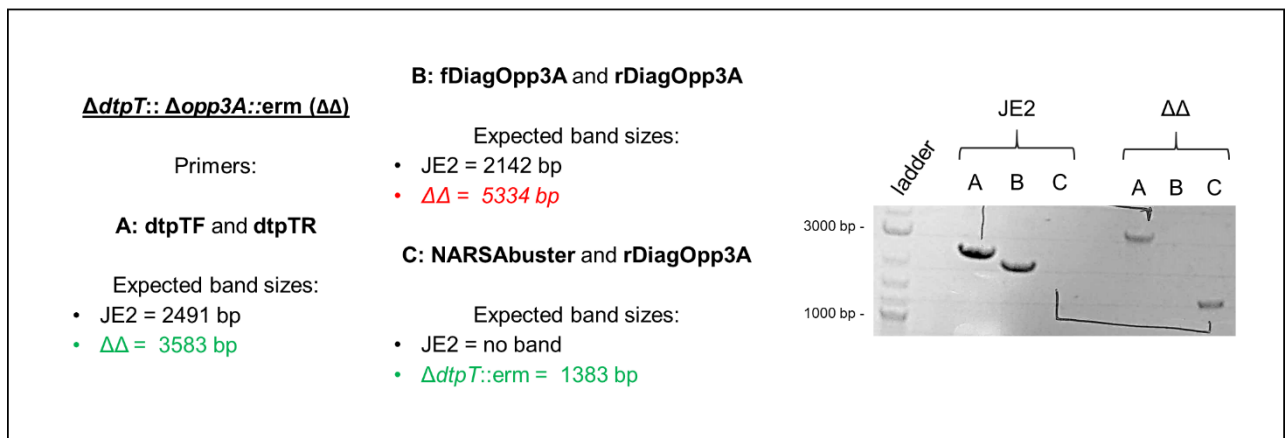
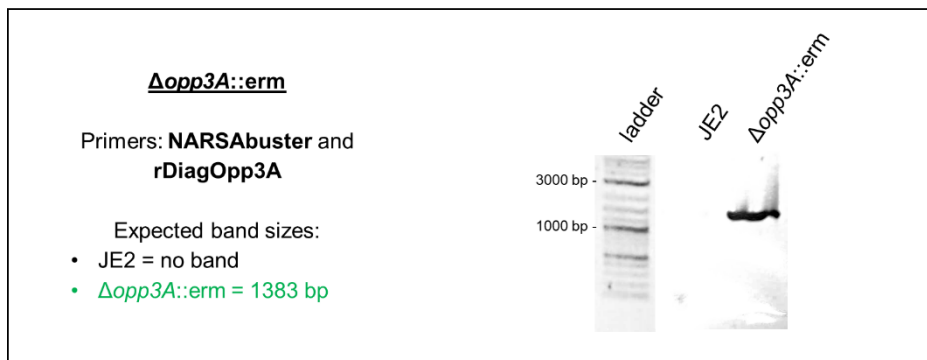
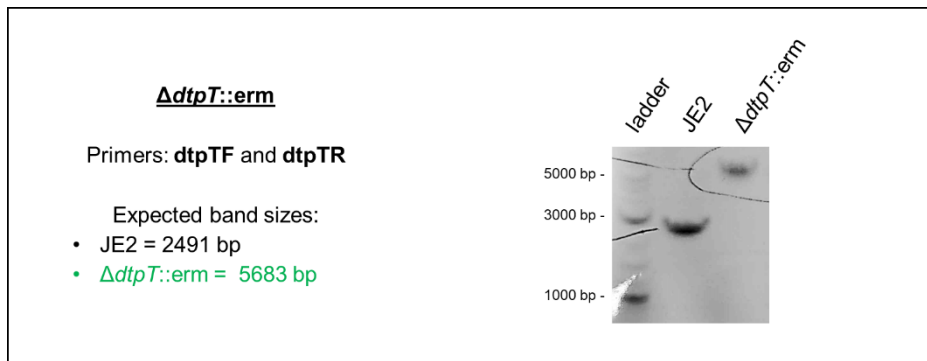
Ingredients	mg/L
Group 1	Adjust pH to 7.2, then autoclave in 700 ml
Na ₂ HPO ₄	8000
KH ₂ PO ₄	3000
L-Aspartic acid	150
L-Alanine	100
L-Arginine	150
L-Cysteine	75
Glycine	100
L-Glutamic acid	150
L-Histidine	100
L-Isoleucine	150
L-Lysine	100
L-Leucine	150
L-Methionine	100
L-Phenylalanine	100
L-Proline	150
L-Serine	100
L-Threonine	150
L-Tryptophan	100
L-Tyrosine	100
L-Valine	150

Ingredients	mg/L
Group 2	Autoclave in 280 ml
d-Glucose	2 000
MgSO ₄ ·7H ₂ O	500

Group 3	
Filter sterile to 100x stock	+ 10 ml
Biotin	0.1
Nicotinic acid	2
D-Pantothenic acid, Ca salt	2
Pyridoxal	4
Thiamine hydrochloride	2
Pyridoxamine dihydrochloride	4

Group 4	
Filter sterile to 100x stock	+ 10 ml
CaCl ₂ ·6H ₂ O	10
MnSO ₄	5
(NH ₄) ₂ SO ₄ ·FeSO ₄ ·6H ₂ O	6

Appendix 6 – PCR confirmation of key chromosomal transposon-interrupted mutant strains utilised in this work:



Appendix 7 – layout of Phenotype Microarray plates PM 6/7/8 (blue = non-standard dipeptides; yellow = tripeptides):

PM6:

Neg.	Pos.	ala-ala	ala-arg	ala-asn	ala-glu	ala-gly	ala-his	ala-leu	ala-lys	ala-phe	ala-pro
ala-ser	ala-thr	ala-trp	ala-tyr	arg-ala	arg-arg	arg-asp	arg-gln	arg-glu	arg-ile	arg-leu	arg-lys
arg-met	arg-phe	arg-ser	arg-trp	arg-tyr	arg-val	asn-glu	asn-val	asp-asp	asp-glu	asp-leu	asp-lys
asp-phe	asp-trp	asp-val	cys-gly	gln-gln	gln-gly	glu-asp	glu-glu	glu-gly	glu-ser	glu-trp	glu-tyr
glu-val	gly-ala	gly-arg	gly-cys	gly-gly	gly-his	gly-leu	gly-lys	gly-met	gly-phe	gly-pro	gly-ser
gly-thr	gly-trp	gly-tyr	gly-val	his-asp	his-gly	his-leu	his-lys	his-met	his-pro	his-ser	his-trp
his-tyr	his-val	ile-ala	ile-arg	ile-gln	ile-gly	ile-his	ile-ile	ile-met	ile-phe	ile-pro	ile-ser
ile-trp	ile-tyr	ile-val	leu-ala	leu-arg	leu-asp	leu-glu	leu-gly	leu-ile	leu-leu	leu-met	leu-phe

