Understanding antibiotic entry into bacteria

Nicole Jackson

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The candidate confirms that the work submitted is her own and that the appropriate credit has been given where reference has been made to the work of others.

Part of the research within Chapter 4 was carried out with the help of, G. Morrison-Williams & T. O'Brien, who were under the candidate's supervision. Credit has been given to the work carried out by these two students within the chapter.

“Sometimes science is more art than science, Morty….A lot of people don’t get that”

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Abstract

The increasing prevalence of multi-drug resistance within clinically relevant bacterial species is threatening the efficacy of our existing antibiotic classes. Compounding the issue is our current lack an effective antibiotic drug discovery platform. One of the main issues hindering the development of novel antibacterials is that there is a lack of knowledge regarding the physico-chemical properties required of compounds to accumulate within the bacterial cytoplasm. In this study, I developed an LC/MS based method which will allowed the screening of chemically diverse small molecules for accumulation within the cytoplasm of *E. coli*. This method could be used in future small molecule screens, from which we may attempt to identify structure activity relationships associated with bacterial penetration and efflux avoidance.

This study also revisited the role of membrane carriers in the entry of antibiotics within bacteria. To assess this, I designed a screen which would allow the identification of membrane transporters which play a putative role in drug uptake, using a library of *S. aureus* strains containing transposon disruptions in non-essential membrane transporter genes. Using this screen, 30 carriers were identified to play a putative role in the uptake of 9 antibiotics from different drug classes. Further characterisation using genetic complementation, competition studies, drug accumulation assays and the generation of strains containing disruptions in multiple genes associated with drug uptake then confirmed the role of membrane carriers in the uptake of gentamicin, ciprofloxacin, chloramphenicol, tetracycline, fosfomycin and D-cycloserine. The results of this study show that membrane transporters play a previously unrealised role in the entry of antibiotics within *S. aureus*; this challenges the idea that drug entry occurs predominately via lipoidal diffusion, within bacterial cells.

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1. General introduction

1.1. An introduction to antibacterial drug discovery

The ‘golden-era’ of antibacterial drug discovery spanned from the 1940s – 1960s, during which around 20 novel classes of antibiotic agents were discovered, including most classes still in clinical use today (Coates *et al.,* 2011). The achievements during this time laid the foundations of modern medicine, revolutionising the treatment of infectious diseases by prompting a 20 - fold reduction in the associated mortality rate (Walsh and Wright, 2005). However, the successes seen throughout this period were not set to last; after the discovery of the quinolone class in 1962 (Lesher *et al.*, 1962), no further classes of broad-spectrum agents have been introduced in the clinic.

Alongside the lack of novel antibacterials in the drug discovery pipeline, the efficacy of our existing antibiotic arsenal is being threatened by the ever increasing prevalence of antibiotic resistance. The ubiquitous antibiotic usage in medicine and agriculture over the past ~ 70 years has engineered a strong selective pressure which las led to the preferential survival and spread of clinically relevant bacterial species harbouring novel or pre-existing antibiotic resistance mechanisms (Laxminarayan *et al.,* 2013, Grave *et al.,* 2012). Resultantly, resistance now affects all antibiotic classes (IDSA, 2010). The prevalence of multi-drug resistant (MDR) pathogens is now a major global problem, recognised by the World Health Organisation (WHO) as one of the greatest current threats to global health (IDSA, 2010). This is perhaps best illustrated by the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* & *Enterobacter* spp.), which are the leading cause of

MDR nosocomial infections worldwide (Kumarasamy *et al.,* 2010, Hsueh *et al.,* 2005, Boucher *et al.,* 2009).

A common cause of nosocomial infections and the most frequent source of MDR isolates (Rice, 2010), the ESKAPE pathogens are a considerable challenge for clinicians. As the therapeutic options for the treatment of infections involving MDR pathogens are limited, this is necessitating the revival of drugs which are associated with significant adverse effects in comparison to modern antibiotics. For instance, the polymyxins, were abandoned in the 1960s but are nowadays often the last line agent of choice for infections involving MDR Gram-negative pathogens (Li *et al.,* 2006). Worryingly, in 2015 the identification of the plasmid-borne gene *mcr1* marked the beginning of the fall of the polymyxins, as this was the first instance recorded of a horizontally acquired polymyxin resistance determinant. Encoding a phosphoethanolamine transferase, this resistance gene modifies the lipid A moiety within the lipopolysaccharide, mediating polymyxin resistance (Liu *et al.,* 2016). Originally discovered in a colistin resistant strain of *E. coli* isolated from a Shanghai pig farm (Liu et al., 2016), the *mcr1* gene has subsequently been identified in a number of clinical cases worldwide (McGann et al., 2016, Nordmann et al., 2016, Elnahriry et al., 2016, Zhi et al.). Annually around 700,000 deaths globally can already be attributed multidrug resistance (O'Neill, 2016). Thus, if antibiotic development fails to keep pace with the continual rise in antibiotic resistance, this toll will inevitably increase as unmanageable bacterial infections become more prevalent.

The blockbuster antibiotic compounds which gave the Golden-Era of antibiotic discovery its name, had mainly been uncovered through empiric discovery methods. The platform which delivered the most hits was termed the ‘Waksman platform’; this method involved the extraction of secondary metabolites from soil actinomycetes that were then screened against whole cells for antibacterial activity

(Schatz *et al.,* 1944, Lewis, 2013). In the years following, the drugs discovered in the Golden Era were extensively modified by medicinal chemistry approaches in order to improve the pharmacology of these agents, in addition to overcoming emerging antibiotic resistance mechanisms. However, after several decades of this process, the returns of this discovery model began to diminish and the outcome often involved the re-isolation of previously identified agents (Silver, 2011).

In the mid-1990s, advances in the field of molecular biology and particularly whole genome sequencing, allowed bacterial genomes to be 'mined' for previously unexploited targets. This prompted the development of a novel antibiotic drug discovery platform, the 'genes to drug' discovery model. Using this novel approach, it was hoped that novel antibiotic classes with unique modes of action would be readily identifiable in a high-throughput manner. By this method, genes could be identified and chosen as potential targets via genomic screening. After validation for essentiality, the corresponding protein target could then be expressed and subsequently employed in an enzyme-based, *in vitro* screen. Synthetic chemical libraries would be screened for potential lead compounds that exhibited activity against the chosen target, and these leads could be subsequently improved for potency and target affinity by structure based drug design (SBDD) (Silver, 2011, Payne *et al.,* 2007). Genomic screening methods generated a wealth of potential antibacterial targets; for instance, GlaxoSmithKline (GSK) identified more than 350 potential target genes by comparing genome sequences of the organisms, *P. aeruginosa*, *S. aureus* and *H. influenzae* (Payne *et al.,* 2007). However, this method of drug discovery ultimately proved fruitless. For example, from 70 high-throughput screening campaigns by GSK, only 5 lead compounds were identified, none of which have subsequently been developed into a novel drug (Payne *et al.,* 2007). One of the main issues with this discovery platform lay in the inability of these compounds to accumulate within bacteria (Silver, 2011). For instance, compounds

displaying activity within the *in vitro* screening often did not possess whole cell activity. Additionally, lead compounds with an inadequate spectrum of activity, could not be altered to allow broad-spectrum activity. As such, none of the potential leads were developed further (Payne *et al.,* 2007).

Despite iterative research efforts, the innovation gap in antibacterial drug discovery extended until the year 2000, when the first member of the novel oxazolidinone class of antibiotics, linezolid, was approved by the Food and Drug Administration (FDA) for clinical use (Brickner *et al.,* 2008). This however, was thirteen years after the discovery of the oxazolidinone family, first described in 1987.(Slee *et al.,* 1987) Five additional novel classes have subsequently been approved by the FDA; the lipopeptides, pleuromutilins, macrocycles and the diarylquinolones in 2003, 2007, 2011 and 2013, respectively (Rybak, 2006, Novak, 2011, Johnson and Wilcox, 2012, Jones, 2013). With the exception of the diarylquinolones, these novel classes were initially developed in the 1950s (pleuromutilins) (Novak, 2011) and the 1980s (lipopeptides and macrocycles) (Boeck et al., 1990, McAlpine *et al.,* 1990). However, the antibiotic classes introduced within the past decade are not enough to address the problem of resistance, as all possess a narrow spectrum of action. Due to this, some would argue that we are still experiencing this 'innovation gap' and will continue to do so until the scientific, regulatory and funding hurdles facing antibiotic discovery are addressed (O'Neill, 2016, Shlaes *et al.,* 2013, Bragginton and Piddock, 2014, Kelly *et al.,* 2016). Meanwhile, the antibiotic arsenal that we possess against Gram-negative pathogens continues to dwindle, as it has now been half a century since the discovery of the last class broad spectrum agents, the quinolones (Ball, 2000).

Combined, the various hurdles (scientific, funding & regulatory) facing antibiotic drug discovery have prevented the development of an effective discovery platform (Projan, 2003, Piddock, 2012). This has led to a failure in the replenishment

of the antibiotic pipeline with broad-spectrum antibacterials, possessing novel modes of action (Silver, 2011, Piddock, 2012). It has been proposed that between 5 and 20 novel antibacterial drugs would need to enter the development pipeline in order to effectively contend with the current resistance problem. However, given the attrition rate within the existing drug discovery model, a minimum of 200 discovery programmes would optimistically be needed in order to achieve this outcome (Coates *et al.,* 2011). Hence, the issues regarding our current discovery efforts must be addressed if we are to contend with the ever-increasing problem of antibiotic resistance. Evident from the penetration abilities of our existing antibiotic arsenal (Figure 1) and the failures of the 'Genes to drug' antibiotic discovery model (Payne et al., 2007), one of the primary issues facing the development of broad-spectrum antibiotics is ensuring the accumulation of drugs within bacterial cells. Addressing this issue would therefore allow one of the major hurdles facing novel antibacterial drug discovery to be overcome, providing knowledge that could allow the antibiotic pipeline to be re-stocked with broad-spectrum inhibitors. In the subsequent sections, I will discuss the barriers of the bacterial cell membrane which antibiotics must overcome in order to reach and interact with their intracellular targets; the outer membrane, cytoplasmic membrane and efflux mechanisms.

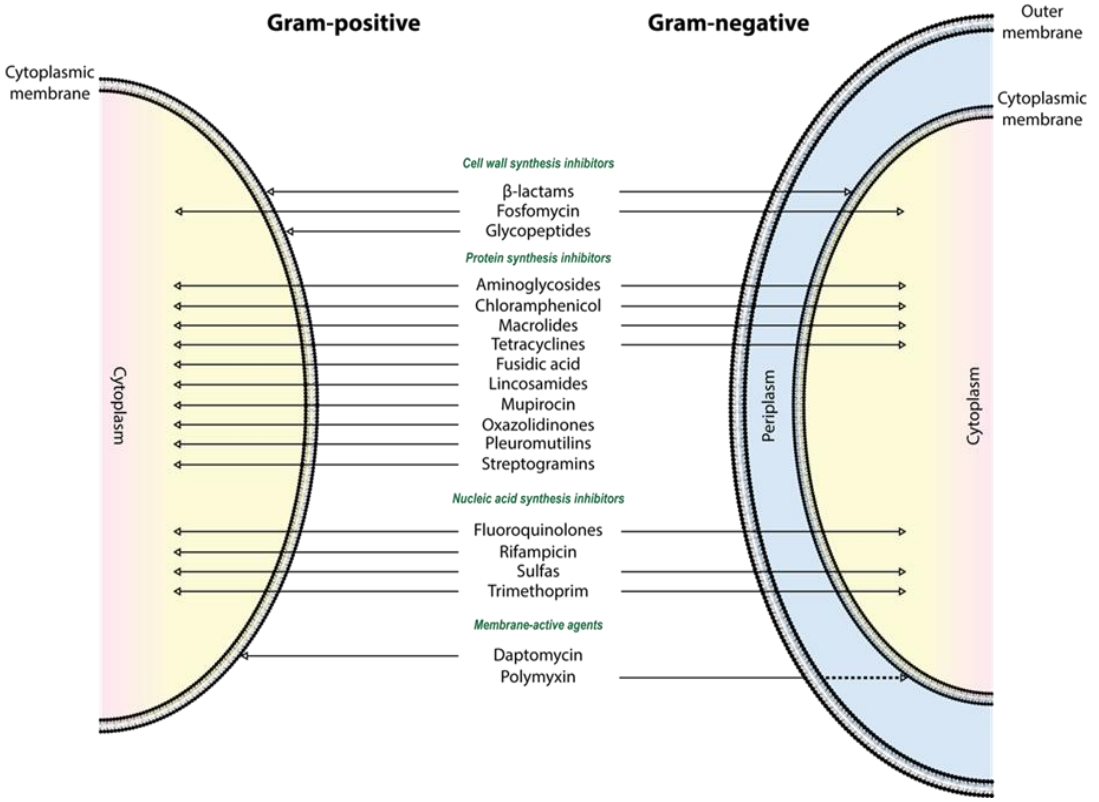


Figure 1: Antibiotic activity spectra.

Image adapted from (Silver, 2008) by O'Neill & Sharkey (personal communication). Gram-negative active agents are only a fraction of those available with activity against Gram-positive pathogens; this highlights that influx, not target differences, is the major factor affecting the spectrum of antibiotic activity.

1.2. The outer membrane

With the exception of daptomycin (Randall et al., 2013), antibiotic agents with activity against Gram-negative pathogens are only a subset of those active against Gram-positives (Silver, 2008) (Figure 1). Responsible for these susceptibility differences, are the architectural differences between the Gram-negative and Gram-positive cell envelopes. Gram-negative bacteria possess an outer membrane (OM) which serves as an additional, highly-complex selective barrier to the passage of molecules into the bacterial cell. This is in comparison to Gram-positive bacteria, as

their cell wall consists of a single cytoplasmic membrane surrounded by a thick layer of peptidoglycan (Delcour, 2009, Navarre and Schneewind, 1999).

The characteristic OM of Gram-negative bacteria is responsible for the intrinsic resistance that these organisms possess against a range of chemically diverse, antimicrobial compounds. Composed of an asymmetric bilayer, consisting of lipopolysaccharide (LPS) and various phospholipids and proteins, the OM of Gram-negative organisms forms an additional selective barrier against the permeation of toxic compounds into the bacterial cell (Figure 2) (Ruiz *et al.*, 2009). This is in contrast to the cytoplasmic membrane (CM), which allows the passive diffusion of neutral and hydrophobic compounds into the cell, independent of molecular weight restraints (Weiner and Rothery, 2001, Silver, 2008).

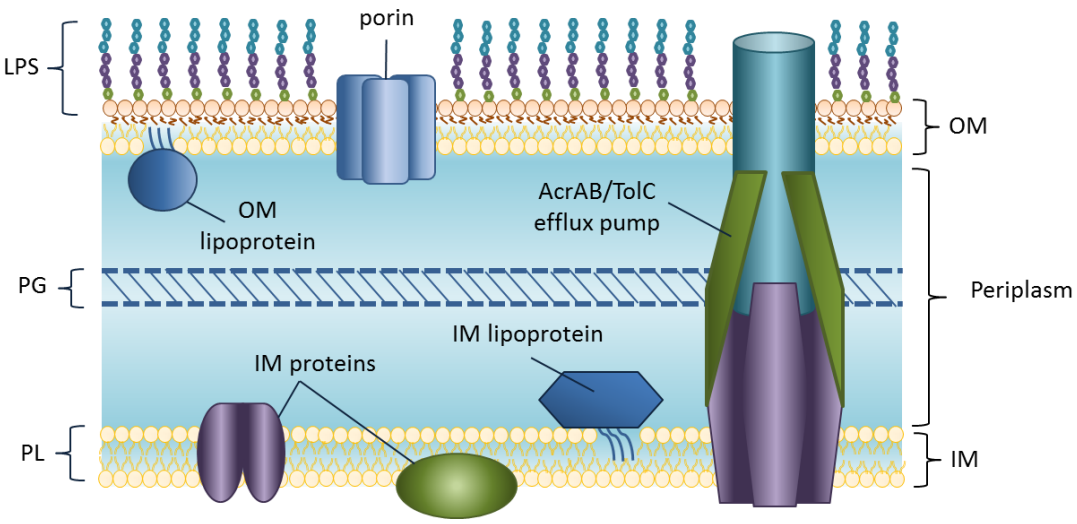


Figure 2: The envelope of Gram-negative bacteria.

Gram-negative bacteria contain two membranes within their cell envelope; the inner membrane (IM), which surrounds the cytoplasm and the outer membrane (OM) on the surface of the cell. The IM and the OM are separated by an aqueous layer termed the periplasm, which contains soluble proteins and a thin layer of peptidoglycan (PG). The IM is composed of composed entirely of phospholipid (PL), whereas the OM is an asymmetric bilayer containing PL on the periplasmic side, and lipopolysaccharide (LPS) on the outer monolayer. The IM is covalently anchored to the cell through interactions of outer membrane lipoproteins with the PG layer, and contains various outer membrane proteins. Tripartate pumps, such as the AcrAB/TolC efflux pump span both membranes and the periplasm.

The permeability properties of the OM are linked both to its molecular composition and overall structure. Found exclusively on the outer leaflet of the structure the complex glycolipid lipopolysaccharide (LPS), forms the basis of the Gram-negative permeability barrier (Funahara and Nikaido, 1980, Hancock and Nikaido, 1978) (Figure 3). Essential for cell viability in most Gram-negative bacteria (excluding organisms possessing glycosphingolipids in place of LPS, in addition to *Neisseria meningitides & Acinetobacter baumannii*), the proposed primary role of LPS is in the maintenance of the barrier function (Vuorio and Vaara, 1992a, Gronow and Brade, 2001, Moffatt *et al.,* 2010). The LPS molecule is comprised of three covalently linked parts; a hydrophobic glycolipid known as 'lipid A', a core oligosaccharide and repeating O-antigen units (Raetz and Whitfield, 2002). The arrangement of the fatty acyl groups attached to the Lipid A moiety promotes a gel-like state of the outer monolayer, while interactions between the O-chain antigens have the ability to decrease membrane fluidity, stabilising the OM further (Nikaido, 2003).

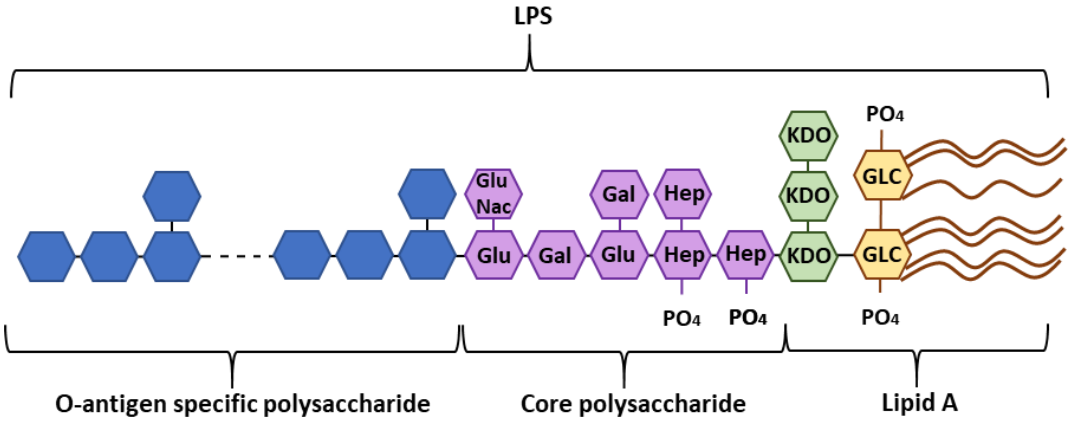


Figure 3: Schematic showing the basic structure of lipopolysaccharide.

LPS consists of three regions: the external O-antigen chain, a core polysaccharide region and lipid A, which anchors the molecule to the bilayer. (Glu Nac) N-acetyl glucosamine, (Glu) glucose, (Gal) galactose, (Hep) heptose, (KDO) 2-keto-3-deoxyoctonic acid, (GLC) glucosamine.

The hydrophobic exterior of the OM is further maintained by lateral interactions between neighbouring LPS molecules, mediated through ionic interactions between phosphate groups and the divalent cations, Mg^{2+} and Ca^{2+}, in the OM. Disruption of these lateral interactions can affect the permeability of the OM, highlighted by the action of the polycationic antimicrobials, polymyxins and the aminoglycoside class of antibiotics. Initial uptake studies in *P. aeruginosa* have shown that polycationic antibiotics influence their own uptake by causing displacement of the Mg^{2+} cross linking adjacent LPS molecules (Hancock and Wong, 1984, Hancock, 1981). Consequently, the stabilising effect which bound Mg^{2+} provides is lost. This leads to disruption of the OM and allows the entry of the polycationic antibacterial agents into the periplasm. The importance of LPS in the maintenance of barrier function can be highlighted by mutants possessing defects in the LPS core, which have shown increased susceptibility to hydrophobic antibiotics, detergents and bile salts (Sirisena *et al.,* 1992, Vuorio and Vaara, 1992b, Picken and Beacham, 1977, Tamaki *et al.,* 1971). Moreover, disruption of the OM by cationic agents can increase the permeability of the outer membrane to both hydrophobic antibiotics (which are normally excluded) and those which are too large to enter via porin channels, such as vancomycin. For instance, colistin displays synergy with vancomycin in the killing of the MDR organism, *A. baumanni* (Gordon *et al.,* 2010), while *E. coli* and *S. typhimurium* display increased sensitivity to vancomycin and the hydrophobic agents, rifampin, fusidic acid, novobiocin and erythromycin after treatment with polymyxin B nonapeptide (PMBN) (Vaara, 1992). Although hydrophobic compounds are not entirely excluded from passive uptake through the OM, their uptake directly through the membrane is very slow (Plesiat and Nikaido, 1992). As such, many species of Gram-negative bacteria, including *Salmonella* spp. and *E. coli*, are said to be devoid of a hydrophobic route of antibiotic entry (Plesiat and Nikaido, 1992). This is with the exception of some *Neisseria*

strains, which contain areas of exposed phospholipid on the outer monolayer of the OM, through which hydrophobic compounds may diffuse (Lysko and Morse, 1981).

The major route of entry through the OM is provided by abundant protein structures, known as porins. These 'pore' forming proteins allow the transmembrane passage of selected nutrients and small, hydrophilic compounds into the bacterial cell. Porins are present in all Gram-negative bacterial species and organisms of the Gram-positive, Corynebacterium-Nocardia-Mycobacterium complex (Hünten *et al.,* 2005, Kläckta *et al.,* 2011, Speer *et al.,* 2013). In regards to their structure and function, the best characterised of these channels are OmpF, OmpC and PhoE, the three major porins of the model organism, *E. coli* (Cowan *et al.*, 1992). Possessing a distinct structure, porins consist of an amphipathic β-barrel structural motif, in which antiparallel β-strands fold to form hydrophilic channels in the OM (Nikaido, 2003, Fernández and Hancock, 2012). Characterised according to their substrate range, porins may be involved in the maintenance of the permeability barrier (the fairly unspecific, 'general porins') or act as routes of uptake for specific nutrients, such as sugars or as iron siderophores (Delcour, 2003, Sandrini *et al.,* 2013, Van Gelder *et al.,* 2002).

Irrespective of porin characterisation, substrate specificity is dependent mainly on the charge and size of the compound, not chemical structure. General porins possess a size exclusion limit of around 600 Da, excluding the passage of substrates above this size range.(Silver, 2008) Most porins which are involved in antibiotic uptake are of the general porin subfamilies, including the cation-selective OmpC and OmpF channels in *E. coli*, and the OmpD subfamily in *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium (Pagès *et al.,* 2008). A range of hydrophilic antibiotics utilise porins to transverse the OM of Gram-negative bacteria, including agents of the β-lactam class, together with the penems and members of the third and fourth generation cephems (James *et al.,* 2009, Huang and Hancock,

1996). Through the study of porin deficient mutants, it appears that quinolones, beta-lactams and tetracycline compounds utilise porins to transverse the OM also (Hirai *et al.,* 1986, Richardot *et al.,* 2015, Peak *et al.,* 2014). As porins function as hydrophilic gateways across the OM for a range of chemically unrelated antibacterial classes, it is unsurprising that alterations in expression can contribute to the intrinsic resistance of some organisms to antibacterials. This example is highlighted by the ESKAPE organism, *P. aeruginosa*, in which a downregulation of the OprD alongside an increase in expression of a range of efflux mechanisms has also been detected within intensive care isolates of *P. aeruginosa* (Fournier *et al.,* 2013). A similar case has also been documented in a clinical isolate of *Klebsiella pneumoniae*, wherein a defective OmpK impacting Ceftazidime-avibactam susceptibility was identified (Nelson *et al.,* 2017).

1.3. The cytoplasmic membrane

The cytoplasmic membrane (CM) is the second barrier which drugs must overcome in order to enter into the cytoplasm of bacterial cells. The prokaryotic CM is comprised of a symmetric phospholipid bilayer, with those phospholipids consisting of a glycerol backbone which is esterfied to a phosphate. This forms the hydrophilic head group which is attached to two hydrophobic fatty acyl chains (Huijbregts *et al.,* 2000). In Gram-negative species, phosphatidyl ethanolamine & phosphatidyl glycerol predominate and in Gram-positives, o-aminoacyl phosphatidylglyerols (Raetz and Dowhan, 1990, Goldfine, 1982). This membrane serves to enclose the contents of the cell and houses the protein machinery which is responsible for the cellular processes necessary for life, such as energy production, lipid biosynthesis, transport of nutrients, secretion of proteins and export of toxins (Silhavy *et al.,* 2010). Furthermore, bacteria survive in hostile environments in which they are surrounded by a plethora of compounds which could potentially be toxic if they gained entry into

the cell. Therefore, the cytoplasmic membrane must also serve as a selective barrier to the entry of substances into the cells interior.

In terms of the passage of compounds across membranes, it is believed that transport predominately occurs by passive diffusion through the membrane (lipoidal diffusion), with membrane transporters contributing marginally to drug entry (Figure 4) (Al-Awqati, 1999, Sugano *et al.,* 2010, Di *et al.,* 2012). In the case of antibiotics, there are few documented examples which support the case of carrier mediated transport of these drugs into bacterial cells. Of the antibiotics known to use transporter mediated uptake, these agents hijack membrane transporters by mimicking the natural substrates of those carriers. For instance, by mimicking the natural substrates of glycerol-3-phosphate permease (GlpT) (Lemieux *et al.,* 2004) or glucose-6-phosphate (UhpT) (Kahan *et al.,* 1974) and the D-alanine-glycine system (Wargel *et al.,* 1970), both fosfomycin and D-cycloserine respectively have been shown to hijack these native transporters in order to permeate the cytoplasmic membrane.

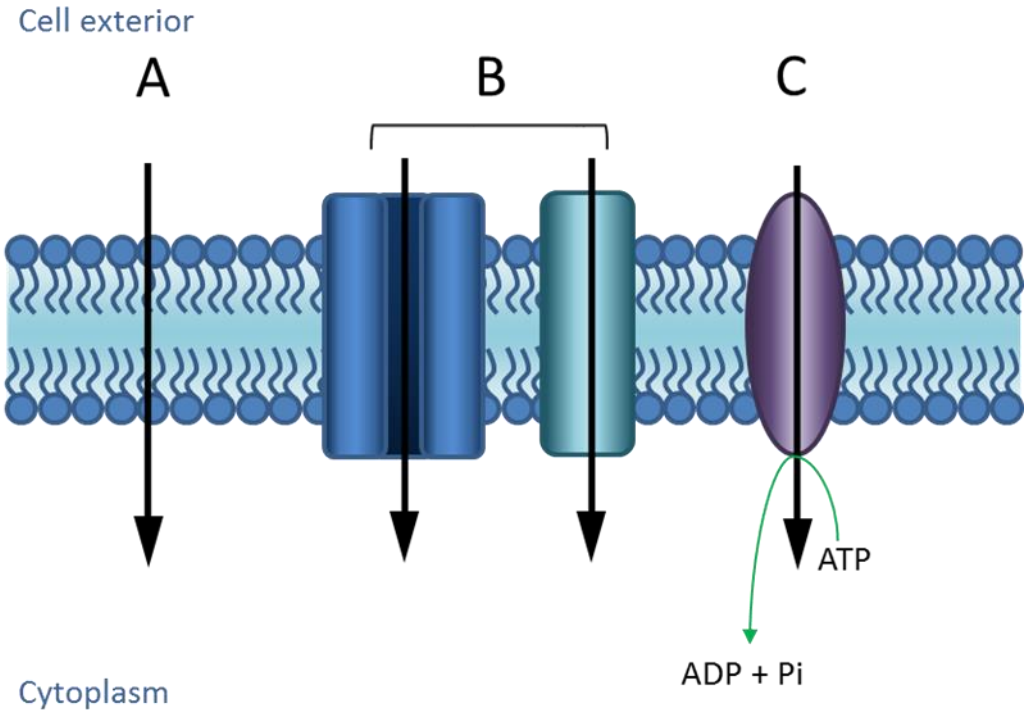


Figure 3: Different views of drug transport mechanisms across bilayers.

(A) lipoidal diffusion (B) the passive diffusion of drugs through protein channels or membrane carriers or (C) the active uptake of compounds.

The evidence which has led to the idea of lipoidal diffusion being the predominant route of drug passage across bilayers, has been based mainly upon correlations between lipophilicity (log P), drug absorption within eukaryotic cell lines and the study of drug permeability using artificial membrane systems (Di *et al.,* 2012). In terms of logP, for a long time it has been known that there is some association between the hydrophobicity of a compound and its cellular permeation (Pade and Stavchansky, 1998, Liu et al., 2011). As a result this is presented as evidence supporting the entry of drugs by the lipoidal diffusion model. This notion is further supported by the fact that experimentally, the uptake of many drugs across biological membranes is non-saturable or concentration dependent (Sugano *et al.,* 2010). These uptake kinetics are suggestive of passive diffusion and in the case of

many drugs, is assumed to be occurring through the lipid portion of the bilayer. For example, in a model system using rat hepatocytes, 14 out of 16 tested displayed uptake kinetics which were suggestive of passive diffusion. Moreover, the authors noted the correlation of uptake by passive diffusion of these agents, with log $D_{7.4,}$ the measurement of drug lipophilicity at physiological pH (Yabe *et al.,* 2011). Since, this study has been cited in support of evidence supporting the lipoidal diffusion model of drug uptake (Di *et al.,* 2012). Moreover, drug uptake can also be studied using model systems which include artificial membranes (such as the Parallel Artificial Membrane Permeation Assay) (Peetla *et al.,* 2009, Kansy *et al.*, 1998) and whole cell permeability assays, using Madin-Darby canine kidney (MDCK) or the human intestinal, Caco-2 cell lines (Volpe, 2008, Pade and Stavchansky, 1998).

Conversely, in recent years the importance of this process in biological systems has been questioned by some (Kell *et al.,* 2011, Dobson and Kell, 2008). This is in regards to the suitability of the experimental methods used to model drug flux across bilayers, in addition to the conclusions drawn from the resulting evidence. Regarding lipophilicity and correlations with compound uptake, correlation cannot solely provide evidence for a mechanism. Therefore, the hydrophobic nature of a compound doesn't rule out transporter mediated uptake. This has been demonstrated with anaesthetics, drugs for which potency has strong correlations with lipophilicity. This was once thought to be the physico-chemical property linking compound entry into cells with their ability to solubilise across membranes (Gupta, 1991). However, it is now known that this property is linked to the drugs binding to hydrophobic regions in their target proteins (Franks, 2008). Concerning Caco-2 & MDCK cell based assays, authors have stated that the 'background' rates of different drug uptake within these cell lines is comparable, therefore is suggestive of lipoidal diffusion for those agents (Sugano *et al.,* 2010, Di *et al.,* 2012). Both of these cell lines encode hundreds of membrane transporters,

therefore alternatively, some correlation in drug entry may be the result of the promiscuous diffusion of drugs through multiple membrane transporters (Dobson *et al.,* 2009a).

Furthermore, there are several downfalls of comparing drug permeability measured using synthetic membrane systems to the flux of compounds across biological membranes. One main issue is that many of these models to not contain proteins. The composition of cytoplasmic membranes varies between organisms, but the mass of proteins can be 1.5 – 4 x the mass of the lipid component (Singer and Nicolson, 1972, Salton and Freer, 1965, Steck, 1974), consequently protein free membrane models are not representative of living bilayers as native transporters may facilitate the entry of a drug. Proteins also have a role in stabilising the bilayer. As such, synthetic bilayers can be 'leaky' due to transient pore formation (Leontiadou *et al.,* 2004, Leontiadou *et al.,* 2007). The spontaneous formation of these pores could facilitate the passage of hydrophilic compounds through the membrane, so it could be argued that permeability measured using these systems may not be comparable to that in living membranes.

These issues discussed regarding the lipoidal diffusion model do not rule it out as a method of drug transport across biological membranes; however the issues addressed above have led to the re-examination of the role of membrane transporters in drug uptake. There is now a growing body of new evidence implicating carriers in the entry of xenobiotics within cells, with this evidence being reviewed in several articles which provide an extensive overview regrading eukaryotic membrane carriers involved in drug uptake (Dobson and Kell, 2008, Kell *et al.,* 2013). Perhaps one of the most convincing studies was carried out by Lanthaler *et al.,* in 2011 within the model organism, *Saccharomyces cerevisiae*. In this study, the authors identified transporters that facilitated the uptake of 18 drugs, previously thought to enter cells via lipoidal diffusion.

For many classes of antibiotics (such as the tetracyclines and the aminoglycosides) (Chopra, 1988, McMurry and Levy, 1978) the involvement of transporters in their uptake has been suggested in the past. However, no membrane carriers have subsequently been identified in facilitating the entry of these antibacterial classes into bacteria, leading to the consensus that these agents probably enter the cell via lipoidal diffusion (Hancock, 1981, Schnappinger and Hillen, 1996). The role of carriers in antibiotic efflux has been extensively studied, however to date there has been no comprehensive study into the role of membrane carriers in the entry of antibiotics into bacteria. Increasing our knowledge regarding antibiotic entry could have major implications on drug discovery, as we may currently be overlooking properties which would be beneficial in the discovery of novel antimicrobials. If antibiotics do indeed mimic natural substrates of membrane carriers to gain entry into the cell, this information could be used in future drug disovery settings in order to enrich screening libraries with fragments bearing structural similarities to natural products, exhibiting ‘metabolite likeness’. This may increase the potential bacterial permeability of lead compounds, allowing them to serve as transporter substrates. Lipoidal diffusion cannot be ruled out in the uptake of antibiotics and in some cases may still be the predominating route of entry; however, it may be time to revisit the role of transporters which may facilitate the passive entry of some antibiotic classes.

1.4. Efflux

Regardless of the pathway involved in antibiotic entry, if an agent is to exhibit an effect on the bacterial cell it must be able to accumulate within the organism at a concentration high enough to exert its desired effect. While alterations in membrane permeability add to the problem of resistance within Gram-negative bacteria, a major contributor to both intrinsic and acquired multi-drug resistance is efflux (Wong *et al.,* 2014). Chromosomally encoded efflux mechanisms are ubiquitous among Gram-negative organisms and according to their architecture, can be characterised into five broad classes (Wong *et al.,* 2014). The resistance nodulation division (RND) type family of efflux pumps predominate within Gram-negative species, existing as tripartite systems and members of this class are composed of an inner membrane (IM) anchored transporter which is brought into contact with an OM channel through interactions with periplasmic adapter proteins (Blair and Piddock, 2009). The IM pump component has the ability to recognise and capture substrates within the lipid bilayer, which are consequently expelled to the cell exterior through translocation along the associated protein channel (Figure 5) (Wong *et al.,* 2014, Piddock, 2006a).

The primary physiological function of this machinery is to aid bacterial survival within their ecological niche, an example of this being the protective effect against bile salts exerted by the AcrAB pump of *E. coli* (Piddock, 2006b). The combined substrate range possessed by the five families of protein pumps is vast, encompassing a variety chemically and structurally unrelated compounds and unfortunately, including agents of various antibiotic classes. Consequently, the expression of efflux mechanisms can contribute greatly to both intrinsic and acquired resistance within Gram-negative bacteria, as the expression of a single species of MDR pump can confer resistance to a range of antimicrobial agents (Poole, 2004). As a result, the presence of efflux mechanisms can radically reduce the efficacy of the therapeutic options available for the treatment of Gram-negative

infections (Fernández and Hancock, 2012). A classic example of this is the opportunistic human pathogen, *P. aeruginosa*, to which whole genome sequencing has revealed the presence of 12 potential RND efflux systems (Stover *et al.*, 2000). This includes MexAB–OprM, a transporter with one of the most extensive substrate profiles of all efflux systems currently characterised. Expression of MexAB–OprM can confer resistance to tetracyclines, macrolides, quinolones, chloramphenicol, novobiocin and the β-lactam class, as well as non-antibiotic agents such as dyes, detergents and organic solvents (Masuda *et al.,* 2000, Middlemiss and Poole, 2004, Zhao *et al.,* 1998, Stoitsova *et al.,* 2008). The intrinsic resistance properties of the Gram-negative OM are complemented by the presence of MDR efflux systems, functioning together to prevent a range of chemically diverse antibiotics from reaching their intracellular targets at concentrations sufficiently high enough to inhibit growth (Middlemiss and Poole, 2004).

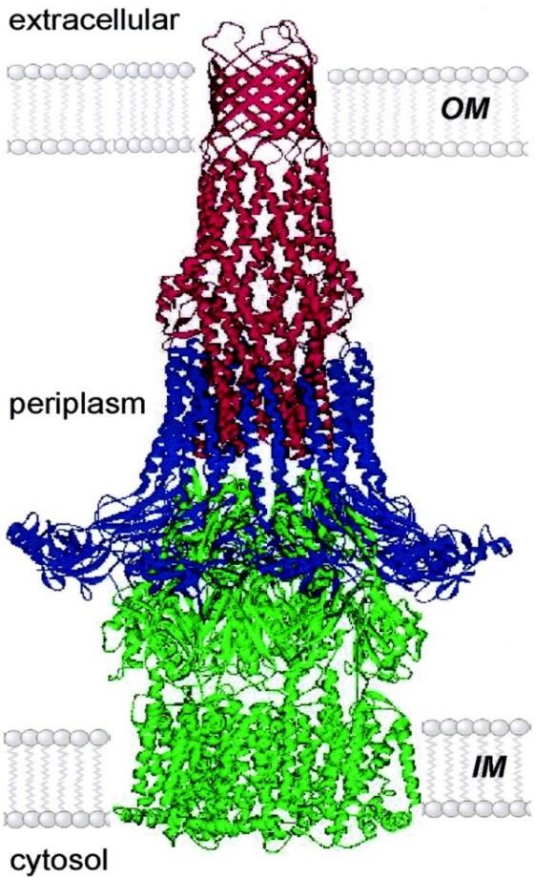


Figure 4: A model of an RND-type efflux pump (Piddock, 2006a).

