

**Using a zebrafish model to examine the importance of the stringent response for*Staphylococcus aureus* pathogenesis**

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# Abstract

Staphylococcus aureus is a human commensal organism with the potential to become opportunistic given the right conditions. During infection, bacteria are exposed to stresses like nutrient limitation, which they respond to by inducing the stringent response. This is a conserved bacterial reaction to stress that ultimately shuts down macromolecular processes to promote bacterial survival, achieved by the production of the nucleotide signalling molecule guanosine penta-/tetraphosphate ((p)ppGpp). The S. aureus genome encodes three (p)ppGpp synthetases: Rel, RelP and RelQ. (p)ppGpp produced by these synthetases contributes to bacterial pathogenesis in a number of species. In this study, a toolbox of *S. aureus* strains was constructed to improve understanding of how the stringent response is important for the survival and virulence of *S. aureus* *in vitro* and in vivo. To elucidate the mechanism behind the requirement of (p)ppGpp for intracellular survival *in vitro*, the *S. aureus* strains were exposed to stress conditions typically found in a phagolysosome. A (p)ppGpp0 mutant was observed to be more susceptible to hydrogen peroxide, itaconic acid and hypochlorous acid whereas an overproduction of (p)ppGpp increased the tolerance of *S. aureus* to these stressors. RelP alone could complement itaconic acid stress while both Rel and RelP could have a role in the tolerance of *S. aureus* to hydrogen peroxide. Systemic infection of zebrafish embryos with a (p)ppGpp0 mutant revealed increased survival of embryos when compared to embryos infected with the wildtype, which is likely due to a virulence defect as the (p)ppGpp0 mutant replicated at a similar rate to the wildtype *in vivo*. (p)ppGpp overproduction also resulted in an increased survival of embryos. Complementation experiments revealed that Rel is a key (p)ppGpp synthetase during infection, but that RelP and RelQ may be sufficient in the absence of Rel. Taken together, these results demonstrate a role of (p)ppGpp for *S. aureus* pathogenesis within a zebrafish embryo systemic infection model, with *in vitro* experiments showing that the stringent response is required for survival during stress.

# Publications

Choudhury, NR., Urwin, L., Salamaga, B., Prince, LR., Renshaw, SA., Corrigan, RM. Determining the importance of the stringent response for *Staphylococcus aureus* pathogenesis using a zebrafish model of infection. **Manuscript in preparation.**

Carrilero, L., Urwin, L., Ward, E., Choudhury, NR., Monk, IR., Stinear, TP., Corrigan, RM. Stringent response-mediated control of GTP homeostasis is required for long-term viability of *Staphylococcus aureus*. *Microbiology Spectrum.* 2023. doi: [10.1128/spectrum.00447-23](https://doi.org/10.1128/spectrum.00447-23).

Irving, SE., Choudhury, NR., Corrigan, RM. The stringent response and physiological roles of (pp)pGpp in bacteria. *Nature Reviews Microbiology*. 2021. 19(4):256-271. doi: 10.1038/s41579-020-00470-y.