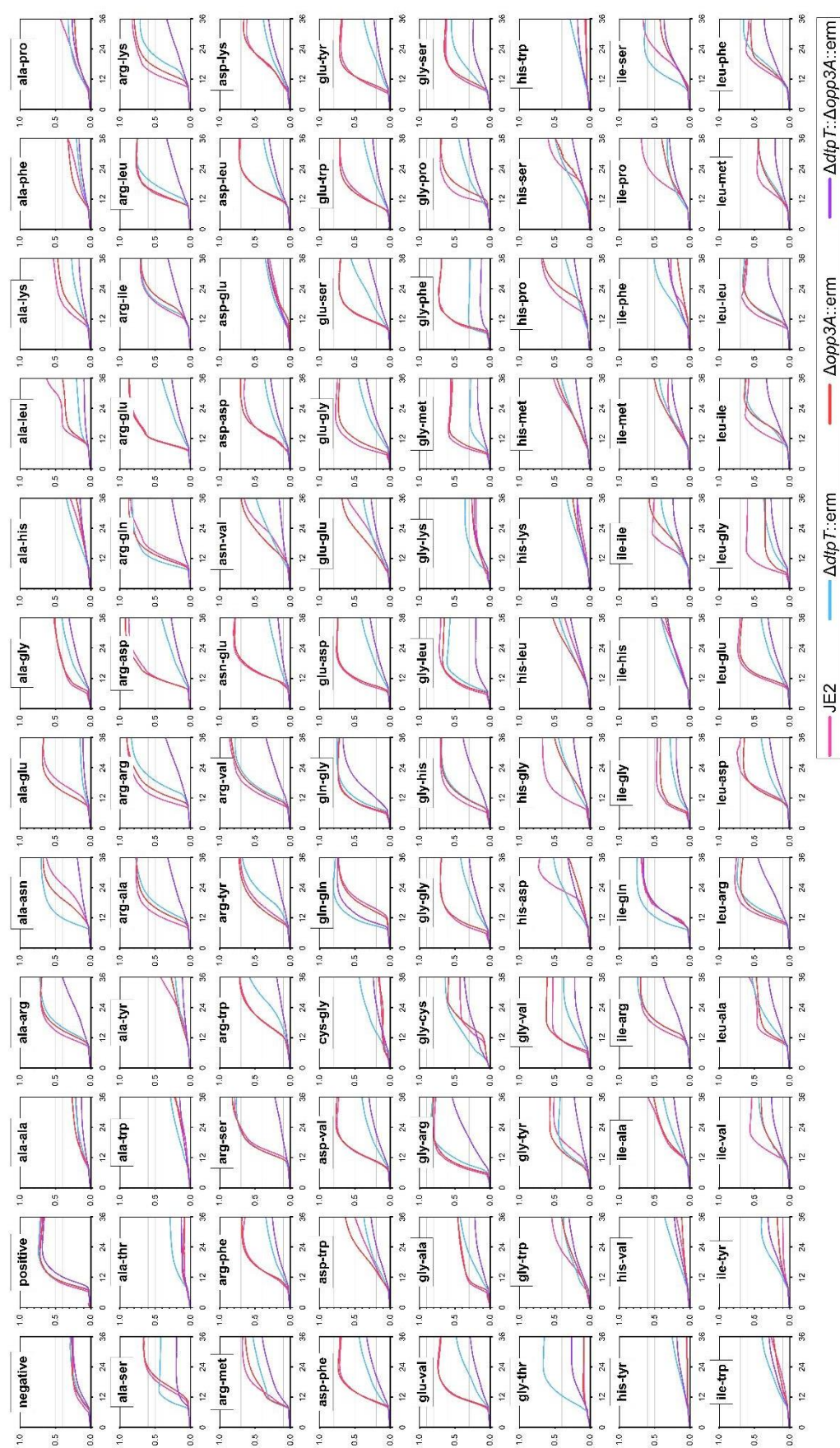
PM7:

Neg.	Pos.	leu-ser	leu-trp	leu-val	lys-ala	lys-arg	lys-glu	lys-ile	lys-leu	lys-lys	lys-phe
lys-pro	lys-ser	lys-thr	lys-trp	lys-tyr	lys-val	met-arg	met-asp	met-gln	met-glu	met-gly	met-his
met-ile	met-leu	met-lys	met-met	met-phe	met-pro	met-trp	met-val	phe-ala	phe-gly	phe-ile	phe-phe
phe-pro	phe-ser	phe-trp	pro-ala	pro-asp	pro-gln	pro-gly	pro-hyp	pro-leu	pro-phe	pro-pro	pro-tyr
ser-ala	ser-gly	ser-his	ser-leu	ser-met	ser-phe	ser-pro	ser-ser	ser-tyr	ser-val	thr-ala	thr-arg
thr-glu	thr-gly	thr-leu	thr-met	thr-pro	trp-ala	trp-arg	trp-asp	trp-glu	trp-gly	trp-leu	trp-lys
trp-phe	trp-ser	trp-trp	trp-tyr	tyr-ala	tyr-gln	tyr-glu	tyr-gly	tyr-his	tyr-leu	tyr-lys	tyr-phe
tyr-trp	tyr-tyr	val-arg	val-asn	val-asp	val-gly	val-his	val-ile	val-leu	val-tyr	val-val	y-glu-glu

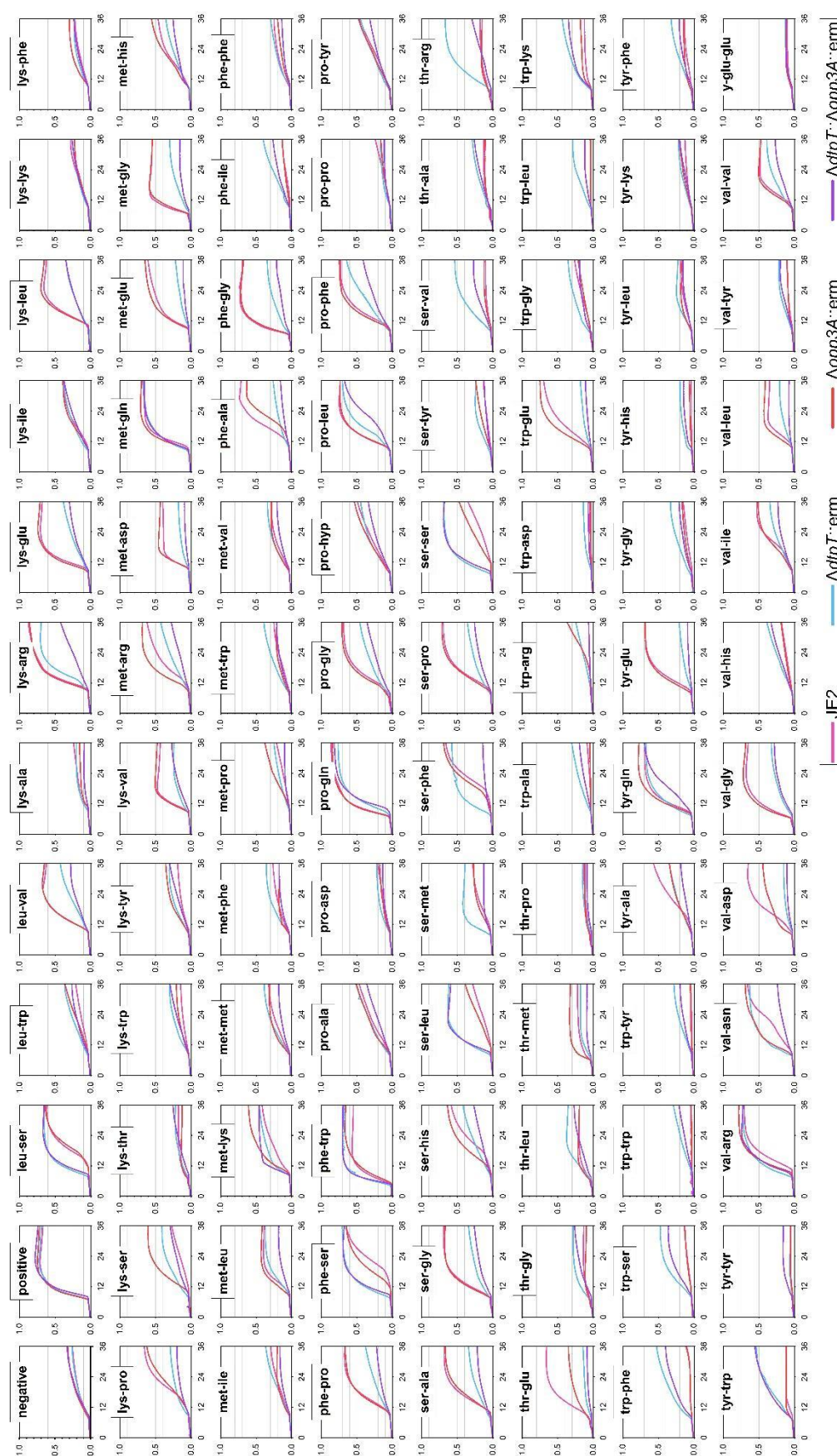
PM8:

Neg.	Pos.	ala-asp	ala-gln	ala-ile	ala-met	ala-val	asp-ala	asp-gln	asp-gly	glu-ala	gly-asn
gly-asp	gly-ile	his-ala	his-glu	his-his	ile-asn	ile-leu	leu-asn	leu-his	leu-pro	leu-tyr	lys-asp
lys-gly	lys-met	met-thr	met-tyr	phe-asp	phe-glu	gln-glu	phe-met	phe-tyr	phe-val	pro-arg	pro-asn
pro-glu	pro-ile	pro-lys	pro-ser	pro-trp	pro-val	ser-asn	ser-asp	ser-gln	ser-glu	thr-asp	thr-gln
thr-phe	thr-ser	trp-val	tyr-ile	tyr-val	val-ala	val-gln	val-glu	val-lys	val-met	val-phe	val-pro
val-ser	β-ala-ala	β-ala-gly	β-ala-his	met-β-ala	β-ala-phe	d-ala-d-ala	d-ala-gly	d-ala-leu	d-leu-d-leu	d-leu-gly	d-leu-tyr
γ-glu-gly	γ-d-glu-gly	gly-d-ala	gly-d-asp	gly-d-ser	gly-d-thr	gly-d-val	leu-β-ala	leu-d-leu	phe-β-ala	ala-ala-ala	d-ala-gly-gly
gly-gly-ala	gly-gly-d-leu	gly-gly-gly	gly-gly-ile	gly-gly-leu	gly-gly-phe	val-tyr-val	gly-phe-phe	leu-gly-gly	leu-leu-leu	phe-gly-gly	tyr-gly-gly

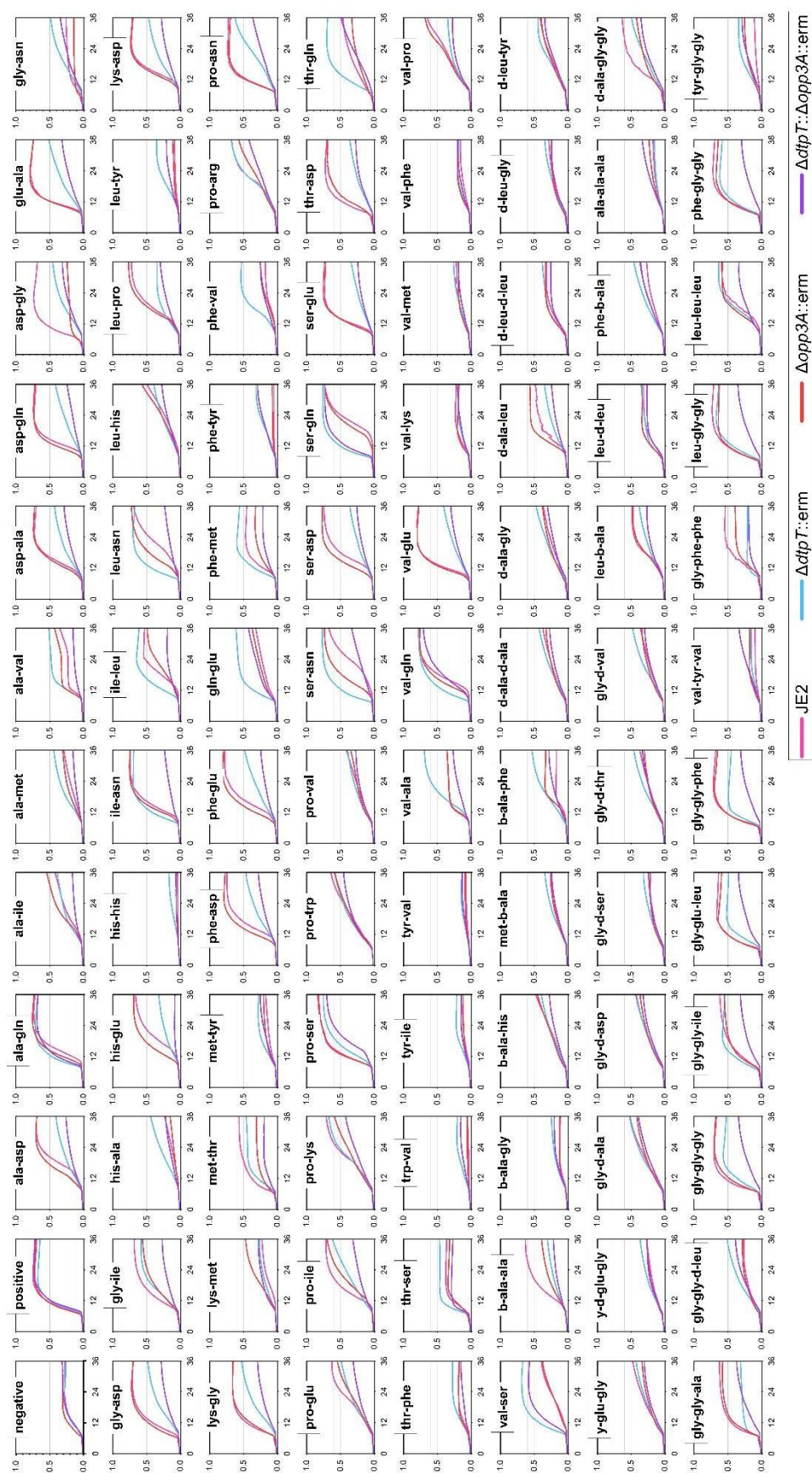
Appendix 8 – Complete signal curves of all strains on PM 6:



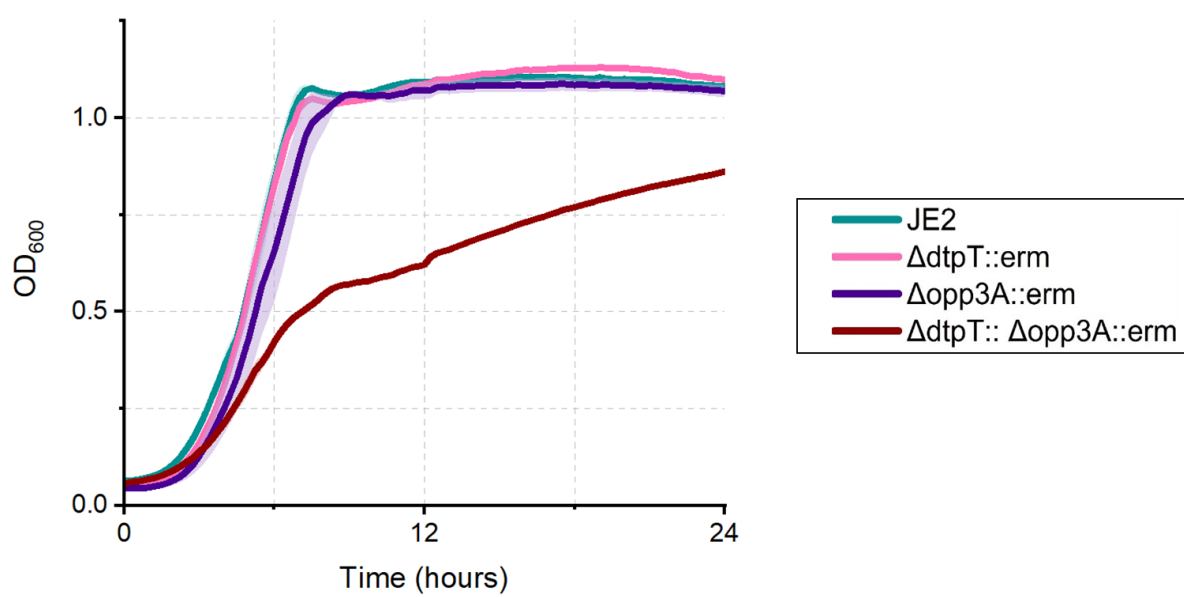
Appendix 9 – Complete signal curves of all strains on PM 7:



Appendix 10 – Complete signal curves of all strains on PM 8:



Appendix 11 – Arginine auxotrophy assay in JE2 and dtpT/opp3A mutant-derivatives in the presence of 1 mM Arg-Ala



Appendix 12 – Expression and purification of DtpT in *E. coli* from a pBAD-based expression vector

Expression trials

Initial attempts to recombinantly express DtpT utilised the arabinose-inducible vector pBADcLIC, previously generated by Geertsma and Poolman and applied for the overexpression of diverse membrane proteins in *E. coli* (Geertsma et al., 2008; Geertsma & Poolman, 2007). Notably, this vector incorporates the *araBAD* promoter (P_{araBAD}) and AraC repressor protein to enable high-level expression of a target protein in the presence of arabinose and absence of glucose (Guzman et al., 1995). pBADcLIC also incorporates a C-terminal 10xHis tag into the protein coding sequence, enabling detection of the target protein by anti-His Western blot and purification via immobilised metal-affinity chromatography (IMAC).

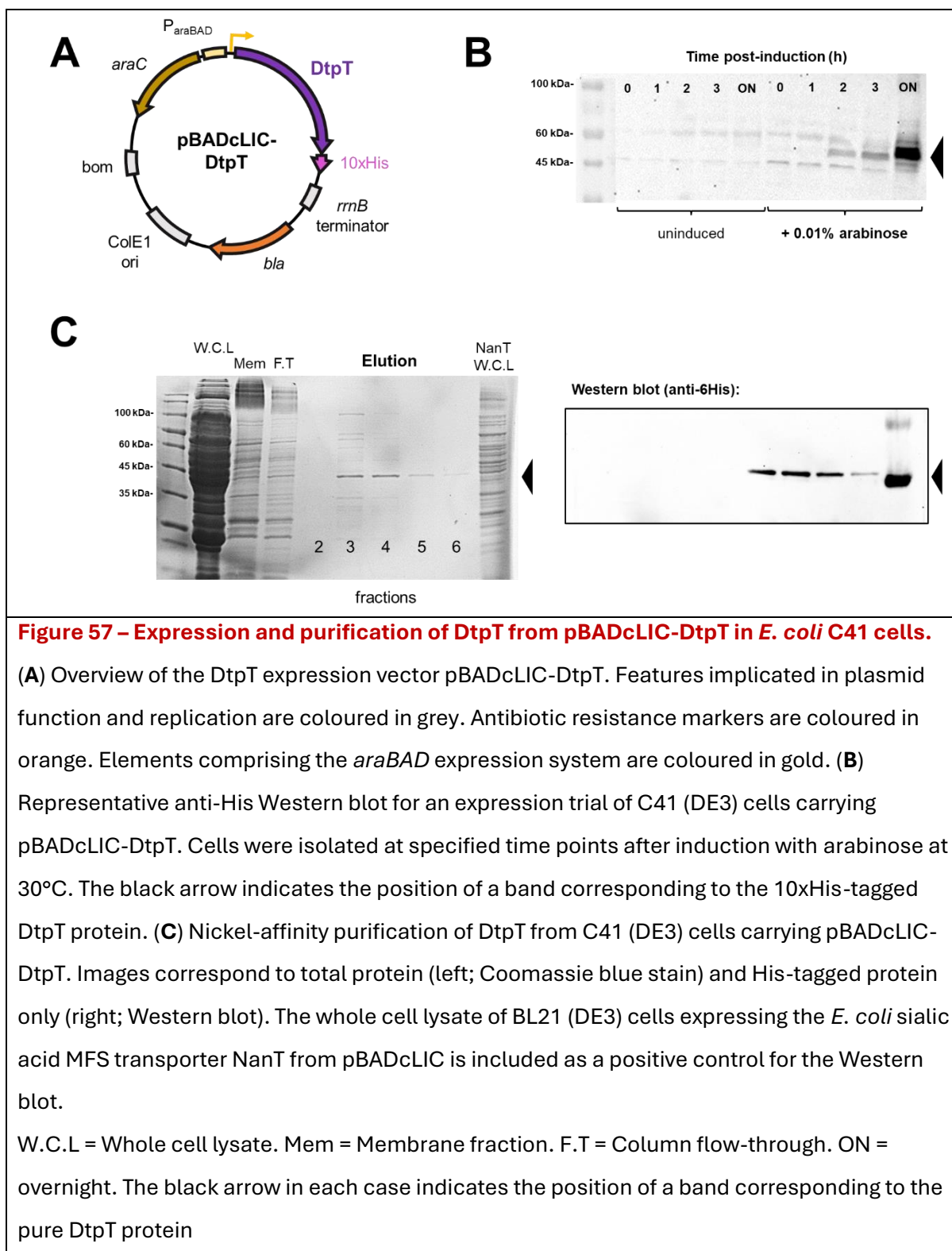
Here, the entire *dtpT* gene was amplified from the genome of *S. aureus* JE2 and inserted downstream of the P_{araBAD} promoter to produce the expression vector pBADcLIC-DtpT (Figure 57A). This was then transformed into various expression strains of *E. coli* – namely BW25113, BL21 (DE3), C41 (DE3) and C43 (DE3) (Miroux & Walker, 1996) – and expression in each case was assessed in LB medium following induction with 0.01% arabinose at 30°C and 37°C. Strong expression of the target protein was apparent only in the C41 (DE3) after overnight growth in the presence of the inducer at 30°C (Figure 57B, other strains/conditions not show). This condition was therefore scaled up and taken forward for an attempted purification

Purification

Having determined optimal DtpT expression conditions from pBADcLIC-DtpT, we next attempted to purify the protein from 2 L of bacterial culture expressing the protein under optimal conditions. Briefly, cells expressing the target protein were lysed and membrane fractions were isolated via ultracentrifugation. Solubilised membranes were applied to a nickel affinity column and washed before elution of the bound His-tagged target protein via the application of excess imidazole.

Here, we observed the elution of a single band corresponding to the tagged DtpT protein as confirmed by anti-His Western blot (Figure 57C). Though the final protein yield from this purification attempt was never quantified, crude gel analysis suggests that < 0.5 mg of DtpT was yielded from 2 L of expression culture (calculated by comparing band intensity to the 250 µg/ml protein ladder). We also note that tagged DtpT was below the limit of detection via Western blot in the whole-cell lysate and membrane fractions, indicating poor expression overall (particularly when compared to the whole-cell lysate of cells expressing the *E. coli* sialic acid transporter NanT from an equivalent vector, included here as a positive control) (Figure 57C). Overall, while

this purification strategy demonstrated partial success, the pWaldo expression system (trialled concurrently) consistently provided much greater yields of the target protein and was ultimately taken forward for use in future purifications.



Appendix 13 – Progress toward structural characterisation of the *S. aureus* oligopeptide binding SBP Opp3A

The oligopeptide transporter Opp3 facilitates the utilisation of diverse oligopeptides in *S. aureus*, with sizes ranging from dipeptides (as demonstrated in this study) to $n = 9$ residues in length ([Hiron et al., 2007](#)). Several studies have now implicated Opp3 in the utilisation of peptides as a major nutrient source in relevant growth environments, including those derived from collagen ([Lehman et al., 2019](#)) and casein in milk ([Borezée-Durant et al., 2009](#)). However, the biochemical basis of substrate recognition and preference in this transport system remains poorly understood.