This is a model of the MexA-AcrB-TolC pump of *E.coli*. The channel, formed by TolC (in red) forms an interaction with six hairpins of the adapter protein, AcrB (blue), which surrounds the MexA pump (green, a homologue of AcrA, the native partner of AcrB and TolC).

1.5. Toward understanding the parameters of antibiotic entry – our current knowledge

In order to challenge the problem of resistance through the discovery and development of novel broad-spectrum antibacterials, we must begin to understand the intricate chemical properties which are required for drugs to successfully transverse the OM and accumulate within the cytoplasm of Gram-negative pathogens. With this knowledge, the principal issue facing antibiotic drug discovery

could be addressed. By fulfilling the potential requirements of OM penetration, through the informed modification of novel inhibitors, the delivery of lead compounds to their intracellular targets could be ensured. This in turn would ensure the efficacy of antibiotic drug candidates against Gram-negative pathogens.

The first comprehensive attempt to characterise the chemical properties required for the influx of drugs into the bacterial cell was in a study carried out by O'Shea & Moser (O'Shea and Moser, 2008). In this study, 147 antibacterial compounds were selected for analysis, including those that were clinically available and several which were still under clinical investigation. Using computational methods, a set of chemical descriptors thought to be involved in drug entry, were predicted and measured for each of the antibacterial compounds; these parameters included values for molecular weight (MW), the number of hydrogen bond donors and acceptors, calculated log of the octanol-water partition and distribution coefficients at a given pH (clogP & clogD, respectively) and the polar surface area (PSA). The commercially available, Comprehensive Medicinal Chemistry (CMC) database was used to provide a reference set of the same parameters for non-antibacterial drugs. In the same year, Silver reviewed the published literature to produce an overview of the link between uptake mechanisms and the physico-chemical properties of natural product antibiotics (Silver, 2008).

From these studies, it is clear that antibiotics occupy different chemical space depending on their target organism, as well as the cellular location of the system which they interact with (Figure 6). For instance, agents which enter Gram-negative cytoplasm by passive diffusion had an upper mass limit of 600 Da, conforming to the size restraint of porin channels within the OM. In contrast, agents active against Gram-positive organisms had a considerably larger mass range, reaching a maximum value of 1022 Da. This, in part, can be explained by the presence of large cell-wall active compounds, such as the glycolipids and

lipopeptides, which were present in the data set for Gram-positive active compounds. Antibiotics which enter the Gram-negative cytoplasm were also found to be of a higher polarity with comparison antibiotics with Gram-positive efficacy, which tend to more lipophilic, being of low or neutral charge (O'Shea and Moser, 2008, Silver, 2008). Interestingly, Silver noted that the lincosamides and oxazolidinones, two classes showing activity against Gram-positive bacteria which also occupy the chemical space shared by Gram-negative active antibiotics, were subject to efflux by the AcrAB-TolC pump (Silver, 2008). This demonstrates that despite fulfilling the parameters required to transverse the OM, the activity of agents may also be limited by the presence of efflux mechanisms.

The chemical properties of antibiotics were distinct from non-antibiotic compounds also. Non-antibiotic drugs are designed to conform to 'Lipinski's Rule of Five', a set of physico-chemical parameters that were outlined by the medicinal chemist, Christopher Lipinski, which could be used to determine the oral-bioavailability of potential human drugs. These were outlined as no more than 5 hydrogen bond donors or 10 hydrogen bond acceptors, a molecular weight of ≤ 500 Da and an octanol-water partition coefficient (log P) ≤ 5 (Lipinski, 2004, Lipinski *et al.,* 2001a). Non-antibiotic drugs displayed a similar lipophilicity to Gram-positive antibiotics. Additionally, both categories of antibacterials occupied a larger MW range than the limit of 500 Da set out in Lipinski's rules (O'Shea and Moser, 2008, Silver, 2008). With respect to H-bonding interactions, Gram-positive drugs possessed higher average values (7.1 H-bond donors & 16.3 H-bond acceptors) than the values laid out in Lipinski's rule of five, while, in this respect, Gram-negative active compounds conformed with Lipinski's parameters (O'Shea and Moser, 2008). The rules put forward by Lipinski have important implications for antibacterial drug discovery and design, as many of the compound libraries which have been developed for high-throughput screening (HTS) campaigns were made to satisfy

these parameters (Silver, 2011). If the properties required of drugs to enter bacterial cells differ from those laid out in Lipinski's rules, we may have limited past discovery efforts due to the unsuitable design of libraries for bacterial accumulation.

As shown by the studies discussed above there are clear differences between the chemical-space which is occupied by antibiotic and non-antibiotic drugs (Silver, 2008, O'Shea and Moser, 2008). To ensure the successful discovery and development of novel broad-spectrum antibacterials, it is imperative that we further the understanding of the chemical space occupied by existing antibiotics. Simultaneously, antibiotics must also obtain a reasonable level of oral bioavailability in humans if they are to be used systemically. There is some overlap between the physico-chemical properties needed for Gram-negative activity and oral-bioavailability in humans, as both of these requirements can be overcome by compounds (with a mass less than 600 Da) which exist in both zwitterionic and uncharged states at physiological pH. An example of this being the fluoroquinolones & tetracyclines (Piddock, 1991). The ability to interchange between both charged and uncharged species would ensure that the compound could, respectively, traverse the OM of Gram-negative organisms through hydrophilic porin channels, as well as the CM of Gram-positive bacteria or mammalian membranes (Nikaido and Thanassi, 1993, Tam *et al.,* 2010).

In terms of entry across the CM, this is assuming that zwitterionic agents cross the bilayer via lipoidal diffusion. Although few antibiotics are known to make use of solute carriers in their uptake, it could be possible that there is a previously unrealised role for protein carriers in antibiotic entry. As discussed in section 1.3. The cytoplasmic membrane, there is an increasing body of evidence supporting the carrier mediated uptake of drugs for other therapeutic areas (Lanthaler *et al.,* 2011, Kell *et al.*, 2011, Dobson and Kell, 2008). Compounds which contain structural similarities to natural products may serve hijack membrane transporters by

mimicking natural substrates, therefore a return to natural products in antibacterial drug discovery may yield antibiotics which can overcome the permeability issue (Dobson *et al.,* 2009c, Silver, 2008). As such, this idea has been touched on in a previous review regarding natural product antibiotics by Silver (Silver, 2008). Furthermore, there has been a renewed interest in natural products in regards to antimicrobial drug discovery. This is due to increases in our understanding of natural products chemistry (Moloney, 2016) and improvements in bacterial culture techniques, allowing the isolation of environmental organisms which may be a source of novel, secondary metabolites (Nichols *et al.,* 2010). No studies as yet however have attempted to reassess the role of protein carriers in the uptake of antibiotics within bacterial cells. Therefore, in order to increase our understanding regarding antibiotic entry within bacteria, this may warrant further investigation.

In past years, the majority of studies which have attempted to outline physico-chemical parameters associated with compound accumulation within bacteria were based on retrospective studies. The study to directly measure the accumulation of small-molecules in order to gain information regarding the parameters for bacterial entry was carried out earlier this year, by Richter *et al.,* (2017). In this study, the authors screened a library of 100 chemically diverse small molecules for accumulation within *E. coli*. Computational analysis of the results revealed that that flexibility (measured by the number of rotatable bonds) and the shape of a compound are also important factors when considering the Gram-negative permeability, in contrast to simply size and polarity. Specifically, compounds which were most likely to accumulate were rigid in nature with low globularity, were amphiphilic and most contained a primary amine. These findings formed the basis for a set of predictive guidelines for accumulation, which were used to guide the derivatisation of the natural product, deoxynybomycin (6DNM). This agent only possesses activity against Gram-positive bacteria due to poor

permeability within Gram-negative organisms. Due to its low globularity and lack of rotatable bonds, 6DNM was a good candidate for modification and an analogue containing a primary amine was subsequently developed (6DNM-NH3). Unlike its unmodified counterpart, 6DNM-NH3 accumulated within *E. coli* and possessed activity against a range of clinical isolates of ESKAPE pathogens (Richter *et al.,* 2017). This study has formed a basis for understanding some of the properties which may aid compound accumulation within Gram-negative bacteria, however the rules for compound entry and efflux avoidance remain to be fully elucidated.

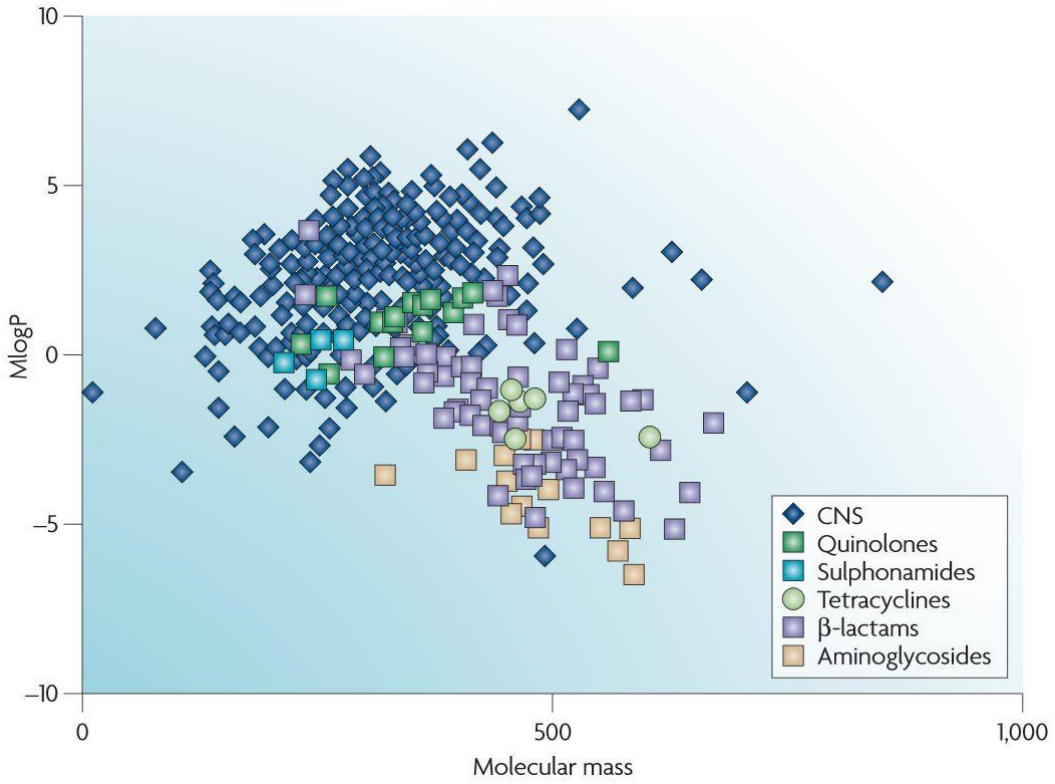


Figure 5: The chemical space occupied by antibiotics differs from that occupied by drugs of other therapeutic classes (Payne et al., 2007).

The calculated logP values plotted against the molecular mass of marketed antibiotics and drugs for central nervous system (CNS) disorders. The CNS drugs possessed similar observations of those in other mammalian drug classes and conform to Lipinski's Rule of Five.

1.6. Objectives

The main aim of this project was to develop a screening platform which could be used to study the chemical properties required for entry of small molecules into the Gram-negative cytoplasm. Liquid chromatography-mass spectrometry (LC/MS) may be used to measure the accumulation of small molecules, which enter the cytoplasm of *E. coli* by passive diffusion and lack an intracellular 'sink'. Once established, this technique could be used to screen libraries of small compounds for their ability to transverse the Gram-negative envelope and accumulate within the cytoplasm. The physico-chemical properties of these agents could be subsequently characterised using computational analysis and the parameters required for entry and accumulation realised. The effect of efflux on the accumulation of the test compounds should also be measured, by analysing accumulation within well characterised, efflux deficient *E. coli* mutants, possessing deletions in the AcrAB-TolC efflux pump.

LC/MS has been used extensively in HTP drug development as an effective means to detect and quantify small molecules (Espada *et al.,* 2008). Accordingly, this technique has been applied in the identification of novel ligands for resistance proteins and in the discovery of novel natural product antibiotics, through the identification and purification of secondary metabolites possessing antibacterial activity (Chen *et al.*, 2007, Gavrish *et al.,* 2014). Indeed, this method has also been used previously to quantify the accumulation of the quinolone, ciprofloxacin, within the cytoplasm of, *P. aeruginosa* (Cai *et al.,* 2009). As discussed previously (1.5. Toward understanding the parameters of antibiotic entry – our current knowledge) earlier this year LC/MS has been utilised for the first time in screening the intracellular accumulation capabilities of a chemically diverse compound set within *E. coli,* in order increase our knowledge regarding the phyisco-chemical properties required for compound entry within Gram-negative bacteria (Richter *et al.,* 2017).

Although the results of this study have identified some properties which aid small molecule accumulation within these organisms, the ‘rules’ for compound entry and efflux avoidance are not yet fully characterised.

The properties governing the uptake of compounds into the bacterial cell are likely to be complex; therefore by undertaking further screening studies to measure the accumulation capabilities compound libraries within *E. coli*, the chemical properties which are important for penetration of the OM can be elucidated. This, in turn, will address one of the main problems in antibiotic drug discovery; overcoming the Gram-negative permeability barrier. The successful development of a formula for intracellular accumulation would enable the optimisation of chemical libraries used for HTS assays and guide the optimisation of potential lead compounds and synthetic drug design, to ensure that inhibitors have the ability to reach their respective intracellular targets within Gram-negative pathogens.

Furthermore, the role of membrane transporters in the uptake of our existing antibiotic classes should be re-assessed. The lipoidal diffusion model has become an established idea in the uptake of several clinically important antibiotic classes. However, it may be possible that there are membrane transporters which facilitate the uptake of antibiotics which are yet to be identified. If antibiotics do hijack membrane carriers in their uptake, this could allow us to enrich screening libraries with fragments which display ‘metabolite likeness’, improving permeabilities of lead compounds. Increasing our knowledge in regards to the properties required of drugs to accumulate within bacteria, in addition to how antibiotics are transported into the bacterial cell could work toward the restoration of a successful antibiotic drug discovery platform. This could improve our future successes in the production of novel therapeutic strategies against Gram-negative MDR pathogens.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2, respectively. For long term maintenance, bacterial strains were stored as saturated cultures at –80˚C in 20 % glycerol. Purified plasmid DNA was stored at –20˚C.

2.1.2. Chemicals and reagents

All chemicals and reagents included in this study were purchased from Sigma Aldrich (St. Louis, USA), unless stated otherwise.

2.1.2.1. Compound library set included in the accumulation assay

This bespoke screening set was compiled using compounds selected from a range of libraries available within the Chemistry Department at the University of Leeds. This set was chosen to evenly sample a range of prominent physicochemical parameters, including; molecular weight, polarity (polar surface area, cLogP), pka/charge state (ionic/cationic at physiological pH), aromatic surface area/ratio of Sp3 vs Sp2 carbon centres and number of rotational bonds.

Table 1: Bacterial strains used in this study.

***Escherichia coli* strains**	***Information***	***Reference / Source***
DH5α	For routine cloning. Genotype: *fhuA2 lac*Δ*U169 phoA glnV44 Φ80' lacZ*Δ*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen (Paisley, UK)
SA08B	This strain allowed cloning of DNA from *E. coli* SA08B directly into NTML library strains of *S. aureus*. Genotype: DC10BΩP_{help}-hsdMS (CC8-2) (SAUSA300_1751) of NRS384 integrated between the *atpI* and *gidB* genes	(Monk *et al.,* 2012)
CopyCutter EPI400	Used to maintain the shuttle vector, pSK5487 containing the *Col*E1 origin of replication at a low copy number. Genotype: F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *araD*139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G trfA *ton*A Δ*pcn*B4 *dhfr*	Epicentre (Madison, WI, USA)
BW25113	Parental strain of the Keio collection and derivative of the *E.coli* strain, BD792 (*lacIq* $rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$)	(Datsenko and Wanner, 2000)
BW25113 - Δ***acrA***	BW25113 derivative deficient in AcrA (*acrA::kan*)	(Baba *et al.,* 2006)
BW25113 - Δ***acrB***	BW25113 derivative deficient in AcrB (*acrB::kan)*	(Baba *et al.,* 2006)

Table 1 continued: Bacterial strains used in this study.

***Staphylococcus aureus* strains**	***Information***	***Reference / Source***
RN4220	For routine cloning. Restriction deficient derivative of *S. aureus* 8325-4.	(Fairweather *et al.,* 1983)
USA300 JE2	Parent strain of the NTML library collection. A plasmid cured derivative of the community acquired strain, *S. aureus* USA300 LAC, a highly characterized community-acquired MRSA.	(Kennedy *et al.,* 2008) This strain was acquired from BEI resources (https://www.beiresources.org/)
Nebraska Transposon Mutant Library (NTML) collection	A sequence defined *bursa aurealis* mariner-based transposon library. Strains within this collection are derived from *S. aureus* USA300 JE2 and contain disruptions in non-essential genes, which have been replaced by an erythromycin resistance cassette.	(Bae and Schneewind, 2006), (Fey *et al.,* 2013) This collection was acquired from BEI resources (https://www.beiresources.org/)

Table 2: List of plasmids used in this study.

Plasmid	***Information***	***Reference / Source***
pUC19	High copy number cloning vector containing a *lac* promoter.	(Yanisch-Perron *et al.*, 1985)
pET28aTetM	The cloning vector, pET28a, containing a codon-optimised insert of *tet(M)*.	Synthesised and cloned by GenScript (New Jersey, USA)
pRAB11	An *E. coli* / *S. aureus* shuttle vector. A modified variant of the pRMC2 expression vector, allowing the regulated expression of cloned genes under the control of the tetracycline inducible, Pxyl/tet promoter.	(Helle *et al.,* 2011)
pSK5487	Amp^r Cm^r, *E. coli* / *S. aureus* pSK41-based shuttle vector. This plasmid was engineered by cloning the *cat* gene from pSK5299 into pSK5473, which contains a constitutive pQacR promoter.	Randall (Personal communication)
pLOW	A low copy number *E. coli* / *S. aureus* shuttle vector with the IPTG regulated promoter, P*spac.* This plasmid has been engineered to contain a kanamycin resistance cassette for selection in *S. aureus.*	Gupta (Personal communication)
Nebraska Transposon Mutant Library (NTML) Genetic Toolbox – pKAN, pSPC, pTET	Plasmids for the allelic exchange of the Erm^r cassette within the *bursa aurealis* transposon, with the selectable marker for either kanamycin, spectinamycin or tetracycline resistance within NTML library strains of *S. aureus*.	BEI resources (https://www.beiresources.org/) (Fey *et al.,* 2013)

2.2. Bacteriological methods

2.2.1. Routine bacterial culture and growth media

E. coli was cultured in either Luria-Bertani broth (LB) (Oxoid, Cambridge, UK) or on LB agar (LBA). *S. aureus* strains were grown on either Mueller-Hinton broth (MHB) or agar (MHA). Bacteria were routinely grown at 37˚C for 18 hours, broth cultures were aerated by vigorous agitation.

2.2.2. Antibacterial susceptibility testing

Susceptibility testing was carried out in accordance with CLSI standards (Cockerill et al., 2012). Briefly, bacteria were diluted in cation-adjusted Mueller-Hinton broth (MHB-2) (Oxoid, Cambridge, UK) to a final inoculum of 5.5 x 10^5 cfu/ml. This culture was added to a 96 well plate, with wells containing two-fold serial dilutions of each antibiotic. For agar MICs, cultures were spotted (10^4 CFU per spot) onto a series of plates containing doubling dilutions of antibiotic in Mueller-Hinton agar (Oxoid, Cambridge UK). The MIC value was defined as the lowest concentration of an antibiotic to inhibit bacterial growth, after incubation at 37˚C for 18 hours. MIC determinations carried out in triplicate to ensure reproducibility.

2.2.3. Phage transduction

Phage transduction was performed as previously described by. Foster, (1998). Briefly, an overnight culture of either the propagation strain or the donor strain, was grown in phage broth (20 g/L Oxoid nutrient broth No. 2). Cultures were diluted 1:100 into fresh phage broth which had been supplemented with 10 mM calcium chloride, before 300 µl of this culture was decanted and approximately 10^5 ϕ80α phage particles were added. Cultures were incubated with phage at room

temperature for 30 minutes, before being added to 10 ml molten phage top agar (phage broth and 3.5 g/L oxoid agar No.1) supplemented with 10 mM calcium chloride. This suspension was poured over set phage bottom agar plates (phage broth and 7 g/L oxoid agar No.1) supplemented with 10 mM calcium chloride. Plates were then placed in sealed bags and incubated overnight at 37 °C. To harvest phage, the top agar from plates containing the highest dilution to give confluent lysis were removed and centrifuged. The remaining supernatant was filtered twice through 0.45 µM filters. Phage suspensions were kept at 4 °C until use.

For the transduction of recipient strains, 500 µl aliquots of recipient cells (concentrated from 20 ml of overnight culture in Tryptic Soy Broth [TSB]) to 50 µl of phage, suspended in 1450 µl LB supplemented with 10 mM calcium chloride. The recipient-phage suspension was then incubated statically for 30 minutes at 37 °C, then incubated for a further 15 minutes at 37 °C with shaking. Cells were centrifuged and washed once in 1 ml of ice cold 0.02 mM sodium citrate, before being re-suspended and incubated on ice for 2 hours in 1 ml 0.02 mM sodium citrate. Cultures were then spread into Tryptic Soy Agar (TSA) supplemented with selection for the marker of interest.

2.2.4. Accumulation assay for the quantification of compounds entering the cytoplasm of *Escherichia coli*

2.2.4.1. Measurement of cell lysis and viability

Briefly, strains to be tested were cultured overnight before being diluted 1:100 into 300 ml of Iso-sensitest broth (ISB) (Oxoid, Cambridge, UK), contained within 500 ml flasks. Cultures were then incubated at 37 ˚C with shaking until an OD_{660} value of 0.7 was reached. This was in accordance with the cell density used in the accumulation assay (Section 2.5.4). Cells were then harvested by centrifugation at

7000 x *g* for 30 minutes and re-suspended in 15 ml of 50 mM sodium phosphate buffer pH 7. Cell suspensions were then equilibrated at 37 ˚C with shaking for 10 minutes. A 500 µl aliquot was removed before the addition of ciprofloxacin, which was diluted to a working concentration of 10 µg/ml.

2.2.4.2. Cell viability

Aliquots were taken and diluted in phosphate buffered saline (PBS) at time intervals up to 1 hour, before being plated onto LBA (BW25113 with no selection, BW25113 Δ*acrA* & BW25113 Δ*acrB* with 25 µg/ml^{-1} kanamycin). Plates were incubated overnight at 37˚C in aerobic conditions, before enumerating colonies.

2.2.4.3. Cell lysis

Aliquots were taken and diluted in 50 mM sodium phosphate buffer (pH 7), at time intervals up to 1 hour. Lysis was monitored spectrometrically by reading OD at 600 nm.

2.5.5. *E. coli* accumulation assay: practical aspect

The accumulation assay was modified from the protocol outlined by, Mortimer & Piddock (Mortimer and Piddock 1991). Briefly, strains to be tested were cultured overnight before being diluted into Iso-sensitest broth. Cultures were then incubated at 37 ˚C with shaking (200 rpm) until an OD_{660} value of 0.7 was reached. Cells were then harvested by centrifugation at 7000 x g for 30 minutes and re-suspended in 15 ml of 50 mM sodium phosphate buffer pH 7. Cell suspensions were then equilibrated at 37 ˚C with shaking for 10 minutes.

To calculate the dry weight of bacterial cells, 1.5 ml of this culture was decanted into a sterile 2 ml glass vial of known weight. Bacterial suspensions were then frozen at –80˚C for 2 hours before freeze drying. Afterward, the weight of the

contents was determined. An average was then taken from the dry weights of four biological replicates to give a final weight, which was used to calculate concentration of antibiotic in ng per mg dry weight of bacterial cells.

When measuring drug accumulation, a 500 µl aliquot was removed as a blank (time zero) before the addition of drug. During method validation, the control compound ciprofloxacin was added with time points being removed for analysis up to 20 minutes after addition of drug. When being used as part of the compound screen, an adaption was made at this point; in this case, library compound was added alongside the internal control, tetracycline to a volume of 1 ml of bacterial culture. Aliquots of 500 µl were then removed and diluted into 1 ml of ice-cold 50 mM sodium phosphate buffer (pH 7). Cells were collected immediately by centrifugation at 7000 *x g* at 4 ˚C. Cell pellets were washed twice in 1 ml of ice-cold 50 mM sodium phosphate buffer (pH 7) before being centrifuged under the same conditions above and the supernatant removed.

2.2.5.1. Accumulation assay analysis – fluorescence based assay

Cells were then lysed by overnight incubation of the pellet in 1 ml 100 mM glycine hydrochloride (pH 3) at room temperature. Cell debris was removed by centrifugation at 17,000 *x g* for 5 minutes and the fluorescence of the supernatant was measured using an Analyst 100 luminescence spectrometer (Perkin Elmer, Cambridge, UK), using an excitation filter of 279 nm and emission filter of 447 nm. Absolute ciprofloxacin concentrations were determined by comparison of the relative fluorescence units (RFU) values against a standard curve, generated using known concentrations of ciprofloxacin in 100 mM glycine hydrochloride (pH 3). For comparison to established methods, final ciprofloxacin concentrations were expressed in ng per mg dry weight of bacterial cells. These values were converted to µM for comparison to the results obtained via LC-MS analysis (Section 2.2.4.5).

2.2.5.2. Accumulation assay analysis – LC/MS based assay

Samples were re-suspended in either 100 mM glycine hydrochloride (pH 3) and lysed overnight, or for the modified protocol, 20:80 (v/v) acetonitrile:ultrapure water, before being homogenised using FastPrep Lysing matrix B from MP Biomedicals. When using mechanical lysis, the supernatant was subsequently filtered to remove cellular debris and lysing matrix. Aliquots of 500 µL were removed from the filtrate and decanted into glass sample vials for analysis. Volumes of 10 µL were injected by the LC/MS autosampler. Quantification of ciprofloxacin or library compound in accumulation samples was achieved by comparison with a calibration curve, containing serial fourfold dilutions of 5 uM to 0.31 uM. For the calibration curves, drugs were prepared in 20:80 (v/v) acetonitrile:ultrapure water. Calibration samples were kept under the same conditions as the experimental samples. The HPLC employed a Dionex Ultimate3000 UPLC system with a Waters ACQUITY UPLC CSH130 C18 1.7 µ 2.1 x 100 mm column using a gradient of acetonitrile and water each with 0.1% formic acid as the eluent system. This was linked to a Bruker MaXis Imapct mass spectrometer equipped with electrospray ionisation (ESI).

2.2.6. Baclight™ assay

An adapted method of the BacLight™ assay carried out in a 96 well plate format (Boulos *et al.,* 1999). Briefly, after an inoculation from an overnight culture, *S. aureus* SH1000 was grown to an OD_{600} 0.5 in Mueller-Hinton broth (MHB). The culture was centrifuged at 6000 x *g* for 10 minutes and washed once in dH2O, before undergoing a second centrifugation step. Pelleted cells were then re-suspended in the desired amount of dH2O; 70 µl total volume is required in each well. The bacterial suspension was added to a conical bottom, 96 well microtitre plate, each well containing a 2 mM stock of test compound. Upon addition of the bacterial suspension, the test compounds were diluted to a working concentration of 100 µM.

Wells containing 5 % SDS and 5 % DMSO were included to serve as the 100 % loss of membrane integrity control or drug free control, respectively. Cells were incubated with drug for 10 minutes, with shaking at 37 ºC. Plates were then centrifuged at 1500 x *g* for 15 minutes and washed once in 70 µl dH2O. The contents of the first plate were then transferred to a black 96 well microtitre plate, with a volume of 50 µl being added to each well. Maintaining the reagents and plate in the dark, the Baclight™ reagent was prepared according to the manufacturer's instructions and a volume of 150 µl was added to each well. The plate was incubated in the dark for 15 minutes. Using an excitation wavelength of 470 nm, the red (620-650 nm) and green (510-540 nm) fluorescence was measured for each drug condition, using a microtitre plate reader.

2.5.7. Identification of membrane transport proteins involved in the uptake of antibiotics within *Staphylococcus aureus* USA300

2.5.6.1. Identification of membrane transporter knockouts within the NTML library

To produce a screening collection of strains with deletions in membrane transporters, strains from the NTML library with deletions in known and putative membrane transporters were identified and compiled. The relational database Transport DB 2.0 (http://www.membranetransport.org/transportDB2/index.html [last accessed 11-09-2017]) was used to predict membrane transporter genes within the genome of *S. aureus* USA300 FRP5737. This information was then used to identify strains of the NTML library collection with transposon insertions in both known and putative transport proteins. In total, 257 strains were identified. To produce the screening collection index plates, strains were inoculated into 96 well plates in LB broth, containing 20 % glycerol and 5 µg/ml erythromycin. Plates were grown for 18 hours at 37 ºC with shaking, before being sealed and frozen at – 80 ºC until use.

2.5.6.2. Screening transposant collection for identification of membrane transporters involved in antibiotic uptake

Strains were grown overnight in 96 well plates containing LB broth (5 µg/ml erythromycin). A volume of 0.2 µL overnight culture (around 3 x 10^{10} CFU/ml) was then stamped onto LB agar plates containing dilutions of the test antibiotic at varying multiples of the agar MIC value recorded for the parental strain (2.2.2. Antibacterial susceptibility testing). Plates were incubated overnight at 37 ºC. Potential hits were identified as colonies which displayed increased resistance to the test antibiotic. This was evident by colonies growing reproducibly on a concentration of drug on which the parental strain, JE2 did not. Putative hits were further confirmed by broth MIC (Section 2.2.2. Antibacterial susceptibility testing).

2.5.6.3. Complementation of hits in knockout strains

Primers used for the amplification of the genes to be complemented can be found in Appendix 1. Constructs which led to successful complementation experiments were created as follows; the gene corresponding to the confirmed tetracycline hit, SAUSA300_0615, was cloned into the BstBI site of the pSK5478 pQacR vector, generating pSK5478: *0615.* The gene 0846, corresponding to the trimethoprim hit, SAUSA300_0846 was cloned into the pRAB11 shuttle vector. This was via the ligation of sticky-end DNA products which had been generated via the digestion of PCR products with the restriction enzymes, *KpnI* & *EcoRI*.

The ciprofloxacin hits, SAUSA300_0202, SAUSA300_0308, and the chloramphenicol hits, SAUSA300_1300, SAUSA300_2587 & SAUSA300_1628 were complemented using the pLOW vector. This was via the ligation of sticky-end DNA products which had been generated via the digestion of PCR products, with the *Sal*I/ *BamH*I sites of pLOW.

The gentamicin hits, SAUSA300_1883 & SAUSA300_2576, the ciprofloxacin hits, SAUSA300_0718 & SAUSA300_0171, and the D-cycloserine hits, SAUSA300_2358 & SAUSA300_2286, were all successfully complemented via expression of their corresponding Tn disrupted genes, under control of their native promoters from the shuttle vector, pRB474. The genes *1883*, *2576, 0718, 0171 &* (alongside the native promoters) were cloned into the *EcoRI* and *BamHI* sites of pRB474. The gene, *2358*, was not encoded at the start of an operon, therefore the cloning of the promoter region and the gene into pRB474 was carried out in two stages. Firstly, the promoter region was cloned into the *EcoRI* and *BamHI* sites, followed by ligation of the gene, *2358,* into the *BamHI* and *SalI* sites.

Recombinant plasmids were then transformed into the corresponding knockout NTML library strains by electroporation (see 2.4.3. Transformation of *S. aureus* strains). Complementation was confirmed by broth MIC (Section 2.2.2. Antibacterial susceptibility testing). In *S. aureus* strains containing the pRAB11 plasmid, MHB was supplemented with 100 ng/ml anhydrotetracycline to induce expression of cloned genes from the reverse TetR regulated promoter, Pxyl/tet. Expression of cloned genes within the pLOW vector were induced by supplementation of MHB with 0.125 mM IPTG, during susceptibility testing.

2.5.6.4. Creation of multiple transporter knockout strains

In cases for which more than one transporter had been confirmed to be involved in the uptake of a drug, strains containing multiple transporter knockouts were generated. Firstly, the erythromycin resistance cassette was switched for either a kanamycin, spectinomycin or tetracycline cassette via allelic exchange using the pKAN, pSPC or pTET plasmids respectively, provided by the NTML Library Toolkit (Table 2). The protocol for allelic exchange was adapted from the method devised by Bose *et al.,* (2013) and was carried out as follows; pKAN, pSPC or pTET was transformed into the strain of interest via electroporation (see 2.4.3. Transformation

of *S. aureus* strains). Strains were recovered for 1 hour at 30 °C with shaking before being plated onto TSA containing 10 µg/ml chloramphenicol, plates were then grown overnight at 30 °C. The following day colonies were picked and inoculated into 3 ml TSB containing 10 µg/ml chloramphenicol. The culture was grown at 30 °C with shaking until the end of the day, before being streaked for single colonies onto TSA containing 10 µg/ml chloramphenicol and grown overnight at 44 °C to promote plasmid integration. Large colonies were chosen as likely single recombinants, these were inoculated into 3 ml TSB containing 10 µg/ml chloramphenicol. Cultures were grown at 44 °C overnight with shaking. This was repeated the following day under the same conditions.

To promote loss of the plasmid, fresh TSB containing no selection was then used for subculture for the following two days. Cultures were grown at 30 °C with shaking. Cell suspensions were then sub cultured into fresh TSB containing 100 ng/ml anhydrotetracycline, and grown overnight at 30 °C with shaking. The following day cultures were diluted in PBS to a concentration of 10^{-7} and plated onto TSA containing 100 ng/ml anhydrotetracycline, plates were grown overnight at 37 °C. Colonies were picked and inoculated into a 96 well plate containing TSB and incubated at 37 °C until the end of the day. Cultures were replica plated into three additional 96 well plates containing TSB supplemented with 10 µg/ml chloramphenicol, 5 µg/ml erythromycin and a third plate containing the selection introduced by the allelic exchange plasmid of choice (kanamycin 50 µg/ml, spectinomycin 1000 µg/ml or tetracycline 5 µg/ml). Plates were grown overnight at 37 °C with shaking. Single recombinants were identified as strains which displayed susceptibility to erythromycin and chloramphenicol, while being resistant to either kanamycin, spectinomycin or tetracycline.

To create strains containing multiple transporter knockouts, the single recombinant strain was engineered to contain additional resistance cassettes

(kanamycin, spectinomycin or tetracycline) by phage transduction (see 2.2.3. Phage transduction).

2.3. DNA manipulation

2.3.1. Polymerase chain reaction (PCR)

PCR protocols were in accordance with the methodology described by (Sambrook and Russell, 2001), and were performed using a Techne TC-3000 thermal cycler (Bibby Scientific, Staffordshire, UK). Oligonucleotide primers from Eurofins MWG Operon (Ebersberg, Germany), and used with Q5 Polymerase and Q5 reaction buffer (New England Biolabs; Hitchin, UK) for amplification of DNA. Reactions were carried out in volumes of 50 µl, and reaction conditions were optimised as appropriate.

For colony PCR, the 2 x MyTaq™ Red Mix from Bioline (London, UK) was used in reaction volumes of 25 µl. PCR cycling conditions were in accordance with manufacturer's instructions.

2.3.2. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in accordance to the method described by (Sambrook and Russell, 2001). The standard approach used gels containing 0.8% agarose, suspended in Tris-acetate-EDTA (TAE) buffer and SYBR® Safe gel stain (Invitrogen, Paisley, UK) was included to visualise DNA. Pre-prepared DNA ladders (Promega; Fitchburg, USA) containing markers of an appropriate molecular weight were added to each gel. Gels were ran at 90V for 30 minutes, before visualisation of DNA fragments under UV light.

2.3.3. DNA extraction and purification

Plasmid DNA from *E. coli* strains DH5α, SA08B and CopyCutter EPI400 was prepared using the ISOLATE II Plasmid Mini Kit from Bioline (London, UK). Briefly, plasmid DNA was isolated from 9 ml of overnight culture and subsequently purified in accordance to the protocol provided by the manufacturer. For isolation of plasmid DNA from *S. aureus* RN4220, buffer P1 was supplemented with 100 µg/ml recombinant lysostaphin (affinity purified in house) in order to degrade the staphylococcal cell wall. The suspension was then incubated at 37 ºC for 30 minutes, before following the subsequent steps for alkaline lysis in accordance with the manufacturer's protocol.

DNA amplified via PCR was purified using the QIAquick PCR purification kit (Quiagen). To purify DNA following restriction digests, digested DNA products were first separated by gel electrophoresis (Section 2.3.2. Agarose gel electrophoresis). The relevant bands were then excised from the gel and subsequently extracted using an ISOLATE II PCR and Gel kit from Bioline. The protocol was carried out in accordance with manufacturer's instructions.

The PurElute Bacterial Genomic Kit (Edge BioSystems, Gaithersburg, MD, USA) was used to isolate genomic DNA from both *E. coli* and *S. aureus* strains. For isolation of staphylococcal DNA, the spheroplast buffer was supplemented with 100 µg/ml recombinant lysostaphin.