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# List of Abbreviations

ACT/RRM Aspartokinase, Chorismite Mutase and TyrR/RNA Recognition Motif

ACP Acyl-carrier Protein

AhpC Alkylhydroperoxide Reductase

AIP Autoinducing Peptide

AMP Antimicrobial Peptide

APC Antigen-presenting Cell

Atc Anhydrotetracycline

ATP Adenosine Triphosphate

Bap Biofilm Associated Protein

BCAA Branched-chain Amino Acids

BMDMs Bone marrow-derived Macrophages

C5aR C5a Receptor

CA-MRSA Community-Associated Methicillin Resistant *Staphylococcus aureus*

c-di-AMP Cyclic di-AMP

CHIPS Chemotaxis Inhibitory Protein of Staphylococcus

ClfA/B Clumping Factor A/B

CLR C-type Lectin Receptor

Cna Collagen adhesin

CR3/4 Complement Receptor 3/4

Ccr Cassette Chromosome Recombinase

CTD C-Terminal Domain

CWA Cell wall-associated

DAMP Danger Associated Molecular Pattern

DksA DnaK Suppressor

Eap Extracellular adherence protein

EEA1 Early Endosome Antigen 1

Efb Extracellular Fibrinogen-binding protein

EF-G Elongation factor G

EF-Tu Elongation factor Tu

EHEC Enterohaemorrhagic *E. coli*

EPEC Enteropathogenic *E. coli*

EPS Extracellular Polymeric Substance

ETA/B Exfoliative Toxin A/B

FnBPA/B Fibronectin-binding Protein A/B

FPR Formylated Peptide Receptor

GFP Green Fluorescent Protein

Gmk Guanylate Kinase

GMP Guanosine Monophosphate

GppA Guanosine Pentaphosphate Phosphohydrolase A

GTP Guanosine Triphosphate

GuaA Guanosine monophosphate Synthase

GuaB IMP Dehydrogenase

HA-MRSA Hospital-Associated Methicillin Resistant *Staphylococcus aureus*

HD Hydrolase Domain

HNP1-4 Human Neutrophil α-defensin 1-4

Hpf Hours Post Fertilisation

Hpf Hibernation Promoting Factor

Hpi Hours Post Infection

HprT Hypoxanthine Phosphoribosyltransferase

HSC Haematopoietic Stem Cell

HTE Human Tonsillar Epithelial

IEC Intestinal Epithelial Cell

IFN-γ Interferon-γ

IMP Inosine monophosphate

iNOS Inducible Nitric Oxide Synthase

iNTP Initiating Nucleotide Triphosphate

IL-6 Interleukin-6

IF2 Initiation Factor 2

LAMP-1/2 Lysosome-associated membrane proteins 1 and 2

LEE Locus of Enterocyte Effacement

LPS Lipopolysaccharide

LTA Lipoteichoic Acid

LukAB Leukocidin AB

MBL Mannose-binding Lectin

MDR Multi-Drug Resistance

MGE Mobile Genetic Element

MIP-3α Macrophage Inflammatory Protein-3 α

MPO Myeloperoxidase

MRSA Methicillin-Resistant *Staphylococcus aureus*

MSCRAMM Microbial Surface Component Recognising Adhesive Matrix Molecules

MSSA Methicillin-Sensitive *Staphylococcus aureus*

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NOX Nicotinamide Adenine Dinucleotide Phosphate Oxidase