Here, we have demonstrated that Opp3 can facilitate the uptake of dipeptides in *S. aureus*, with an apparent preference for those containing arginine (see [Chapter 3](#)). Generally, the SBP components of ABC importer systems are implicated in substrate recognition and thereby responsible for selectivity in regard to transport targets ([Thomas & Tampé, 2020](#)). Based on this, we hypothesised that structural characterisation of ligand binding in Opp3A may provide novel insights into the substrate preferences of Opp3 and reveal the molecular basis of Arg-peptide selectivity in this system.

Expression and purification of Opp3A*

Work carried out by a project student in our group (Steven Kurniawan; *University of York*) demonstrated that the full-length Opp3A protein from *S. aureus* JE2 was generally insoluble but could be stably express in a soluble form in *E. coli* via the utilisation of an N-terminal glutathione S-transferase (GST) solubility tag. However, we found that attempts to cleave the GST tag (via overnight incubation with 3C protease) resulted in a loss in protein solubility.

Many SBPs in Gram-positive bacteria are natively expressed as lipoproteins and feature a highly hydrophobic N-terminal signal peptide, followed shortly by a characteristic cysteine which functions as a lipid anchor site ([Hayashi & Wu, 1990](#)). For Opp3A, the signal peptide recognition tool SignalP-5.0 (DTU Health Tech) predicts that a signal peptide encompasses the first 20 residues of the full-length protein, immediately followed by a lipid anchor site at Cys21 ([Figure 58A](#)). Given that signal peptides are generally cleaved during lipoprotein secretion and processing, this sequence is probably not present in the native *S. aureus* Opp3A lipoprotein. Indeed, the inclusion of such a hydrophobic sequence may explain the observed insolubility of Opp3A observed in our experiments. To circumvent this, a new expression construct was prepared in which Opp3A had been truncated to remove the complete signal peptide and lipid anchor site (Opp3A₂₅₋₅₄₉, or Opp3A*; [Figure 58B](#)). This protein expressed at high levels in *E. coli*

and was demonstrated to be highly soluble following cleavage of the N-terminal GST tag, allowing for effective purification via IMAC and SEC (**Figure 58C-E**). Unexpectedly, we found that the GST tag utilised here became entirely insoluble following overnight cleavage, forming a precipitant which could be conveniently removed via centrifugation to yield pure Opp3A* in the soluble fraction (**Figure 58D**). We believe that this precipitation is likely to be driven by oxidation of the GST under prolonged incubation with rotation, leading to the formation of disulphide bonds and thereby resulting in large insoluble protein aggregates ([McDuffee et al., 1997](#)). Such a model would explain why Opp3A* remains in the soluble fraction, given that this truncated protein lacks any cysteine residues which would contribute to disulphide bond formation.

Design and purification of Opp3A*W438S

Bacterial SBPs typically possess high binding affinities for their cognate substrates and are therefore often purified in a ligand-bound state when a suitable ligand is present in the expression culture (often referred to as “the retention effect”, as described by [Silhavy et al., 1975](#)). Whilst these ligand-bound complexes can shed new light on the basis of ligand binding, we hypothesised that the presence of diverse native peptides in the purified Opp3A* protein may cause issues during structural elucidation; specifically, heterogeneity in the bound ligands may prevent the clear elucidation of a single ligand binding mode in the final structure. Previous work on Opp homologues from diverse bacteria has revealed the presence of a conserved tryptophan residue in these proteins which is seemingly required for strong protein-ligand interactions ([Hughes et al., 2019](#); [Yang et al., 2024](#)). In the Mycobacterial OppA protein, mutation of this tryptophan residue (Trp491) was sufficient to prevent the retention of native ligands during purification and thereby allowed for the elucidation of an *apo* structure of the pure protein ([Yang et al., 2024](#)). Here, we applied a similar methodology in an attempt to purify Opp3A* in the absence of natively bound peptide ligands. A DNA sequence alignment suggested that Trp438 in the *S. aureus* Opp3A protein was a direct orthologue of Trp491 in the Mycobacterial protein (**Figure 59A**). Hence, a mutant variant of Opp3A* was generated for which Trp438 had been substituted for to serine (W438S). This novel variant was then purified using the same methodology as the original Opp3A* protein (**Figure 59B-C**).

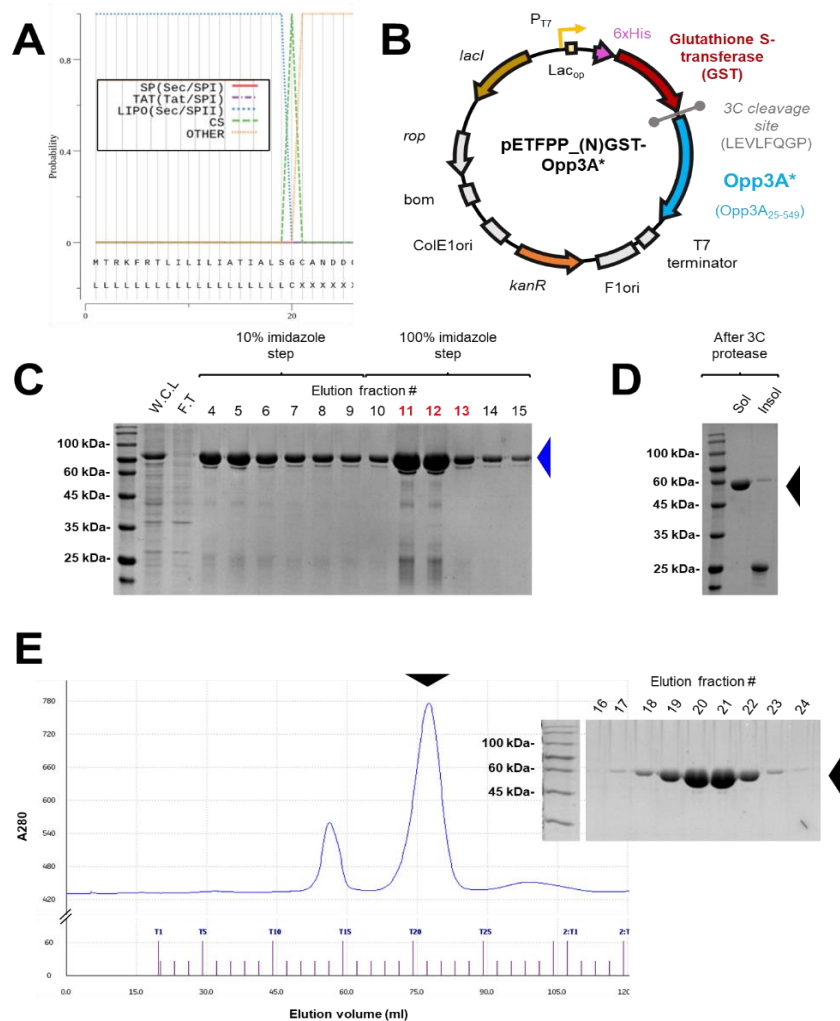
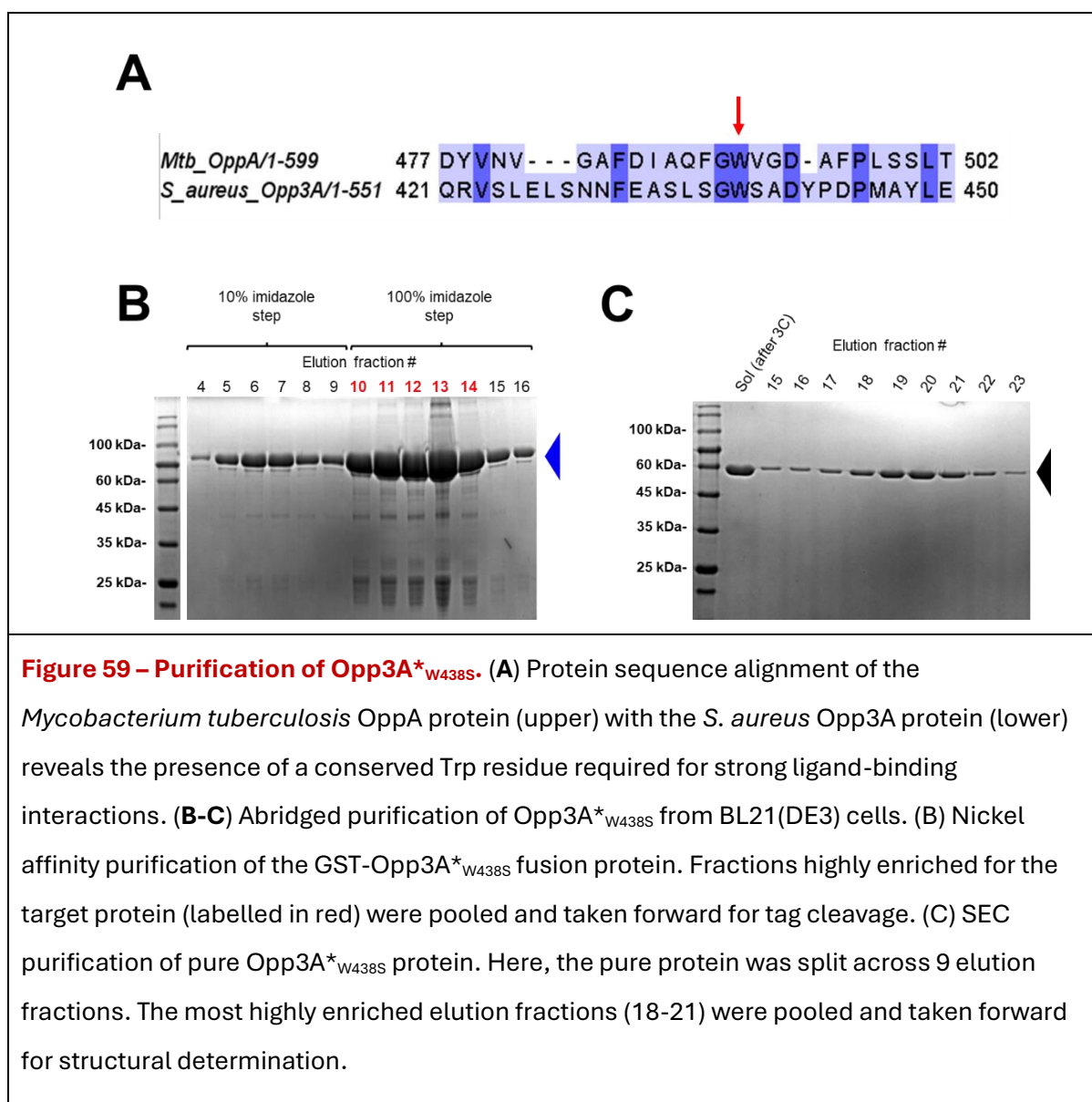