2.3.4. Cloning

PCR products and plasmid DNA were adapted for cloning using restriction enzymes purchased from New England Biolabs (Hitchin, UK). Restriction digests were carried out in reaction volumes of 50 µl. Ligations of digested DNA products was performed

using the Quick Ligation kit™ from the same manufacturer. All reactions were performed in accordance with manufacturer's instructions.

2.4.5. Transformation of *E. coli* strains

Chemically competent *E. coli* strains were prepared in accordance with the Ionue method (Sambrook and Russell, 2001). For transformation, cells were thawed on ice and 1-100 ng of DNA was added. Cell suspensions were incubated on ice for 30 minutes, heat shocked at 42 °C for 45 seconds, and returned to ice for 2 minutes. Bacteria were recovered in SOC media (Hanahan, 1983) and grown for 1 hour at 37 ºC before being plated onto LB agar containing appropriate selection. Plates were incubated overnight at 37 °C before colonies were screened by colony PCR for the presence of ligated insert.

2.3.6. Transformation of *S. aureus* strains

Electrocompetent *S. aureus* strains were prepared in accordance with the protocol devised by (Monk et al., 2012) For transformation, cells were thawed on ice for 5 minutes before resting on the bench at room temperature for a further 5 minutes. These aliquots were then centrifuged for 1 minute at 5000 x *g*, before the supernatant was removed and the cell pellet resuspended in 10% glycerol, 500 mM sucrose (filter sterilized). DNA was then added at a concentration of 50-100 ng/µl. The DNA-cell suspensions were added to a 1 mm electroporation cuvette (Geneflow, Elmhurst, UK), and pulsed at 21 kV/cm, 100 Ω & 25 µF. Cells were recovered in 1 ml TSB supplemented with 500 mM sucrose (filter sterilized) and grown for 1 hour at 37 °C. Aliquots of 100 µl culture were then plated onto MHB containing the appropriate selection and grown overnight at 37 °C.

2.3.7. DNA sequencing

Inserts from cloned plasmids were sequenced by Beckman Coulter Genomics (Takeley, UK). Sequence data was analysed using Sequencer software, version 4.9 (Gene Codes Corporation, Michigan, USA).

3. Development of an LC/MS based assay to measure small molecule accumulation within *E. coli*

3.1. Abstract

One of the main issues facing the discovery of novel, broad-spectrum antibiotics through either structure based drug design or via the screening of library compounds, is delivering drug candidates to their respective intracellular targets. Our knowledge is lacking regarding the physico-chemical properties required of a compound to accumulate within the Gram-negative cytoplasm. Therefore, defining 'rules' for drug entry could guide future antibacterial drug discovery. In this study, an LC/MS method was developed for screening chemically diverse, small molecules for accumulation within the cytoplasm of *E. coli*. This method could be used in future studies to measure small molecule accumulation within the bacterial cytoplasm in an attempt to identify structure activity relationships associated with bacterial penetration and efflux avoidance.

3.2. Introduction

From the late 1990s onwards, antibiotic drug discovery efforts consisted of a genomics driven discovery model, which involved the high-throughput screening of (predominantly synthetic) compounds for the ability to inhibit purified protein targets *in vitro* (Payne *et al.,* 2007, Miesel *et al.,* 2003). This approach however, proved unsuccessful in the discovery of any new antibiotic classes. One of the main issues which has hindered the conversion of *in vitro* potency to *in vivo* antibacterial activity, was often the inability of hit of compounds to penetrate the cell and avoid efflux mechanisms (Silver, 2011, Payne *et al.,* 2007, Shore and Coukell, 2016). In order to challenge the problem of resistance, we must begin to characterise the physico-chemical properties required of small molecules to traverse the cellular envelope and evade efflux. This in turn would enable the informed modification of novel inhibitors and the rational tailoring of drug libraries, ensuring that candidate drugs have the ability to reach their intracellular targets, increasing our chances of discovering novel compounds with Gram-negative efficacy.

The first comprehensive attempt to characterise the chemical properties required for the entry of antibiotics into the bacterial cell was carried out by (O'Shea and Moser, 2008). In this article, the authors selected 147 antibacterial compounds and analysed the chemical space occupied by compounds, by assessing physico-chemical descriptors which are thought to be important in drug entry (molecular weight, lipophilicity, hydrogen bond donors and acceptors). The commercially available CMC database served as a reference of drugs from other therapeutic areas. The results of this study revealed that the chemical space occupied by antibiotics differs from that of other therapeutic areas. Overall, the two major properties which differed between the two groups of compounds was molecular weight and lipophilicity. The average MW of antibiotics was higher than that recorded for drugs in the CMC database. This was most pronounced regarding

agents with Gram-positive activity, due to the presence of large envelope targeting agents, such as the glycopeptides, in this subset. In regards to drugs with Gram-negative activity, there was a defined cut-off at 600 Da, this finding corresponding with the putative porin size exclusion limit (Nakae, 1976). Regarding lipophilicity, antibiotics were more polar than drugs from the CMC subset. Compounds with Gram-negative activity were markedly more polar, with on average, double the relative polar surface area of that recorded for drugs from other therapeutic areas (O'Shea and Moser, 2008).

Other notable studies which have attempted to analyse the chemical space occupied by antibiotics include a study by, L. Silver, wherein she provided an overview of the link between uptake mechanisms and the physico-chemical properties of natural product antibiotics (Silver, 2008). Additionally, Cronin *et al.,* carried out a broad bioinformatical analysis in an attempt to identify quantitative structure-activity relationship (QSAR) for the prediction of antibacterial activity (Cronin *et al.,* 2002). The findings of these studies complement those from O'Shea and Moser's research (O'Shea and Moser, 2008), highlighting the differences in chemical space occupied by antibiotics versus drugs from other therapeutic classes. Overall, broad-spectrum antibiotics are more hydrophilic when in comparison to either those targeting only Gram-positive organisms or those of other therapeutic areas, measured by clogP and $clogD_{7.4}$. This is reflected also by a large polar-surface area and greater number of hydrogen-bond donors and acceptors in compounds with Gram-negative activity, when comparison to other drugs (O'Shea and Moser, 2008, Silver, 2008, Cronin *et al.,* 2002). However, there are currently too few antibiotic classes in order to establish a comprehensive set of descriptors in relation to bacterial permeation and efflux avoidance. Moreover, studying the accumulation capabilities of only drugs possessing antibacterial activity limits the diversity of the compounds studied.

As discussed above, previous studies which have attempted to outline the physico-chemical parameters which drugs must fulfil in order to accumulate within bacteria, are based on retrospective analyses. As proposed in the aims of this study, one way in which the physico-chemical properties which are required for bacterial entry may be characterised, is by screening small molecules for accumulation within whole bacterial cells. However, unknown to us, there was another group which was undertaking this research at the same time as ourselves. Published earlier this year, Richter and colleagues carried out a study wherein the ability of library of around 180 diverse small molecules to accumulate within the cytoplasm of *E. coli* was assayed using an LC/MS/MS based method (Richter *et al.*, 2017). Computational analysis of the results revealed that in contrast to polarity, flexibility (measured by the number of rotatable bonds) and shape of a compound are important factors that govern accumulation within Gram-negative bacteria. Their results revealed that molecules most likely to accumulate were rigid in nature with low globularity, were amphiphilic and in most cases contained a primary amine. They subsequently applied these rules in the modification of deoxynybomycin, a natural product which inhibits DNA-gyrase activity. This agent normally possesses activity solely against Gram-positive bacteria, due to limited accumulation within Gram-negative organisms. By modifying the agent by the addition of a primary amine, the accumulation capabilities of deoxynybomycin was improved and resultantly was afforded with Gram-negative activity.

The findings of the study by Richter and colleagues provides a step towards understanding the properties required for small molecule permeability within *E. coli*. There is however still much to learn about the rules of compound entry within this classification of bacteria. Although this study identified properties which can aid the accumulation of a compound within *E. coli*, the physico-chemical properties for bacterial accumulation have still not been fully characterised. One of the main issues

facing previous antibacterial drug discovery studies was finding compounds which accumulate within bacteria cells at all; the problem of entry is only exacerbated in Gram-negative bacteria (Payne *et al.*, 2007). Regarding the study by Richter *et al.*, (2017), deoxynybomycin already possesses the properties required to cross the cytoplasmic membrane (as it possessed activity against Gram-positives), therefore this study identified some properties which aid passage across the OM.

Further studies of this sort could make steps toward the full characterisation of the properties required of compounds for accumulation in both Gram-negative and Gram-positive organisms. This would enable synthetic drug libraries to be tailored to fulfil properties which are associated with bacterial accumulation. It may also enable the modification of synthetic compounds (that may possess poor permeability), in a way which enhances their accumulation within the cell. This could open up the area of structure based drug design in the discovery of novel antibiotics, a method which has proved unsuccessful in the past due to the difficulty in designing an inhibitor which also possesses good cell permeation. Moreover, the role of efflux in compound entry is yet to be assessed. By comparing the accumulation of small molecules within efflux proficient and deficient strains, properties which are associated with recognition by efflux machinery may be realised.

3.3. Aims

To study the properties required of small molecules for entry into the Gram-negative cytoplasm, this study aimed to develop an assay based on liquid chromatography-mass spectrometry (LC/MS) which would be used to measure the accumulation of compounds within the cytoplasm of *E. coli*. Once established by validation against a characterised accumulation assay, the LC/MS based technique was employed to screen a chemically diverse compound set for their ability to successfully accumulate within the cytoplasm of, *E. coli* BW25113. This method could be utilised

in future screens compound libraries for uptake, broadening our currently limited knowledge of the physico-chemical properties required for influx and efflux avoidance within Gram-negative bacteria.

3.4. Results

3.4.1. Measuring the accumulation kinetics of ciprofloxacin within *Escherichia coli* BW25113, using a fluorescence based assay

We first sought a characterised assay to measure drug influx and efflux within bacterial cells, the results of which would then be used to validate an LC/MS based method of screening the accumulation of small molecules within the cytoplasm of *E. coli*. An early method developed by, Chapman and Georgopapadakou, (1988), utilises the intrinsic fluorescent properties of fluoroquinolones in order to measure their accumulation within the cytoplasm of Gram-negative bacteria. Therefore, this characterised fluorescence based assay was chosen to measure the accumulation kinetics of the fluoroquinolone, ciprofloxacin, within the cytoplasm of the *E. coli* strain, BW25113.

Drug accumulation kinetics were assayed by measuring the intracellular concentration of ciprofloxacin at time points for up to one hour, after addition of the drug to cells to give a final concentration of 10 µg/ml (33 µM). Ciprofloxacin is an effective inhibitor of *E. coli* BW25113 with a minimal inhibitory concentration (MIC) value of 0.015 µg/ml. This characterised, fluorescence based accumulation assay requires a high concentration of drug to be added to the cells (33 µM), therefore, in order to ensure that any observations were not the consequence of the antibacterial action of the drug, cell lysis and viability were monitored in the presence of ciprofloxacin throughout the 60 minute time course of the experiment (Figure 7). As results showed that no lysis or a loss in cell viability occurred under the conditions

tested, the accumulation assay was carried out. A steady state concentration (SSC) of ciprofloxacin was reached within 5 minutes after drug exposure. This plateaued at around 2.9 µM (± 0.2) for parental strain, and 4.8 µM (± 0.2) & 4.2 µM (± 0.4) in the efflux deficient strains, Δ*acrA* & Δ*acrB*, respectively (Figure 8).

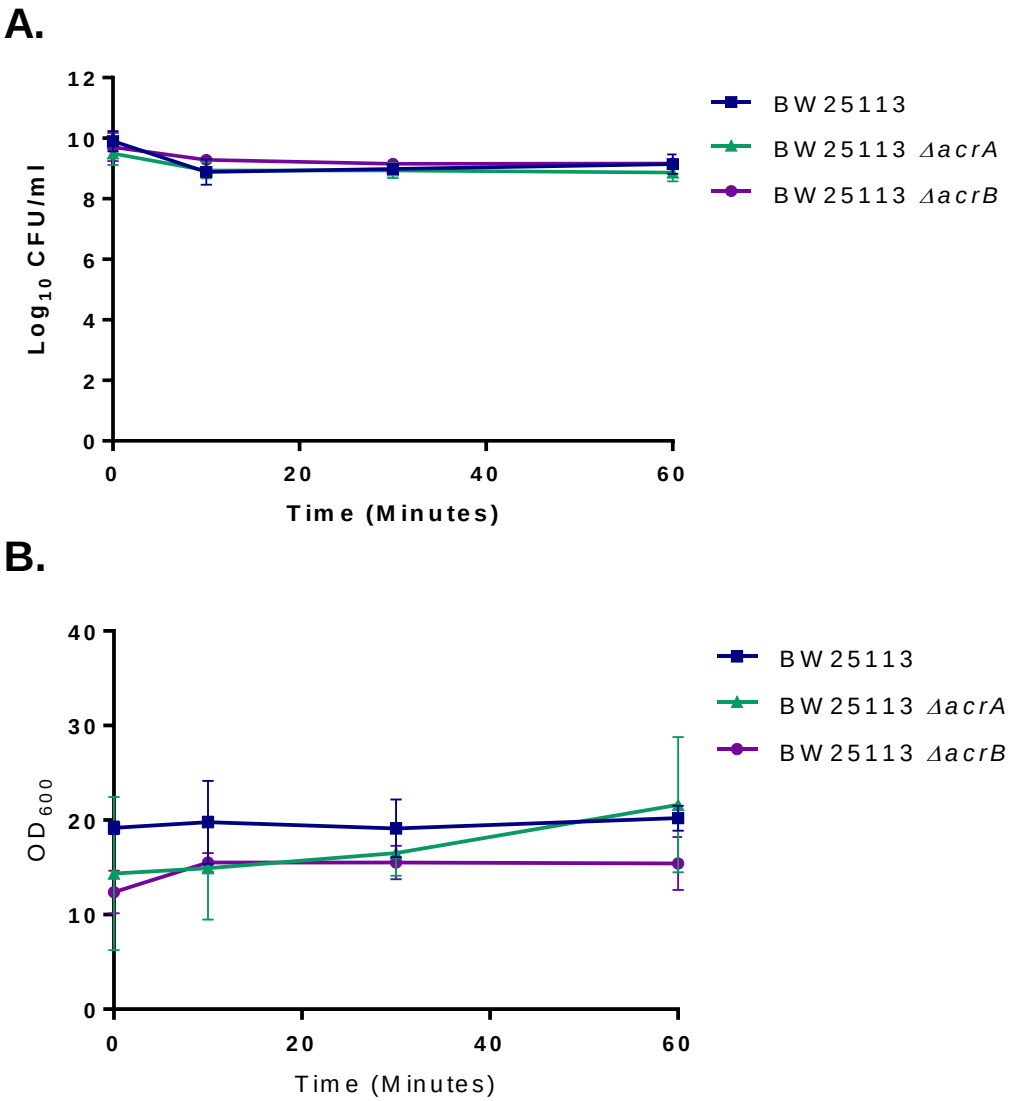


Figure 6: Cell viability and lysis following ciprofloxacin exposure (in *E. coli* BW25113, BW25113 Δ*acrA* & Δ*acrB*).

Cell viability (A.) and lysis (B.) was monitored after the addition of ciprofloxacin. Ciprofloxacin was added to the cultures to achieve a final concentration of 10 µg/ml (33 µM), with time points being recorded up to 1 hour after drug exposure. The values plotted were an average of three biological replicates, with the standard deviation being determined from these values.

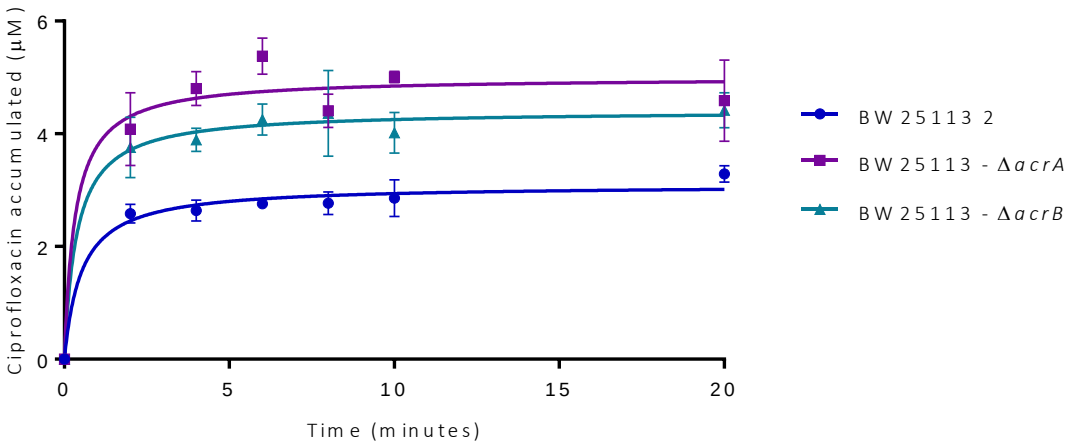


Figure 7: Accumulation kinetics of ciprofloxacin within *E. coli* BW25113, BW25113 Δ*acrA* & Δ*acrB*, measured using the fluorescence based assay

Drug accumulation is shown in µM. Ciprofloxacin was added to the cultures to achieve a final concentration of 10 µg/ml (33 µM), with time points being recorded up to 20 minutes after drug exposure. The values plotted were an average of three biological replicates, with the standard deviation being determined from these values.

3.4.2. LC/MS can be used to measure the concentration of ciprofloxacin within the cytoplasm of *Escherichia coli* BW25113, in a way that is comparable to established methods.

Ciprofloxacin accumulation kinetics were then measured under the same experimental conditions as described for the characterised fluorescence method; however, LC/MS was substituted in place of the fluorescence measurement in the analysis step. The results gained from the LC/MS analysis were then compared to those observed for the fluorescence based quinolone accumulation assay. The trends observed for the accumulation kinetics of ciprofloxacin were comparable between both methods of analysis (Figure 9) with the LC/MS analysis potentially exhibiting slightly improved sensitivity when in comparison to the fluorescence assay. The detection limits for ciprofloxacin were recorded as 0.01 µM & 0.75 µM respectively for the two methods of analysis.

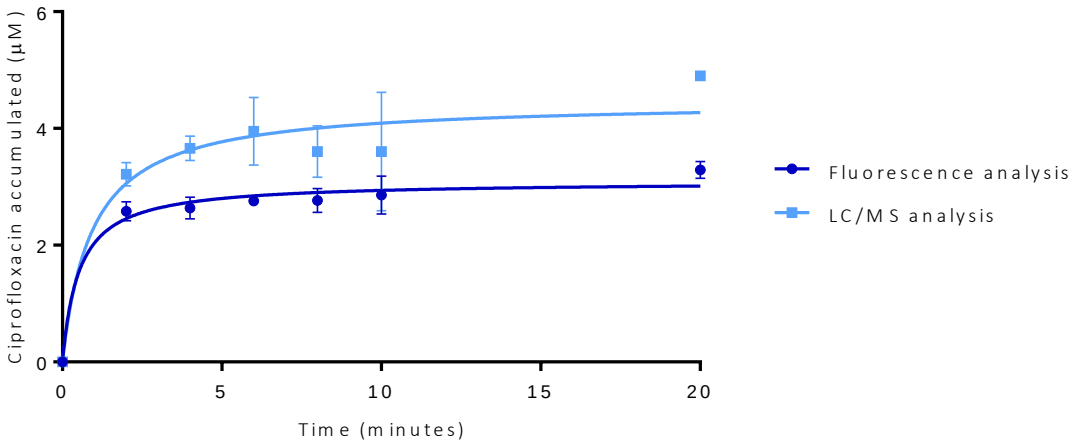


Figure 8: The LC/MS based accumulation assay is comparable to established methods in quantifying intracellular ciprofloxacin within *E. coli* BW25113.

Ciprofloxacin was added to the cultures to achieve a final concentration of 10 µg/ml (33 µM). The values plotted were an average of three biological replicates, with the standard deviation being determined from these values.

3.4.4. Modifying the LC/MS based accumulation assay for its use in the development of a small molecule screening platform.

Established methods of measuring quinolone accumulation are not amenable to the screening of small molecules. The volumes of the library compounds which we could obtain were limited (20 µl, at a concentration of 10 mM), therefore these compounds would not be conserved if they were used at the concentrations and culture volumes required for the established, fluorescence based quinolone accumulation assay. Before the screening of the drug library could be carried out, the assay was therefore miniaturised to make it suitable for use in a small molecule screening platform. Firstly, I needed to assess whether the assay could still confidently detect intracellular ciprofloxacin if the initial inoculated drug concentration was lowered and culture volume

was reduced. The compound library was provided at a concentration of 10 mM, therefore a dilution to 10 µM was chosen as a suitable concentration for screening.

An accumulation assay was carried out with samples being removed for LC/MS analysis at intervals up to 20 minutes following drug exposure (Figure 10). When the external concentration of ciprofloxacin was reduced from 33 µM to 10 µM, intracellular ciprofloxacin could still be detected. The same trends in accumulation kinetics of ciprofloxacin were observed, however the SSC reached was around 1/3 of that as when drug was added at the higher concentration of 33 µM. These results were as expected, as an extensive study on quinolone accumulation by Piddock *et al.,* described that the majority of quinolones accumulate within *E. coli* via simple diffusion which is indicated by an increase in accumulated drug linear to that of the external drug concentration (Piddock *et al.,* 1999). Furthermore, ciprofloxacin detection was reproducible after the volume of culture to which drug was added was reduced from 15 ml to 1 ml. After both the drug concentration and culture volume was reduced, the concentration of intracellular ciprofloxacin was recorded as 1.06 µM (± 0.27) after 10 minutes of drug exposure, across 6 biological replicates. As the results of the miniaturised assay were reproducible, the method was deemed suitable for screening library compounds for accumulation within the cytoplasm of *E. coli*.

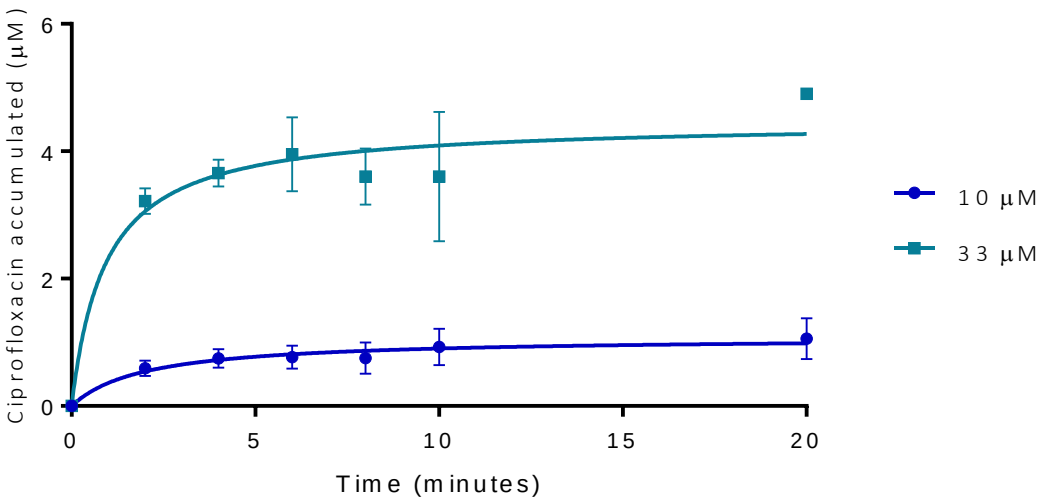


Figure 9: Accumulation kinetics of ciprofloxacin within *E. coli* BW25113, measured by LC/MS.

Ciprofloxacin was added to the cultures to achieve a final concentration of either 33 µM or 10 µM, with time points recorded up to 20 minutes following drug exposure. The values plotted were an average of three biological replicates, with the standard deviation being determined from these values.

As this assay could confidently detect quantify intracellular ciprofloxacin, I then went on to test if the assay could confidently detect another antibiotic which is known to enter the cytoplasm. Therefore, an accumulation assay was carried out with the ribosomal inhibitor, tetracycline. When added at a concentration of 10 µM, tetracycline was detected in the cytoplasmic fraction at a concentration of 0.65 µM (± 0.06) after 10 minutes of drug exposure in the parental strain, *E. coli* BW25113. This is comparable to ciprofloxacin, which accumulated to a concentration of 0.94 µM (± 0.42) under the same experimental conditions.

The incidence of potential background from drug binding to either residual cellular debris after lysis, as well as drug which may enter the periplasm without accumulating in the cytoplasm was assessed by the inclusion of two negative controls, vancomycin and ampicillin. The glycopeptide antibiotic, vancomycin was included as this large, hydrophobic compound is unable to pass through the intact OM of Gram-

negative bacteria (Vaara *et al.,* 2008). Additionally, the penicillin binding proteins which are targeted by the beta-lactam antibiotic, ampicillin, lie within the inner membrane of *E. coli* (Curtis *et al.,* 1979). When included in the accumulation assay, neither vancomycin nor ampicillin could be detected in the cytoplasmic fraction, despite displaying clear detection by LC/MS in calibration samples (Figure 11).

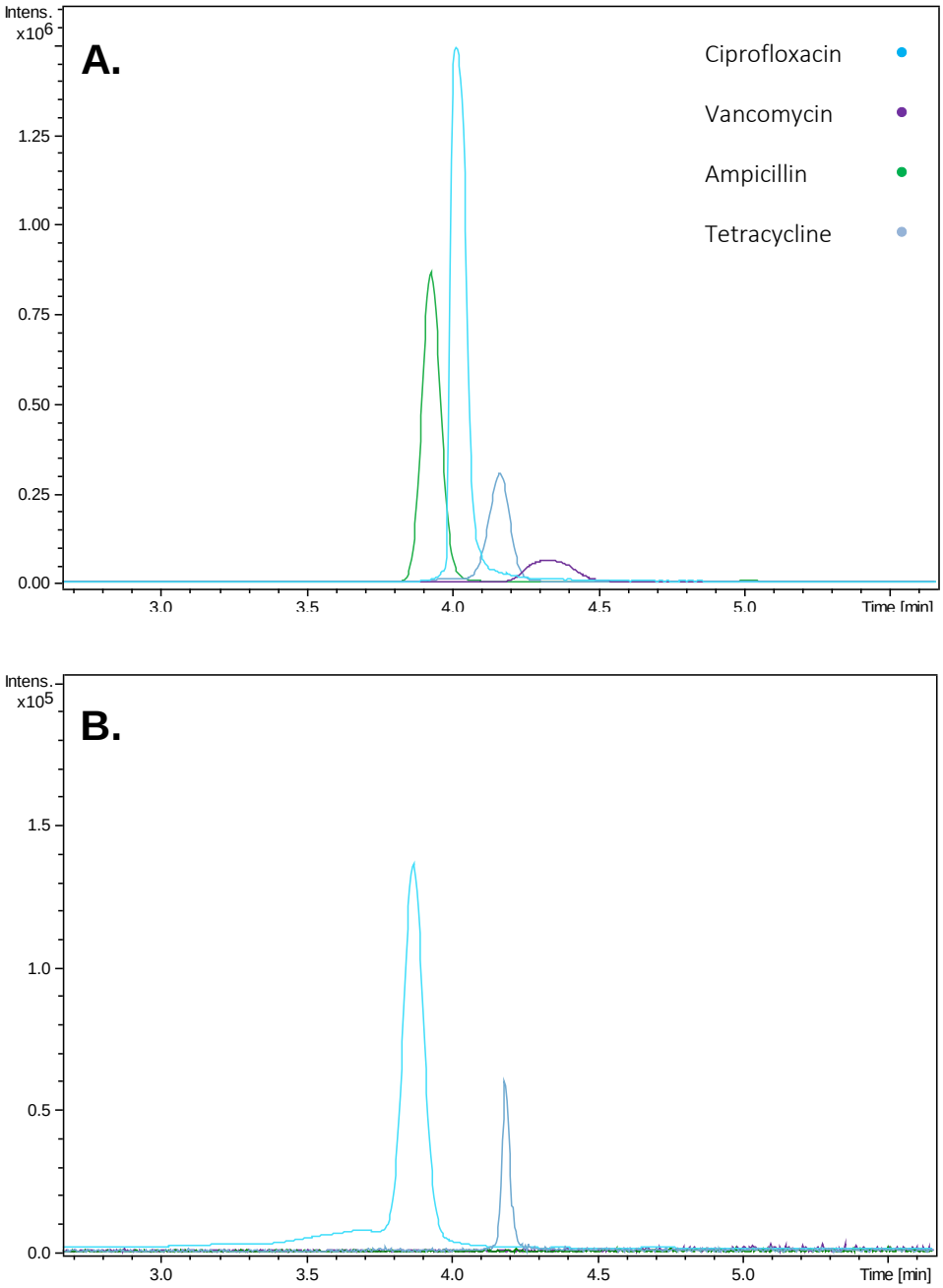


Figure 10: LC/MS analysis of positive and negative controls within the cell lysate of *E. coli*, BW25113.

Extracted ion chromatograms showing the detection of ciprofloxacin, tetracycline, vancomycin and ampicillin in (A.) calibration samples (5 μM) & (B.) in cell lysate. Antibiotics were added to 1 ml volumes, to achieve a working concentration of 10 μM. Samples were removed for analysis after 10 minutes of drug exposure.

3.4.5. The cytoplasmic accumulation of chemically diverse compounds can be measured within *E. coli*, using LC/MS.

The LC/MS based accumulation assay was then used to detect the presence of a diverse set of compounds within the cytoplasm of *E. coli*. The bespoke screening set contained 138 library compounds with a diverse range of chemical properties, possessing a range of molecular weights below 600 Daltons (Appendix 2). This MW cut-off was chosen to correlate with the OM porin size exclusion limits of *E. coli*, which prevents the passive diffusion of compounds larger than this MW limit entering the cell via passive diffusion.(Cowan et al., 1992, Nikaido, 1994a)

Before the screen was carried out, the membrane damaging activity of the compound set needed to be assessed to remove the possibility of false 'hits' which may arise from the self-promoted uptake of compounds entering the cell through a damaged cell wall. The compound set was assessed for membrane damaging activity using the BacLight™ bacterial viability kit.(Boulos et al., 1999) This kit allows membrane damage to be assayed by measuring the signal of two nucleic acid dyes, SYTO9 and propidium iodide, whose abilities to penetrate an intact cell membrane differ. Any compounds showing membrane damaging activity, which was defined as a loss of membrane integrity greater than 30 % against *S. aureus* SH1000, were omitted from further screening. Of the screening set, 14 compounds (~ 10 % of the set) were identified as membrane damaging compounds were excluded (Appendix 2).

After removing compounds with membrane damaging activity, the remaining 124 compounds were screened for accumulation within *E. coli* BW25113, using the LC/MS based assay. During screening, each test compound was added to the culture alongside ciprofloxacin, to provide an internal positive control for accumulation. From these, 22 accumulating hits were generated. Of the 14 confirmed hits, 5 compounds accumulated to a similar concentration of the ciprofloxacin control (1 – 2 µM). The majority of test compounds accumulated to a concentration below 1 µM, with only one compound

accumulating at a concentration higher than that of the control compound (LDS-016657-1). However, these data must be considered preliminary, as during this stage of the study I began to notice increased variation in the accumulation of the control compound, ciprofloxacin. The troubleshooting of the method will now be discussed in further detail.

3.4.6. Method development

3.4.6.1. Troubleshooting the issues which arose during the development of the LC/MS based accumulation assay

During the compound screening, it was noticed that there was an increase in error regarding the accumulation of the control compound, ciprofloxacin. In the first instance, this was assumed to be a result of miniaturising the assay. Therefore, I thought it best to determine confidence limits for the modified method. To obtain confidence limits, 60 miniaturised accumulation assay samples were ran, where cytoplasmic ciprofloxacin was measured within the parental strain, BW25113. The results of this experiment showed substantial variation, as within this run of 60 samples the concentration of intracellular ciprofloxacin measured varied from < 1 µM to > 5 µM (Figure 12). Furthermore, during the analysis of these samples, I noticed that the area under the curve (AUC) values recorded for ciprofloxacin from the calibration samples varied also, with raw values being twofold or higher between samples. In mass-spectra traces, the AUC in an extracted ion chromatographic trace represents the total signal from that ion. The sensitivity of the accurate LC/MS varies from day to day, therefore a slight variation in raw values should be expected. However, the variation between calibration samples should not be as substantial as were noted here. Moreover, in some instances the relationship between the calibration samples was not linear (Figure 13).

To pinpoint whether this was an issue with sample preparation or with the LC/MS itself, I went back to the raw data and compared extracted ion chromatograms (EICs)

for ciprofloxacin detected in the 5 µM calibration samples. Not only was there a variation in peak area and shape, but retention time varied from 3.8 minutes to 4.1 minutes (Figure 14). There are a few reasons that could lead to this observation. The variation between absolute area under the curve (AUC) values could be the result of an issue with the sample preparation (for instance, dilutions not being made to the correct concentration) or the drug coming out of solution. Variation in AUC values, or drifting retention times suggests that there may be an issue with the LC/MS itself. This could stem from variations in sample injection, damage to the column or a blockage, resulting in the drug reaching the detector at different times.

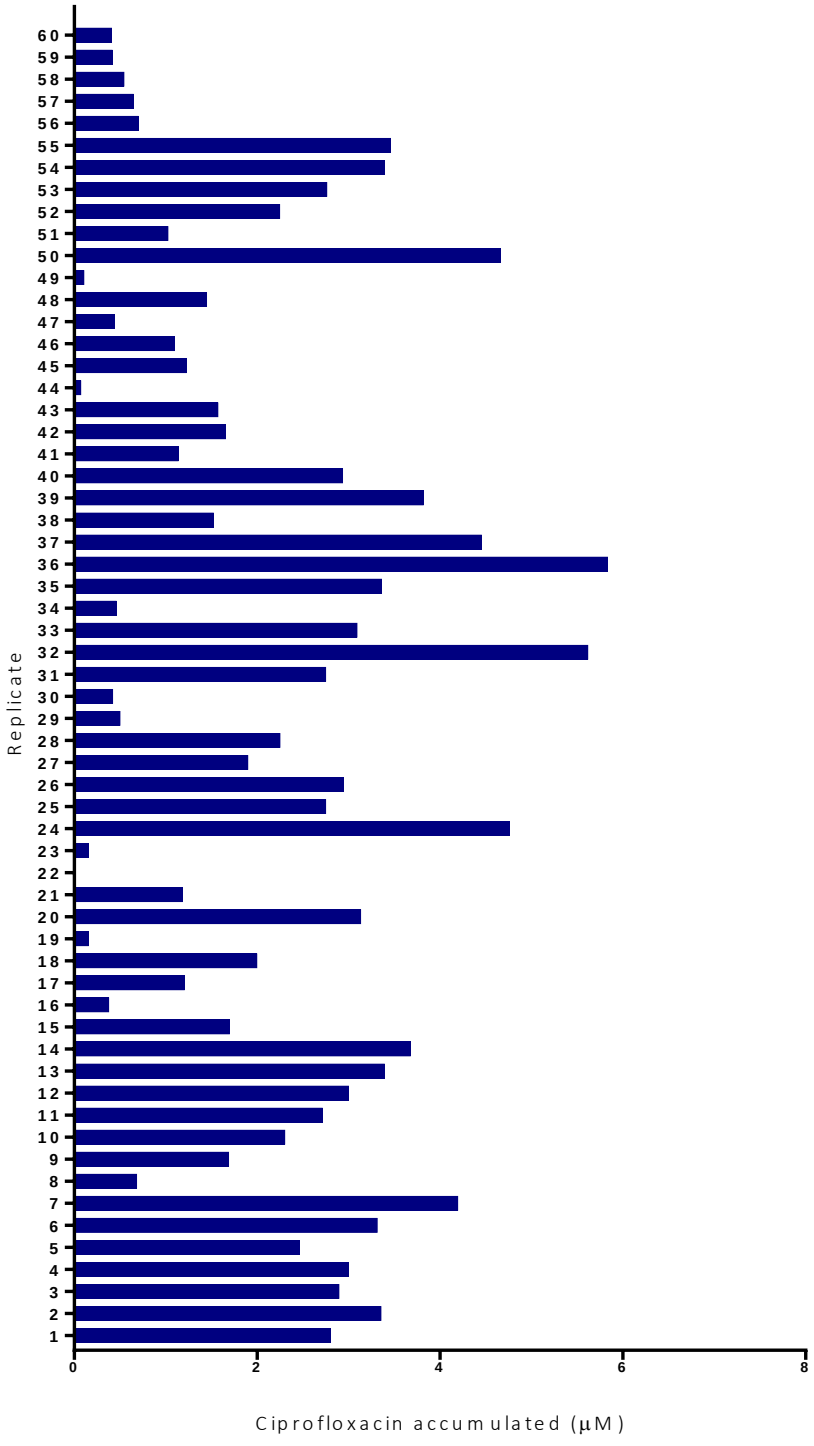


Figure 11: Variability in ciprofloxacin accumulation within *E. coli* BW25113.