NE Neutrophil Elastase

NLR NOD-like Receptor

Ndk Nucleoside Diphosphate Kinase

NO Nitric Oxide

NST Neutrophil Serine Protease

NTD N-terminal Domain

NuDix Nucleoside Diphosphate linked moiety X

ORPL1 Oxysterol-binding protein–Related Protein 1

PAMP Pathogen Associated Molecular Pattern

PBP Penicillin-Binding Protein

PBP2a Penicillin-Binding Protein 2a

PG Phosphatidylglycerol

PI(3)P Phosphatidylinositol 3-phosphate

PMN Polymorphonuclear leukocyte

(p)ppGpp Guanosine penta/tetraphosphate

PRA 5-phosphoribosylamine

PRPP Phosphoribosyl Pyrophosphate

PRR Pattern Recognition Receptor

PSM Phenol-Soluble Modulin

PurF Inosine monophosphate amidophosphoribosyl transferase

PVL Panton-Valentine Leukocidin

RaiA Ribosome-associated Inhibitor A

RbgA Ribosome Biogenesis GTPase A

RF1/2/3 Release Factor 1/2/3

RILP Rab7-interacting Lysosomal Protein

RLR RIG-I-like Receptor

RMF Ribosome Modulating Factor

RNAP RNA Polymerase

Rot Repressor of Toxins

rRNA Ribosomal RNA

RsgA Ribosome Small Subunit-dependent GTPase A

RSH RelA/SpoT Homologue

*sae S. aureus* exoprotein expression

SAH Short Alarmone Hydrolase

SAS Short Alarmone Synthetase

SasG *S. aureus* surface protein G

Sbi *S. aureus*Binder of IgG protein

SCIN Staphylococcal Complement Inhibitor

SCC*mec* Staphylococcal Chromosome Cassette *mec*

SCV Small Colony Variant

SNARE N-ethylmaleimide-sensitive factor attachment protein receptor

SNP Single Nucleotide Polymorphism

SOD Superoxide Dismutase

*spa* Staphylococcal Protein A

SPI-1 *Salmonella* pathogenicity Island 1

SPIN Staphylococcal Peroxidase Inhibitor

SSTI Skin and Soft Tissue Infection

SYNTH Synthetase Domain

T3SS Type III Secretion System

TA Toxin-Antitoxin

TCR T-cell Receptor

TCS Two-component System

TGS Threonyl-tRNA Synthetase GTPase and SpoT

TIR Toll/interleukin-1 Receptor

TLC Thin Layer Chromatography

TLR Toll-like Receptor

TRAF3 Tumour Necrosis-associated Factor 3

TRM Tissue-resident macrophage

TT Transcriptional Terminator

V-ATPase Vacuolar-Type ATPase

VAMP7/8 Vesicle-associated Membrane Proteins 7/8

VraS/R Vancomycin-resistance Associated Sensor/Regulator

VRSA Vancomycin Resistant *Staphylococcus aureus*

WTA Wall Teichoic Acid

XMP Xanthosine 5’-phosphate

ZFD/CC Zinc Finger Domain/Conserved Cysteine

# Chapter 1: Introduction

## 1.1. *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive, non-motile, facultative anaerobic bacterium and member of the phylum, Firmicutes. *S. aureus* is named according to its clustered appearance similar to grapes, spherical shape (coccal) and golden colony colour (*aureus*). Its pigment is owed to staphyloxanthin, a membrane-bound carotenoid that provides the characteristic golden colour and also functions as an antioxidant to resist oxidative stress **(Pelz *et al.*, 2005; Clauditz *et al.*, 2006)**. The *Staphylococcus* genus consists of 47 species and 23 subspeciesand while most are coagulase-negative such as *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*, *S. aureus* is among the few that are coagulase-positive **(Becker, Heilmann and Peters, 2014)**. Coagulase is an enzyme and virulence factor that promotes clotting by modulating the host clotting cascade and is useful for the establishment of infection **(Cheng *et al.*, 2010; McAdow, Missiakas and Schneewind, 2012)**, and testing for coagulase is a common method of identifying *S. aureus* in the laboratory for differentiation from coagulase-negative staphylococci **(Foster, 1996)**.

Based on a comparative analyses of different staphylococcal genomes, the *S. aureus* genome consists of an approximately 2.8 Mbp circular chromosome with low G+C content of around 32% **(Baba *et al.*, 2008)**. While most of the *S. aureus* genome is well conserved, particular sequences can be highly variable which is most likely due to horizontal gene transfer **(Baba *et al.*, 2008)**. Varying mobile genetic elements (MGE) are also present including plasmids, transposons and pathogenicity islands, with many of these encoding for antibiotic resistance and virulence factors **(Malachowa and DeLeo, 2010; Mlynarczyk, Mlynarczyk and Jeljaszewicz, 1998)**.

### 1.1.1. Epidemiology of *S. aureus*

*S. aureus* isa human commensal organism colonising many niches including the skin and mucosal membranes such as the anterior nares and upper respiratory tract. It was first isolated in 1880 from an abscess produced by a surgical wound infection **(Ogston, 1880)**. The ubiquity of *S. aureus* highlights its ability to survive in diverse environments. Approximately 20-30% of the human population carry *S. aureus* in the nares and 5% on the skin **(Tong *et al.*, 2015)** and persistent carriage increases the risk of developing infection **(Wertheim *et al.*, 2005; Gosbell and van Hal, 2013)**, particularly if a hospital patient is colonised or immunocompromised. Thus, it is important to consider the colonisation status of patients prior to surgery to prevent surgical site infections. For example, studies have reported that prior to total joint arthroplasty (joint replacement), preoperative decolonisation of the nares and skin using intranasal mupirocin administration and chlorhexidine showers respectively, had markedly reduced both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) colonisation **(Chen *et al.*, 2013; George, Leasure and Horstmanshof, 2016)**. This then reduces the likelihood of surgical site infections. Further research has demonstrated that preoperative decolonisation regimes in Yorkshire and Humber region in the UK have not affected the resistance rates of *S. aureus,* further emphasising its advantage **(Horner *et al.*, 2017)**. As *S. aureus* is an opportunistic pathogen, it has the ability to cause a plethora of infections, affecting most body systems. Upon breach of skin and mucosal barriers, *S. aureus* can cause skin and soft tissue infections (SSTIs), pneumonia, medical device-related infections and sepsis. Furthermore, in the event of persistent carriers developing an infection, the causative agent is usually a commensal *S. aureus* strain **(Williams *et al.*, 1959)**.