Figure 58 – Purification of the truncated *Staphylococcus aureus* peptide-binding SBP Opp3A*. (A) Trimmed SignalP-5.0 output for the *S. aureus* JE2 Opp3A coding sequence reveals the presence of a lipoprotein signal peptide comprising the first 20 residues of the complete protein. (B) Overview of the GST-Opp3A* expression vector pETFPP_(N)GST-Opp3A*. Features implicated in plasmid function and replication are coloured in grey. Antibiotic resistance markers are coloured in orange. Elements comprising the *E. coli* *lac* expression system are coloured in gold. (C-E) Complete purification of Opp3A* from BL21(DE3) cells. (C) Nickel affinity purification of the GST-Opp3A* fusion protein. Fractions highly enriched for the target protein (labelled in red) were pooled and taken forward for tag cleavage. W.C.L = Whole cell lysate. F.T = Column flow-through. (D) Following overnight cleavage, GST was effectively removed to yield a single soluble protein (sol) corresponding to the cleaved Opp3A*. Conversely, GST became entirely insoluble and was effectively removed by centrifugation (insol). (E) SEC purification of pure Opp3A* protein. A single sharp peak is seen in the A280 trace (left) corresponding to the pure Opp3A* protein split across four elution fractions (right).



Crystallisation

Following purification, both Opp3A* variants were subjected to crystallisation trials under diverse buffer conditions using commercially available crystal screening platforms. Within a week, the appearance of proto-crystals, needles and single crystals was observed for a handful of conditions in both Opp3A* and the W438S variant. Based on these conditions, we devised more targeted screens in which only the salt and precipitant concentrations were varied around those utilised in the initial screen. As a result, we identified conditions for which large, ordered crystals were formed within one week for both protein variants (Table 8; Figure 60A).

Table 8. Optimised crystallisation conditions for Opp3A and the W438S variant*

Protein	Crystallisation conditions			
	Salt		Precipitant	
Opp3A*	Lithium citrate tetrahydrate	200 mM	PEG 3350	24% w/v
Opp3A* _{W438S}	Ammonium tartrate	200 mM	PEG 3350	22% w/v

Upon addition of the cryoprotectant glycerol, we observed rapid dissolution of the formed crystals. Hence, crystals were instead resuspended in a cryoprotectant solution consisting of 35% PEG 3350 (that is, the precipitant utilised for crystallisation in each case). Crystals were then isolated, and samples were sent for diffraction using the i03 beamline at the Diamond Light Source (Oxfordshire). Diffraction patterns clearly indicate the presence of ordered protein structures within the tested samples (Figure 60B). However, the diffraction data was of an overall poor quality, and we were therefore unable to confidently build a structural model for the protein at a useful resolution from this dataset. Hence, we conclude that further optimisation is needed to improve crystal quality and protein packing before a high-resolution structure can be elucidated. Such optimisation may involve testing an expanded pool of reagent conditions and timeframes. It may also be advantageous to reattempt structural elucidation for the crystals generated here utilising alternative cryoprotectant solutions, which may better maintain crystal integrity during storage and transit.