Intracellular ciprofloxacin was measured within the parental strain, BW25113. Drug was added to 1 ml volumes of culture to a working concentration of 10 µM. Cells were incubated with drug for 10 minutes with before samples were removed for analysis via LC/MS

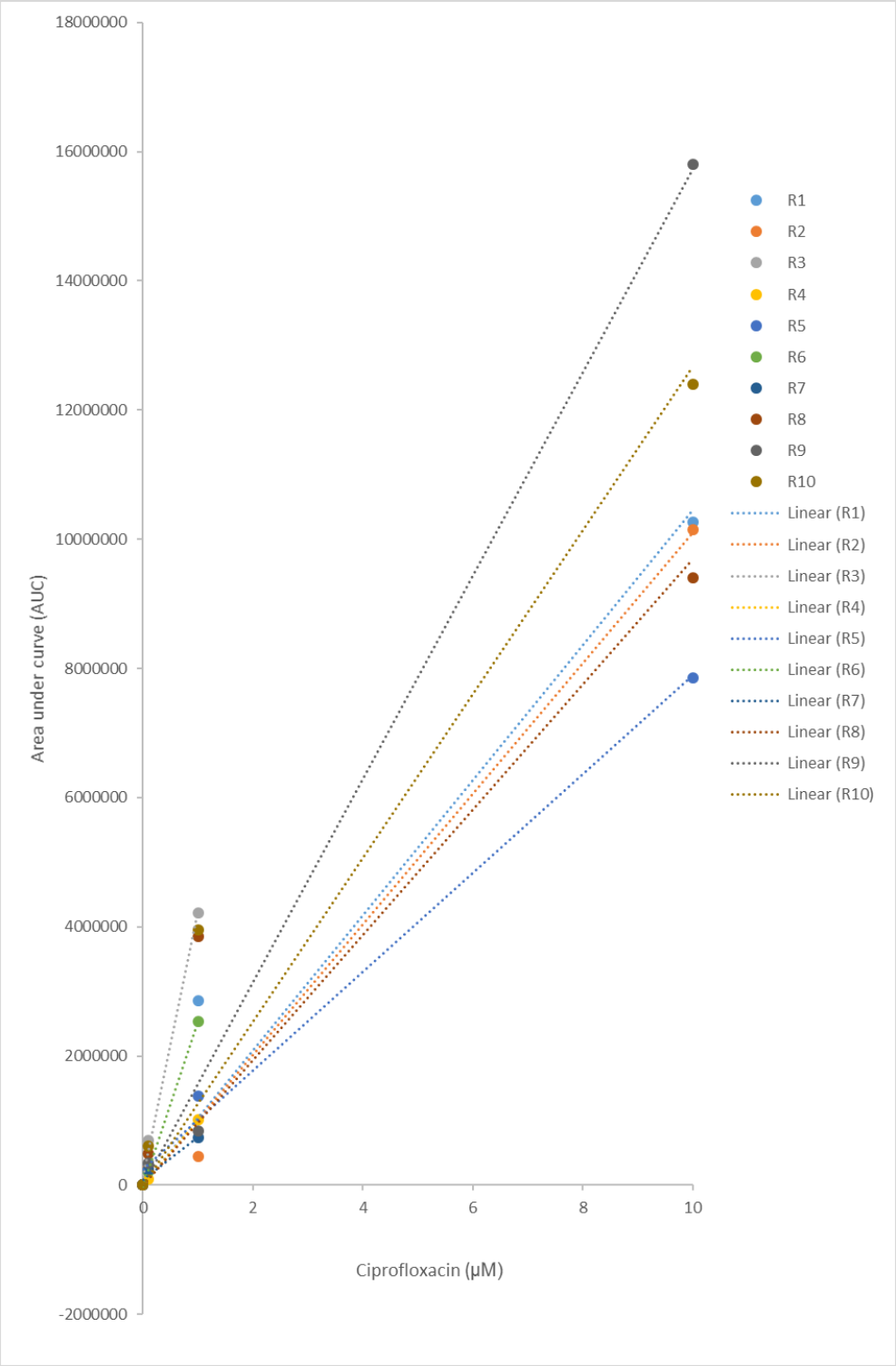


Figure 12: Ciprofloxacin standard curves, measured by LC/MS.

Ciprofloxacin standard curves were prepared in glycine hydrochloride (pH 3) and were serially diluted to give a concentration range of 10 µM, 1 µM and 0.1 µM.

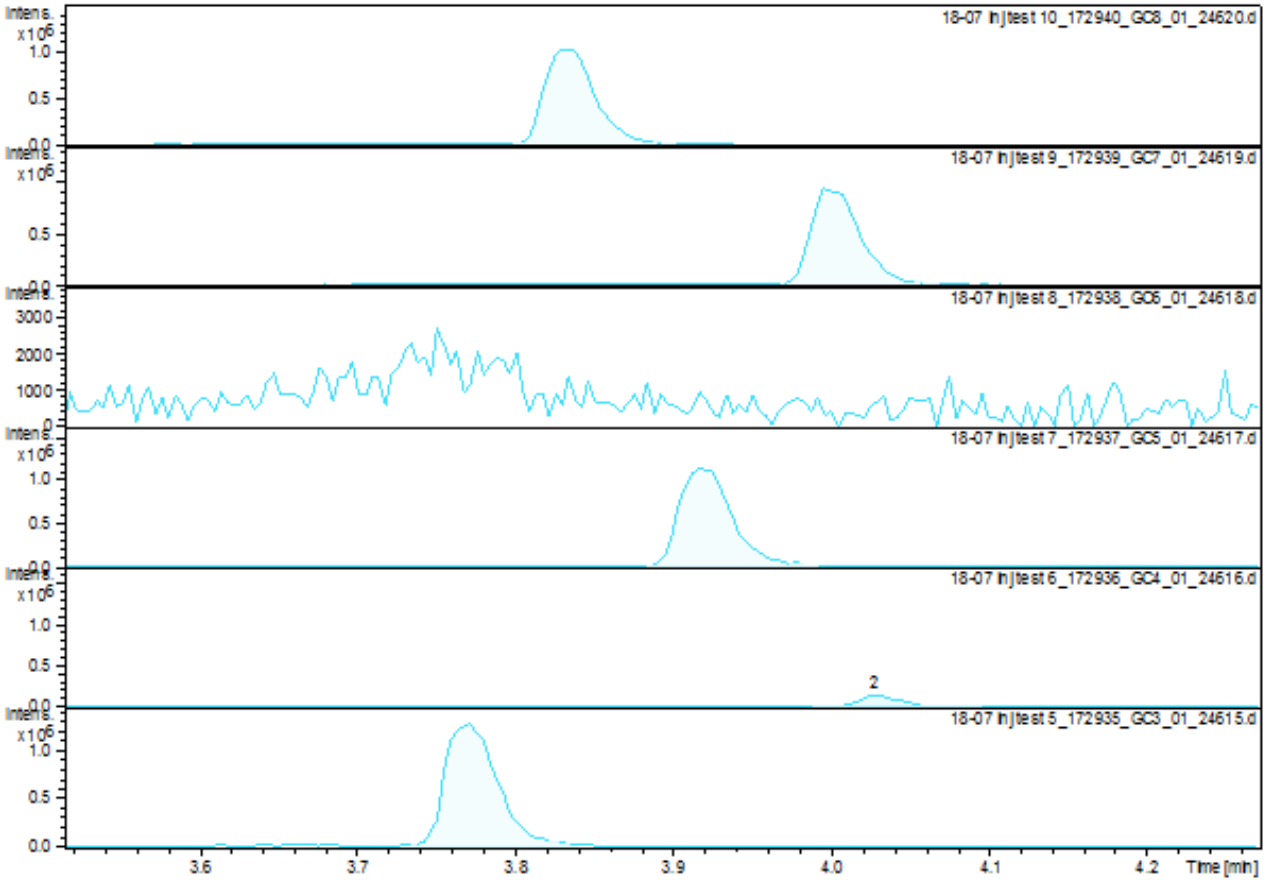


Figure 13: Extracted ion chromatograms of ciprofloxacin within accumulation assay samples.

Intracellular ciprofloxacin was measured within the parental strain, BW25113. Drug was added to 1 ml volumes of culture to a working concentration of 10 µM. Cells were incubated with drug for 10 minutes with before samples were removed for analysis via LC/MS.

To pinpoint the issue, a single calibration sample (5 µM drug dissolved in glycine hydrochloride, pH 3) was analysed in triplicate via LC/MS (Figure 15). As seen previously, peak area and shape varied, in addition to retention times drifting between 3.75 and 4 minutes. In the second run of analysis, ciprofloxacin could not be detected despite being clearly detected in the first and third run (green, red and blue peaks in Figure 15, respectively). As the peak area varied for multiple rounds of analysis for this single sample, it was unlikely that the drug was coming out of solution between these runs and confirms that the variation was not due to error introduced during sample preparation.

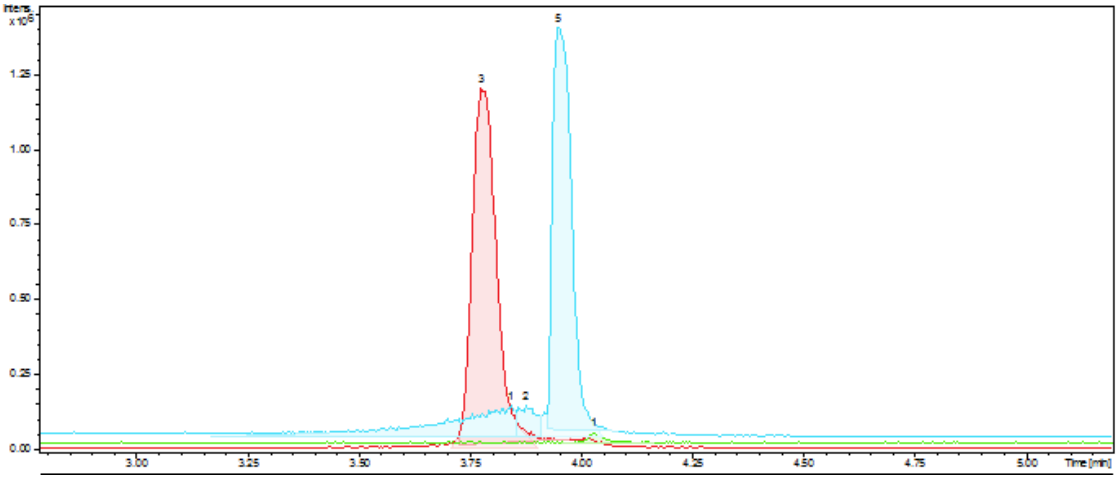


Figure 14: Extracted ion chromatograms of ciprofloxacin from multiple analysis of a single calibration sample.

A single calibration sample (5 µM ciprofloxacin in glycine hydrochloride, pH3) was analysed via LC/MS three times consecutively. The image shows the extracted ion chromatograms (EIC) for ciprofloxacin detected in each round of sample analysis. The red EIC was the first run, green second and blue third.

As the issues of the drug coming out of solution or sample preparation were ruled out, this suggests the problem lies in the functioning of some element of the LC/MS itself. Common issues which may lead to variable peak shape during LC/MS analysis can be the result of blockages in the lines or an uncalibrated injection needle. At this point, there were no leakages from the HPLC or the MS, therefore a blockage

somewhere in the system was ruled out. In order to test the reproducibility of injections, a 10 ml solution was prepared of ciprofloxacin (2 µM) in glycine hydrochloride (pH 3), which was then split into 10 separate samples and analysed by LC/MS (Figure 16). There was a high degree in variability between the 10 replicates, with around an 8-fold difference between the highest and lowest AUC values recorded for the extracted ion chromatograms of ciprofloxacin. Furthermore, ciprofloxacin could not be detected in two of the samples and retention times were variable. As these samples were prepared from the same stock, the AUC values of the ciprofloxacin extracted ion chromatograms should have been uniform across all ten samples. This suggested that there may be an issue with the volumes being introduced into the HPLC, therefore the auto-injection needle was recalibrated which temporarily improved both retention time and peak shape.

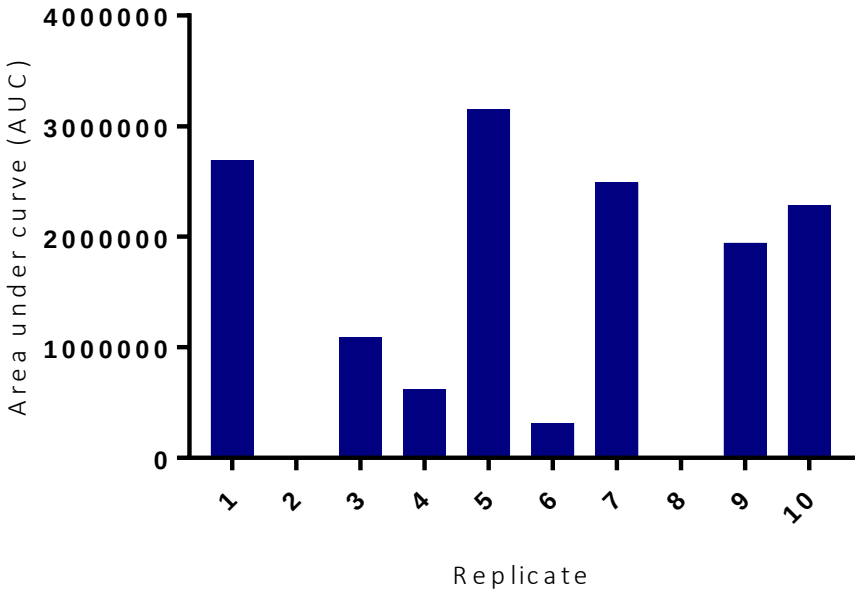


Figure 15: An injection test of 10 ciprofloxacin calibration samples.

A series of 10 samples, prepared from the same stock (2 µM ciprofloxacin) were analysed via LC/MS. Ciprofloxacin was diluted in glycine hydrochloride (pH 3) and an injection volume of 10 µl was selected.

The improvements in regard to reproducibility in detection of the control compound were, however, short-lived. In subsequent runs, peak drifting occurred as retention time increased within runs, as additional samples were added to the processing queue. Leaking also became an issue, specifically downstream of the column. It became apparent that the use of glycine hydrochloride (pH 3) was likely stripping silica from the column. This silica residue was causing blockages downstream of the HPLC, building up in lines entering the mass spectrometer. Additionally, the damaged column was responsible for the variation in peak size and retention time, as drug was not binding uniformly to the column. This resulted in poor chromatography of the drug and increased error within and between experiments. A new column was purchased and as I was unable to use acid lysis in this experiment, an alternative method for cell lysis was sought. Other tested lysis methods included freeze thawing and sonication. However complete lysis of cells was not achieved with either of the aforementioned methods, after samples checked with microscopy revealed living cells (data not shown). The only method which achieved full lysis of the culture was mechanical lysis; therefore this method was chosen for further studies.

Afterwards, an alternative solvent was sought in which I could re-suspend the cellular extract before LC/MS analysis. A suitable solvent must dissolve the compound of interest, be compatible with electrospray ionisation (ESI), in addition to being of HPLC grade. In regards to the latter, this is to prevent the addition of any residue to the system (which I had experienced previously) as this can lead to instrument downtime. Therefore, I sought an HPLC grade solvent which would give a reproducible retention times, peak size and shape, when uniform calibration samples were ran. Methanol and acetonitrile (ACN) are common LC/MS compatible solvents which, have been used previously in the preparation of biological samples for the detection of ciprofloxacin (Richter *et al.,* 2017, Wang *et al.*, 2017). Therefore calibration sample reproducibility was tested in both methanol and varying concentrations of acetonitrile in ultra-pure

water. When either methanol or concentrations above 40:60 (v/v) ACN – H_2O were used, there was poor chromatography of ciprofloxacin. In conditions using high organic solvent, complete drug binding to the column was not achieved as some of the compound was washed from the column with the solvent front. This can be visualised by the presence of two peaks in the EIC (Figure 17). Despite the use of an inappropriate solvent in these experiments, retention time and peak area remained uniform, highlighting that the issues with injection volume and column damage had been overcome.

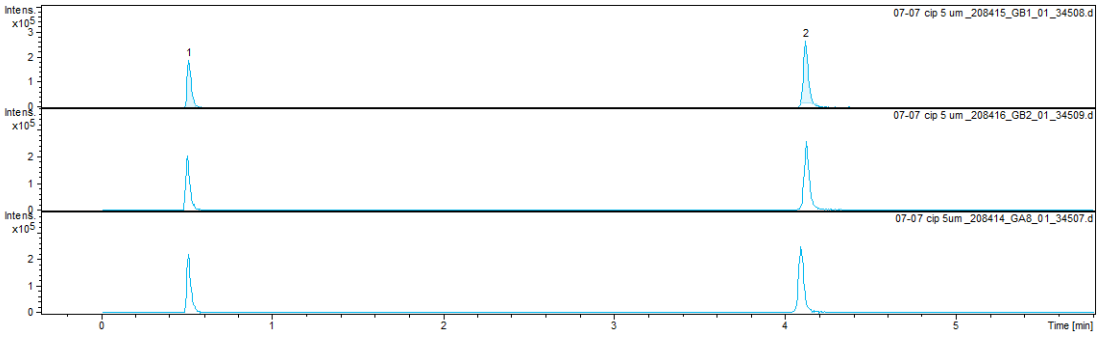


Figure 16: Extracted ion chromatograms of ciprofloxacin in three calibration samples, prepared in methanol.

Typical EICs for three calibration samples of ciprofloxacin (5 µM) in methanol. Poor chromatography of ciprofloxacin also occurred when samples were prepared in samples containing 40:60(v/v) ACN – H_2O.

Subsequently, several accumulation samples were analysed, dissolved in varying concentrations of ACN in ultra-pure water. The cleanest peaks were achieved with concentrations between 10:90 and 20:80 (v/v) ACN – H_2O. Retention times remained uniform, with ciprofloxacin being detected at 4.5 minutes, and calibration samples displayed a linear correlation when plotted in a standard curve. The presence of protein in biological samples has been known to reduce the ionisation of compounds of interest in samples analysed by LC/MS (termed, ‘matrix effects’) and can reduce the sensitivity and accuracy of subsequent experiments. Excess protein in biological

samples can form also form residues, which in turn can lead to blockages in the HPLC. Acetonitrile has been used in past studies to precipitate protein out of biological samples before LC/MS analysis (Polson *et al.,* 2003) therefore I chose the higher concentration of 20:80 (v/v) ACN – H_2O for future experiments. Furthermore, samples were centrifuged at 30,000 x g for 20 minutes and as an extra precaution and subsequently filtered through a 0.22 µM nylon filter, to remove any residual precipitate before LC/MS analysis.

Using the adapted method, the issues with variation in the absolute values of calibration samples had been resolved (Figure 18). However, after plotting multiple calibration curves which had been ran over several weeks, it was evident that there were still minor changes in the sensitivity of the accurate LC/MS when detecting ciprofloxacin from week to week (Figure 19). This highlighted the importance of running calibration curves alongside each set of samples to be analysed during future experiments to account for any changes in sensitivity.

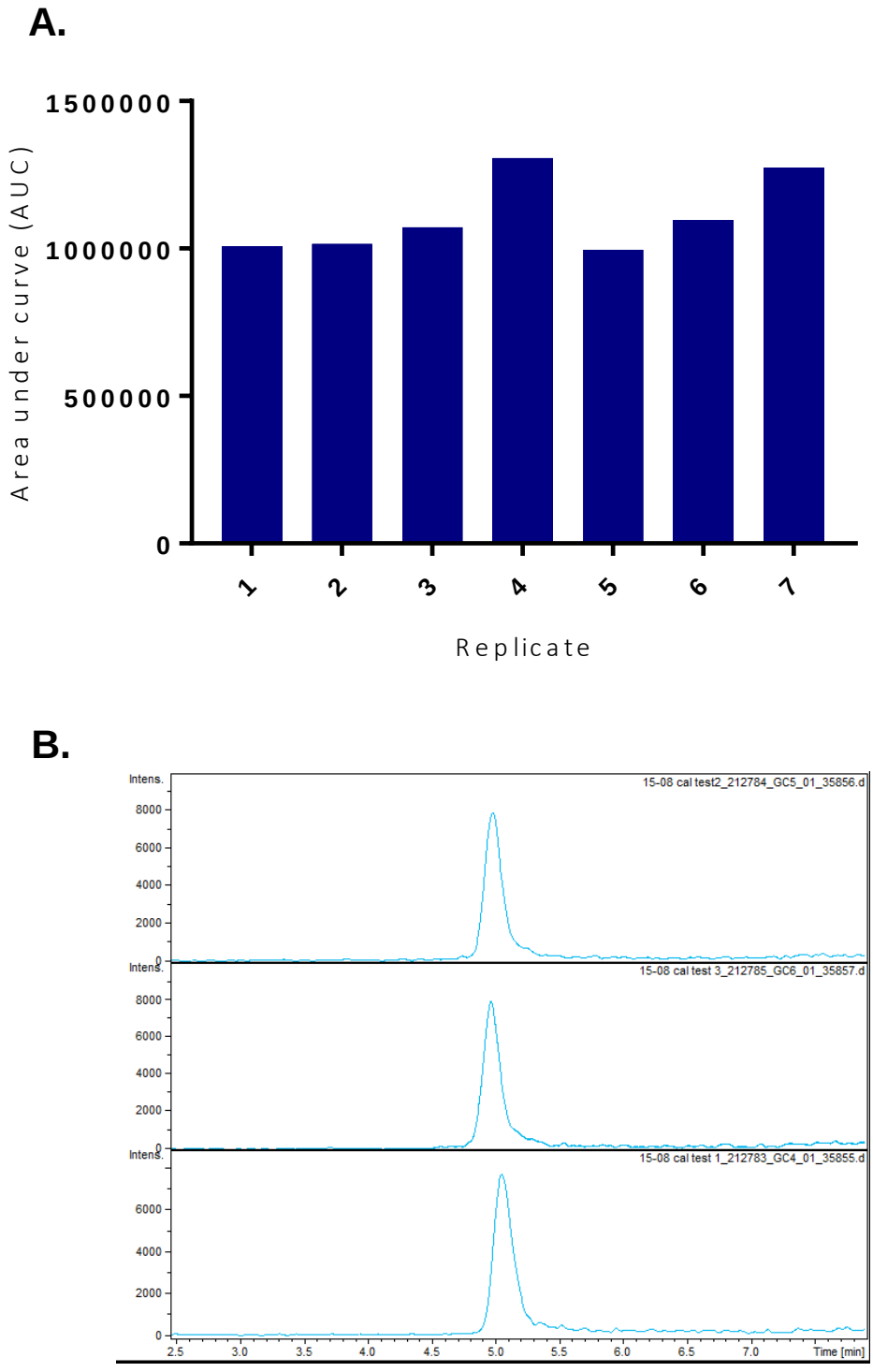


Figure 17: Comparisons of AUC values and extracted ion chromatograms for calibration samples, using the modified method.

(A) A series of 7 calibration samples were made from separate top stocks of ciprofloxacin and analysed on separate days. Ciprofloxacin was dissolved in ACN - H2O (20:80 v/v) to achieve a final concentration of 5 µM. (B) Extracted ion chromatograms of ciprofloxacin in three separate calibration samples, concentration 5 µM.

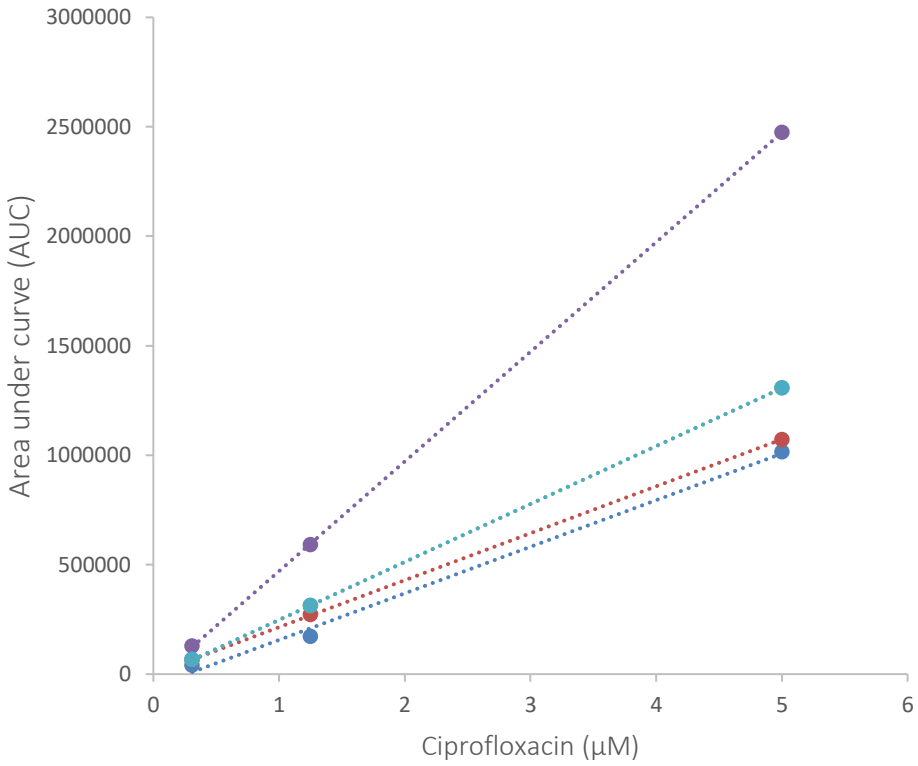


Figure 18: The sensitivity of the accurate LC/MS in the detection of ciprofloxacin can vary over time.

Graph displays a series of seven standard curves (5 µM, 1.25 µM & 0.31 µM) with ciprofloxacin prepared in ACN – H2O (20:80 v/v), which were analysed over separate weeks by LC/MS. Drug concentration displays a linear correlation with the area under the curve values for ciprofloxacin extracted ion chromatograms, however the gradient of the line varies.

3.4.6.2. Repeating the validation experiments under the modified solvent & lysis conditions

To ensure that the modified method was robust, I aimed to repeat the initial validation experiments. This included repeating the initial full-sized assay, to which the results would be compared to the characterised fluorescence method (Figure 20), in addition to the miniaturised assay. Under the improved lysis, solvent and column conditions, results of the full-size accumulation assay revealed that ciprofloxacin accumulation was comparable to established methods. Furthermore, ciprofloxacin could be confidently and reproducibly detected whenever the assay was miniaturised to make it suitable for screening purposes (ciprofloxacin added to a 1 ml volume of culture to achieve a working

concentration of 10 µM). Using the miniaturised assay, the average concentration of ciprofloxacin accumulated was recorded as 1 µM (± 0.2) from 10 biological replicates of *E. coli* BW25113. Furthermore, the negative controls, vancomycin and ampicillin, as well as the additional positive control, tetracycline, was re-tested using the new method (Figure 21). Tetracycline was detected at a concentration of 0.2 µM. In line with our previous results, neither vancomycin nor ampicillin could be detected in the cytoplasmic fraction.

When accumulation samples were plotted and standard deviation calculated, the error in the assay was on average ± 0.2 µM when measuring drug accumulation within the parental strain and the efflux mutant, BW25113 - Δ*acrB*. This was an improvement from the error experienced previously, when using glycine hydrochloride as a solvent (± 2.3 µM). However, when using the modified method, error was slightly higher when measuring drug accumulation within the efflux mutant BW25113 - Δ*acrA*, recorded as ± 0.5 µM. In this case, although the intrinsic error within the assay had been improved fourfold, it could be improved in future experiments. Adding to the problems described previously, the accurate LC/MS itself was offline for several weeks during this study. During this time, samples were lyophilised and frozen until they could be analysed at a later date; this was the case for the samples taken for, BW25113 - Δ*acrA*. Accumulation samples were stored alongside calibration samples which were made on the same date, to account for any possible degradation of the drug over time. When re-suspending samples for LC/MS analysis, vials were sonicated and washed thoroughly to ensure that the majority of drug was recovered. Therefore, this error could have been the result of insufficient washing during sample resuspension. Overall, as error within the assay had been improved since the modifications to the method were made, the assay validation was considered complete.

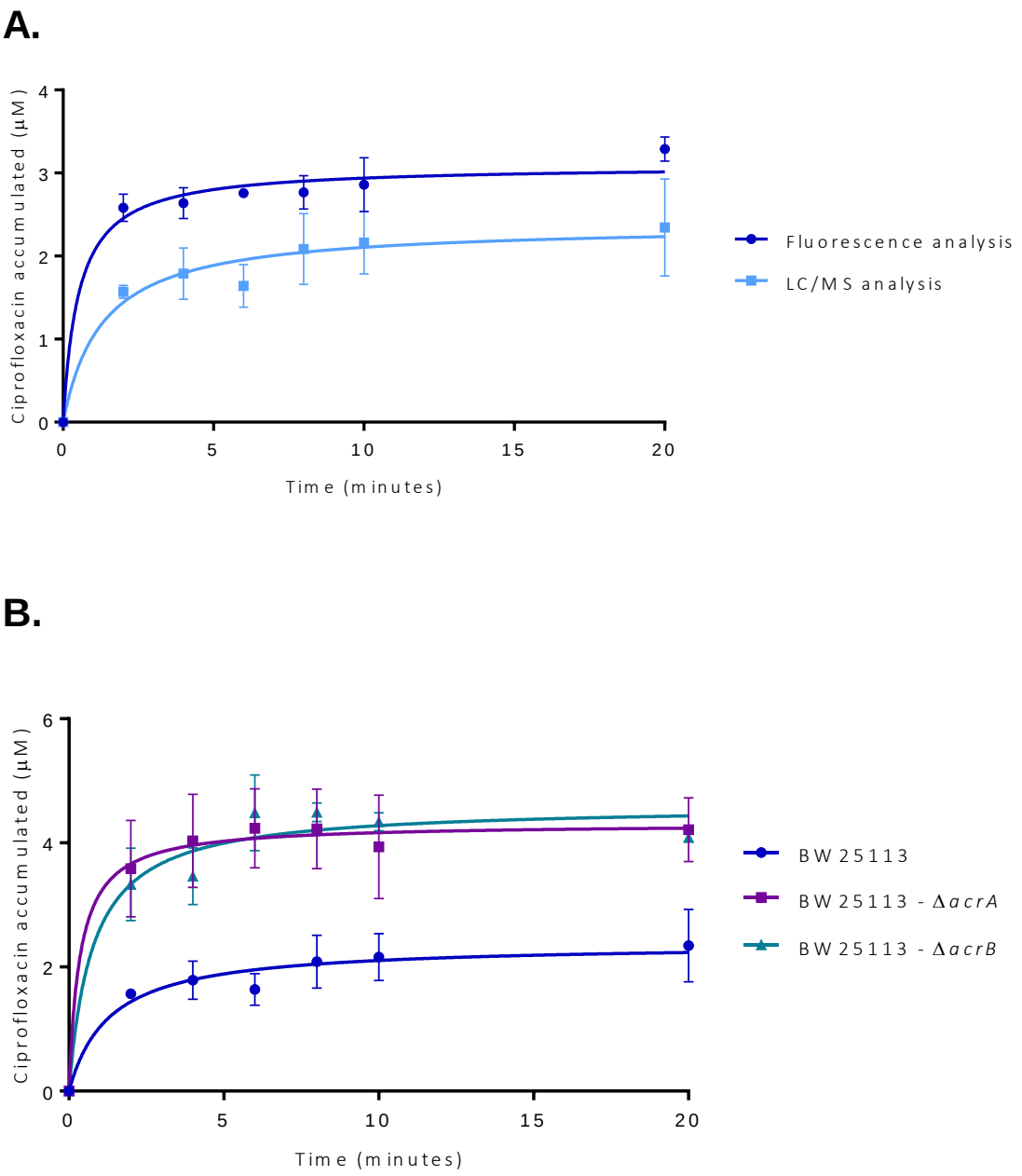


Figure 19: Repeated validation of the ciprofloxacin accumulation assay shows that this method is comparable to established methods in quantifying intracellular ciprofloxacin within *E. coli* BW25113.

(A.) Comparison of the accumulation kinetics of ciprofloxacin, measured using ether the characterised fluorescence based assay or LC/MS analysis and (B.) accumulation kinetics of ciprofloxacin measured via LC/MS in the parental and efflux deficient strains, BW25113 Δ*acrA* & Δ*acrB.*

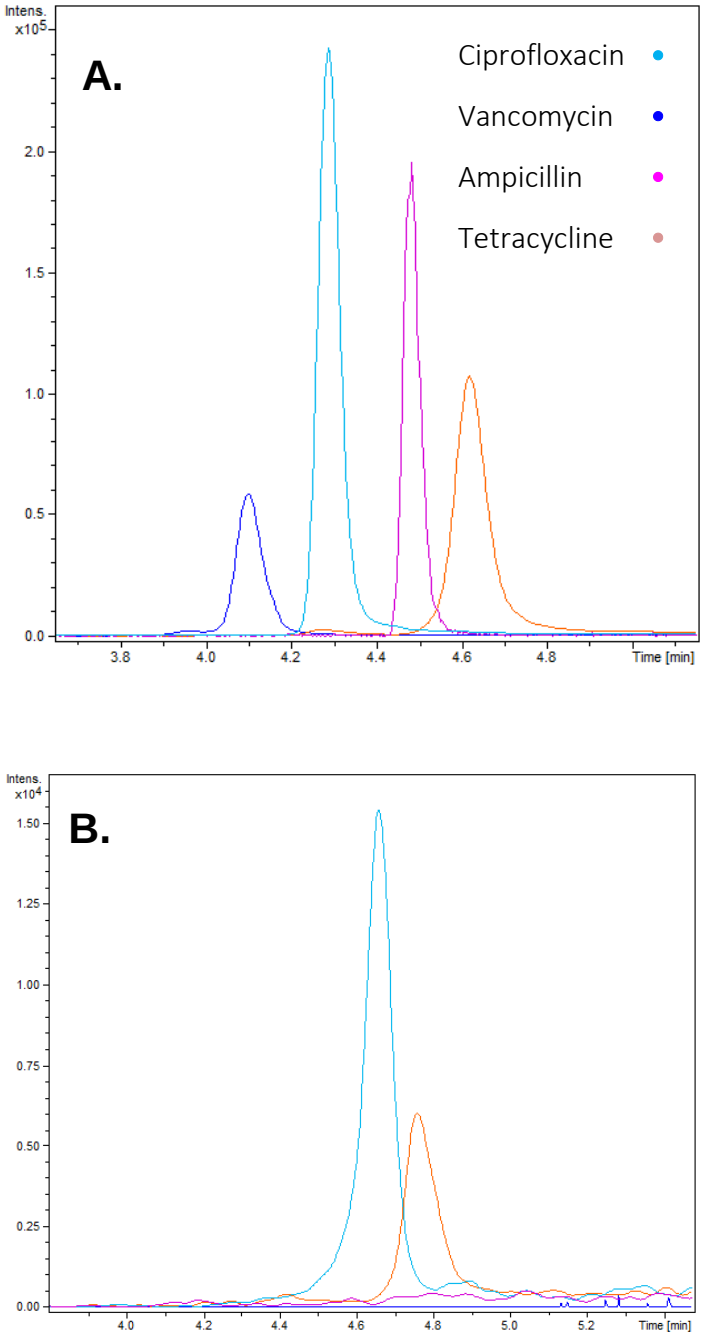


Figure 20: LC/MS analysis of positive and negative controls within the cell lysate of *E. coli*, BW25113, under the modified solvent and lysis conditions.

Extracted ion chromatograms showing the detection of ciprofloxacin, tetracycline, vancomycin and ampicillin in (A.) calibration samples (5 µM) & (B.) in cell lysate. Antibiotics were added to 1 ml volumes, to achieve a working concentration of 10 µM. Samples were removed for analysis after 10 minutes of drug exposure.

3.4.6.3. Repeating the compound library screen using the modified solvent and lysis conditions

After the modified method had been validated, I sought to re-screen some of the compounds which had been included in accumulation assays previously. This was in order to ensure the validity of the results obtained previously, as the compound screen was carried out on a damaged column initially. I sought to re-screen a selection of compounds which were run in the previous screen; this included compounds which were considered hits (positive accumulators) and those which could not be detected in the cell lysate previously.

Of the compounds included in the initial screen, 20 were chosen to be included in a second accumulation assay. Of these compounds, 16 accumulated initially and 4 did not. The ability of these compounds to accumulate within the cytoplasm of the parental strain, BW25113, was then assayed. Of the 16 compounds identified as positive accumulators initially, 8 were validated in the second round of screening (Table 3). Of these remaining 8 compounds, 6 could not be detected despite being present in the calibration samples. Additionally, 4 compounds couldn't be detected in calibration samples when dissolved in 20:80 (v/v) ACN-H_2O. None of the 4 compounds which did not accumulate initially, could be detected in the cell lysate during the second round of screening (Figure 22).

As 8 of the compounds which accumulated originally could not be validated as hits in the second round of screening, this suggests that the damage to the column previously may have given rise to false hits. This could potentially have resulted from poor elution of analytes from the calibration samples, leading to carryover and elution of samples later in the run. All four compounds which were identified as non-accumulators in the initial screen remained so in the second round of screening. Before any conclusions can be drawn from the hits identified in the initial screen, all compounds should be re-run using the modified method.

Furthermore, this screen highlighted that the issues regarding the high variation in the detection of the control compound which was experienced in previous screens, had been overcome. During this screen, the control compound, ciprofloxacin, accumulated to an average concentration of 1.4 µM (± 0.3). This level of error was comparable to what was measured previously in the full size accumulation assay (Figure 20) and during validation of the miniaturised method.

Several important physico-chemical properties which are associated with cell penetration, were also characterised for each of the compounds screened (Table 3). An important property associated with the ability of a compound to penetrate cells is lipopholicity, therefore the logD at pH 7.4 and polar surface area (PSA) were calculated. Most hits can be defined as being within the mid-polar ($logD_{7.4}$ 1 – 3), to non-polar range ($logD_{7.4} > 3$). One compound fell within the polar range ($logD_{7.4} < 1$). Corresponding to this, most hits possessed a low PSA (< 75 $Å^2$), with the average being 67 $Å^2$. This was also reflected in the number of hydrogen bond donor (HBD) and acceptors (HBA) for hit compounds. The HBD values recorded were generally low, between 0-2, with the HBA values slightly higher, with the maximum HBA value of 6 being identified. Compounds which accumulated also contained a low number of rotatable bonds (0-2). Regarding the compounds which did not accumulate, these compounds lay within the mid-polar ($logD_{7.4}$ 1 – 3), to non-polar range ($logD_{7.4} > 3$), and possessed an average PSA of 91 $Å^2$. For compounds which did not accumulate, the HBD values recorded were between 0-5 and the HBA values between 4-6. Compounds which did not accumulate possessed a slightly higher number of rotatable bonds (3-6) than what was recorded for compounds which could enter the cytoplasm of *E. coli*.