Of the different *S. aureus* infections, particularly important are MRSA strains that have the ability to cause detrimental healthcare- and community- associated (HA-MRSA/CA-MRSA) infections. MRSA isolates are classified as such due to the presence of *mecA*, encoding for an alternative penicillin-binding protein 2a (PBP2a). MRSA infections were predominantly healthcare-associated, affecting both in- and outpatients, however during the late 20th century, there was an increase in MRSA infections within the general community **(Li *et al.*, 2009)**. Since then, both HA-MRSA and CA-MRSA cases appear to be declining **(Wyllie *et al.*, 2011)**, however the mortality associated with these infections remain high **(Turner *et al.*, 2019)** and even with the advent of antibiotics, the mortality rates have been reported as 15-50% **(van Hal *et al.*, 2012; Lam and Stokes, 2023).** Risk factors of HA-MRSA include long-term hospitalisation, presence of an invasive medical device, surgery and being previously colonised by *S. aureus* or MRSA **(Gajdacs, 2019)**. Importantly, *S. aureus* bacteraemia is increasingly becoming a concern **(Thwaites and Gant, 2011; Turner *et al.*, 2019).** Once *S. aureus* enters the bloodstream, if not treated in a timely manner, it can be introduced to various organs leading to metastasis **(Raineri, Altulea and van Dijl, 2022)** and can thus further complicate treatment, in addition to the issue of antibiotic resistance.

### 1.1.2. MRSA and its treatment

In the 1940s, after the discovery and wide administration of penicillin, resistance to this drug had been documented. An extracellular penicillin-inactivating enzyme named penicillinase (β-lactamase) was first reported in an *Escherichia coli* strain **(Abraham and Chain, 1988)**. Resistance became a significant issue by the 1960s, where over 80% of infections were caused by strains producing penicillinase **(Lowy, 2003)**, highlighting how resistance can develop very shortly after antibiotic use as bacteria are constantly evolving. After the introduction of methicillin (a β-lactam resistant to β-lactamases), MRSA was first described in *S. aureus* in 1961 and was found to employ a different method of resistance in 1981. Methicillin-resistant bacteria produced an altered version of the PBP named PBP2a, encoded on staphylococcal chromosome cassette *mec* (SCC*mec*), an MGE, which is part of the *S. aureus* chromosome **(Katayama, Ito and Hiramatsu, 2000)**. In addition to *mecA*, the *mec* gene complex also contains the *mecA* regulatory genes, *mecRI and mecI* **(Tesch et al., 1990; Sharma et al., 1998)***.* SCC*mec* also encodes for the *ccr* gene complex encoding for two site-specific cassette chromosome recombinases (CcrA and CcrB), that are required for the excision and insertion of SCC into the *S. aureus* genome at the 3’ end of *rlmH* (*orfX*) **(Ito, Katayama and Hiramatsu, 1999)**. While the presence of the *mec* and *ccr* complexes are a prerequisite for SCC*mec* **(Zong, Peng and Lu, 2011)**,there are 13 variations of SCC*mec* depending on the *mec* type and *ccrAB* allotype. HA-MRSA predominantly carry type I-III, while CA-MRSA tends to carry IV and V **(Yamaguchi, Ono and Sato, 2020)**. Antibiotic resistance can be acquired via MGEs as demonstrated with SCC*mec* or through horizontal gene transfer but can also be acquired through mutations that occur randomly and through selection pressure **(Hawkey, 1998)**.