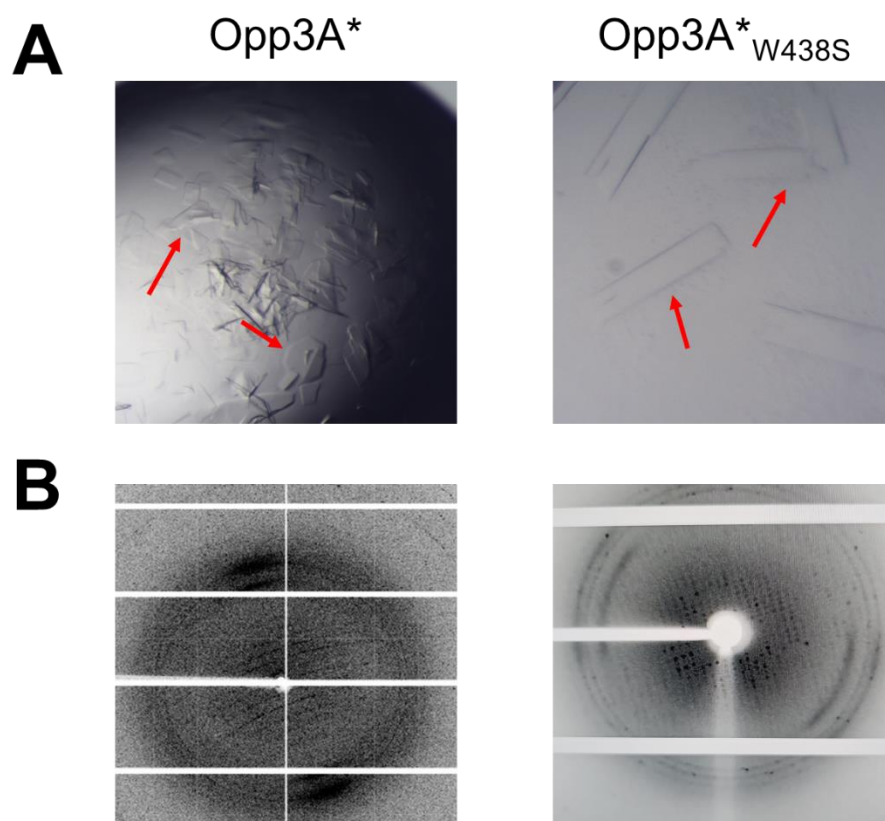
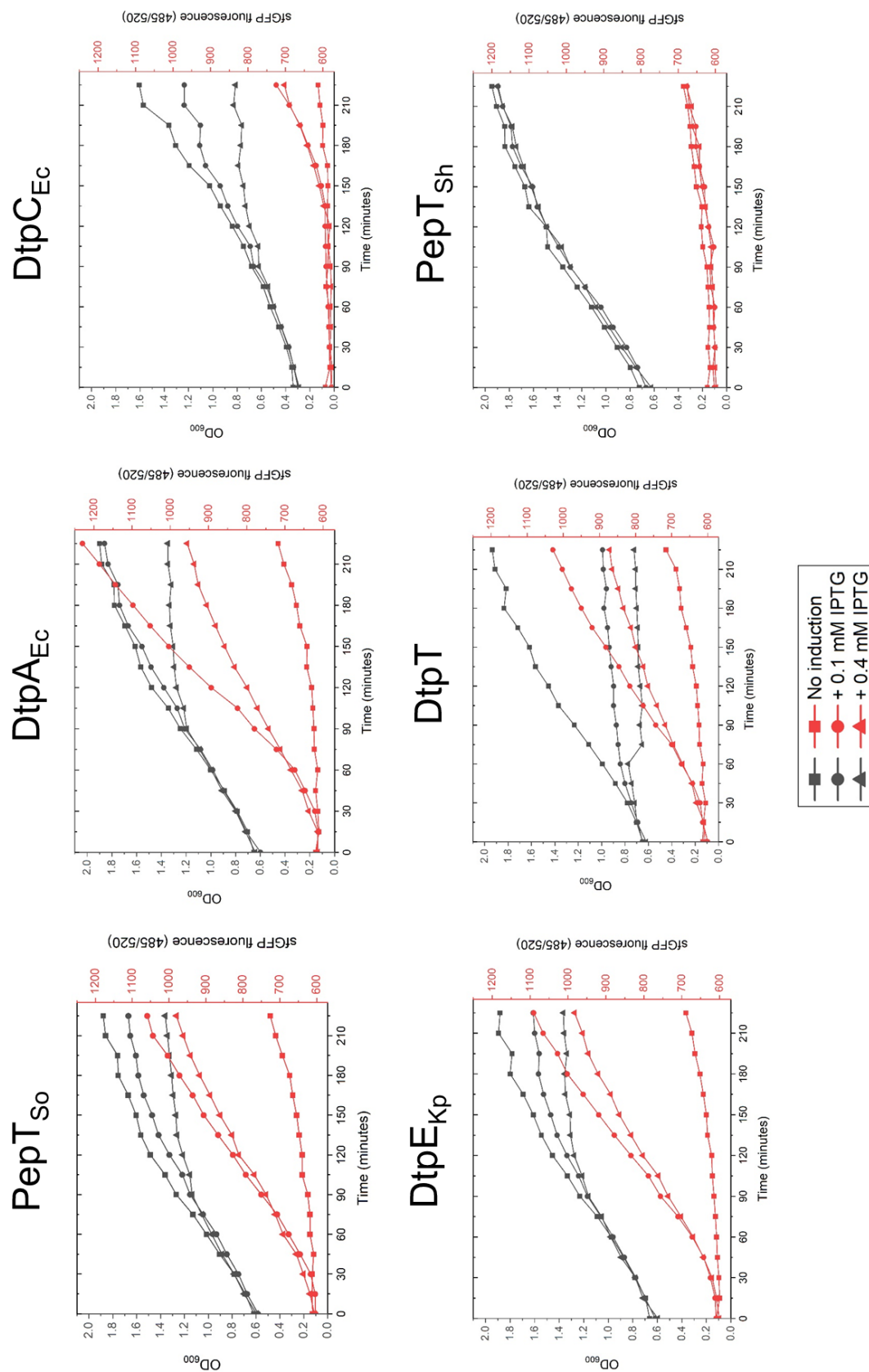
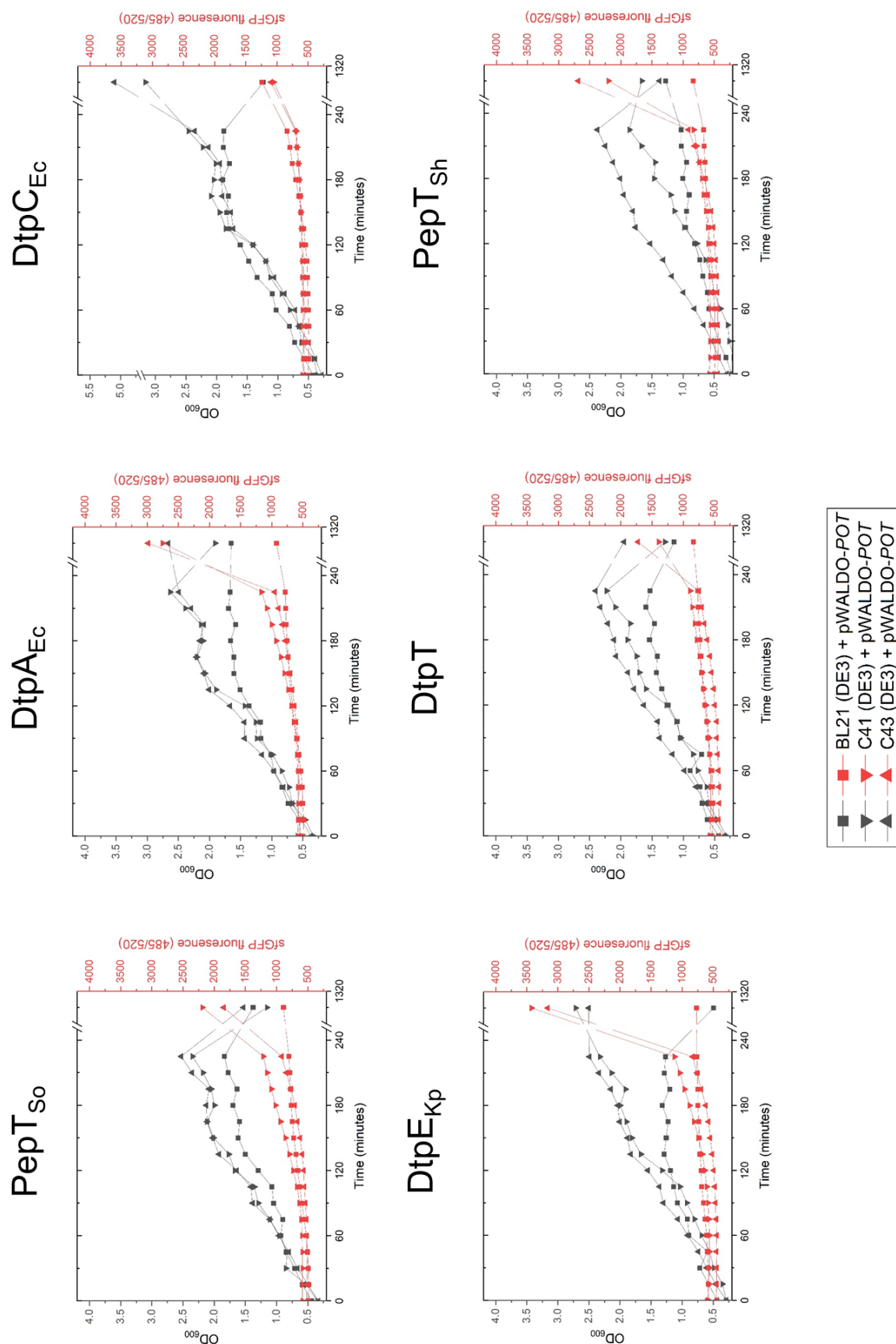


Figure 60 – Crystallisation of Opp3A* and the W438S variant. (A) Representative images of protein crystals generated from 20 mg/ml solutions of Opp3A* (left) and Opp3A*_{W438S} (right) under optimised crystal screening conditions. Red arrows indicate examples of large, ordered protein crystals. (B) Diffraction patterns generated from the crystals generated here, supplied by Diamond Light Source (Oxford).