Recent research by Richter *et al.,* (2017) has shown that charge is the predominating factor in compound entry within *E. coli*. Furthermore, accumulation may be aided by the presence of amines, (particularly primary amines) within *E. coli*. Regarding the charge at physiological pH of the compounds which accumulated, one

was positively charged. The remaining 6 compounds were neutral. None of the hit compounds possessed primary amines, whereas, LDS-016648 contained a secondary amine. Both, LDS-019819 & LDS-015715 contained tertiary amines. Five compounds contained amide functional groups, with, LDS-019668 containing a primary amide. Secondary amines were present in the compounds, LDS-019721, LDS-019560 & LDS-016393, with, LDS-019819 containing a tertiary amine. At physiological pH, the majority of the non-accumulating compounds were neutral. The compound, LDS-031151 was negatively charged, whereas LDS-019406 was positively charged. None of the compounds which did not accumulate contained amines, however 4 out of the 6 non-accumulating compounds contained amides (LDS-031151, LDS-011446, LDS-016660 & LDS-018429).

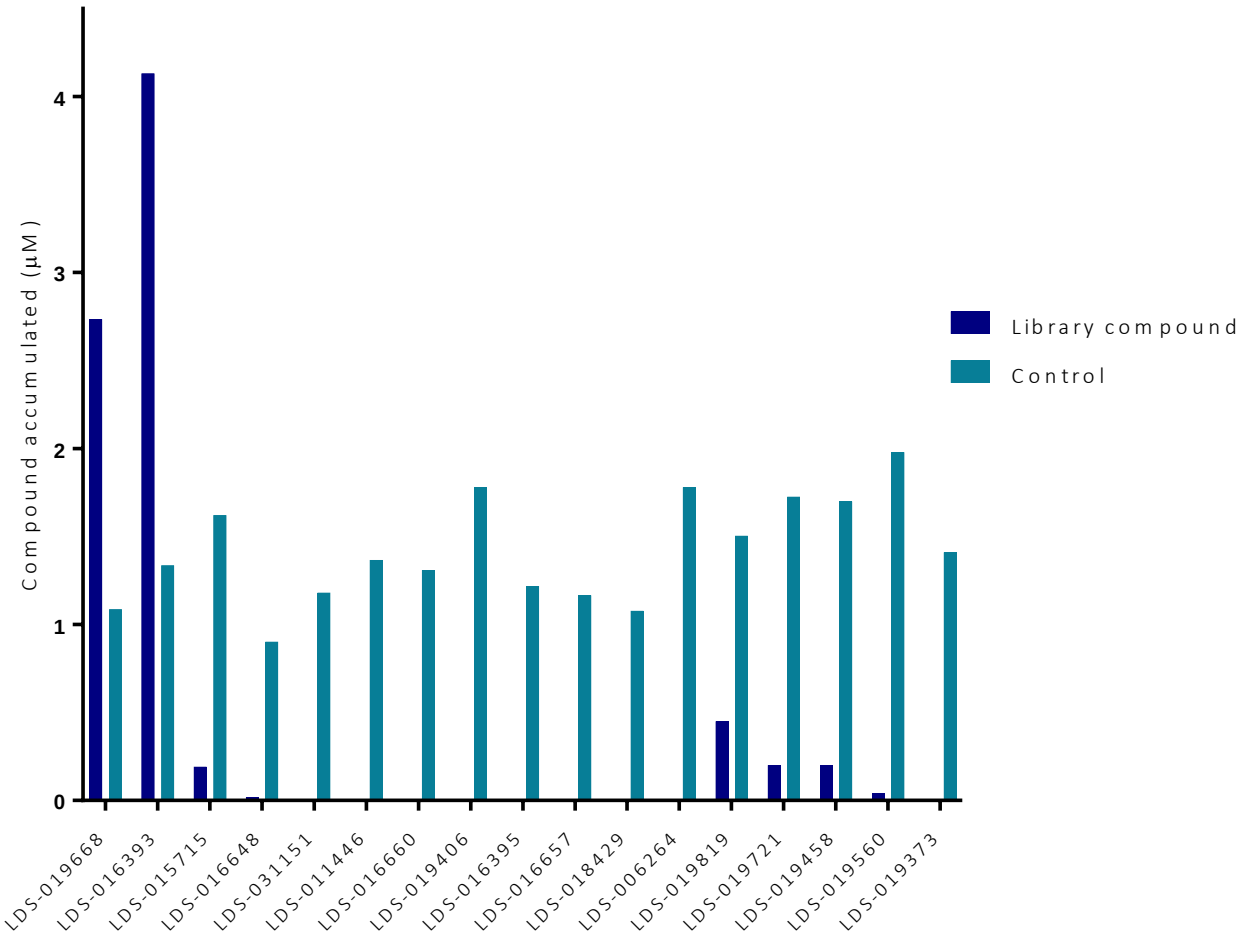
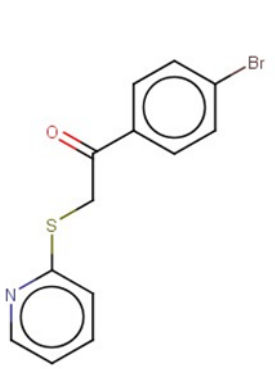
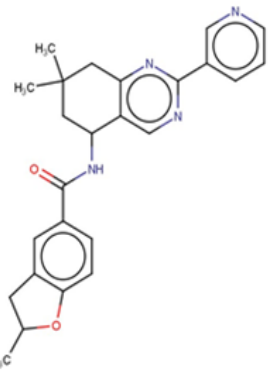
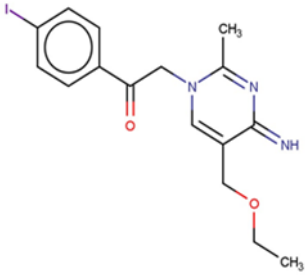


Figure 21: Selection of library compounds which were re-screened for accumulation within *E. coli*, BW25113.

Graph displaying the cytoplasmic accumulation of various library compounds and the positive control compound, ciprofloxacin within *E. coli* BW25113. The positive control was added alongside each of the library compounds, with both agents being added to the culture to achieve a final concentration of 10 µM. Cultures were incubated with drug for 15 minutes before samples were removed for analysis.

Table 3: Library compounds which accumulated within E. coli.

			
Compound:	LDS-019458	LDS-016393	LDS-015715
MW:	308.1936	414.4995	411.2374
Rotatable bonds:	1	0	1
HBD:	0	0	2
HBA:	2	5	6
clogD$_{7.4}$:	3.6	3.7	0.2
PSA:	55	77	67
Accumulated (µM):	0.2	4.13	0.19

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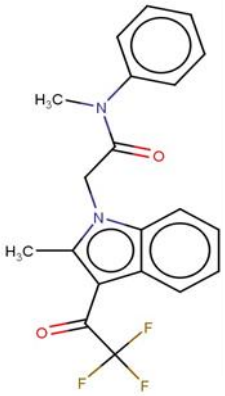
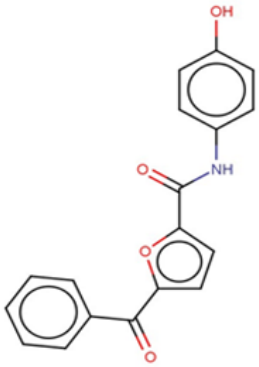
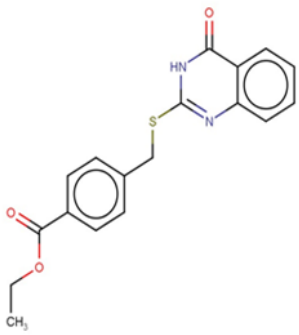
			
Compound:	LDS-019819	LDS-019560	LDS-019721
MW:	374.3563	307.3001	340.3962
Rotatable bonds:	2	2	2
HBD:	4	2	5
HBA:	0	5	3
logD$_{7.4}$:	3.9	3.2	4.1
PSA:	42	79	93
Accumulated (µM):	0.45	0.04	0.2

Table 3: Library compounds which accumulated within *E. coli* (continued).

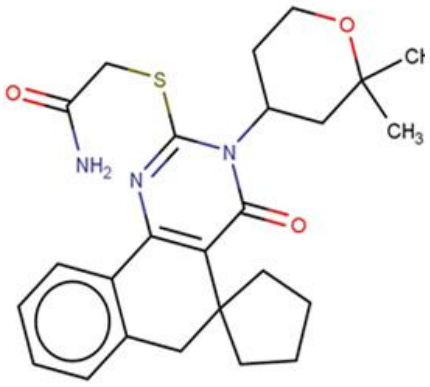
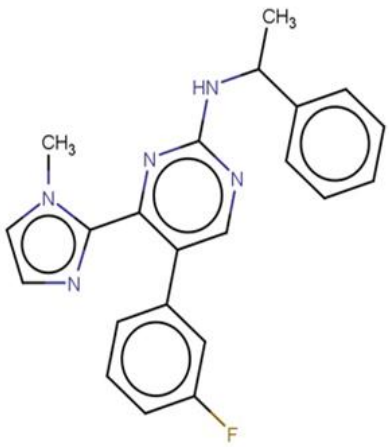
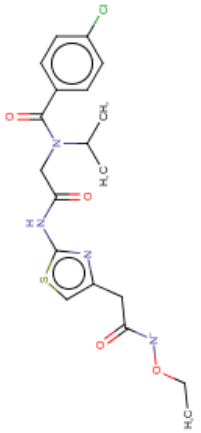
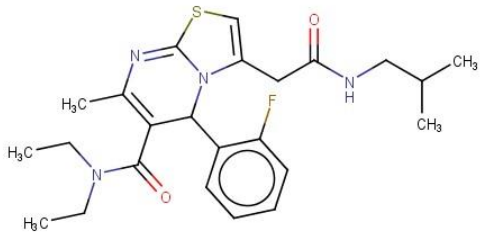
		
Compound:	LDS-019668	LDS-016648
MW:	453.5969	373.4261
Rotatable bonds:	1	0
HBD:	2	1
HBA:	6	4
$logD_{7.4}$:	2.1	4.6
PSA:	110	55
Accumulated (µM):	2.7	0.02

Table 4: Library compounds which did not accumulate within *E. coli*.

		
Compound:	LDS-031151	LDS-011446
MW:	438.9283	458.591
Rotatable bonds:	6	6
HBD:	5	1
HBA:	5	4
clogD$_{7.4}$:	2.3	2.7
PSA:	126	90

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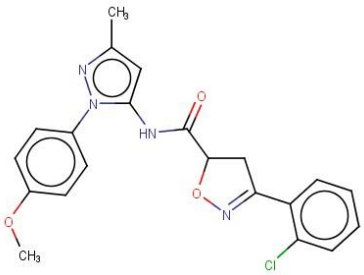
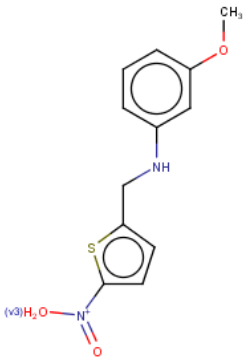
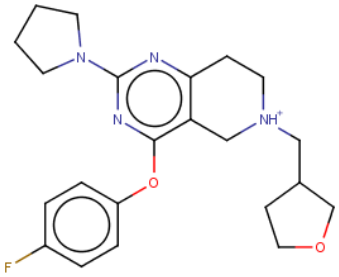
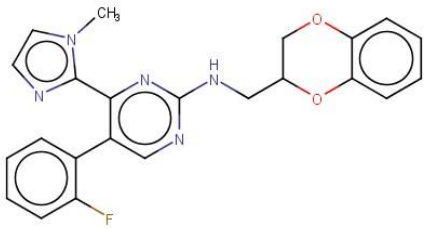
		
Compound:	LDS-016660	LDS-019406
MW:	410.8535	264.3002
Rotatable bonds:	3	3
HBD:	1	3
HBA:	5	4
logD$_{7.4}$:	3.7	1.4
PSA:	78	83

Table 4 continued: Library compounds which did not accumulate within *E. coli*.

		
Compound:	LDS-016395	LDS-016657
MW:	398.4737	417.4356
Rotatable bonds:	3	4
HBD:	0	1
HBA:	5	6
clogD$_{7.4}$:	2.8	3.8
PSA:	126	74

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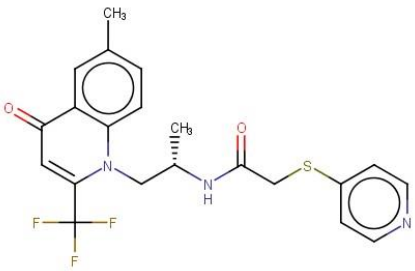
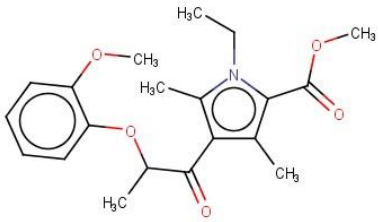
		
Compound:	LDS-018429	LDS-019406
MW:	435.4626	264.3002
Rotatable bonds:	3	4
HBD:	1	0
HBA:	4	4
logD$_{7.4}$:	3.3	3.7
PSA:	88	67

3.5. Discussion

The LC/MS based accumulation assay developed in this study, could be used in future research when attempting to gain detailed information regarding small molecule penetration and accumulation within Gram-negative bacteria. Further studies of this type may eventually be used to characterise 'rules of entry' for these organisms.

The LC/MS based accumulation assay was developed in a stepwise manner. To gain data for validation of the novel assay, a characterised accumulation assay was carried out. Utilising the intrinsic fluorescence properties of fluoroquinolone antibiotics, an accumulation assay developed by, Chapman and Georgopapadakou, (1988) has been used extensively in the past to study intracellular fluoroquinolone concentrations within Enterobacteriaceae, Pseudomonas, Streptococcus and Staphylococcus (Piddock *et al.,* 1999, Mortimer and Piddock, 1991). Using this characterised assay, the accumulation kinetics of ciprofloxacin observed in this study followed the same trends as those measured in *E. coli* by other authors, with a similar SSC being observed. For instance, after addition of ciprofloxacin at 33 µM (10 µg/ml) and the conversion of the results from µM to ng/mg dry weight of cells, the SSC recorded in the parental strain was around 50 ng/mg dry weights of cells. This can be compared to SSC values between 53-60 ng/mg dry weights of cells with efflux proficient strains of *E. coli* in previous studies which have used the same accumulation assay method (Piddock *et al.,* 1999, Mortimer and Piddock, 1991). When the accumulation kinetics of ciprofloxacin was then measured using LC/MS, the results were reproducible and comparable to those gained from the characterised fluorescence method. In addition, LC/MS analysis displayed superior sensitivity, with the level of detection for ciprofloxacin by LC/MS being 75-fold lower than that measured by fluorescence. This sensitivity allowed for the modification of the assay for screening small molecules within cell lysates of *E. coli*, which displayed confident detection of the control after reducing both the initial drug concentration and culture volume.

The ability of LC/MS to detect and quantify chemically diverse molecules irrespective of fluorescence or radiolabels makes this technique ideal for the screening purposes required for our assay. Due to the high sensitivity of the LC/MS assay, there was concern that false ‘hits’ would be generated from contaminating compound residue which had not been fully removed in the wash steps, as opposed to compound which had actually accumulated. Firstly, this was controlled for by introducing an additional wash step. Moreover, the accumulation of several the negative controls was assayed within the parental strain. Neither vancomycin nor ampicillin could not be detected in the accumulation sample, despite being detected in calibration samples. This suggests that compound which does not accumulate within the cell, whether it remains bound to the cellular fraction, will not contaminate the lysate and give rise to a false hit.

During the screen, ciprofloxacin was added to each culture as an internal positive control alongside the library compound to be tested. Initially, a screen of 124 compounds was completed and 22 accumulation positive hits identified. Due to the issues described in section, 4.3.6: Method development – troubleshooting the issues which arose during the development of the LC/MS based accumulation assay, these should be considered preliminary hits only. A subset of library compounds were re-screened using the modified method, including 16 of the compounds which were identified as hits previously. After this screen, 8 hits were verified as positive. The library screen should be repeated using the modified method to verify positive accumulators, in addition to uncovering any potential hit compounds which may have been missed due to column damage in the first round of screening. Moreover, the accumulation levels of the control compound remained fairly uniform in the second round of screening, highlighting that the issues previously had been overcome. Although the intrinsic error within the experiment was improved after modification of the method and the purchase of a new column, the production of confidence limits based on the accumulation of ciprofloxacin would be a useful control experiment to ensure the validity of hits.

From the compounds which accumulated in the second round of screening, a limited set of physico-chemical parameters known to be associated with drug accumulation were analysed. Contradicting the study by Richter *et al.,* (2017), the majority of hits identified in this study were neutral at physiological pH. The results of the study by Richter *et al.,* (2017) showed that only compounds with a positive charge accumulated within *E. coli*. Furthermore, none of the compounds which were identified as hits in this study contained primary amines; this functional group was identified as being important but not necessary for accumulation by the research carried out by Richter *et al.,* (2017). Several compounds (LDS-019819, LDS-015715 & LDS-016393) contained either secondary or tertiary amines. However, all compounds which accumulated were rigid in nature, containing a low number of rotatable bonds. This information was in agreement with that described by Richter *et al.,* (2017). When comparing compounds which accumulated to those that didn't, non-accumulating compounds were less rigid, containing on average a higher number of rotatable bonds. This was however the only difference in physicochemical properties which could be identified between accumulating and non-accumulating compounds from this small data set. In order to gain any meaningful information regarding important physico-chemical properties which may aid drug accumulation within *E. coli*, this data set will need to be extended.

In terms of assay limitations, the method proved highly sensitive in the detection of ciprofloxacin, however this was not the case for all library compounds. This was evident by lower AUC values in calibration samples for some of the library compounds, when in comparison to those seen for ciprofloxacin. The limits of detection were not determined for each compound to be screened, therefore some 'hits' may have been missed as they may have fallen below detectable levels. Finally, a few of the compounds included in the screening set could not be detected in the calibration samples,

suggesting that some library compounds may be unsuitable for this accumulation assay due to their inability to be ionised by soft ionisation methods.

No study has yet determined the impact of efflux on the accumulation of small molecules within Gram-negatives. Screening the library set used in this study, for accumulation within either of the efflux deficient strains (BW25113 - Δ*acrA* or Δ*acrB*) could yield more information about the physico-chemical properties of small molecules associated with recognition by efflux mechanisms within *E. coli*. Furthermore, screens could be carried out within problematic organisms which possess high levels of intrinsic drug resistance, such as *Pseudomonas aeruginosa* (Breidenstein *et al.,* 2011). There are past instances where LC/MS has been used to detect and quantify drugs within the cytoplasm of such bacteria, namely Pseudomonas and Mycobacteria (Cai *et al.,* 2009, Bhat *et al.,* 2013). However none of these authors, as yet, have gone so far to use these methods in small molecule screens, akin to that carried out in this study or that described by Richter *et al*., (2017). Compound accumulation screens within such bacteria could enable us to identify properties for drug accumulation within organisms which possess low outer membrane permeability, in addition to a myriad of efflux mechanisms.

The library compounds chosen for this study have no know antibacterial activity; therefore they lack targets, or a binding 'sink' within the bacterial cell. This is in comparison to ciprofloxacin, which targets and binds to cellular gyrases and type II topoisomerases, enzymes responsible for altering DNA topology. The interaction of ciprofloxacin with these enzymes triggers cell death through the formation of lethal double stranded DNA breaks, as a result of the stabilisation of catalytic DNA-enzyme cleavage complexes (Mustaev *et al.*, 2014, Laponogov *et al.,* 2009). Drugs which interact with their respective target are removed from the pool of drug within the cell, maintaining the concentration gradient and driving ingress of free drug into the cell. This 'sink effect' has been documented for the drug erythromycin, as in the presence of the erm(B) ribosomal methylase, the accumulation of this drug is decreased due to the

inability of the drug to bind its target and as a result the presence of a drug ‘sink’ (Barre *et al.,* 1986, Capobianco and Goldman, 1990). Therefore, the presence of a binding sink could account for some of the differences in the level of accumulation of the control when in comparison to the library compounds.

To allow the accumulation of the control to be comparable to that of the library compounds, a control compound which lacks a binding sink within the cell would be required. During this study, a sinkless strain was generated for the compound, tetracycline. This was achieved by transforming the parental strain, BW25113 with a pUC19 construct expressing the tetracycline resistance determinant, *tetM*. It has been established that tetracyclines inhibit bacterial protein synthesis by preventing the interaction of aminoacyl tRNAs with the 70s ribosome. This action is mediated through the high affinity site binding of tetracycline to a single site on the 30s subunit, with the 16s rRNA contributing to binding interactions within this pocket (De Stasio *et al.,* 1989, Chopra and Roberts, 2001, Chukwudi, 2016). The ribosomal protection protein, Tet(M), is a cytoplasmic protein which is part of the translation factor superfamily of GTPases, exhibiting homology to the elongation factor, EF-G. Tet(M) mediates resistance to tetracyclines by facilitating the release of the drug from the ribosome, which is driven by conformational changes upon binding of the ribosomal protection protein (Dönhöfer *et al.,* 2012). Therefore, the expression of Tet(M) within the parental strain, BW25113, would prevent tetracycline from interacting with its target, abolishing the presence of a binding sink within this organism. Using the *tetM* expression strain, alongside tetracycline as a control compound in future screens would enable the accumulation of library compounds to be directly compared to that of the control, and the effect of the drug ‘binding sink’ on cytoplasmic accumulation to be studied.

The miniaturised LC/MS accumulation assay possesses an intrinsic error of around ± 0.2 μM, when used to quantify accumulated ciprofloxacin within the parental strain. The noise of the experiment had been reduced from ± 2.3 μM, after the method

was modified (4.4.6: Method development) as much of the initial error was due to column damage which has been discussed previously. There are however many points within the method of this accumulation assay where error could be introduced. For instance, variation could result from differences between the OD values between biological replicates, or drug being lost before samples were analysed, either during the wash steps or effluxed from the cells during processing. Steps were taken to prevent this, such as OD values being adjusted, in addition to samples and pipette tips being chilled to 4 °C to prevent the loss of drug via efflux during processing. Despite these steps, the assay itself proved to possess some intrinsic noise. This was especially evident in the second round of validation experiments after lyophilised samples had been reconstituted. However, the aim of this research was to develop a method which could simply detect compounds which accumulate in the cytoplasm of *E. coli*, which then could be used in library screens to generate many preliminary 'hits'. From these hits, information regarding bacterial permeation could be extrapolated. As such after the validation stage, quantification (and therefore the associated error) of the compound within the cytoplasm during screening is not paramount. If the internal positive control falls within normal limits when present alongside a control compound in a screening sample, this should be considered a hit and the screen deemed suitable for its purpose. In future studies, the determination of confidence limits in relation to accumulation of the control within the parental strain, BW25113, would be useful in order to ensure the validity of results regarding the accumulation of library compounds.

One of the main hurdles of this assay was generating reliable, reproducible data due to the problems experienced with the accurate mass-spectrometer during the development of this method. Issues ranged from the trivial, such as an uncalibrated auto-injection needle and general leaks, to the more severe, such as a damaged column. Furthermore, the equipment was offline for some time during the study due to various issues which required investigation by an engineer, such as a broken detector

and leaking auto-sampler. For this study, the machine must maintain sensitivity and accuracy over the course of many weeks and months. The aforementioned issues led to variation in retention times & reduced the sensitivity of the LC/MS, meaning that often the equipment was unsuitable for the analysis of samples generated which would form part of the validation experiments. As a result, a large portion of the data generated for this study was unusable, delaying the validation of the assay.

The main issue in this study however, was the damage to the column due to improper solvent use in the early stages of this study. Once a new column had been obtained, all validation experiments were repeated using the modified method and due to this wasted time, the compound library could not be re-screened in full. The issues which arose due to column damage highlights the importance of suitable sample preparation when detecting compounds from complex mixtures. Samples are always best reconstituted in HPLC suitable buffer. Although this adds additional processing steps which may increase the standard deviation within the experiment, it results in less equipment downtime and lengthens the lifespan of the column.

The method developed in this study therefore provides a highly sensitive assay which may be used to detect and quantify the intracellular accumulation of chemically diverse compounds within *E. coli*, without the need for a radiolabel or use of fluorescent compounds. Use of this assay in further screening of chemically diverse small compound libraries could be used to characterise detailed information regarding physic-chemical descriptors that small molecules must adhere to accumulate within the cytoplasm of *E. coli*, avoiding efflux. This information in turn could be used to rationally guide the modification of hit compounds, or the makeup of screening libraries in a drug discovery setting when searching for novel antibacterials with Gram-negative efficacy.

4. Do membrane transporters facilitate the entry of antibiotics into bacteria?

4.1. Abstract

The major method of drug uptake through the cytoplasmic membrane has traditionally been thought to be via passive lipoidal diffusion. However, in recent years there has been a growing body of evidence supporting carrier mediated uptake of drugs, which raises the question as to the extent of this phenomenon in nature. Specifically if membrane carriers play a previously unrealised role in the entry of antibiotics within bacteria. To assess this, I designed a screen which would allow the identification of membrane transporters which play a putative role in drug uptake, using a library of *S. aureus* strains containing transposon disruptions in non-essential membrane transporter genes. Using this screen, 30 carriers were identified to play a putative role in the uptake of 9 antibiotics from different drug classes. Further characterisation using genetic complementation, competition studies, drug accumulation assays and the generation of strains containing disruptions in multiple genes associated with drug uptake then confirmed the role of membrane carriers in the uptake of gentamicin, ciprofloxacin, chloramphenicol, tetracycline, fosfomycin and D-cycloserine. The results of this study show that membrane transporters play a previously unrealised role in the entry of antibiotics within *S. aureus*, adding to the growing body of evidence that carrier mediated transport is a major route of entry for the uptake of compounds into cells.

4.2. Introduction

Conventionally it is believed that for the majority of drugs, the predominating route of entry through the cytoplasmic membrane is passive diffusion directly through the bilayer itself (Lipinski *et al.,* 2012, Lipinski *et al.,* 2001b). This phenomenon has been termed, 'lipoidal diffusion' and with regards to antibiotics, it is with few known exceptions. There are several reasons as to why the lipoidal diffusion model has become the favoured hypothesis in regards to antibiotic passage across biological membranes. Until recent years this has primarily been due to the lack of information regarding transporters in drug uptake. Furthermore, many studies have attempted to correlate properties such as lipophilicity with drug permeation into cells, or the absence of a concentration gradient in the entry of some agents. For the majority of antibiotic classes, carrier mediated transport has traditionally been considered an exception rather than a rule. There are past instances where transporters have been proposed to play a role in the uptake of other antibiotic classes, such as the tetracyclines (McMurry and Levy, 1978) and aminoglycosides (Chopra, 1988). However as no transporters were subsequently identified in the uptake of these classes, this notion of carrier facilitated uptake has been dropped in favour of lipoidal diffusion for the entry of these antibiotics into the cell (Schnappinger and Hillen, 1996, Hancock, 1981).

Two classic examples of antibiotics which permeate the cell via transporters are fosfomycin and D-cycloserine. By mimicking the natural substrates of glycerol-3-phosphate permease (GlpT) (Lemieux *et al.,* 2004) or glucose-6-phosphate (UhpT) (Kahan *et al.,* 1974) and the D-alanine-glycine system (Wargel *et al.,* 1970), both fosfomycin and D-cycloserine respectively have been shown to hijack these native transporters in order to permeate the cytoplasmic membrane. More recently, transporters have been shown to facilitate the uptake of kasugamycin and blasticidin S (Shiver *et al.,* 2016), in addition to the semi-synthetic rifamycin derivative, CGP 4832, across the outer membrane of Gram-negative species (Ferguson *et al.,* 2001).

Therefore, seems reasonable that certain antibiotics may have structural features which enable them to mimic the native substrates of protein carriers as opposed to partitioning through the membrane.

In terms of the predominating route of drug entry through membranes, absence of evidence does not necessarily mean evidence of absence and in recent years, there has been a growing body of data supporting the role of protein carriers in drug uptake (Dobson *et al.,* 2009b, Sai and Tsuji, 2004, Dobson and Kell, 2008, Lanthaler *et al.,* 2011). One particularly compelling study by (Lanthaler et al., 2011) involved the development of a screening platform which allowed the identification of transporters with a putative role in drug uptake within the model organism, *Saccharomyces cerevisiae.* Using their novel method, they were able to identify transporters which may be involved in the entry of 18 out of a total of 26 cytotoxic compounds screened. The results of this study established the previously overlooked importance of transporters, as opposed to lipoidal diffusion, in the cellular ingress of drugs in eukaryotes. It does not seem unlikely therefore, that the transporter mediated uptake of drugs may play a more significant role in the uptake of antibiotics into prokaryotic cells.

To date, the role of lipoidal diffusion in drug entry has governed the design of synthetic compound libraries, as it is assumed that lipophilicity is a key physico-chemical property governing the potential ‘drug-likeness’ of a compound, enabling the compound to partition into the bilayer (Leeson and Springthorpe, 2007, Lipinski *et al.,* 1997, Walters and Namchuk, 2003). This could be problematic if membrane transporters play a greater role in drug uptake than what is thought, as it automatically limits the diversity of the chemical space occupied by testable compounds. Therefore, we may be limiting our successes by narrowing the chemical space occupied by drug libraries for antibiotic drug discovery (O’Shea and Moser, 2008).

In this study, we provide the first comprehensive assessment of the involvement of transporters in the entry of antibiotics within *Staphylococcus aureus*. This Gram-

positive, non-motile coccus is an endemic pathogen associated with both community acquired and nosocomial settings (Otto, 2007, Stryjewski and Corey, 2014). In order to identify staphylococcal membrane transporters with a putative role in drug ingress, a library of transposon (Tn) disrupted strains was employed and screened against a panel of antibiotics from different classes. Transporters involved in drug entry were subsequently validated via the reduction in drug susceptibility which can arise from deleting the drug's route of entry into the cell, resulting in lower cellular accumulation and impacting the drug's efficacy. The library of Tn inactivated mutants consisted of strains derived from the commercially available, Nebraska Transposon Mutant Library (NTML). This library contains strains of the prominent community acquired pathogen, *S. aureus* USA300 JE2, containing *Bursa aurealis* transposon disruptions in non-essential genes (Fey *et al.,* 2013, Bose *et a*l., 2013). The results of this study presents a case for the role of protein carriers in drug entry within bacteria, adding to the growing body of evidence that carrier mediated uptake plays a significant role in the cellular ingress of compounds across all domains of life.

4.3. Aims

The primary aim of this of this study was to assess the role of membrane transporters in the uptake of antibiotics within *S. aureus,* USA300 JE2. By utilising an increase in drug resistance which is associated with gene disruptions, NTML library strains containing Tn disruptions in putative membrane transporter genes were screened for increased resistance against a panel of antibiotics, in comparison to a parental strain*, S. aureus* USA300 JE2. Using this method, transporter proteins which played a putative role in drug uptake were identified and where possible, their roles in drug uptake was confirmed. This was carried out by genetic complementation of Tn disrupted strains with functional copies of the respective gene, competition assays between the native substrate of the transporter and the antibiotic to which increased resistance was

associated, assessing the impact of multiple Tn disruptions on drug susceptibility and drug accumulation studies.

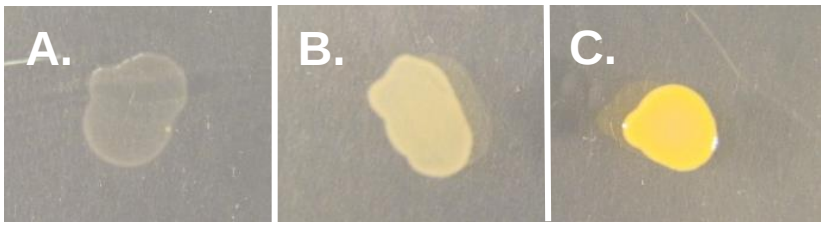
4.4. Results

4.4.1. Validation of a screening assay to enable the identification of membrane transporters in *S. aureus* involved in antibiotic uptake

Firstly I sought to develop a suitable screening technique to identify membrane transporters in *S. aureus* that mediate antibiotic uptake. A model screening method has been developed previously for the identification of drug transporters in yeast (Lanthaler *et al.*, 2011), however, none as yet have been developed to identify bacterial membrane transporters which may facilitate antibiotic entry.

It was reasoned that the loss of the expression of a transporter involved in the uptake of an antibiotic would result in to increased resistance to that antibiotic. Therefore, I sought to screen a library of strains which possessed transposon disruptions in genes encoding membrane transporters (see 2.6.1: Identification of membrane transporter knockouts within the NTML library) for increased resistance to a selection of antibiotics with intracellular targets. Before the screen could take place, the method was first validated by testing strains containing disruptions in genes known to be involved in antibiotic uptake. Fosfomycin was chosen to be included as a positive control, as it is known that both transporters, glycerol-3-phosphate (GlpT) and the hexose phosphate transporter (UhpT) are involved of the uptake of this antibiotic (Kahan *et al.*, 1974, Lemieux *et al.,* 2004). Furthermore, past studies have shown that inactivation of either of these genes results in an increased resistance to fosfomycin (Castañeda-García *et al.,* 2013). To validate the screening method, the two transposants, SAUSA300_0216 (*uhpT::*Tn) & SAUSA300_0337 (*glpT::*Tn), as well as the parental strain, JE2, were spotted onto agar containing fosfomycin at the agar MIC value (4 µg/ml). SAUSA300_0216 & SAUSA300_0337 displayed strong growth at 1 x MIC value, whereas growth of the parental strain, JE2, was diminished. These results were confirmed by broth MIC (See Figure 23). As the Tn disrupted strains displayed a

reduced susceptibility to fosfomycin in comparison to JE2, this validated the screening method for the identification of membrane proteins involved in drug uptake by reduced drug susceptibility.



Strain	Broth MIC values (μg/ml)
USA300 JE2	2
NE1154	64
NE1388	> 64

Figure 22: Strains containing transposon disruptions in genes associated with antibiotic uptake, display reduced drug susceptibility.

Image shows a growth comparison of (A) the control strain, JE2, in comparison to the two transposants containing disruptions in the genes (B) SAUSA300_0216 (*uhpT::*Tn) & (C) SAUSA300_0337 (*glpT*::Tn), on agar containing 1 x agar MIC value (1 μg/ml) of fosfomycin.

4.4.2. Determining antibiotic susceptibilities

A panel of 15 antibiotics possessing antistaphylococcal activity were chosen for this screen. These agents represent a diverse set of drugs from different antibiotic classes, for which their mode of action is the inhibition of an intracellular target. Before screening could take place, agar MIC values of each of these agents were determined for the parental strain, JE2 (Table 5). Agar MICs were determined in accordance with CLSI guidelines. Of the 15 antibiotics tested, USA300 JE2 was susceptible to 14, displaying resistance only to lincomycin. This latter agent was removed from further study. These

susceptibility tests were carried out under my supervision, with the help of the two MSc students, G. Morrison-Williams & T. O'Brien.

Table 5: Agar MIC values of each compound in the chosen antibiotic screening set.

Antibiotic	Agar MIC value (µg/ml)
Rifampicin	0.0156
Fusidic Acid	0.125
Novobiocin	0.125
Retapamulin	0.125
Gentamicin	0.25
Mupirocin	0.25
Tetracycline	0.5
Fosfomycin	1
Trimethoprim	1
Linezolid	2
Streptomycin	4
Chloramphenicol	8
Ciprofloxacin	8
Lincomycin	> 256

4.4.3. Identification of NTML library strains with reduced susceptibilities to the antibiotics included in the screening panel

Once agar MIC values were established, the library of NTML strains possessing transposon disruptions in genes encoding membrane proteins was screened against the chosen panel of antibiotics. This screen was carried out with the help of, G. Morrison-Williams & T. O'Brien. An optimum screening concentration was determined for each drug by replica plating the NTML library strains onto agar containing concentrations of drug at multiples of the agar MIC value. The concentration of drug was either increased or decreased in relation to the growth of the control strain, JE2. A hit was defined as an

NTML library strain which exhibited strong growth across 3 biological replicates, at a drug concentration on which the parental strain, JE2, did not grow. The layout of a typical screening plate (in this case, chloramphenicol) displaying the growth of hits in comparison to JE2, can be seen in Figure 24. As plates were inoculated directly from saturated cultures, the cell density was not normalised for each NTML library strain included in the agar screen. This could give rise to false positives if broths were present which contained a higher density of cells, due to the presence of strains with faster growth rates. In order to account for this, any hits identified in the agar screens were then tested by broth MIC. Strains exhibiting reproducible broth MIC values which were two-fold or higher than that observed for the parental strain, JE2, were considered as validated hits to take forward for genetic complementation. This was to reduce the chance of false positives being taken forward due to experimental error, as for some strains only subtle shifts in drug susceptibility were observed.

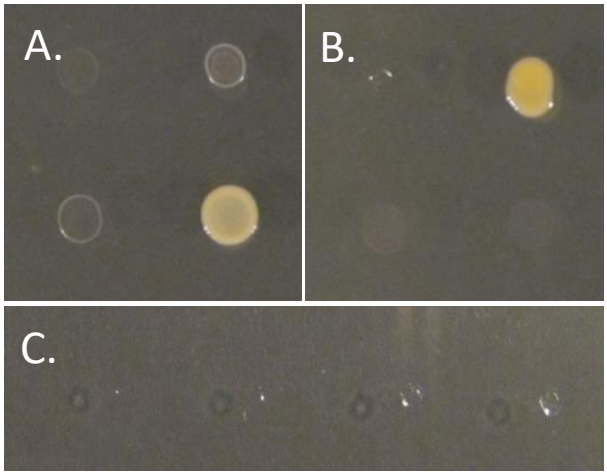


Figure 23: The agar screen allows the identification of strains displaying reduced drug susceptibility to be carried out in a high throughput manner.