With time, MRSA strains became resistant to all types of penicillin and subsequently other classes of antibiotics such as the fluoroquinolones and aminoglycosides. Due to this, MRSA can be classed as the first known multi-drug resistant (MDR) bacterium **(van Duin and Paterson, 2020)**. As MRSA can cause a variety of infections, multiple antibiotics are utilised for treatment depending on the infection type. For example, the protein synthesis inhibitors mupirocin and linezolid can be used for skin infections **(Nandhini *et al.*, 2022; Weigelt *et al.*, 2005)**. The gold standardantibiotics for the treatment of MRSA vary depending on the country, however the last resort antibiotics that are prominently used to treat MDR MRSA include, but are not limited to, the glycopeptide vancomycin and the lipopeptide daptomycin. Vancomycin resistance was not documented until 20 years after use, where resistance in an enterococcal species was first described **(Leclercq *et al.*, 1988; Uttley *et al.*, 1988)**. A patient case study in 1999 found isolates of vancomycin-resistant MRSA **(Sieradzki *et al.*, 1999)**. Since then, vancomycin-resistant *S. aureus* (VRSA) has been found to produce VanA, thought to be acquired from resistant enterococcal species **(McGuinness, Malachowa and DeLeo, 2017)**. *vanA* encodes a ligase required to ligate D-Ala and D-Lac, therefore altering peptidoglycan synthesis and thus affecting vancomycin’s affinity to its target, D-Ala-D-Ala. Fortunately, the prevalence of VRSA is low **(McGuinness, Malachowa and DeLeo, 2017)** however, as with all antibiotics, bacteria will continue to evolve to become resistant. Vancomycin treatment does however carry some disadvantages. Vancomycin is mainly administered parenterally, which means that patients must remain in hospital and also due to side effects such as nephrotoxicity, therapeutic drug monitoring is also necessary **(Gajdacs, 2019)**. Thus, other glycopeptide antibiotics such as teicoplanin have been considered. A meta-analysis has concluded that the efficacy of teicoplanin is similar to vancomycin and while teicoplanin must also be parenterally administered, there is a lower risk of nephrotoxicity **(Cavalcanti *et al.*, 2010)**.

Daptomycin is a cyclic lipopeptide antibiotic, structurally similar to cationic antimicrobial peptides (AMPs). Daptomycin inserts itself into the Gram-positive cell membrane by targeting membrane lipids such as phosphatidylglycerol (PG) and lipid II in a calcium-dependent manner and ultimately acts to depolarise the membrane, leading to by membrane disruption **(Grein *et al.*, 2020; Hobbs *et al.*, 2008)**. Daptomycin is often used as an alternative therapy in the event of vancomycin resistance or nephrotoxicity mediated by prolonged vancomycin treatment. For example, participants in a matched cohort study with MRSA bacteraemia were treated with vancomycin for approximately three days before changing to daptomycin **(Murray *et al.*, 2013)**. Treatment success/failure was compared to a matched group of patients treated with vancomycin only and when daptomycin was administered after vancomycin treatment, there were fewer treatment failure in comparison to the vancomycin only group (20% vs 48.2% respectively) **(Murray *et al.*, 2013)**. This study highlights the benefits of switching to daptomycin therapy. These results were further substantiated recently in a similar study with a larger cohort **(Schweizer *et al.*, 2021)**, providing evidence that daptomycin is a suitable alternative in the event of vancomycin treatment failure. Currently, daptomycin resistance is uncommon however, due to the ability of *S. aureus* to evolve rapidly, the possibility of the widespread development of resistance is almost certain. Mutations in genes encoding for proteins that have been associated with daptomycin resistance include *mprF*, the *dltABCD* operon, *yycG* and *pgsA* **(Tran, Munita and Arias, 2015)**. One of the most common genes involved in resistance to daptomycin encodes the multiple peptide resistance factor (MprF), which is a protein responsible for flipping lysyl-PG to the outer leaflet of the cell membrane **(Andrä  *et al.*, 2011)**. Mutations within *mprF* typically cause single nucleotide polymorphisms (SNPs) and result in fewer lysyl-PG lipids translocated to the outer leaflet, therefore altering the bacterial surface charge **(Sabat *et al.*, 2018; Andrä  *et al.*, 2011)**. Fewer PG molecules within the membrane increase the net positive charge, impairing the functionality of daptomycin **(Heidary *et al.*, 2018)**.