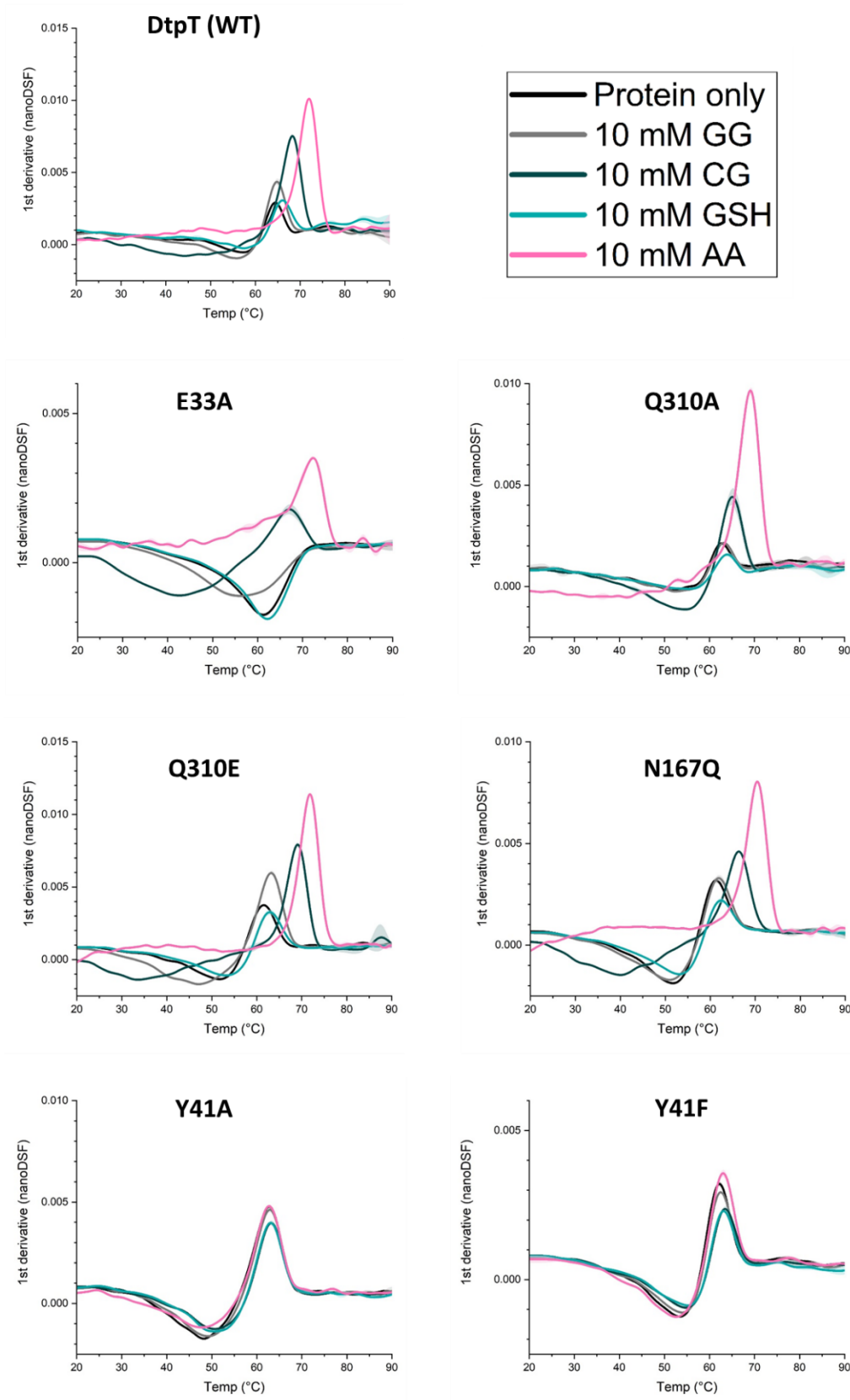
Appendix 14 – Growth and whole-cell fluorescence of BL21 (DE3) + six pWALDO-POT variants induced by the addition of 0.1 or 0.4 mM IPTG at 30°C:



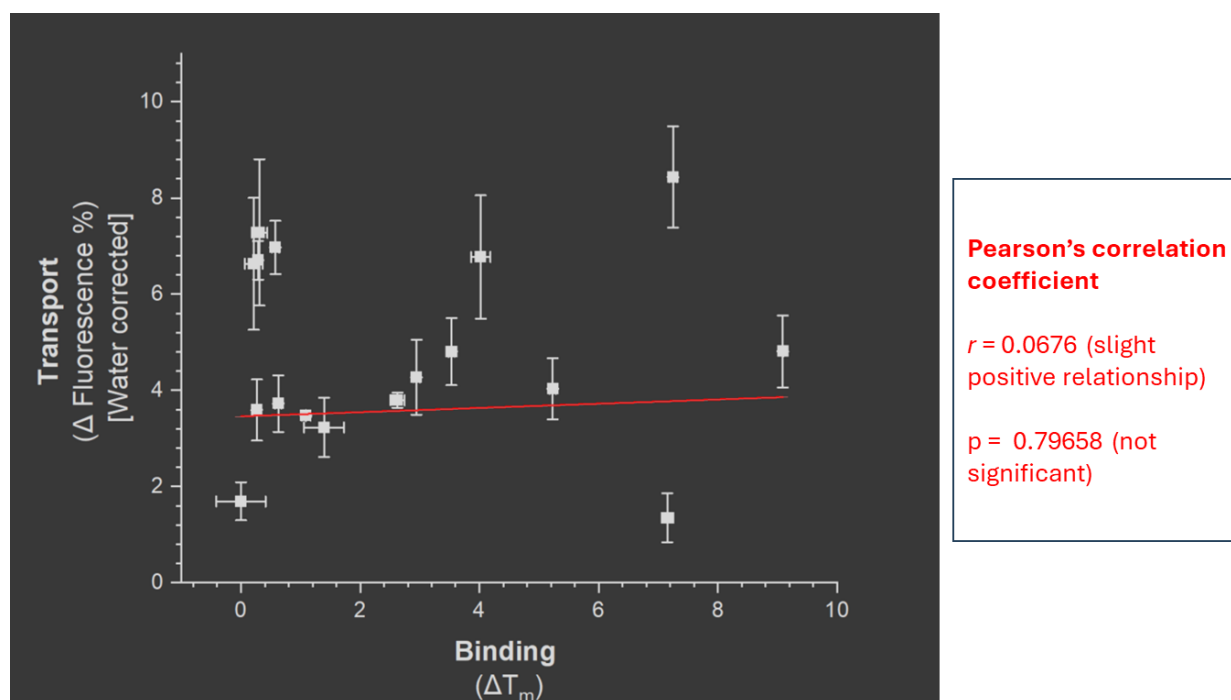
Appendix 15 – Growth and whole-cell fluorescence of BL21 (DE3), C41 (DE3) and C43 (D3) cells induced by addition of 0.1 mM IPTG at 30°C:



Appendix 16 – 1st derivative nanoDSF unfolding curves for six DtpT mutant variants screened against four peptide ligands:



Appendix 17 – Comparison of ligand binding (nanoDSF) data and transport assay data for DtpT with 17 di-/tripeptides



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