Image shows portion of chloramphenicol screening plate, on which 8 NTML library strains and 4 replicates of the control strain, JE2 have been spotted. The 'hit' strains displaying strong growth (A, bottom right) SAUSA300_1628, (B, top right) SAUSA300_0977 & the control strain (C) JE2, which displays no growth. Plate contains chloramphenicol at a concentration of 1.45 x agar MIC

In total, 30 strains which displayed reduced drug susceptibility were validated, across 9 out of the 14 antibiotics screened (Table 6). However, no strains were confirmed to have reduced susceptibility against the following agents: tigecycline, rifampicin or novobiocin. For each of these compounds, preliminary hits were identified in the agar screen, yet these strains could not be validated in broth MICs. This confirmed the likelihood of the agar screen yielding false positives as cell density was not accounted for. Of the antibiotics screened, streptomycin generated the most hits, with 9 confirmed strains displaying reduced drug susceptibility. This is in comparison to fusidic acid, fosfomycin and trimethoprim, for which only one validated hit, SAUSA300_2233, SAUSA300_1255 & SAUSA300_0846 respectively, were identified. Furthermore, when comparing the shifts in MIC values of validated hits with those recorded for JE2, it is also evident that the loss of certain membrane transporters can impact the drug susceptibility to different extents. For instance, the most pronounced shift in MIC value from that recorded for the parental strain was recorded for the validated hits SAUSA300_2587, SAUSA300_0977 & SAUSA300_1628, which were identified in the chloramphenicol

screen. These strains possessed an MIC value which was 4-fold higher (8 µg/ml) than the value recorded for JE2 (2 µg/ml). This is in comparison to the validated streptomycin hits, SAUSA300_2035, SAUSA300_2298 & SAUSA300_2627, for which the MIC value recorded (6 µg/ml) was only moderately higher (1.5 x MIC) than that recorded for JE2 (4 µg/ml). These strains possessed the smallest shift in MIC value which could be confirmed by our screen.

Of the 30 hits identified in this study, 14 strains were identified which displayed reduced drug susceptibility to aminoglycosides (streptomycin and gentamicin). Interestingly, no hits were shared between either of these compounds, despite them being from the same antibiotic class.

Table 6: Identities of verified hits alongside broth MIC values.

*Fold change in relation to the broth MIC value for the parental strain, JE2.

				Broth MIC results (µg / ml)		
Antibacterial agent	Verified hits	Accession number	Strain description	JE2	Strain of interest	Fold change*
Fusidic Acid	NE1541	SAUSA300_2233	BioY family protein	0.031	0.062	2
Gentamicin	NE628	SAUSA300_2349	Formate/nitrite transporter family protein	0.125	0.357	2.9
	NE889	SAUSA300_2135	High affinity proline permease		0.313	2.5
	NE1290	SAUSA300_2576	Phosphotransferase system, fructose-specific IIABC component		0.313	2.5
	NE1516	SAUSA300_0606	Hypothetical protein		0.313	2.5
Tetracycline	NE846	SAUSA300_0615	Putative monovalent cation/H+ antiporter subunit F	0.25	0.5	2
	NE1780	SAUSA300_0568	Hypothetical protein		0.5	2
Fosfomycin	NE1360	SAUSA300_1255	Oxacillin resistance-related FmtC protein	1	2	2
Trimethoprim	NE967	SAUSA300_0846	Na+/H+ antiporter family protein	6	12	2
Streptomycin	NE1034	SAUSA300_0680	norA - multidrug resistance protein	4	10	2.5
	NE1709	SAUSA300_0566	Amino acid permease		10	2.5
	NE773	SAUSA300_2451	Drug transporter		8	2
	NE743	SAUSA300_0180	Integral membrane protein LmrP		8	2
	NE528	SAUSA300_0091	Putative permease		8	2
	NE423	SAUSA300_2035	Sensor histidine kinase, KdpD		6	1.5
	NE622	SAUSA300_0313	Putative nucleoside permease NupC		8	2
	NE781	SAUSA300_2298	Multidrug resistance protein B, drug resistance transporter		6	1.5
	NE1418	SAUSA300_2627	2-oxoglutarate/malate translocator		6	1.5
Chloramphenicol	NE44	SAUSA300_1300	Branched-chain amino acid transport system II carrier protein	2	4	2
	NE45	SAUSA300_2587	Accessory secretory protein Asp1		8	4
	NE123	SAUSA300_0977	Cobalt transport family protein		8	4
	NE610	SAUSA300_1628	Lysine-specific permease		8	4
Ciprofloxacin	NE129	SAUSA300_1547	DNA internalization-related competence proteinComEC/Rec2	8	16	2
	NE144	SAUSA300_0139	Putative tetracycline resistance protein		16	2
	NE146	SAUSA300_0718	Iron compound ABC transporter, permease		16	2
	NE252	SAUSA300_0171	Cation efflux family protein		16	2
	NE457	SAUSA300_0202	Cation efflux family protein		16	2
	NE931	SAUSA300_0308	ABC transporter permease		16	2
D-cycloserine	NE923	SAUSA300_2286	Hypothetical protein	32	62	1.9
	NE1025	SAUSA300_0729	Integral membrane protein		62	1.9
	NE1774	SAUSA300_2358	ABC transporter permease		64	2

4.4.4. Recovery of antibiotic susceptibility via genetic complementation of disrupted membrane transporters

Genetic complementation ensures that the phenotype observed is the direct result of the expression status of a gene of interest, as phenotypic changes can also arise from either mutations (Komp Lindgren *et al.*, 2005) or off target effects of a transposon insertion on the expression of downstream genes (Ciampi *et al.,* 1982). Therefore, to ensure that the phenotype of reduced drug susceptibility in a validated hit was the result of the disrupted expression of genes encoding respective membrane transporters, the NTML library strains were complemented with a functional copy of the native transporter gene. From the hits which were validated, strains which exhibited the largest shift in MIC value when in comparison to that recorded for the parental strain were chosen to be taken forward for complementation, as these results would be the easiest to discern for this initial proof of principle experiment. As such, hits validated for tetracycline, trimethoprim, chloramphenicol and ciprofloxacin were initially taken forward for complementation, as the MIC values recorded for these strains were at least doubling dilution higher than those recorded for the parental strain, JE2 (Table 6). All verified hits which were successfully complemented are shown in Table 7. A list of constructs made, but which proved unsuccessful in the complementation of identified hits can be found in Table 8.

3.4.4.1. Complementation of the trimethoprim hit, SAUSA300_0846 using pRAB11

Initially it was hoped that the shuttle vector, pRAB11 could be used for the complementation of all aforementioned validated hits. The vector, pRAB11 is a well characterised, tetracycline-regulated expression plasmid derived from pRMC2 (Helle *et al*., 2011). Furthermore, this system has been used previously to express staphylococcal membrane proteins, such as the transmembrane protein, VraH (Popella *et al.,* 2016).

For this study, the tetracycline derivative, anhydrotetracycline (ATc) was chosen as an inducer, as this agent is a stronger inducer than tetracycline itself.

Only one validated hit, the Na+/H+ antiporter, SAUSA300_0846 was successfully complemented using this system. When induced with 5 ng/ml ATc, the pRAB11 vector encoding the native *0846* gene successfully restored trimethoprim susceptibility to the level observed for the parental strain, reversing the reduced susceptibility phenotype associated with the Tn disrupted strain. No change in trimethoprim susceptibility was seen in the vector only control. This construct was created and susceptibility testing carried out under my supervision by, G. Morrison-Williams & T. O'Brien.

Complementation of the streptomycin (SAUSA300_2451, SAUSA300_0680 & SAUSA300_0566) and gentamicin hits (SAUSA300_2349, SAUSA300_2135, SAUSA300_2576 & SAUSA300_0606) was also attempted using pRAB11, however this proved unsuccessful. Vectors containing each insert were successfully constructed and were transformed into their appropriate Tn disrupted strains. However, the vector only controls displayed an increase in drug susceptibility during induction at ATc concentrations of 1 – 25 ng/ml, reducing the MIC values of these strains to that of the parental strain. A reduction in drug susceptibility was not observed for JE2, however it is possible that some of the Tn disrupted strains are more susceptible to ATc than the parental strain. Therefore, pRAB11 was deemed an unsuitable plasmid for use in further complementation experiments.

3.4.4.2. Complementation of the tetracycline hit, SAUSA300_0615 using pSK5478

In an attempt to circumvent the issues experienced when using pRAB11, complementation of the remaining streptomycin, gentamicin and tetracycline hits were then attempted using the pSK5478 vector. This plasmid contains the constitutive, low-level *S. aureus* promoter P_{qacR}, therefore it was reasoned that this vector may be an

alternative choice to pRAB11, as no agent is required to induce gene expression. As such, complementation using this vector was successfully achieved for the putative monovalent cation antiporter identified in the tetracycline screen, SAUSA300_0615. Expression of *0615* in the Tn disrupted strain was able to restore the MIC value to that of the parental strain, JE2. Furthermore, there was no change in drug susceptibility in the vector only control. This work was carried out under my supervision by, T. O'Brien.

Complementation of the seven remaining streptomycin and gentamicin hits was also attempted using the pSK5478 P_{qacR} vector. No positive clones in *E. coli* were obtained for the streptomycin hit, SAUSA300_2451, or the gentamicin hits, SAUSA300_2349, SAUSA300_2135 and SAUSA300_0606. This suggests that these genes may be toxic when expressed in *E. coli*, therefore direct ligation into *S. aureus* RN4220 was attempted, but was however unsuccessful. Where transformants were recovered (SAUSA300_0680, SAUSA300_2576 and SAUSA300_0566) the insertion sequence and P_{qacR} promoter was subjected to DNA sequencing. In each instance, sequencing revealed the accumulation of mutations in either the coding sequence, the 5' untranslated region, the P_{qacR} promoter or a combination thereof (data not shown). This supports the idea that expression of these genes may be toxic in *E. coli*. Consequently, complementation using the pSK5478 P_{qacR} vector proved unsuccessful.

3.4.4.3. Complementation using plasmid pLOW

The lack of positive clones obtained using the pSK5478 P_{qacR} vector suggests that even low level expression of certain staphylococcal membrane proteins are toxic when overexpressed in both *E. coli* & *S. aureus*. Therefore, I sought an alternative low-copy number plasmid in which gene expression can be controlled by an inducible promoter. The $pLOW_{kan}$ shuttle vector is a low copy number plasmid which includes a multiple cloning site (MCS) downstream of the IPTG inducible, P_{spac} promoter. This plasmid was a derivative of the pLOW plasmid, in which the erythromycin resistance gene, *ermC* was switched for the kanamycin resistance cassette *aphA-3* from pTM378 (unpublished

data, created in this laboratory by A. Gupta). Furthermore, the P_{spac} promoter is relatively weak in strength, therefore it was thought that this vector may be useful in order to clone the potentially toxic membrane transporter genes.

Complementation of the ciprofloxacin hits, SAUSA300_0202 & SAUSA300_0308, as well as the chloramphenicol hits, SAUSA300_1300, SAUSA300_2587 & SAUSA300_1628, was successfully achieved using $pLOW_{kan}$. In relation to ciprofloxacin, drug susceptibility was restored to parental levels in the completed strains SAUSA300_0202 (pLOW:*0202*) & SAUSA300_0308 (pLOW:*0308*) when induced with 0.125 mM IPTG. In regards to chloramphenicol, drug susceptibility was restored to parental levels in the following complemented strains, SAUSA300_1300 (pLOW:*1300*), SAUSA300_2587 (pLOW:*2587*) & SAUSA300_1628 (pLOW:*1628*) after induction with 0.125 mM IPTG. Additionally, the complementation effect for both the ciprofloxacin and chloramphenicol hits was lost at concentrations of ≥ 0.5 mM IPTG. This suggests that overexpression of these membrane transporter genes are toxic in *S. aureus*.

In contrast to this, complementation of the tetracycline hit, SAUSA300_0568 could not be achieved using $pLOW_{kan}$. Induction of the complemented strain, SAUSA300_0568 (pLOW:*0568*) using concentrations of 0.06 – 0.5 mM IPTG did not alter tetracycline susceptibility. This suggests that downstream effects of the Tn insertion on other genes, or accumulated mutations could be the result of the altered tetracycline susceptibility displayed by this mutant.

For the other validated hits (the chloramphenicol hit, SAUSA300_0977, the ciprofloxacin hits, SAUSA300_0718 & SAUSA300_0171, the gentamicin hits SAUSA300_2349, SAUSA300_2135, SAUSA300_2576 & SAUSA300_0606 and the D-cycloserine hits, SAUSA300_2286, SAUSA300_0729 & SAUSA300_2358) positive pAG01 clones were successfully obtained in *E. coli*. However, no transformants for any of the aforementioned constructs could be recovered in RN4220. In order to tightly

regulate the expression of genes under control of the P_{spac} promoter, the constructs were re-transformed into RN4220 containing the plasmid, pGL485 that constitutively expresses the Lac suppressor, LacI. Despite additional LacI expression from the pGL485 plasmid positive clones could still not be obtained, suggesting that the level of expression of LacI from both pGL485 & $pLOW_{kan}$ was insufficient to suppress the activity of the P_{spac} promoter. The GL485 plasmid was not sequenced, therefore we cannot rule out the possibility of a faulty lac repressor within the pGL485 plasmid.

3.4.4.4. Complementation of genes associated with toxicity, using the low copy number vector, pRB474

An alternative vector was then sought which could overcome the issues of toxicity in relation to permease expression. The shuttle vector pRB474 is a derivative of pRB374 in which the neomycin resistance gene has been replaced with a chloramphenicol resistance cassette. This is a low copy number vector lacks a promoter, allowing genes to be cloned into the MCS alongside their native promoters to achieve a level of expression similar to that seen in wild type cells. Past studies have used this plasmid to complement the staphylococcal penicillin binding protein 2a gene, *mecA*,(Pozzi et al., 2012) a gene which has been associated with toxicicty when cloned into other vectors. Therefore it was reasoned that this vector may be suitable in the complementation of the membrane transporter genes which had displayed toxicity issues in this study.

In regards to ciprofloxacin, drug susceptibility was restored to parental levels in the completed strains SAUSA300_0718 (pLOW:*0718*) & SAUSA300_0171 (pLOW:*0171*). This was also the case for the gentamicin complemented strains SAUSA300_2135 (pLOW:*2135*) & SAUSA300_2576 (pLOW:*2576*), in addition to the D-cycloserine complemented strains SAUSA300_2286 (pLOW:*2286*) & SAUSA300_2358 (pLOW:*2358*). Furthermore, no changes in drug susceptibility were seen in vector only controls.

Table 7: Verified hits which were successfully complemented.

	Accession number	Complementation - Broth MIC (µg / ml)		
Antibacterial agent	of verified hit	JE2	Strain of interest	Construct
Gentamicin	SAUSA300_2135	0.125	0.125	SAUSA300_2349 (pRB474:*2349*)
	SAUSA300_2576		0.125	SAUSA300_2576 (pRB474:*2576*)
Tetracycline	SAUSA300_0615	0.125	0.125	SAUSA300_0615 (pSK5478:*0615*)
Trimethoprim	SAUSA300_0846	6	6	SAUSA300_0846 (pRAB11:*0846*) (+ 5 ng/ml Antet)
Chloramphenicol	SAUSA300_1300	2	2	SAUSA300_1300 (pAG01:*1300*) (+ 0.125 mM IPTG)
	SAUSA300_2587		2	SAUSA300_2587 (pAG01:*2587*) (+ 0.125 mM IPTG)
	SAUSA300_1628		2	SAUSA300_1628 (pAG01:*1628*) (+ 0.125 mM IPTG)
Ciprofloxacin	SAUSA300_0718	8	8	SAUSA300_0718 (pRB474:*0718*)
	SAUSA300_0171		8	SAUSA300_0171 (pRB474:*0171*)
	SAUSA300_0202		4	SAUSA300_0202 (pAG01:*0202)* (+ 0.125 mM IPTG)
	SAUSA300_0308		4	SAUSA300_0308 (pAG01:*0308*) (+ 0.125 mM IPTG)
D-cycloserine	SAUSA300_2358	32	32	SAUSA300_2358 (pRB474:*2358)*
	SAUSA300_2286		32	SAUSA300_2286 (pRB474:*2286*)

Table 8: List of constructs which failed to complement verified hits.

Vector	Accession number of gene included in construct	Issue
pRAB11	SAUSA300_2451 SAUSA300_0680 SAUSA300_0566 SAUSA300_2349 SAUSA300_2135 SAUSA300_2576 SAUSA300_0606	Changes in drug susceptibility observed for vector only control.
pSK5478	SAUSA300_2451 SAUSA300_2349 SAUSA300_2135 SAUSA300_0606 SAUSA300_0680 SAUSA300_2576 SAUSA300_0566	Toxic - no positive clones obtained within *E. coli,* or after direct ligation into *S. aureus,* RN4220.
pLOW$_{kan}$	SAUSA300_0977 SAUSA300_0718 SAUSA300_0171 SAUSA300_2349 SAUSA300_2135 SAUSA300_2576 SAUSA300_0606 SAUSA300_2286 SAUSA300_0729	Toxic- Positive clones obtained in *E. coli,* however no transformants recovered in *S. aureus,* RN4220.

4.4.5. Creation of multiple transposon inactivated strains

If lipoidal diffusion of cytotoxic drugs occurred at a level relative to that occurring through membrane transporters, disruption in the expression of those membrane transporters would not lead to reduced susceptibility to that drug, as the drug would still have a route of entry and accumulate within the cell. Likewise, if a strain were to lose all transporters involved in the uptake of a drug and the susceptibility of the strain to this agent would decrease further, this would suggest that lipoidal diffusion does not occur alongside transporter facilitated uptake for that compound. To investigate whether the disruption of multiple transporters would show a greater reduction in antibiotic susceptibility than a single Tn disrupted strain, we sought to create strains which had Tn disruptions in multiple genes which had been identified as playing a putative role in drug uptake.

Multiple Tn inactivated mutants were created using genes associated with a reduction in drug susceptibility for both chloramphenicol and ciprofloxacin. In the case of ciprofloxacin, the double and triple transposon inactivated strains were generated. Allelic replacement was first used in order to generate the kanamycin resistant strain, SAUSA300_0202$_{kan}$ from the NTML library strain, SAUSA300_0202 (containing the erythromycin resistance cassette). The *0308$_{erm}$* gene was then transduced into the SAUSA300_0202$_{kan}$ strain, forming SAUSA300_0202$_{kan}$/0308$_{erm.}$ This double Tn inactivated strain was found to be equally susceptible to ciprofloxacin compared with SAUSA300_0202 or SAUSA300_0308, both of which have a ciprofloxacin MIC of 16 µg/ml. This strain was created by, G. Morrison-Williams, who was under my supervision. This is in comparison to JE2, which has a ciprofloxacin MIC of 8 µg/ml. The triple Tn inactivated strain, SAUSA300_0202$_{kan}$/0308$_{erm}$/0171$_{spec}$ was also created. This strain displayed a ciprofloxacin MIC of 32 µg/ml.

In the case of chloramphenicol, double and triple Tn inactivated mutants were generated. Allelic replacement was first used to create the kanamycin resistant strain, SAUSA300_2587$_{kan}$ from SAUSA300_2587, and the spectinomycin resistant strain,

SAUSA300_1300$_{spec}$ from SAUSA300_1300. To make the double Tn inactivated strain, the *2587$_{kan}$* gene was then transduced into SAUSA300_1628 creating, SAUSA300_1628$_{erm}$/2587$_{kan}$. Afterward the double Tn inactivated mutant was used to create the triple Tn inactivated strain, SAUSA300_1628$_{erm}$/2587$_{kan}$/1300$_{spec}$, after transduction of *1300$_{spec}$*. Both the double and triple Tn inactivated mutants demonstrated a shift in MIC 4 x greater than their single Tn inactivated counterparts, possessing a ciprofloxacin MIC of 32 µg/ml.

4.4.6. The native substrates of the membrane transporter, BrnQ can provide a protective effect against the antibacterial action of chloramphenicol

Due to the association of the Tn disrupted strains SAUSA300_1300, SAUSA300_2587, SAUSA300_0977 & SAUSA300_1628 with reduced chloramphenicol susceptibility, a competition assay was performed between chloramphenicol and the native substrates of the respective encoded membrane transporters. Checkerboard assays are a common way to assess whether substrates compete for the same transport system. As such, they have been used in the past to characterise substrates of membrane transporters, for instance competition between D-cycloserine and alanine for entry through the amino acid transporter, CycA (Baisa *et al.,* 2013). The gene products of *1300, 2587, 0977 & 1628* are BrnQ, a branched chain amino acid transporter, Asp1, a transporter of serine rich proteins, a cobalt permease and Lys-P, a lysine specific permease respectively, therefore the branched chain amino acids, leucine, isoleucine & valine, as well as serine, cobalt & lysine were included in the assay.

At concentrations of 1.25 – 20 mM, the amino acids leucine, isoleucine & valine decreased the sensitivity of the parental strain, JE2 to chloramphenicol. Concentrations below 1.25 mM had no effect. The addition of serine or lysine (at the same concentrations described above) or cobalt (10 µM – 700 µM) had no impact on the chloramphenicol MIC (Figure 25). These results suggest that in the case of

SAUSA300_1300, branched chain amino acids are in direct competition with chloramphenicol through the respective membrane transporters, BrnQ.

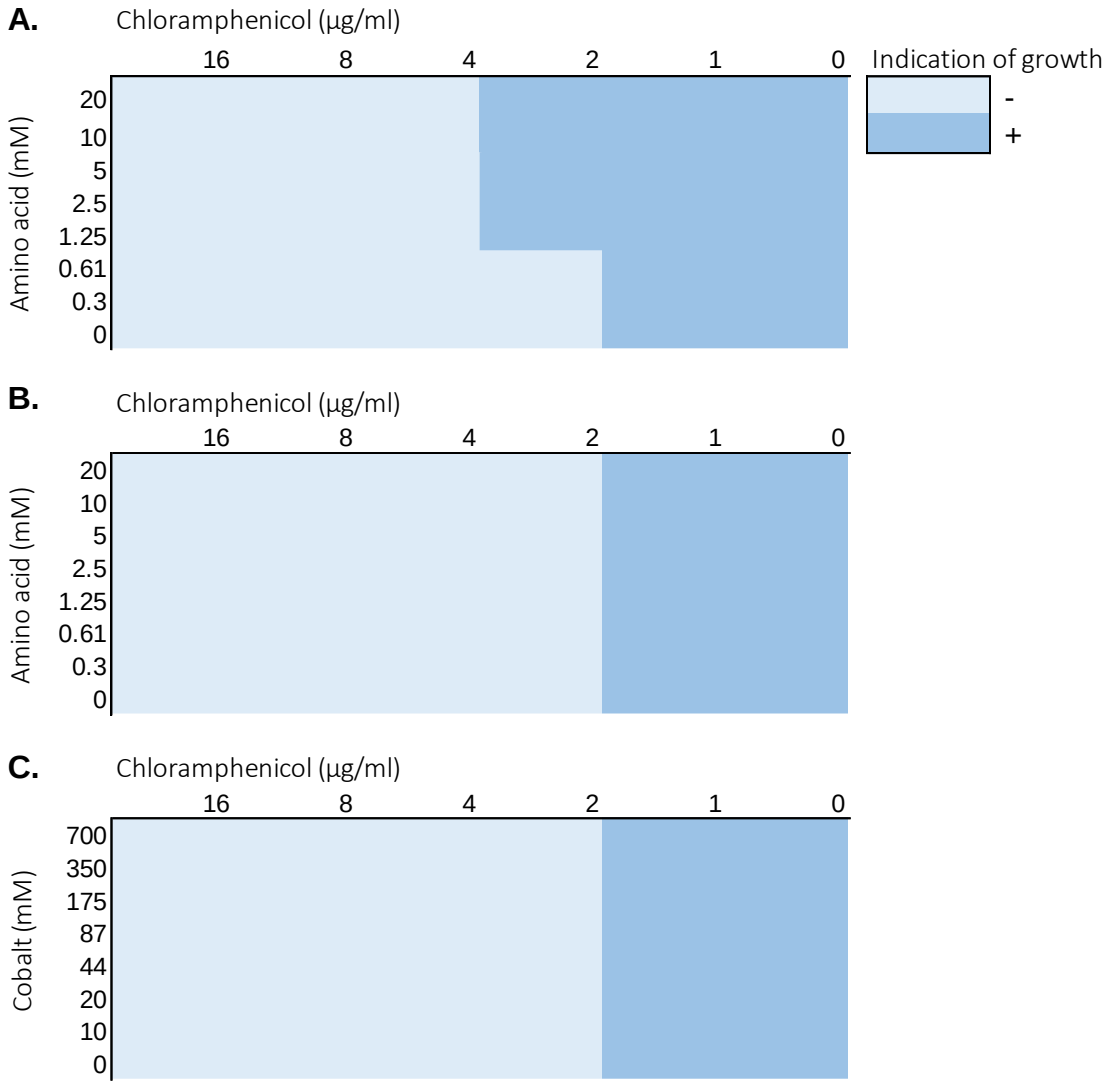


Figure 24: Competition assays between chloramphenicol and the native substrates of the Tn disrupted membrane transporters, which displayed reduced chloramphenicol susceptibility.

Chloramphenicol susceptibility of the parental strain, JE2, in the presence of varying concentrations of amino acids. (A.) Results shown for a chloramphenicol MIC in combination with leucine, isoleucine or valine, and (B.) serine or lysine or (C) in combination with cobalt.

4.4.7. Drug accumulation is lower in Tn inactivated mutants than in the parental strain

For transporters which were confirmed to play a role in drug entry via genetic complementation, it was reasoned that the reduction in drug susceptibility in these strains was the direct result of the disrupted expression of the encoded membrane transporter. As the detectable shift in drug susceptibility suggests, this is most likely due to the reduced ability of the drug to enter the cell, leading to lower intracellular accumulation. In order to test this theory, ciprofloxacin accumulation assays were carried out for JE2, three of the single Tn inactivated strains which displayed reduced ciprofloxacin susceptibility (SAUSA300_0171, SAUSA300_0308, SAUSA300_0202), and the triple Tn inactivated strain (SAUSA300_0202_{kan}/0308_{erm}/0171_{spec}). The significance of drug accumulation of the Tn inactivated mutants against the parental strain, JE2, was determined using an unpaired T test (Figure 26).

When in comparison to JE2, the reduced levels of ciprofloxacin accumulation were significant in the strains, SAUSA300_0171 ($P \leq 0.001$), SAUSA300_0308 ($P \leq 0.01$) and the triple Tn inactivated strain ($P \leq 0.001$). In the strain, SAUSA300_0202, average intracellular ciprofloxacin was 0.19 µM lower than in JE2. However, this reduction was not identified as being significant when included in the T test.

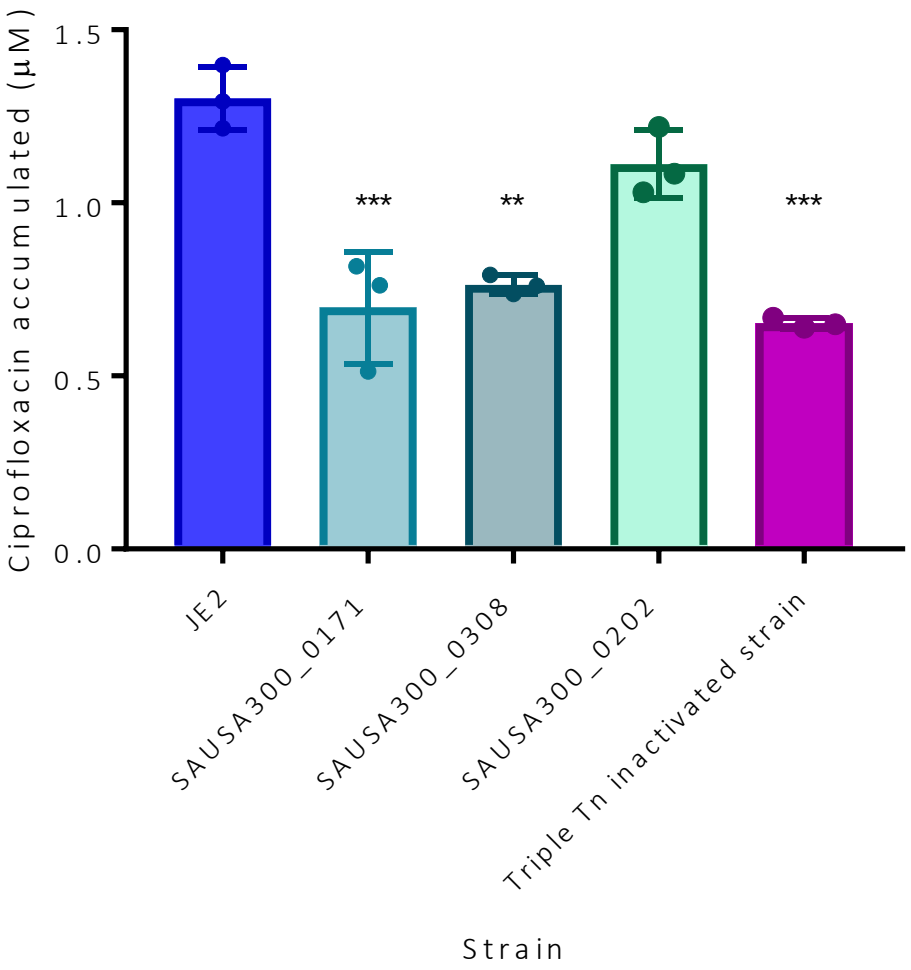


Figure 25: Ciprofloxacin accumulation is lower in the Tn inactivated strains which were identified as having reduced susceptibility to this drug, than in JE2.

Ciprofloxacin was added to cells at a working concentration of 10 µM. Cells were incubated with drug for 10 minutes before samples were removed for LC/MS analysis. Results are expressed as the mean of drug accumulation from 3 biological replicates. Significance was determined using an unpaired T test.

4.5. Discussion

During this study, 30 *S. aureus* strains containing Tn disruptions in membrane transporter genes were identified which displayed an altered phenotype of reduced drug susceptibility against a panel of 9 antibiotics. Where possible, the Tn disrupted strains were complemented with a native copy of the respective transporter gene. In addition to this, the impact of double and triple Tn inactivations on drug susceptibility and drug accumulation were investigated. In some instances where the native substrate of a transporter was known, competition studies were carried out between the native substrate and the drug to which the reduced susceptibility phenotype was associated. Accumulation assays were also carried out. The results of this study have demonstrated that in some of these instances, the respective transporters encoded by the 30 validated genes may facilitate the carrier mediated uptake of antibiotics into *S. aureus*, although some additional characterisation is still needed. Overall, the results of this study have demonstrated that membrane transporters provide an important route of entry for antibiotics into prokaryotic cells. Furthermore, characterisation of the chemical space occupied by the native substrates of the transporters identified in this assay could therefore outline some of the physico-chemical properties required for compounds to accumulate within *S. aureus*.

The identification of multiple validated hits for most of the antibiotics screened in this study suggests that transporter facilitated uptake plays an important role in the entry of drugs into the bacterial cell. During the preliminary screens, many Tn disrupted strains were validated which displayed reproducible shifts in test antibiotic MICs, when in comparison to the parental strain, JE2. A total of 31 strains were identified which displayed reduced drug susceptibility to fusidic acid, gentamicin, streptomycin, tetracycline, fosfomycin, trimethoprim, chloramphenicol, ciprofloxacin or D-cycloserine (Table 5). Furthermore, it became evident that there may be some redundancy in antibiotic uptake. With the exception of fusidic acid, several Tn disrupted transporter

mutants were validated for each of the antibiotics included in the screen. For a limited number of membrane proteins to interact with and transport the many diverse compounds that a cell will experience in nature, a degree of substrate promiscuity is required. As such, the results of this study are unsurprising as this is a well-documented phenomenon among other membrane complexes, such as multidrug resistance pumps (Lewinson *et al.,* 2006) and general porins (Nikaido, 1994a, Delcour, 2003). Moreover, often single amino acid changes in metabolite permease proteins can result in a change of substrate specificity (Olsen et al., 1993, King and Wilson, 1990), further highlighting the favourability of transporter promiscuity. Further supporting this theory was that during the preliminary screens, many strains were identified which displayed subtle changes in antibiotic susceptibility (< 2 x MIC of the parental strain), suggesting the involvement of multiple transporters in the uptake of a single drug. Although there are cases where one documented transport system predominates in the uptake of an antibiotic, such as fosfomycin and D-cycloserine (will be discussed below), the results of this study suggest that in most cases, multiple membrane transporters can contribute weakly to the permeation of a single drug into the bacterial cell. The loss of single transporters are often not recognised to be involved in drug resistance clinically, for instance even the loss of outer membrane porins in Gram-negative pathogens often do not contribute to high level resistance (Nikaido, 1994b). Therefore this may be one of the reasons as to why the lipoidal model of passive drug uptake has been the favoured dogma for drug entry into cells.

The ability to clearly identify strains with Tn disruptions in the genes *glpT* and *uhpT* (SAUSA300_0216 & SAUSA300_0337) from a library of 258 strains in a fosfomycin screen validated this approach, ensuring the suitability of this method for the identification of mutants with reduced drug susceptibility. Interestingly, the preliminary fosfomycin screens also yielded the hit, SAUSA300_1255, possessing a broth MIC value double that of the parental strain. This mutant contained a Tn disruption in the

gene encoding the poorly characterised oxacillin resistance-related protein, FmtC. Furthermore, the successful complementation of the Tn disrupted strain, SAUSA300_1255 with the native gene confirms the role of this membrane protein in fosfomycin susceptibility. It has been postulated that FmtC has a role in cell wall biosynthesis, and BLAST analysis of this gene reveals 99 % sequence identity to provisional lysylphosphatidylglycerol synthetases, proteins involved in the modification of membrane lipids. Since *1255* is poorly characterised, as to how this gene may impact fosfomycin susceptibility remains obscure. However, these results suggest that transporters other than GlpT and UhpT may contribute to the uptake of fosfomycin within *S. aureus*.

Three putative transporters (SAUSA300_2286, SAUSA300_0729 & SAUSA300_2358) were identified to be involved in the uptake of D-cycloserine, suggesting that transporters other than the D-alanine-glycine system could be involved in the influx of this antimicrobial. BLAST analysis of the genes, *2286* & *0729* reveals that the encoded proteins share domains with the uncharacterised membrane proteins, YnfA & YjjP respectively. It has been postulated that YnfA has a role in drug efflux(Sarkar et al., 2015) and the function of YjjP is entirely unknown, therefore the link between these genes and D-cycloserine susceptibility remains obscure. Complementation of the Tn disrupted strain with the *2286* gene under the control of its native promoter did however restore drug susceptibility to that observed in the parental strain. Interestingly, it has been suggested that the YnfA transporter (which displays homology with SAUSA300_2286) may exist adopt a dual topology in cells, therefore this protein could be involved in ingress as opposed to efflux (Rapp *et al.*, 2006). The putative ABC permease, encoded by the gene *2358* was also linked to a reduced susceptibility to D-cycloserine. Moreover, the role of this gene in D-cycloserine entry within *S. aureus* was confirmed by complementation. This protein displays homology with cysteine permeases, in addition to containing domains conserved in the histidine permease,

HisM. It could be possible therefore that D-cycloserine hijacks multiple amino acid transport systems when entering the cell.

Of all the classes of antibiotics screened, the aminoglycosides yielded the most validated hits. In fact, 13 out of the 31 strains identified displayed reduced drug susceptibility to either streptomycin or gentamicin. Aminoglycoside antibiotics contain metabolite moieties such as aminosugar groups and their highly conserved aminocyclitol ring,(Zembower et al., 1998) therefore it could be reasoned that their structural motifs bearing a likeness to metabolites could be the reason for their recognition by bacterial membrane transporters. Several transporters were identified in the screen which recognise similar motifs, such as a fructose-specific phosphotransferase (SAUSA300_2576), the putative nucleoside permease, NupC (SAUSA300_0313) & a high affinity proline permease (SAUSA300_2135). Complementation of the associated Tn disrupted strains is required to confirm the role of the aforementioned transporters in aminoglycoside uptake. Complementation of SAUSA300_2135 & SAUSA300_2576 with native copies of the respective Tn disrupted genes *2135* & *2576* confirms the role of these permeases in the entry of gentamicin within *S. aureus*. Future studies could involve competition assays between the native substrates of these transporters with gentamicin, to assay if the substrates of these transporters can provide a protective effect against the antibacterial action of this aminoglycoside.

The exact mechanism of aminoglycoside uptake is yet to be fully characterised, but it is known to occur via two energy dependent phases across the cytoplasmic membrane. Although energy dependant, the nature of aminoglycoside uptake displays passive diffusion kinetics (Bryan and Van Den Elzen, 1977). Aside from passive diffusion, this phenomenon could be explained by the uptake of drugs through multiple, low affinity transporters. Furthermore, complementation of the *2135* gene encoding a proline permease within the Tn disrupted strain restored susceptibility to the level of the

parental strain. Although complementation of the remaining aminoglycoside hits would be required before we can confirm the role of all the transporters validated during the streptomycin and gentamicin screens, the findings of this study suggest that protein carriers and their significance in uptake of aminoglycosides warrants further investigation.

During this study four membrane transporters were identified which could play a role in the uptake of the synthetic protein synthesis inhibitor, chloramphenicol. When researching the literature, it becomes clear that there is a lack of information regarding the entry mechanism of this antibiotic. The Tn disrupted transporters identified in this study displayed considerable shifts in chloramphenicol resistance, with all but one transporter possessing an MIC value 4-fold higher than that recorded for JE2. BLAST analysis of these genes reveals they possess high sequence identity to amino acid permeases, such as a branched chain amino acid transporter and a lysine specific permease, in addition to a metal ion transporter, cobalt permease. Complementation of *1300, 2587* & *1628* restored susceptibility to the level of the parental strain. Furthermore, competition between chloramphenicol and the native substrates of the branched chain amino acid transporter, BrnQ (SAUSA300_1300) confirms the role of this transporter in the entry of chloramphenicol. Strains with Tn disruptions in the cobalt permease (SAUSA300_0977) and a member of the serine-rich glycoprotein transporter system, Asp1 (SAUSA300_2587) were also identified as having reduced chloramphenicol susceptibility. However, competition assays which included cobalt or serine failed to antagonise the action of chloramphenicol. Therefore, the role of cobalt permease or the Sec system component, Asp1 in the uptake of chloramphenicol still requires further investigation. Accumulation studies could further add to the information supporting the role of SAUSA300_2587, SAUSA300_0977 & SAUSA300_1628 in chloramphenicol entry.