Due to the ability of *S. aureus* to exist as a commensal, hosts may develop tolerance to the strains that they are colonised by **(Proctor, 2012)**, which may affect the efficacy of potential vaccines. Various vaccine candidates have been trialled, for example utilising the toxins that *S. aureus* produces including the pore-forming toxins α-toxin and Panton-Valentine leukocidin (PVL) **(Francois *et al.*, 2018; Magyarics *et al.*, 2019)**. Polysaccharide conjugate vaccines such as StaphVax® have also been developed that consists of purified capsular polysaccharide antigens and PentaStaph®, which also contain teichoic acids, α-toxin and PVL antigens in addition to polysaccharides **(Miller *et al.*, 2020; Tong, Chen and Fowler, 2012)**. The SA4Ag vaccine was also developed, which combined the use of capsular polysaccharides with cell wall-anchored proteins such as MntC and ClfA **(Begier *et al.*, 2017)**. Many of these studies failed during different stages of clinical trials or did not produce high enough titres of antibodies within animal models or human participants. As these vaccines mainly aim to clear bacteria by antibody-mediated opsonisation and thus have not passed clinical trials, new vaccine targets are being developed to exploit T cell-mediated immunity instead **(O'Brien and McLoughlin, 2019; Proctor, 2012)**. Vaccine failure is attributed to the ubiquity of *S. aureus* but also the ability of *S. aureus* to adapt to different environmental conditions. This adaptability allows this remarkable pathogen to infect nearly all body systems and thus cause a variety of disease that has hindered the development of an anti-staphylococcal vaccine. Another limitation in the efforts to produce an anti-staphylococcal vaccine are the animal models used to test their efficacy. For example, neutrophils from different mice are more resistant to PVL than rabbit and human neutrophils **(Löffler  *et al.*, 2010)**, emphasising the necessity of choosing the appropriate animal model when studying the efficacy of vaccines. Moreover, vaccines that have been investigated in murine models did not go on to successfully immunise humans during clinical trials, suggesting that non-human primate models may be more suitable **(Proctor, 2012)**.

### 1.1.3. *S. aureus* virulence factors

*S. aureus* produces a wide range of membrane-bound, secreted and cytoplasmic factors that aid its survival, virulence and evasion of the immune system. These factors contribute greatly to the ability of *S. aureus* to cause infections. While some staphylococcal virulence factors are discussed here, those involved in the evasion of professional phagocytes are discussed in section 1.6.5.

#### 1.1.3.1. *S. aureus* cell wall-associated proteins

The *S. aureus* cell wall-associated (CWA) proteins can be involved in adhesion, colonisation and invasion of *S. aureus*,which is integral for the establishment of infection. CWA proteins are categorised into four different groups: near iron transporters (NEAT) motif, three-helical bundles, G5-E repeats and microbial surface component recognising adhesive matrix molecules (MSCRAMMs), of which the latter is the largest. MSCRAMMs are secreted in a Sec-dependent manner, linked to the cell wall by sortase and are defined by N-terminal Ig-like folds **(Schneewind and Missiakas, 2014; Foster *et al.*, 2014)**. For example, the MSCRAMMs clumping factors A and B (ClfA/B) mediate adhesion of *S. aureus* to fibrinogen **(McDevitt *et al.*, 1994; Eidhin *et al.*, 1998) (Fig. 1.1.3)**. However ClfB can also bind cytokeratin **(Xiang *et al.*, 2012)** and loricrin, which is required for murine nasal colonisation **(Mulcahy *et al.*, 2012)**. Clumping factor-deficient mutants were attenuated in a rat endocarditis model, emphasising the importance of adhesion **(Moreillon *et al.*, 1995)**. Fibronectin-binding proteins A/B (FnBPA/B) bind to fibronectin but can also bind to fibrinogen **(Wann, Gurusiddappa and Hook, 2000)**. FnBPs, like ClfA/B, are able to mediate adhesion but are also involved in invasion of epithelial and endothelial cells as well as fibroblasts via the α5β1 integrin **(Sinha *et al.*, 1999)**. The collagen adhesin (Cna) binds to collagen in the extracellular matrix and thus acts as an adhesin **(Madani, Garakani and Mofrad, 2017) (Fig. 1.1.3)**. A role for Cna in a septic arthritis model was demonstrated where Cna was required for binding to cartilage and for the development of arthritis **(Patti *et al.*, 1994)**. Staphylococcal protein A (*spa*) is the only CWA member of the three-helical bundle family that binds to the Fc region of IgG and blocks IgG hexamerisation and thus complement activation **(Cruz *et al.*, 2021)**, implicating *spa* in immune evasion. The *S. aureus*binder of IgG (Sbi) protein, which is non-covalently linked to lipoteichoic acids (LTA), is also involved in immune evasion by binding to IgG **(Smith *et al.*, 2012)**. The Isd proteins of the NEAT motif family are involved in iron acquisition by binding to haem and have also been involved in invasion of epithelial cells by binding to β3 integrins **(Pilpa *et al.*, 2009; Zapotoczna *et al.*, 2013)**.