It has been proposed that a transport independent mechanism is responsible for the uptake of ciprofloxacin and tetracycline across the cytoplasmic membrane. The quinolones and tetracyclines are thought to traverse the cytoplasmic membrane in a similar manner; as they are both weakly lipophilic agents with multiple protonation sites, their uptake is believed to be facilitated by the pH gradient across the membrane. It is postulated that both classes cross the bilayer in their protonated, neutral forms, however in the cytoplasm they may chelate metal ions, such as Mg^{2+}. The resulting ionic forms are thought to drive the movement of neutral species across the bilayer, achieving an uphill accumulation of these agents within the cytoplasm.(Piddock, 1991, Yamaguchi *et al*., 1991, Nikaido and Thanassi, 1993) The cellular accumulation of fluoroquinolones and tetracyclines also lacks saturation kinetics,(Nikaido and Thanassi, 1993) which is usually suggestive of lipoidal uptake. Again, the passive uptake of these agents, facilitated by multiple membrane transporter complexes could also explain this phenomenon. In line with this, the results of this study suggest that multiple membrane complexes may facilitate the uptake of both ciprofloxacin and tetracycline, as 10 membrane transporters were validated to play a putative role in ciprofloxacin uptake, whereas two were identified with regards to tetracycline. Interestingly, no membrane transporters were identified which displayed a reduced susceptibility to the glycylcycline, tigecycline.

BLAST analysis of the respective ciprofloxacin hits revealed the identification of several transporters which play a role in the transport of cationic amino acids and metal ions, such as nickel and iron (SAUSA300_0139 & SAUSA300_0718 respectively). This is interesting, considering the documented role of metal ion chelation and the formation of cationic drug species in the uptake of quinolones. It has been well documented that an increase in metal ion concentration can inhibit quinolone activity and uptake,(Turel, 2002, Alkaysi *et al.,* 1992, Lecomte *et al.,* 1994) and has been previously attributed to the formation of metal ion-drug complexes. Alternatively, this may be indicative of

competition between metal ions and the drug when interacting with the membrane transporter. Similarly, a monovalent cation importer was recognised to play a putative role in the uptake of tetracycline, with a BLAST analysis giving a hit with 100 % sequence identity to the putative Na+/H+ antiporter, MnhF. Complementation of the ciprofloxacin hits (SAUSA300_0718, SAUSA300_0171, SAUSA300_0202 & SAUSA300_0308) and one tetracycline hit (SAUSA300_0615) restored levels of drug susceptibility to that recorded for JE2, confirming the role of these transporters in drug entry within *S. aureus*.

Moreover, ciprofloxacin accumulation studies revealed reduced drug accumulation in the Tn disrupted strains, SAUSA300_0171, SAUSA300_0202, and the multiple Tn disrupted strain, SAUSA300_0202_{kan}/0308_{erm}/$0171_{spec.}$ The differences in drug accumulation in these strains was significant in comparison to JE2, analysed by an unpaired T test. The ciprofloxacin accumulation in the strain, SAUSA300_0308, was lower when in comparison to JE2, however was not considered significant. These results confirm the role of the transporters, SAUSA300_0171 & SAUSA300_0202 in the entry of ciprofloxacin. These findings also suggest that the loss of different transporters (as may be the case with SAUSA300_0308) contributes to drug influx to different extents.

Complementation of the tetracycline hit, *0615*, encoding a hypothetical protein, was unsuccessful, suggesting that the reduced susceptibility phenotype displayed by this strain is the result of other mutations which have arisen in this strain, or downstream effects of the Tn insertion on the expression of other genes. BLAST analysis of this protein reveals that it contains conserved domains displaying homology to those observed in a putative threonine exporter, ThrE. As the ThrE complex is involved in efflux as opposed to influx, it is likely that the change in susceptibility observed in SAUSA300_0615 is not due to the inactivation of the *0615* gene. Overall, these results suggest that membrane complexes could play a previously overlooked role in the uptake of both quinolones and tetracyclines within bacteria.

Due to transporter redundancy, strains containing Tn disruptions in transporter genes associated with reduced susceptibility to ciprofloxacin and chloramphenicol were created. If lipoidal uptake does not occur alongside membrane facilitated uptake of a drug, the inactivation of multiple transporters thought to be involved in drug uptake would lead to a further reduction in drug susceptibility. The results of this study have shown that in the case of chloramphenicol at least, the generation of a strains containing Tn disruptions in multiple genes associated with drug uptake strengthens the hypothesis that membrane transporters play a role in chloramphenicol entry within *S. aureus.* It is unclear why there is no further reduction in drug susceptibility between the double and triple inactivated mutants. Both single Tn inactivated strains, SAUSA300_2587 & SAUSA300_1628 displayed a greater reduction in drug susceptibility than SAUSA300_1300 when in comparison to JE2, therefore different combinations of transporters may have a different impact on susceptibility. Each transporter may have differing affinities for a compound, therefore Tn inactivated combinations of those with higher drug affinity would lead to a greater change in drug susceptibility. Overall, these results show that the inactivation of multiple genes associated with drug uptake is not always additive, as some drugs may exploit multiple routes of entry.

In the case of ciprofloxacin, there was no change in drug susceptibility between the double Tn inactivated and single inactivated strains when in comparison to the parental, JE2. Again, it is unclear why the combination of Tn inactivated genes in this case did not cause further changes in drug susceptibility. However, the triple Tn inactivated mutant did possess an MIC for ciprofloxacin which was twofold higher than that recorded for the double Tn inactivated strain. There were 10 strains validated to have reduced susceptibility to ciprofloxacin, therefore all encoded transporters may participate in the uptake of the drug. It is uncertain however why only a single Tn disruption in any of these validated genes can cause a twofold shift in drug susceptibility. As was demonstrated with the creation of the triple Tn inactivated mutant, in some

cases, multiple Tn disruptions may have to be combined in a single strain before an additional reductions in susceptibility are seen.

The transporters identified in this study may not be the only membrane proteins involved in the drug uptake within *S. aureus* USA300. Given the number of transporters identified as preliminary hits in the initial agar MICs, it is possible that other transporters may be involved in antibiotic uptake however the level of resistance afforded by them may not be substantial enough to discern from that of the parental strain using standard agar or broth MICs. Furthermore, the NTML library includes disruptions in many genes of unknown function, therefore there may be additional membrane transporter genes yet to be characterised, which were not included in our original screening panel. In fact, during the initial stages of this study, the relational database Transport DB identified 257 strains with Tn insertions in putative membrane transport proteins within *S. aureus* USA300 JE2. An updated search reveals an additional 106 strains. Further characterisation of the Tn disrupted genes within the NTML library, and the addition of any further identified membrane transporter genes to the screening panel could reveal additional transporters in *S. aureus* USA300 which may have a putative role in antibiotic uptake. In addition to this, the NTML library contains disruptions in non-essential genes, therefore conclusions to role of essential membrane complexes in the entry of antibiotics could not be made.

An evident problem in the expression of bacterial membrane transporters was the toxicity associated with the overexpression of many of these proteins. This is a well-documented issue in regards to the heterologous expression of membrane proteins (Wagner *et al.,* 2008, Miroux and Walker, 1996, Zoonens and Miroux, 2010), as overexpression can lead to competition between desired and native proteins for cellular machinery and insertion into the membrane. Often, this results in the formation of insoluble protein aggregates termed inclusion bodies within the cytoplasm, the formation of which is associated with toxicity and cell death (Zoonens and Miroux, 2010). In

hindsight, it was clear that many of the genes identified in this study needed to be expressed at very low levels in order to prevent toxicity issues. The most success was had if genes of interest were then cloned under the control of their native promoters into the shuttle vector, pRB474. However, even some strains (SAUSA300_2286, SAUSA300_0729 & SAUSA300_2349) could not be complemented using this vector. Use of a single copy integrative vector may be useful in future studies in the complementation of the remaining hits validated in this study. Expression of these genes as a single copy, under the control of their native promoters may allow the previous issues of toxicity associated with the expression of membrane proteins to be avoided.

4.6. Conclusions

In this study, the role of membrane transporters in the uptake of antibiotics within *S. aureus* was assessed. The commercially available, NTML library, was included in a screen which was developed to allow the identification of membrane carriers with a putative role in drug uptake. This screen enabled the identification of membrane transporters with a putative role in drug entry by using the reduction in drug susceptibility associated with the disrupted expression of a transporter involved in the entry an antibiotic. From the screen, 30 strains were identified which displayed reduced drug susceptibility, across 9 out of the 14 antibiotics screened. To confirm the role of these carriers in drug entry, Tn disrupted genes were complemented with a functional copy of the native gene, competition studies were carried out between antibiotics and the transporter substrates, accumulation assays were performed and strains containing Tn disruptions in multiple genes associated with drug uptake were made.

Further research is needed in order to provide a comprehensive analysis of the identified membrane proteins in the uptake of antibiotics within *S. aureus*, which would require complementation of the remainder of the validated hits, as well as performing additional competition and accumulation studies However, this study has provided a starting point in assessing the role of membrane carriers in the uptake of antibiotics within prokaryotes. Overall, the results have established that membrane transporters have a previously unrealised role in the entry of antibiotics within *S. aureus*. These results shed some light onto previously unrealised mechanisms of antibiotic entry within bacterial cells, supporting the growing body of evidence that carrier mediated uptake plays a significant role in the cellular ingress of compounds across all domains of life.

5. Discussion

There is currently no successful platform for the discovery of novel antimicrobials. The hurdles facing antibiotic drug discovery has led to a half-century long, 'innovation gap' during which there has been a severe lack of novel drug scaffolds in the development pipeline. Meanwhile, the efficacy of our existing agents is continually being eroded by the ever-growing prevalence of antibiotic resistance. Multi-drug resistance among clinically relevant pathogens is now commonplace, with a number of strains identified possessing resistance to all common antibiotic classes (Falagas *et al.,* 2005, Qureshi *et al.,* 2015, Rice, 2008). Antibiotics underpin modern medicine; unless the pipeline is stocked with novel classes of antimicrobial agents, we face the arrival of a 'post antibiotic era'. There is therefore a pressing need to re-invigorate antibiotic drug discovery; however, the scientific hurdles facing the discovery process must first be addressed if we are to be successful.

The overall aims of this study were to increase our understanding in regards to how antibiotics enter bacteria. In turn, this information could allow us to make rational steps towards addressing one of the major bottlenecks in antibiotic drug discovery. The primary issue that has hindered past discovery efforts (specifically when screening synthetic drug libraries for novel inhibitors or through structure based drug design) is overcoming the bacterial permeability barrier to deliver lead compounds to their intracellular targets (Payne *et al.,* 2007). There is a dearth of knowledge regarding the physico-chemical properties that are required of compounds to accumulate within bacteria in general, and in terms of Gram-negatives, avoid efflux.

A suitable way to obtain this information would be in the screening of large numbers of chemically diverse small molecules, for accumulation within bacteria. Analysis of positive accumulating ‘hits’ could reveal structural properties that are associated with bacterial entry and efflux avoidance. The first aim of this assay was to develop an LC/MS based assay which would allow us to carry out such an experiment. However, many of these rely on either a radiolabel or compound fluorescence for detection. In the past, multiple methods have been developed to measure compound accumulation within bacteria. The use of LC/MS based accumulation assay enables the detection of compounds within complex samples, without being limited to compounds containing fluorescent moieties or radiolabels. As previously discussed, during the course of this study a paper by Richter *et al.,* (2017) was published which described the use of an LC/MS based assay in a small molecule accumulation screen, within *E. coli*.

By screening the accumulation capabilities of 180 compounds within *E. coli*, the study by Richter *et al*, (2017) identified some physico-chemical properties that influence the ability of a compound to accumulate within this organism. The findings of Richter & colleagues study have revealed that the primary factor dictating accumulation was charge. Compounds that were positively charged are the most likely to accumulate, with the presence of a primary amine being important, but not essential for accumulation. In regards to compound shape, accumulating compounds were rigid, with low globularity. These findings contradict our current understanding, which is that the most important features that govern compound accumulation within Gram-negatives is polarity and molecular weight (Richter *et al.,* 2017).

Although this study provides an initial insight into some of the properties that may be required for accumulation within Gram-negatives, there is still much to learn concerning the properties required of small molecules to accumulate within the bacterial cytoplasm. Future studies of this nature will work towards characterising a comprehensive set of descriptors that small molecules must fulfil in order to accumulate within bacteria.

The accumulation assay developed in this study provides a sensitive, reliable method for the detection of small molecules within the cytoplasm of *E. coli*. As discussed above, this method should be employed in assessing small molecules for accumulation within bacteria. In this study, a small, proof of principle screen was carried out, validating the suitability of this method. Of the 8 compounds which accumulated within *E. coli*, analysis revealed that they were rigid, containing low numbers of rotatable bonds. This was in line with the study carried out by Richter *et al.,* 2017. However, the majority of the compounds found to accumulate in this study were neutral in charge, and none contained a primary amine. Before any conclusions can be drawn linking the physico-chemical properties of these hits to their accumulation within *E. coli*, this data set will need to be extended.

In regards to future studies, the impact of efflux on the accumulation of small molecules should also be assessed. This could be carried out using the efflux deficient mutants included in the validation stages of this study; BW25113 – Δ*acrA* & Δ*acrB*. By comparing hits from library screens in efflux proficient and deficient mutants, structural properties of compounds that are associated with efflux may be realised. This method can also be adapted to measure drug accumulation within different bacterial species; this idea is illustrated in chapter

4, where the method was employed in the quantification of ciprofloxacin with *S. aureus*. Future studies could involve the screening of small molecules within bacterial species that are associated with reduced drug permeability, (eg. *P. aeruginosa* & *K. pneumoniae*). Due to their associated multi-drug resistance, these organisms represent a significant clinical challenge. Understanding features associated with compound permeability within these organisms could aid the modification of existing antimicrobial agents for accumulation within these organisms, as demonstrated by the modification of deoxynybomycin described by Richter *et al.,* (2017).

The second aim of this study was to re-examine the role of membrane transporters in antibiotic uptake within bacteria. . It is assumed that the majority of drugs traverse lipid bilayers by lipoidal diffusion, with antibiotics being no exception. From recent research into drug uptake within eukaryotic cells, there has been a growing body of evidence which suggests that drug uptake through protein carriers may be more common than previously thought (Lanthaler et al., 2011, Dobson and Kell, 2008). Some have also challenged the suitability of existing experimental methods and evidence supporting the lipoidal model of drug diffusion (Dobson and Kell, 2008, Kell *et al.,* 2011). This study was the first to provide a comprehensive assessment of the role of membrane transporters in the entry of antibiotics within *S. aureus.*

The results of this study challenge the widely believed idea that the carrier facilitated uptake of antibiotics is an ‘exception to the rule’ (Sugano *et al.,* 2010, Di *et al.,* 2012, Dobson and Kell, 2008). Previously thought to enter cells via lipoidal diffusion, the results of this study provide evidence supporting the role of transporters in the entry of 10 antibiotics within *S. aureus*, from 8 different

antibiotic classes (tetracyclines, quinolones, aminoglycosides, trimethoprim, fosfomycin, chloramphenicol and fusidic acid). This work complements the landmark study by (Lanthaler et al., 2011) wherein the authors identified transporters with a putative role in the entry of 18 drugs within the model organism, *Saccharomyces cerevisiae.* Combined, this evidence suggests that the role of protein carriers in drug uptake has previously been overlooked, supporting the notion that membrane carriers contribute significantly to cellular drug entry across all domains of life.

Of the 33 strains identified to play a putative role in drug entry, 13 were confirmed via genetic complementation. This was carried out for Tn disrupted strains identified with reduced susceptibility to the compounds chloramphenicol, ciprofloxacin, tetracycline, trimethoprim and D-cycloserine. Competition studies between the native substrates of certain transporters and antibiotics, in addition to an accumulation study provided further evidence for carrier facilitated entry of chloramphenicol and ciprofloxacin, respectively. Due to time limitations, complementation could not be carried out for all of the Tn disrupted strains displaying reductions in drug susceptibility. Future studies involving the complementation of these Tn disrupted strains (those identified for fusidic acid, fosfomycin & streptomycin) should confirm the roles of carriers in the uptake of these drugs within the bacterial cell.

This evidence could be further supported by competition studies between antibiotics and characterised substrates of the associated Tn disrupted transporter strains. This includes the amino acid permeases, SAUSA300_0566 (streptomycin) & SAUSA300_1883 (gentamicin), biotin transporter, SAUSA300_2233 (fusidic acid), formate transporter, SAUSA300_2349

(gentamicin) and iron transporter, SAUSA300_0718 (ciprofloxacin). Accumulation assays were also carried out, confirming the roles of the transporters, SAUSA300_0171 & SAUSA300_0308 in ciprofloxacin ingress. Additional accumulation assays should also be carried out for the strains identified displaying reduced susceptibility to chloramphenicol, in addition to the corresponding multiple Tn inactivated mutants.

Lastly, this study also provides an example of a high-throughput method that may be used identify membrane transporters with a role in the entry of cytotoxic drugs within bacterial cells. Using the Keio collection, the same method could be used in the identification of membrane transporters that are involved in the entry of drugs within *E. coli*. This may provide an insight into the role of carriers in antibiotic uptake within Gram-negatives.

Additional characterisation of the mechanisms of drug uptake within bacteria, and subsequent characterisation of the ‘parameters for drug entry’ could have major implications for future antibiotic discovery efforts. Understanding moieties associated with compound accumulation, whether they enable the compound to avoid efflux or gain entry to the cell via hijacking membrane carriers by mimicking natural substrates, could guide the rational tailoring of compound libraries and the modification of existing agents for increased accumulation within bacterial cells. Addressing the major bottlenecks in antibiotic drug discovery is essential if we are to address the ever pressing issue of antibiotic resistance. If we aren’t successful, we will lose a luxury that we have enjoyed for much of the past century; the control of bacterial disease.

Appendix 1

Primers used in this study

Primer	Sequence (5' to 3') and description	Enzyme/condition
0615 F	GAGCTTCGAAAT*AAGAGG*CTGAGAAAATATGATACAAACAATAACACATATTATGATTATTAG Forward cloning primer containing *BstBI* site for amplifying the *0615* gene from JE2	Q5 Anneal at 64 °C 35 cycles
0615 R	GAGCGTTCGAACTAAAGATTTCTTTTGTTATTTCCATTAAAC Reverse cloning primer containing the *BstBI* site for amplifying the *0615* gene from JE2	Q5 Anneal at 64 °C 35 cycles
0846 F	GAGCGGTACCATAAGAGGCTGAGAAAATATGATAAATGCAGTAGTAATAGCAGTAATTTTAATG Forward cloning primer containing *KpnI* site for amplifying the *0846* gene from JE2	Q5 Anneal at 68 °C 35 cycles
0846 R	GAGCGGAGCTCTTATAGTACCATAGCAGCAATAGTACCGAAAATC Reverse cloning primer containing the *BstBI* site for amplifying the *0615* gene from JE2	Q5 Anneal at 68 °C 35 cycles
1300 F	GAGCGTCGACATAAGAGGCTGAGAAAATATGAATAAAAATACATGGGTCATTGG Forward cloning primer containing *SalI* site for amplifying the *1300* gene from JE2	Q5 Anneal at 61 °C 35 cycles
1300 R	GAGCGGGATCCTTATTCCTGTTGATATTTAATTGGATCTTG Reverse cloning primer containing the *BamHI* site for amplifying the *1300* gene from JE2	Q5 Anneal at 61 °C 35 cycles
2587 F	GAGCGTCGACATAAGAGGCTGAGAAAATATGAAATACTTTATTCCAGCTTGGTAC Forward cloning primer containing *SalI* site for amplifying the *2587* gene from JE2	Q5 Anneal at 63 °C 35 cycles
2587 R	GAGCGGGATCCTTACGTGGCATCATTTTCACC Reverse cloning primer containing the *BamHI* site for amplifying the *2587* gene from JE2	Q5 Anneal at 63 °C 35 cycles
1628 F	GAGCGTCGACATAAGAGGCTGAGAAAATATGTCAAAAGTTCAAAATGAAAGTAAC Forward cloning primer containing *SalI* site for amplifying the *1628* gene from JE2	Q5 Anneal at 62 °C 35 cycles
1628 R	GAGCGGGATCCTTATTTATTTGATTTAGCAGCG Reverse cloning primer containing the *BamHI* site for amplifying the *1628* gene from JE2	Q5 Anneal at 62 °C 35 cycles
1883 F	GAGCGAATTCATAAAAGCCTCCTTTAAGTCATTC Forward cloning primer containing *EcoRI* site for amplifying the *1883* gene from JE2	Q5 Anneal at 62 °C 35 cycles

Primer	Sequence (5’ to 3’) and description	Enzyme/condition
1883 R	GAGCGGGATCCTTATTTTTCTCTAACGATGTCACG Forward cloning primer containing *BamHI* site for amplifying the *1883* gene from JE2	Q5 Anneal at 62 °C 35 cycles
0718 F	GAGCGAATTCCTTCTCAGTAAAAGGTGATG Forward cloning primer containing *EcoRI* site for amplifying the *0718* gene from JE2	Q5 Anneal at 57 °C 35 cycles
0718 R	GAGCGGGATCCTTATTGTTGCGCATAATTTTTC Reverse cloning primer containing *BamHI* site for amplifying the *0718* gene from JE2	Q5 Anneal at 57 °C 35 cycles
0171 F	GAGCGAATTCTAAAGGTGCGTTAAGTAACT Forward cloning primer containing *EcoRI* site for amplifying the *0171* gene from JE2	Q5 Anneal at 58 °C 35 cycles
0171 R	GAGCGGGATCCTTATGTTAATTTGTCGAAGTATGG Reverse cloning primer containing *BamHI* site for amplifying the *0171* gene from JE2	Q5 Anneal at 58 °C 35 cycles
0202 F	ATCGTTATGTCGACTGAAATCAACGAAAGGAAGT Forward cloning primer containing *SalI* site for amplifying the *0202* gene from JE2	Phusion Anneal at 57 °C 35 cycles
0202 R	TGCTTAGTGGATCCCCTTTTTAATGAATTCTTGGATC Reverse cloning primer containing *BamHI* site for amplifying the *0202* gene from JE2	Phusion Anneal at 57 °C 35 cycles
0308 F	ATCGTTATGTCGACCATAGTAGTAGGGAGAGAAAT Forward cloning primer containing *SalI* site for amplifying the *0308* gene from JE2	Phusion Anneal at 57 °C 35 cycles
0308 R	TGCTTAGTGGATCCCCTTTTTAATGAATTCTTGGATC Reverse cloning primer containing *BamHI* site for amplifying the *0308* gene from JE2	Phusion Anneal at 57 °C 35 cycles
2286 F	GAGCGAATTCATTGTAATGGTAGCTGTCTTGATATGGA Forward cloning primer containing *EcoRI* site for amplifying the *2286* gene from JE2	Q5 Anneal at 66 °C 35 cycles
2286 R	GAGCGGGATCCTCAAGCTCTGCTGGGTAGC Reverse cloning primer containing *BamHI* site for amplifying the *2286* gene from JE2	Q5 Anneal at 66 °C 35 cycles
2358p F	GAGCGAATTCTCAATAAATATAAGTTGCTAGCTATATAAAG Forward cloning primer containing *EcoRI* site for amplifying the promoter region of the *2256* gene from JE2	Q5 Anneal at 57 °C 35 cycles

Primer	Sequence (5' to 3') and description	Enzyme/condition
2358p R	GAGCGGGATCCTCATAGAATATATCTCCTTATTCTTATTATTC Reverse cloning primer containing *BamHI* site for amplifying the promoter region of the *2258* gene from JE2	Q5 Anneal at 57 °C 35 cycles
2358 F	GAGCGGGATCCATGTTTCTAAATCTAAATAGCGAACAAC Forward cloning primer containing *BamHI* site for amplifying the *2258* gene from JE2	Q5 Anneal at 62 °C 35 cycles
2358 R	GAGCGTCGACTCATGAGCGATACCCTCTTTC Reverse cloning primer containing *SalI* site for amplifying the *2258* gene from JE2	Q5 Anneal at 62 °C 35 cycles
pRMC2 seq F	ATTACGTAAAAAATCTTGCC Forward primer used for sequencing across pRAB11 MCS and beyond	Phusion Anneal at 55 °C 35 cycles
pRMC2 seq R	GTGAAAACCTCTGACACATG Reverse primer used for sequencing across pRAB11 MCS and beyond	Phusion Anneal at 55 °C 35 cycles
tet(M) F	GGCAAAGCTTATAGGAGGATCAGAAAATATGAAAATTATCAACATTGGTGT Forward primer for amplification of the *tet(M)* gene. Contains the *HindIII* restriction site	*Q5 Polymerase* Annealing 59˚C 25 cycles
tet(M) R	GGCAGGATCCTTAGGTGATTTTATTGAACATGTAACGG Reverse primer for amplification of the *tet(M)* gene. Contains the *BamHI* restriction site	*Q5 Polymerase* Annealing 59˚C 25 cycles

Appendix 2

Plate ID: **30000124**

	1	2	3	4	5	6	7	8	9	10	11	12
A	LDS-019726-1	LDS-019912-1	LDS-019697-1	LDS-019440-1	LDS-019335-1	LDS-019328-1	LDS-019526-1	LDS-020167-1	LDS-027765-1	LDS-025855-1		
B	LDS-019806-1	LDS-020073-1	LDS-019321-1	LDS-019560-1	LDS-019443-1	LDS-019338-1	LDS-019546-1	LDS-020586-1	LDS-028806-1	LDS-024361-1		
C	LDS-019857-1	LDS-019824-1	LDS-019341-1	LDS-019700-1	LDS-019513-1	LDS-019348-1	LDS-023364-1	LDS-020429-1	LDS-027586-1	LDS-029251-1		
D	LDS-019977-1	LDS-019905-1	LDS-019361-1	LDS-019324-1	LDS-019553-1	LDS-019358-1	LDS-021197-1	LDS-020171-1	LDS-027738-1	LDS-029184-1		
E	LDS-019848-1	LDS-019378-1	LDS-019380-1	LDS-019344-1	LDS-019424-1	LDS-019396-1	LDS-021690-1	LDS-020452-1	LDS-028039-1	LDS-029146-1		
F	LDS-019819-1	LDS-019686-1	LDS-019409-1	LDS-019354-1	LDS-019337-1	LDS-019406-1	LDS-021243-1	LDS-020674-1	LDS-025682-1	LDS-029077-1		
G	LDS-019721-1	LDS-019458-1	LDS-019322-1	LDS-019373-1	LDS-019405-1	LDS-019426-1	LDS-021873-1	LDS-028723-1	LDS-025163-1	LDS-005582-2		
H	LDS-019901-1	LDS-019668-1	LDS-019332-1	LDS-019552-1	LDS-019425-1	LDS-019436-1	LDS-020156-1	LDS-027764-1	LDS-024674-1	LDS-029010-1		

Plate ID: **30000125**

	1	2	3	4	5	6	7	8	9	10	11	12
A	LDS-029517-1	LDS-006654-2	LDS-013682-2	LDS-016402-1	LDS-016648-1	LDS-017100-1	LDS-017438-1	LDS-019015-1				
B	LDS-005920-2	LDS-011054-2	LDS-013822-2	LDS-016393-1	LDS-016640-1	LDS-017162-1	LDS-017520-1	LDS-019008-1				
C	LDS-031151-1	LDS-011446-2	LDS-015652-1	LDS-016395-1	LDS-016660-1	LDS-017172-1	LDS-017743-1					
D	LDS-031291-1	LDS-014237-2	LDS-015635-1	LDS-016405-1	LDS-016773-1	LDS-017115-1	LDS-017688-1					
E	LDS-031393-1	LDS-011770-2	LDS-015715-1	LDS-016534-1	LDS-016739-1	LDS-017396-1	LDS-018076-1					
F	LDS-007166-2	LDS-013840-2	LDS-015826-1	LDS-016528-1	LDS-016809-1	LDS-017427-1	LDS-018120-1					
G	LDS-005851-2	LDS-014962-2	LDS-015963-1	LDS-016567-1	LDS-016860-1	LDS-017452-1	LDS-018429-1					
H	LDS-006264-2	LDS-015004-2	LDS-016248-1	LDS-016657-1	LDS-016963-1	LDS-017496-1	LDS-018941-1					

Compound identities (LDS ID) and plate locations of the compound library set used in this study. Compounds highlighted in grey were identified as having membrane damaging activity.

Appendix 3

Sources, formula, molecular weight and SMILES information of the compound set used in this study.

Supplier	ID	LDS ID	RMM	Formula	Canonical SMILES
ChemBridge	5139189	LDS-019328-1	357.4234	C19H19NO4S	COc1ccc(cc1)OCCNS(=O)(=O)c1cc2ccccc2cc1
ChemBridge	4003147	LDS-019321-1	151.2056	C9H13NO	CC(C)Oc1ccccc1N
ChemBridge	5215298	LDS-019322-1	320.3419	C19H16N2O3	COc1ccc(cc1)C1CC(=NN1)C1C(=O)c2ccccc2C1=O
ChemBridge	5224354	LDS-019324-1	328.154	C12H11Cl2N5O2	COC(=O)CNc1[n]c(Nc2ccc(Cl)cc2)[n]c(Cl)[n]1
ChemBridge	5210644	LDS-019337-1	283.1284	C9H8Cl2O4S	OC(=O)CCS(=O)(=O)c1cc(Cl)c(Cl)cc1
ChemBridge	5144053	LDS-019338-1	226.1828	C10H10O6	OC(=O)COc1ccccc1OCC(O)=O
ChemBridge	5217550	LDS-019332-1	289.3711	C11H15NO4S2	O=S1(=O)CC(CC1)S(=O)(=O)NCc1ccccc1
ChemBridge	5260188	LDS-019335-1	298.3397	C16H18N4O2	O=[N+]([OH2+255])c1cc(CN2CCN(CC2)c2cccc[n]2)ccc1
ChemBridge	5144416	LDS-019348-1	286.2826	C15H14N2O4	CCOC(=O)c1ccc(cc1)NC(=O)c1c[n+]([OH2+255])ccc1
ChemBridge	4015121	LDS-019341-1	174.5849	C6H7ClN2O2	Nc1cc(cc[n]1)C(O)=O.HCl
ChemBridge	5229962	LDS-019344-1	322.3578	C19H18N2O3	Nc1c2ccccc2[n]c2CCCc21.Oc1ccccc1C(O)=O
ChemBridge	5146695	LDS-019358-1	269.2506	C12H15NO6	COc1c(cc(cc1OC)C(=O)NCC(O)=O)OC
ChemBridge	5229979	LDS-019354-1	346.4438	C18H22N2O3S	Cc1cc(ccc1)NC(=O)c1cc(ccc1)S(=O)(=O)N(CC)CC
ChemBridge	5100477	LDS-019361-1	207.2688	C12H17NO2	COC(=O)CCNC(C)c1ccccc1
ChemBridge	5230085	LDS-019373-1	348.2393	C10H10BrN3O2S2	Nc1[n][n]c(CCS(=O)(=O)c2ccc(Br)cc2)[s]1
ChemBridge	5115300	LDS-019378-1	231.2074	C11H9N3O3	O=C(NNC(=O)c1c[n]ccc1)c1ccco1
ChemBridge	5106728	LDS-019380-1	183.1613	C8H9NO4	OC(=O)CCNC(=O)c1ccco1
ChemBridge	5155468	LDS-019396-1	271.1027	C10H8Cl2N4O	O/N=C(\C[n]1c[n]c[n]1)/c1ccc(Cl)cc1Cl
ChemBridge	5523518	LDS-019405-1	259.3186	C16H18FNO	COc1cc(CCNCc2ccc(F)cc2)ccc1
ChemBridge	5460440	LDS-019406-1	264.3002	C12H12N2O3S	COc1cc(ccc1)NCc1ccc([s]1)[N+](=O)[OH2+255]
ChemBridge	5214769	LDS-019409-1	297.3053	C17H15NO4	COc1ccc(cc1)C(=O)CC1(O)c2ccccc2NC1=O
ChemBridge	5528485	LDS-019425-1	234.2694	C13H15FN2O	Fc1ccc(cc1)OCCCC[n]1cc[n]c1
ChemBridge	5473649	LDS-019426-1	219.2365	C12H13NO3	C/C(=C/C(=O)NCc1ccccc1)/C(O)=O
ChemBridge	5543209	LDS-019424-1	213.2535	C9H11NO3S	NC(=O)CCS(=O)(=O)c1ccccc1
ChemBridge	5480967	LDS-019436-1	257.2447	C13H11N3O3	Cc1cc(ccc1[N+](=O)[OH2+255])C(=O)Nc1c[n]ccc1

Supplier	ID	LDS ID	RMM	Formula	Canonical SMILES
ChemBridge	5571013	LDS-019443-1	334.2077	C16H16BrNO2	Brc1ccc(CNCCc2cc3OCOc3cc2)cc1
ChemBridge	5281112	LDS-019458-1	308.1936	C13H10BrNOS	O=C(CSc1cccc[n]1)c1ccc(Br)cc1
ChemBridge	5175142	LDS-019513-1	367.2496	C16H12Cl2N2O2S	Cc1ccc(cc1)S(=O)(=O)Nc1c2[n]cccc2c(Cl)cc1Cl
ChemBridge	5734687	LDS-019526-1	203.2371	C12H13NO2	CCOCc1ccc(O)c2[n]cccc21
ChemBridge	5752433	LDS-019546-1	275.3262	C13H13N3O2S	C=CC[n]1c([n][n]c1SCC(O)=O)-c1ccccc1
ChemBridge	5349504	LDS-019552-1	296.7924	C15H21ClN2O2	CC1C/C(=N\OC(=O)c2ccccc2)/C(C)CN1C.HCl
ChemBridge	5191687	LDS-019553-1	317.7701	C16H16ClN3O2	O=C(CCc1ccccc1)NNC(=O)Nc1ccc(Cl)cc1
ChemBridge	5933927	LDS-019560-1	307.3001	C18H13NO4	Oc1ccc(cc1)NC(=O)c1ccc(o1)C(=O)c1ccccc1
ChemBridge	5914430	LDS-019668-1	453.5969	C25H31N3O3S	CC1(C)CC(CCO1)N1C(=NC2=C(C1=O)C1(Cc3ccccc32)CCCC1)SCC(N)=O
ChemBridge	6664590	LDS-019686-1	337.2501	C16H10F3NO4	O=C(O/N=C/c1ccc2OCOc2c1)c1cccc(c1)C(F)(F)F
ChemBridge	5923464	LDS-019697-1	310.3901	C19H22N2O2	CC(C)c1ccc(cc1)OCC(O)C[n]1c[n]c2ccccc12
ChemBridge	6996505	LDS-019700-1	426.5069	C27H26N2O3	CC1(C)CC(=O)C2C(Nc3ccc4ccccc4c3C=2C1)c1ccc(cc1)OCC(N)=O
ChemBridge	7733378	LDS-019721-1	340.3962	C18H16N2O3S	CCOC(=O)c1ccc(CSC2NC(=O)c3ccccc3N=2)cc1
ChemBridge	7728552	LDS-019726-1	326.3034	C17H14N2O5	COC(=O)c1ccc(CON2C(=O)C=[N+]([OH2+255])c3ccccc23)cc1
ChemBridge	7746306	LDS-019806-1	274.2438	C15H11FO4	O=C(OCc1ccc(F)cc1)c1ccc2OCOc2c1
ChemBridge	7820226	LDS-019824-1	378.4162	C20H26O7	CC(=O)C1C(C(C(C)=O)C(C)(O)CC1=O)c1cc(OC)c(OC)c(c1)OC
ChemBridge	7608802	LDS-019819-1	374.3563	C20H17F3N2O2	CN(c1ccccc1)C(=O)C[n]1c2ccccc2c(C(=O)C(F)(F)F)c1C
ChemBridge	7862121	LDS-019848-1	315.3238	C16H17N3O4	COCCNc1ccc(cc1C(=O)Nc1ccccc1)[N+](=O)[OH2+255]
ChemBridge	7843996	LDS-019857-1	329.3256	C17H16FN3O3	CC1CCc2cc(F)ccc2N1C(=O)Nc1cc(ccc1)[N+](=O)[OH2+255]
ChemBridge	7910532	LDS-019905-1	304.321	C14H12N2O4S	CN(c1ccccc1)S(=O)(=O)c1cc2OC(=O)Nc2cc1
ChemBridge	7891362	LDS-019901-1	354.4012	C23H18N2O2	CC(=O)c1ccc(COc2cc(ccc2)-c2c[n]c3ccccc3[n]2)cc1
ChemBridge	7905606	LDS-019912-1	364.4161	C17H20N2O5S	CC(Oc1ccc(cc1OC)C1NC(=S)NC(C)=C1C(C)=O)C(O)=O
ChemBridge	7924864	LDS-019977-1	356.2117	C15H18BrNO4	Cc1cc(Br)c(OCCCON2C(=O)CCC2=O)c(C)c1
ChemBridge	7937914	LDS-020073-1	371.3157	C14H15BrN2OS2	CN(C)C(=O)CCSc1[n]c(c[s]1)-c1ccc(Br)cc1
ChemBridge	7952935	LDS-020156-1	349.4246	C16H15NO4S2	CCCSc1oc([n]c1S(=O)(=O)c1ccccc1)-c1ccco1
ChemBridge	7953747	LDS-020167-1	409.5013	C22H23N3O3S	CCCC1=Nc2c([s]c3[n]c4CC(C)(C)OCc4cc23)C(=O)N1Cc1ccco1
ChemBridge	7921731	LDS-020171-1	393.2367	C19H13BrN4O	Cc1c[n][n](c1)C(=O)c1cc([n]c2ccc(Br)cc21)-c1c[n]ccc1
ChemBridge	7968218	LDS-020429-1	345.3696	C21H16FN3O	Oc1c2[n]cccc2ccc1C(Nc1c[n]ccc1)c1ccccc1F
ChemBridge	7969320	LDS-020452-1	335.383	C17H13N5OS	CCSc1[n]c2OC(=Nc3ccccc3-c2[n][n]1)c1cc[n]cc1
ChemBridge	7974778	LDS-020586-1	293.3382	C14H15NO4S	CC(=O)c1ccc(cc1)N1C(=O)CC(SCCO)C1=O
ChemBridge	7997530	LDS-020674-1	352.4102	C18H16N4O2S	NC1=CC(=O)NC(=N1)SCC(=O)N(c1ccccc1)c1ccccc1
ChemBridge	9018556	LDS-021197-1	319.1533	C14H11BrN2O2	Brc1ccc(o1)-c1[n]oc(CCc2ccccc2)[n]1
ChemBridge	9033490	LDS-021243-1	303.3529	C17H21NO4	COC(=O)c1ccc(cc1)-c1ccc(CNC(CO)CC)o1

Supplier	ID	LDS ID	RMM	Formula	Canonical SMILES
ChemBridge	9064676	LDS-021690-1	302.3251	C16H18N2O4	CC1NC(=O)CC(C=1C(=O)OC)C(=O)NCc1ccccc1
ChemBridge	9056966	LDS-021873-1	256.2533	C15H12O4	Cc1ccc(o1)C(=O)CC1OC(=O)c2ccccc21
ChemBridge	9099355	LDS-023364-1	206.241	C11H14N2O2	Cc1cc([n]o1)C(=O)N(CC=C)CC=C
ChemBridge	9119197	LDS-024361-1	341.3578	C19H19NO5	CC1(C)CC(=O)c2cc(CC(O)=O)[n](-c3ccc(cc3)C(O)=O)c2C1
ChemBridge	9122419	LDS-024674-1	316.3154	C16H19F3O3	CC1(C)CC(CCO1)(CC(O)=O)c1cccc(c1)C(F)(F)F
ChemBridge	5349504	LDS-019552-1	296.7924	C15H21ClN2O2	CC1C/C(=N\OC(=O)c2ccccc2)/C(C)CN1C.HCl
ChemBridge	9133926	LDS-025682-1	246.2667	C15H10N4	c1c[n]ccc1-c1cc[n]c2[n]c3ccccc3[n]12
ChemBridge	9135149	LDS-025855-1	220.2261	C14H8N2O	c1cccc2oc3[n]c4ccccc4[n]c3c21
ChemBridge	9148754	LDS-027586-1	263.2906	C17H13NO2	Oc1ccc(cc1)/C=C/c1cc(O)c2ccccc2[n]1
ChemBridge	9189975	LDS-027738-1	214.2615	C10H18N2O3	CC1C(=O)NCCN1C(=O)OC(C)(C)C
ChemBridge	9188693	LDS-027764-1	224.6834	C12H13ClO2	OC(=O)C1(CCCC1)c1ccccc1Cl
ChemBridge	9188814	LDS-027765-1	353.3389	C17H18F3N3O2	CC(C)N1C(=O)C(NC(=O)C2CC2)(N=C1c1ccccc1)C(F)(F)F
ChemBridge	9151097	LDS-028039-1	210.2363	C8H14N6O	CC(C)=NOc1[n]c(N)[n]c(NCC)[n]1
ChemBridge	9155192	LDS-028723-1	203.709	C10H18ClNO	CCC(C)(C)NCc1ccco1.HCl
ChemBridge	9156240	LDS-028806-1	261.2748	C8H7NO5S2	COC(=O)C1NS(=O)(=O)c2cc[s]c2C=1O
ChemBridge	9199007	LDS-029010-1	290.3342	C15H14O4S	OC(=O)C1C2C3CC1C(Sc1ccccc1)C3OC2=O
ChemBridge	9199742	LDS-029077-1	201.2212	C12H11NO2	Cc1cc(C)[n]c2cc(ccc12)C(O)=O
ChemBridge	9199722	LDS-029146-1	193.6329	C9H8ClN3	Nc1cc(Cl)ccc1-[n]1cc[n]c1
ChemBridge	9199782	LDS-029184-1	159.1878	C9H9N3	NNc1[n]ccc2ccccc21
ChemBridge	9193434	LDS-029251-1	202.1728	C8H6N6O	OC1=NNC=Cc2c1c[n]c1[n]c[n][n]21
AMRI	ALB-H01785442	LDS-005582-2	468.5852	C22H33FN4O4S	CC(C)(C)NC(=O)N(Cc1c[n]c([n]1C(C)C)S(=O)(=O)Cc1ccc(F)cc1)CCOC
AMRI	ALB-H11775114	LDS-005851-2	331.815	C14H18ClNO4S	CCS(=O)(=O)Oc1ccc(CN(C2CC2)C(=O)CCl)cc1
AMRI	ALB-H01470323	LDS-005920-2	395.2548	C19H17Cl2FN2O2	CCN(CC1CC(=NO1)c1ccc(Cl)c(Cl)c1)C(=O)c1cccc(F)c1
AMRI	ALB-H04808136	LDS-006264-2	359.4162	C20H25NO5	CC[n]1c(C(=O)OC)c(C)c(C(=O)C(C)Oc2ccccc2OC)c1C
AMRI	ALB-H05329240	LDS-006654-2	347.8358	C19H22ClNO3	CC1=C(C(CC(=O)N1CC=C)c1ccc(Cl)cc1)C(=O)OC(C)C
AMRI	ALB-H09829420	LDS-007166-2	281.3522	C17H19N3O	Cc1cc(ccc1)Oc1cc([n]c[n]1)N(CC=C)CC=C
AMRI	ALB-H00890876	LDS-011054-2	276.3739	C16H24N2O2	Cc1cc(/C=N/NC(=O)CCCCCCC)c(O)cc1
AMRI	ALB-H01811116	LDS-011446-2	458.5919	C24H31FN4O2S	CC1N=C2SC=C(CC(=O)NCC(C)C)N2C(C=1C(=O)N(CC)CC)c1ccccc1F
AMRI	ALB-H04361507	LDS-011770-2	370.3974	C19H15FN2O3S	Cc1cc([n]c([n]1)SCc1ccccc1F)Oc1cc2OCOc2cc1
AMRI	ALB-H02167129	LDS-013682-2	146.1875	C6H14N2O2	CCOC(OCC)C(N)=N
AMRI	ALB-H02173234	LDS-013822-2	350.3349	C18H17F3N2O2	CO/C(/NC(C)c1ccccc1)=N/C(=O)c1cc(ccc1)C(F)(F)F
AMRI	ALB-H02176144	LDS-013840-2	266.336	C14H22N2O3	CC(C)C(C)N/C(=N\C(=O)c1ccco1)/OCCC

Supplier	ID	LDS ID	RMM	Formula	Canonical SMILES
AMRI	ALB-H10045695	LDS-014237-2	282.2509	C15H10N2O4	OC(=O)c1c[n]2cc(ccc2[n]1)-c1cc2OCOc2cc1
AMRI	ALB-H00145372	LDS-014962-2	238.2844	C15H14N2O	CC[n]1c2ccc(/C=N/O)cc2c2ccccc12
AMRI	ALB-H03208816	LDS-015004-2	223.3128	C16H17N	C(NC1C[C@H]1c1ccccc1)c1ccccc1
Asinex	LMK 17350849	LDS-016393-1	414.4995	C25H26N4O2	CC1Cc2cc(ccc2O1)C(=O)NC1CC(C)(C)Cc2[n]c([n]cc21)-c1c[n]ccc1
Asinex	AOP 20660975	LDS-016395-1	398.4737	C22H27FN4O2	Fc1ccc(cc1)Oc1[n]c([n]c2CCN(Cc21)CC1COCC1)N1CCCC1
Asinex	ADM 19806637	LDS-016402-1	422.4042	C19H21F3N6O2	Cc1[n]oc2[n]c(cc(c21)C(F)(F)F)C1CN(CCC1)C(=O)CCC[n]1c[n]c[n]1
Asinex	AOP 19384363	LDS-016405-1	366.4136	C20H22N4O3	Cc1cc(C)[n][n]1CCNC(=O)[C@H]1C2C(=O)Nc3ccccc3C(=O)[C@]21C
Asinex	AOP 22094622	LDS-016528-1	397.4906	C21H23N3O3S	CN1CCN(CC1)C1=NS(=O)(=O)C(=C1c1ccccc1)c1ccc(cc1)OC
Asinex	AOP 21238360	LDS-016534-1	342.3922	C18H22N4O3	CC(CO)(CO)C(=O)N1C[C@@H]2[C@@H](Cc3c([nH][n]c32)-c2cc[n]cc2)C1
Asinex	AOP 22025518	LDS-016567-1	449.9326	C24H24ClN5O2	Cc1[n]c(O)cc(CC(NCc2c[n](CC(N)=O)c3ccccc23)c2ccc(Cl)cc2)[n]1
Asinex	SYN 22849056	LDS-016640-1	355.3878	C14H18FN5O3S	CN(C)S(=O)(=O)NCC(=O)Nc1[n]cc(-c2ccc(F)cc2)[n]1C
Asinex	LMK 22833444	LDS-016648-1	373.4261	C22H20FN5	C[n]1cc[n]c1-c1[n]c(NC(C)c2ccccc2)[n]cc1-c1cc(F)ccc1
Asinex	SYN 23019532	LDS-016660-1	410.8535	C21H19ClN4O3	Cc1cc(NC(=O)C2CC(=NO2)c2ccccc2Cl)[n]([n]1)-c1ccc(cc1)OC
Asinex	LMK 22833386	LDS-016657-1	417.4356	C23H20FN5O2	C[n]1cc[n]c1-c1[n]c(NCC2COc3ccccc3O2)[n]cc1-c1ccccc1F
Asinex	AEM 10299700	LDS-016739-1	392.3863	C20H17FN6O2	CN(C)c1ccc(cc1)-c1[n]c(C(N)=O)c2NC(=O)N(c2[n]1)c1ccccc1F
Asinex	AEM 07463373	LDS-016773-1	306.3568	C16H22N2O4	Cc1cc2c(cc(C(=O)OC(C)C(=O)NC(C)(C)C)[n]2C)o1
Asinex	AEM 12409092	LDS-016809-1	311.3318	C18H17NO4	O=C(c1ccco1)N1C(=O)c2ccccc2OC21CCCCC2
Asinex	AEM 12226983	LDS-016860-1	300.3323	C15H12N2O3S	CN(C(=O)c1ccco1)c1[n]c2-c3ccoc3CCc2[s]1
Asinex	SYN 15355143	LDS-016963-1	305.3655	C17H21F2N3	CN(C)CCN(Cc1cc[n]cc1)Cc1cc(F)c(F)cc1
Asinex	SYN 15411887	LDS-017100-1	323.3841	C17H25NO5	COc1ccc(CN(CC(C)O)CC2=COCCO2)cc1OC
Asinex	ART 11721874	LDS-017115-1	227.1858	C10H8F3N3	Cc1cc([n]c2[n]c(N)ccc21)C(F)(F)F
Asinex	ART 11723153	LDS-017162-1	217.3067	C14H19NO	NC1CC2(CCCCC2)Oc2ccccc21
Asinex	ART 11115230	LDS-017172-1	287.332	C16H18FN3O	O=C(C[n]1cc[n]c1)NC1(CCCC1)c1ccc(F)cc1
Asinex	ART 13978822	LDS-017396-1	255.3149	C15H17N3O	Cc1ccc(CN(C)Cc2[nH]c3ccccc3[n]2)o1
Asinex	ART 13087396	LDS-017427-1	239.2709	C11H17N3O3	CC1NC(=O)C2CN(CCC2N=1)C(=O)OCC
Asinex	ART 15395789	LDS-017438-1	296.3636	C18H20N2O2	C(C1=COCCO1)N(Cc1cccc[n]1)Cc1ccccc1
Asinex	ART 13850307	LDS-017452-1	237.2566	C14H11N3O	Cc1[n]c(c(c[n]1)-c1cc[n]cc1)-c1ccco1
Asinex	ART 15394060	LDS-017496-1	277.3818	C15H19NO2S	Cc1ccc(cc1)C1=CCC2CN(CC12)S(C)(=O)=O
Asinex	LMK 12712023	LDS-017520-1	416.4739	C22H17FN6S	Fc1ccc(cc1)-c1c[n]c2Sc3[n]c([n][n]3C(NC3CC3)c2c1)-c1cc[n]cc1
Asinex	AOP 14765864	LDS-017688-1	292.3303	C15H20N2O4	CC(C)C(=O)NCCOCCN1c2ccccc2OC1=O
Asinex	AOP 13302054	LDS-017743-1	391.4429	C21H17N3O3S	CC1OCC2=CC(=O)N(CC(=O)N3c4ccccc4Sc4ccccc34)N=C21
Asinex	ART 18687270	LDS-018076-1	232.2999	C9H16N2O3S	CC(C)NC(=O)C1CN(CC=1)S(C)(=O)=O

Supplier	ID	LDS ID	RMM	Formula	Canonical SMILES
Asinex	AEM 16188036	LDS-018120-1	405.3704	C20H18F3N3O3	Cc1cc2c(cc1)N(CCNC(=O)c1cc([n]cc1)OC)C(=CC2=O)C(F)(F)F
Asinex	AOP 15779984	LDS-018429-1	435.4626	C21H20F3N3O2S	Cc1cc2c(cc1)N(C[C@H](C)NC(=O)CSc1cc[n]cc1)C(=CC2=O)C(F)(F)F
Asinex	SYN 15572366	LDS-018941-1	321.373	C19H19N3O2	O=C(Nc1c[nH]c2ccccc21)C1=CC2CCCCCC=2NC1=O
Asinex	SYN 17673326	LDS-019008-1	406.5421	C18H18N2O3S3	CS(=O)(=O)c1ccc(CC(=O)NC(Cc2ccc[s]2)c2[n]cc[s]2)cc1
Asinex	SYN 15599643	LDS-019015-1	313.3047	C17H15NO5	Cc1cc2OCCOc2cc1NC(=O)C1Oc2ccccc2O1
ChemDiv	2332-0313	LDS-015635-1	322.1755	C10H12BrNO4S	CN(C1CCS(=O)(=O)C1)C(=O)c1ccc(Br)o1
ChemDiv	0416-0100	LDS-015652-1	272.3819	C18H24O2	COc1ccc(cc1)C(O)C12CC3CC(C1)CC(C2)C3
ChemDiv	3647-1859	LDS-015715-1	411.2374	C16H18IN3O2	CC1=NC(=N)C(COCC)=CN1CC(=O)c1ccc(I)cc1
ChemDiv	7706-0179	LDS-015826-1	280.3642	C18H20N2O	Cc1[nH]c2ccccc2c1C(OC(C)C)c1cccc[n]1
ChemDiv	8017-6838	LDS-015963-1	309.3855	C17H15N3OS	Cc1ccc[n]2cc(COCc3[n]c4ccccc4[s]3)[n]c21
ChemDiv	F454-0096	LDS-016248-1	336.3844	C20H20N2O3	COc1ccc(CN2C3CC(C)(C)CC(=O)C=3C=C(C#N)C2=O)cc1
AMRIa	ALB-H10744687	LDS-029517-1	327.2057	C15H16Cl2N2O2	O=C(CC1CC(=NO1)c1ccc(Cl)cc1Cl)NCC1CC1
AMRIa	ALB-H03225117	LDS-031151-1	438.9283	C19H23ClN4O4S	CCONC(=O)Cc1c[s]c(NC(=O)CN(C(C)C)C(=O)c2ccc(Cl)cc2)[n]1
AMRIa	ALB-H00180306	LDS-031291-1	291.2893	C12H12F3NO2S	COC(=O)C1CSC(N1)c1ccc(cc1)C(F)(F)F
AMRIa	ALB-H02026943	LDS-031393-1	314.3358	C17H18N2O4	Cc1[n]c([n]c(c1C(=O)OCC(C)C)C(O)=O)-c1ccccc1

Appendix 4

Compounds identified as positively accumulating 'hits', during the first library screen.

Plate ID:

30000124	Hits	MW	Formula
	LDS-019819-1	374.3563	C20H17F3N2O2
	LDS-019721-1	340.3962	C18H16N2O3S
	LDS-019458-1	308.1936	C13H10BrNOS
	LDS-019668-1	453.5969	C25H31N3O3S
	LDS-019560-1	307.3001	C18H13NO4
	LDS-019373-1	348.2393	C10H10BrN3O2S2
	LDS-019328-1	357.4234	C19H19NO4S
	LDS-019406-1	264.3002	C12H12N2O3S
	LDS-019526-1	203.2371	C12H13NO2
	LDS-020167-1	409.5013	C22H23N3O3S
	LDS-025855-1	220.2261	C14H8N2O

Plate ID:

30000125	Hits	MW	Formula
	LDS-006264-2	359.4162	C20H25NO5
	LDS-015715-1	411.2374	C16H18IN3O2
	LDS-016248-1	336.3844	C20H20N2O3
	LDS-016393-1	414.4995	C25H26N4O2
	LDS-016395-1	398.4737	C22H27FN4O2
	LDS-016657-1	417.4356	C23H20FN5O2
	LDS-016648-1	373.4261	C22H20FN5
	LDS-016963-1	305.3655	C17H21F2N3
	LDS-017688-1	292.3303	C15H20N2O4
	LDS-018120-1	405.3704	C20H18F3N3O3
	LDS-018429-1	435.4626	C21H20F3N3O2S

Appendix 5

NTML library strain identities and plate locations.

Strain Name	plate	Well	Gene name	gene discription	Accession Number
NE4	1	A7		ABC transporter ATP-binding protein	SAUSA300_0309
NE12	1	A23		drug resistance transporter, EmrB/QacA subfamily	SAUSA300_2126
NE13	1	C1		ribose transporter RbsU	SAUSA300_0264
NE14	1	C3		putative transporter	SAUSA300_2406
NE17	1	C9		putative drug transporter	SAUSA300_1705
NE34	1	E19		L-lactate permease	SAUSA300_2313
NE39	1	G5	ptsG	phosphotransferase system, glucose-specific IIABC component	SAUSA300_2476
NE41	1	G9		cation transport family protein	SAUSA300_1979
NE44	1	G15	brnQ	branched-chain amino acid transport system II carrier protein	SAUSA300_1300
NE45	1	G17		accessory secretory protein Asp1	SAUSA300_2587
NE48	1	G23		maltose ABC transporter, permease protein	SAUSA300_0210
NE54	1	I11		ABC transporter, ATP-binding/permease protein	SAUSA300_2375
NE61	1	K1	lacE	PTS system, lactose-specific IIBC componen	SAUSA300_2150
NE68	1	K15		ABC transporter ATP-binding protein	SAUSA300_0620
NE70	1	K19		ABC transporter, permease protein	SAUSA300_0648
NE82	1	M19		putative membrane protein	SAUSA300_2472
NE91	1	O13	kdpA	K+-transporting ATPase, A subunit	SAUSA300_2034
NE102	1	B11		arginine/ornithine antiporter	SAUSA300_2568
NE107	1	B21		putative ABC transporter protein EcsB	SAUSA300_1785
NE112	1	D7		sucrose-specific PTS tranporter protein	SAUSA300_0194
NE123	1	F5		cobalt transport family protein	SAUSA300_0977
NE129	1	F17		DNA internalization-related competence proteinComEC/Rec2	SAUSA300_1547
NE132	1	F23		putative transcriptional regulator	SAUSA300_2640
NE142	1	H19		amino acid carrier protein	SAUSA300_1252
NE144	1	H23		putative tetracycline resistance protein	SAUSA300_0139
NE146	1	J3		iron compound ABC transporter, permease	SAUSA300_0718
NE154	1	J19		putative membrane protein	SAUSA300_2385
NE168	1	L23	opp-2B	tide ABC transporter, permease protein / oligopeptide permease, channel-forming pro	SAUSA300_1276
NE172	1	N7	ptsG	PTS system, glucose-specific IIBC component domain protein	SAUSA300_0191
NE176	1	N15		anion transporter family protein	SAUSA300_0676
NE177	1	N17		putative membrane protein	SAUSA300_2448
NE179	1	N21		multidrug resistance protein	SAUSA300_2360
NE187	1	P13		phosphate transporter family protein	SAUSA300_0650
NE192	1	P23		transporter gate domain protein	SAUSA300_2520
NE197	1	A10		amino acid permease	SAUSA300_2383
NE199	1	A14		cation efflux family protein	SAUSA300_2099
NE200	1	A16		iron compound ABC transporter, permease protein	SAUSA300_2134
NE206	1	C4		amino acid permease family protein	SAUSA300_2538
NE211	1	C14		amino acid permease	SAUSA300_1329
NE215	1	C22		putative lipoprotein	SAUSA300_0203
NE226	1	E20	amt	ammonium transporter	SAUSA300_1996
NE231	1	G6		sodium dependent transporter	SAUSA300_0432

NE240	1	G24	tcaB	teicoplanin resistance associated membrane protein TcaB protein	SAUSA300_2301
NE251	1	I22		polysaccharide biosynthesis protein	SAUSA300_0482
NE252	1	I24		cation efflux family protein	SAUSA300_0171
NE271	1	M14	opuCd	glycine betaine/carnitine/choline transport system permease	SAUSA300_2390
NE273	1	M18		putative transporter	SAUSA300_2449
NE280	1	O8	pbuX	xanthine permease	SAUSA300_0387
NE283	1	O14		xanthine/uracil permease family protein	SAUSA300_2207
NE298	1	B20		putative membrane protein	SAUSA300_2398
NE308	1	D16		transporter, monovalent cation:proton antiporter-2 (CPA2) family protein	SAUSA300_0911
NE317	1	F10		putative membrane protein	SAUSA300_0881
NE323	1	F22		conserved hypothetical protein	SAUSA300_0362
NE333	1	H18		putative maltose ABC transporter,maltose-binding protein	SAUSA300_0209
NE337	1	J2		integral membrane domain protein	SAUSA300_0109
NE339	1	J6		LysE/YggA family protein	SAUSA300_0784
NE357	1	L18		Na/Pi cotransporter family protein	SAUSA300_0107
NE366	1	N12		putative Na+/H+ antiporter	SAUSA300_2384
NE374	1	P4	feoB	ferrous iron transport protein B	SAUSA300_2487
NE378	1	P12		putative membrane protein	SAUSA300_0010
NE388	2	A7		sodium-dependent transporter	SAUSA300_1897
NE405	2	C17		MATE efflux family protein	SAUSA300_0335
NE412	2	E7		conserved hypothetical protein	SAUSA300_1374
NE418	2	E19		cation efflux family protein	SAUSA300_2363
NE423	2	G5	kdpD	sensor histidine kinase, KdpD	SAUSA300_2035
NE426	2	G11	tatC	Sec-independent protein translocase TatC	SAUSA300_0347
NE430	2	G19		putative transporter	SAUSA300_2417
NE433	2	I1	pstC	phosphate ABC transporter, permease protein PstC	SAUSA300_1282
NE440	2	I15	oppA	oligopeptide ABC transporter, substrate-binding protein	SAUSA300_0891
NE450	2	K11		PTS system, IIBC components	SAUSA300_0236
NE457	2	M1		peptide ABC transporter, permease protein	SAUSA300_0202
NE461	2	M9		polysaccharide extrusion protein	SAUSA300_0134
NE462	2	M11		putative membrane protein	SAUSA300_0917
NE470	2	O3		putative membrane protein	SAUSA300_0346
NE478	2	O19	phnE	phosphonate ABC transporter, permease protein	SAUSA300_0142
NE484	2	B7		amino acid ABC transporter, permease/substrate-binding protein	SAUSA300_1808
NE496	2	D7		transporter, TRAP family	SAUSA300_1064
NE521	2	H9		ABC transporter, permease protein	SAUSA300_2307
NE528	2	H23		putative permease	SAUSA300_0091
NE531	2	J5		putative drug transporter	SAUSA300_2128
NE533	2	J9		ABC transporter, permease protein	SAUSA300_0436
NE541	2	L1		peptide ABC transporter, permease protein	SAUSA300_0201
NE544	2	L7	nupC	pyrimidine nucleoside transport protein	SAUSA300_0506
NE548	2	L15		putative transporter	SAUSA300_2139
NE560	2	N15	gltS	sodium/glutamate symporter	SAUSA300_2291
NE561	2	N17		copper-translocating P-type ATPase	SAUSA300_2494
NE566	2	P3	gltT	proton/sodium-glutamate symport protein	SAUSA300_2329
NE578	2	A4		iron compound ABC transporter, permease protein	SAUSA300_0599
NE584	2	A16	narK	nitrite extrusion protein	SAUSA300_2333

NE588	2	A24	srrB	staphylococcal respiratory response protein, srrB	SAUSA300_1441
NE589	2	C2		sodium/bile acid symporter family protein	SAUSA300_2268
NE590	2	C4	copA	ATPase copper transport	SAUSA300_0078
NE592	2	C8	atpA	ATP synthase F1, alpha subunit	SAUSA300_2060
NE605	2	E10	brnQ	branched-chain amino acid transport system II carrier protein	SAUSA300_0306
NE607	2	E14	phnE	phosphonate ABC transporter, permease protein	SAUSA300_0143
NE610	2	E20	lysP	lysine-specific permease	SAUSA300_1628
NE618	2	G12	phoR	sensory box histidine kinase PhoR	SAUSA300_1638
NE622	2	G20		putative nucleoside permease NupC	SAUSA300_0313
NE625	2	I2		sodium:dicarboxylate symporter family protein	SAUSA300_0382
NE626	2	I4	sdhA	succinate dehydrogenase, flavoprotein subunit	SAUSA300_1047
NE627	2	I6		putative membrane protein	SAUSA300_2211
NE628	2	I8		formate/nitrite transporter family protein	SAUSA300_2349
NE636	2	I24		exfoliative toxin A	SAUSA300_1065
NE645	2	K18		ABC transporter, ATP-binding protein	SAUSA300_0647
NE648	2	K24	glvC	PTS system, arbutin-like IIBC component	SAUSA300_2270
NE658	2	M20	potD	spermidine/putrescine ABC transporter, spermidine/putrescine-binding protein	SAUSA300_1002
NE661	2	O2		putative Na+/H+ antiporter, MnhG component	SAUSA300_0616
NE663	2	O6		maltose ABC transporter, permease protein	SAUSA300_0211
NE675	2	B6	sirB	iron compound ABC transporter, permease protein SirB	SAUSA300_0116
NE677	2	B10	bccT	choline/carnitine/betaine transporter, BCCT family	SAUSA300_2549
NE681	2	B18	kdpB	K+-transporting ATPase, B subunit	SAUSA300_2033
NE683	2	B22	oppB	oligopeptide ABC transporter, permease protein	SAUSA300_0895
NE719	2	H22	corA	magnesium and cobalt transport protein	SAUSA300_2293
NE724	2	J8		iron compound ABC transporter, permease protein	SAUSA300_0719
NE736	2	L8	mgtE	magnesium transporter	SAUSA300_0910
NE737	2	L10		ABC transporter permease protein	SAUSA300_0797
NE743	2	L22		integral membrane protein LmrP	SAUSA300_0180
NE749	2	N10		putative drug transporter	SAUSA300_0121
NE752	2	N16		putative membrane protein	SAUSA300_2287
NE758	2	P4		putative transport protein SgaT	SAUSA300_0330
NE767	2	P22		PTS system, sucrose-specific IIBC component	SAUSA300_2324
NE768	2	P24	fruA	fructose specific permease	SAUSA300_0685
NE771	3	A5	-	putative transporter protein	SAUSA300_2379
NE773	3	A9	-	drug transporter	SAUSA300_2451
NE775	3	A13	-	ABC transporter permease	SAUSA300_2634
NE779	3	A21	-	hypothetical protein	SAUSA300_1254
NE781	3	C1	-	multidrug resistance protein B, drug resistance transporter	SAUSA300_2298
NE782	3	C3	opuCb	glycine betaine/carnitine/choline ABC transporter	SAUSA300_2392
NE803	3	E21	cap5M	capsular polysaccharide biosynthesis protein Cap5M	SAUSA300_0164
NE810	3	G11	-	D-serine/D-alanine/glycine transporter	SAUSA300_1642
NE813	3	G17	nixA	high-affinity nickel-transporter	SAUSA300_2630
NE818	3	I3	-	ABC transporter permease	SAUSA300_0176
NE829	3	K1	-	cobalt transport family protein	SAUSA300_2174
NE832	3	K7	-	ComE operon protein 1	SAUSA300_1549

NE846	3	M11	-	putative monovalent cation/H+ antiporter subunit F	SAUSA300_0615
NE854	3	O3	-	membrane spanning protein	SAUSA300_2454
NE866	3	B3		AcrB/AcrD/AcrF family protein	SAUSA300_2213
NE869	3	B9	-	iron compound ABC transporter permease	SAUSA300_2135
NE870	3	B11	opp-3C	oligopeptide permease, channel-forming protein	SAUSA300_0075
NE884	3	D15	-	oligopeptide ABC transporter permease	SAUSA300_2409
NE889	3	F1	putP	high affinity proline permease	SAUSA300_1883
NE891	3	F5	-	sodium transport family protein	SAUSA300_0924
NE901	3	H1	-	putative drug transporter	SAUSA300_0268
NE923	3	J21	-	hypothetical protein	SAUSA300_2286
NE929	3	L9	mtlF	PTS system, mannitol specific IIBC component	SAUSA300_2105
NE931	3	L13	-	ABC transporter permease	SAUSA300_0308
NE945	3	N17	brnQ	branched-chain amino acid transport system II carrier protein	SAUSA300_0188
NE951	3	P5	-	putative abrB protein	SAUSA300_1613
NE952	3	P7	gntP	gluconate permease	SAUSA300_2442
NE967	3	A14	-	Na+/H+ antiporter family protein	SAUSA300_0846
NE969	3	A18		sodium:solute symporter family protein	SAUSA300_0314
NE971	3	A22		amino acid/peptide transporter (Peptide:H+ symporter)	SAUSA300_0712
NE979	3	C14		sugar efflux transporter	SAUSA300_0659
NE992	3	E16	-	PTS system, sorbitol-specific IIC component	SAUSA300_0241
NE996	3	E24	-	putative transport protein	SAUSA300_2397
NE1000	3	G8	-	putative monovalent cation/H+ antiporter subunit C	SAUSA300_0612
NE1007	3	G22	-	putative proline/betaine transporter	SAUSA300_0558
NE1015	3	I14	-	formate/nitrite transporter family protein	SAUSA300_0305
NE1023	3	K6	ptsI	phosphoenolpyruvate-protein phosphotransferase	SAUSA300_0984
NE1025	3	K10		integral membrane protein	SAUSA300_0729
NE1030	3	K20	rarD	RarD protein	SAUSA300_2628
NE1033	3	M2		putative membrane protein	SAUSA300_2466
NE1034	3	M4	norA	multi drug resistance protein	SAUSA300_0680
NE1041	3	M18	-	putative drug transporter	SAUSA300_2217
NE1048	3	O8	pyrP	uracil permease	SAUSA300_1092
NE1049	3	O10	atpD	F0F1 ATP synthase subunit beta	SAUSA300_2058
NE1052	3	O16	-	peptide ABC transporter permease	SAUSA300_1275
NE1068	3	B24	-	citrate transporter, permease	SAUSA300_2552
NE1074	3	D12	oppB	oligopeptide ABC transporter permease	SAUSA300_0887
NE1083	3	F6	potC	spermidine/putrescine ABC transporter permease	SAUSA300_1001
NE1097	3	H10	-	manganese transport protein MntH	SAUSA300_1005
NE1105	3	J2	-	ABC transporter protein	SAUSA300_2556
NE1114	3	J20	-	perfringolysin O regulator protein	SAUSA300_2471
NE1115	3	J22	-	transporter protein	SAUSA300_2434
NE1125	3	L18	arcD	arginine/oirnithine antiporter	SAUSA300_0064
NE1130	3	N4	-	hypothetical protein	SAUSA300_1561
NE1131	3	N6	-	amino acid permease	SAUSA300_2395
NE1135	3	N14	opuD	glycine betaine transporter opuD	SAUSA300_1245
NE1137	3	N18	-	glycine betaine transporter	SAUSA300_2145
NE1139	3	N22	-	putative monovalent cation/H+ antiporter subunit D	SAUSA300_0613
NE1154	4	A3	uhpT	sugar phosphate antiporter	SAUSA300_0216
NE1161	4	A17	-	integral membrane protein	SAUSA300_0714

NE1183	4	E13	arlS	sensor histidine kinase protein	SAUSA300_1307
NE1184	4	E15	-	putative drug transporter	SAUSA300_1328
NE1187	4	E21	-	hypothetical protein	SAUSA300_0321
NE1197	4	G17	-	cobalt transport family protein	SAUSA300_2616
NE1204	4	I7	-	antibiotic transport-associated protein-like protein	SAUSA300_2489
NE1209	4	I17		oligopeptide ABC transporter, permease protein	SAUSA300_2410
NE1214	4	K3	-	Na+/H+ antiporter family protein	SAUSA300_2273
NE1229	4	M9	-	ABC transporter permease	SAUSA300_1515
NE1234	4	M19	lctP	L-lactate permease	SAUSA300_0112
NE1254	4	B11	pfoR	perfringolysin O regulator protein	SAUSA300_0310
NE1270	4	D19	-	hypothetical protein	SAUSA300_0913
NE1274	4	F3	pstA	phosphate ABC transporter permease PstA	SAUSA300_1281
NE1280	4	F15		ABC transporter, substrate-binding protein	SAUSA300_0231
NE1282	4	F19	-	PTS system, IIA component	SAUSA300_0332
NE1290	4	H11		phosphotransferase system, fructose-specific IIABC component	SAUSA300_2576
NE1292	4	H15		putative Na+/H+ antiporter, MnhE component	SAUSA300_0614
NE1314	4	L11	oppC	oligopeptide ABC transporter permease	SAUSA300_0896
NE1327	4	N13	-	hypothetical protein	SAUSA300_0393
NE1351	4	A14		conserved hypothetical protein	SAUSA300_1846
NE1356	4	A24		putative urea transporter	SAUSA300_2237
NE1360	4	C8	fmtC	oxacillin resistance-related FmtC protein	SAUSA300_1255
NE1373	4	E10		putative homoserine O-acetyltransferase	SAUSA300_0012
NE1376	4	E16	cobI	transporter, CorA family	SAUSA300_2323
NE1378	4	E20		oligopeptide ABC transporter, oligopeptide-binding protein	SAUSA300_0892
NE1388	4	G16		glycerol-3-phosphate transporter	SAUSA300_0337
NE1398	4	I12		conserved hypothetical protein	SAUSA300_1084
NE1400	4	I16		putative drug transporter	SAUSA300_2389
NE1405	4	K2	glcU	probable glucose uptake protein"	SAUSA300_2210
NE1411	4	K14	mscL	large conductance mechanosensitive channel protein	SAUSA300_1244
NE1418	4	M4		2-oxoglutarate/malate translocator	SAUSA300_2627
NE1419	4	M6		putative nucleoside transporter	SAUSA300_0631
NE1421	4	M10		PTS system, fructose-specific enzyme II, BC component	SAUSA300_0239
NE1435	4	O14		putative amino acid permease	SAUSA300_2265
NE1436	4	O16	oppC	oligopeptide ABC transporter, permease protein	SAUSA300_0888
NE1457	4	D10	nagE	phosphotransferase system, N-acetylglucosamine-specific IIBC component	SAUSA300_1672
NE1463	4	D22		sodium:alanine symporter family protein	SAUSA300_0914
NE1468	4	F8	fhuG	ferrichrome transport permease protein fhuG	SAUSA300_0635
NE1470	4	F12	nhaC	Na+/H+ antiporter NhaC	SAUSA300_2250
NE1484	4	H16	arsB	aresenical pump membrane protein	SAUSA300_1718
NE1490	4	J4	treP	PTS system, trehalose-specific IIBC component	SAUSA300_0448
NE1499	4	J22	opp-3B	oligopeptide permease, channel-forming protein	SAUSA300_0074
NE1501	4	L2	hemX	hemA concentration negative effector hemX	SAUSA300_1618
NE1504	4	L8	-	Na+/H+ antiporter	SAUSA300_0617
NE1509	4	L18	-	ABC transporter ATP-binding protein	SAUSA300_0630
NE1514	4	N4	-	gamma-aminobutyrate permease	SAUSA300_1231
NE1516	4	N8	-	hypothetical protein	SAUSA300_0606
NE1541	5	A9	-	BioY family protein	SAUSA300_2233

NE1548	5	A23	atpB	F0F1 ATP synthase subunit A	SAUSA300_2064
NE1566	5	E11	sirC	iron compound ABC transporter permease SirC	SAUSA300_0115
NE1580	5	G15	glpF	glycerol uptake facilitator	SAUSA300_1191
NE1584	5	G23	-	PTS system, IIA component	SAUSA300_0259
NE1592	5	I15	-	amino acid ABC transporter amino acid-binding protein	SAUSA300_2359
NE1609	5	M1	opp-2F	oligopeptide permease, ATP-binding protein	SAUSA300_1273
NE1620	5	M23	-	PTS system, galactitol-specific enzyme II, B component	SAUSA300_0240
NE1629	5	O17	-	hypothetical protein	SAUSA300_2618
NE1650	5	D11	-	twin arginine-targeting protein translocase	SAUSA300_0348
NE1706	5	N3	-	hypothetical protein	SAUSA300_1870
NE1709	5	N9	-	amino acid permease	SAUSA300_0566
NE1737	5	A18	mtlA	PTS system, mannitol specific IIA component	SAUSA300_2107
NE1751	5	C22	-	hypothetical protein	SAUSA300_0979
NE1763	5	E22	-	putative traG membrane protein	SAUSA300_1480
NE1774	5	G20	-	ABC transporter permease	SAUSA300_2358
NE1780	5	I8	-	hypothetical protein	SAUSA300_0568
NE1784	5	I16	-	ABC transporter permease	SAUSA300_0619
NE1804	5	M8	-	putative drug transporter	SAUSA300_0106
NE1817	5	O10	potB	spermidine/putrescine ABC transporter permease	SAUSA300_1000
NE1822	5	O20	-	ABC transporter permease	SAUSA300_1218
NE1823	5	O22	-	Tat-translocated protein	SAUSA300_0345
NE1839	5	D6	-	cadmium-exporting ATPase, truncation	SAUSA300_0068
NE1847	5	D22	-	putative hemolysin	SAUSA300_0687
NE1850	5	F4	atpG	F0F1 ATP synthase subunit gamma	SAUSA300_2059
NE1877	5	J10	-	hypothetical protein	SAUSA300_0824
NE1881	5	J18	fhuB	ferrichrome transport permease fhuB	SAUSA300_0634
NE1889	5	L10	atpH	F0F1 ATP synthase subunit delta	SAUSA300_2061

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