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| **Figure 1.1.3. The *S. aureus* virulence factors. a)** *S. aureus* use CWA proteins ClfA/B, FnBPA/B and Cna to facilitate adhesion to host factors such as fibrinogen, fibronectin and collagen respectively in order to establish infection. Isd, an iron acquisition protein will scavenge iron from haem. FnBPA/Band Isd have also been implicated in immune cell invasion and are represented by the green arrows. Protein A and Sbi are IgG binding proteins and thus contribute to immune evasion. CWA proteins are outlined in light blue. **b)** The secreted protein Efb is secreted when *S. aureus* is intracellular and inhibits C3b. CHIPS prevents chemotaxis by interacting with the C5aR and FPR. SCIN inhibits C3 convertase which prevents all three complement cascades, thus preventing opsonisation and the formation of the membrane attack complex (MAC). Eap inhibits the NSP and SPIN inhibits MPO. Both MPO and NSP are components of azurophilic granules found in neutrophils. Secreted proteins except for the toxins are outlined in red. **c)** The exfoliative toxin ETA/B inhibits desmoglein-1 which leads to breakdown of the skin layers. The α-toxin, PSMs, LukAB and PVL function by forming pores in the host cell membrane, thus causing cell lysis. The TSST superantigen crosslinks the TCR on T cells with MHCII on dendritic cells, leading to a cytokine storm. Toxins are outlined in purple. **d)** Biofilm formation requires adhesins for establishment. FnBPA/B are upregulated by SarA and are necessary for initial adhesion along with Bap. Cell-cell contact within biofilms is mediated by SasG and Bap. |

#### 1.1.3.2. Secreted proteins and toxins

*S. aureus* also secretes many proteins that act locally of the bacterial colonisation site, and contribute to immune evasion as well as virulence and pathogenesis, enabling *S. aureus* to thrive both extracellularly and intracellularly. The staphylococcal complement inhibitor (SCIN) is an antiphagocytic molecule, regarded as the most efficient factor at preventing the complement cascade. SCIN targets the C3 convertase, preventing complement deposition by inhibiting the three complement pathways which has resulted in reduced phagocytosis by human neutrophils **(Rooijakkers *et al.*, 2005) (Fig. 1.1.3)**. Another protein involved in complement inhibition is chemotaxis inhibitory protein of *Staphylococcus* (CHIPS), which competitively inhibits formylated peptide receptor (FPR) and C5a receptor (C5aR), preventing these receptors from binding to their cognate ligands (bacterial formylated peptides and C5a respectively), thus preventing neutrophil recruitment *in vivo* **(de Haas *et al.*, 2004; Postma *et al.*, 2004)**. Furthermore, *S. aureus* secretes extracellular fibrinogen-binding protein (Efb) within the macrophage cytoplasm to inhibit pro-inflammatory cytokine expression and was shown to bind the tumour necrosis-associated factor 3 (TRAF3) regulator, which ultimately leads to activation of the pro-inflammatory transcription factor NF-κB **(Zhang *et al.*, 2022; Fournier and Philpott, 2005)**. Efb has also been implicated in preventing complement deposition by interacting with C3b **(Hammel *et al.*, 2007)**. The staphylococcal serine proteases, extracellular adherence proteins (Eap), were shown to prevent granular neutrophil serine proteases (NSPs), such as neutrophil elastase, from degrading the phenol soluble modulins (PSMs) (PSMs are further discussed below) **(Stapels *et al.*, 2014; Kretschmer *et al.*, 2021) (Fig. 1.1.3)**. The staphylococcal peroxidase inhibitor (SPIN) binds to and inhibits myeloperoxidase (MPO), another component of neutrophil granules **(de Jong *et al.*, 2017a)**. Increased expression and activity of SPIN was observed upon uptake by neutrophils and SPIN-deficient *S. aureus* demonstrated decreased survival **(de Jong *et al.*, 2017a)**. Thus, both Eap proteins and SPIN are able to target degradative enzymes released by neutrophils.

Staphylococcal secreted proteins also extend to the arsenal of toxins it produces. Toxins are categorised as exfoliative, pore-forming or superantigens. The exfoliative toxins A/B (ETA/B) are involved in SSTIs such as bullous impetigo and staphylococcal scalded skin syndrome. ETA/B are serine proteases that cleave desmoglein 1, an adhesive protein that maintains the structure of the skin layers **(Amagai *et al.*, 2000) (Fig. 1.1.3)**, which results in skin blisters. The pore-forming toxins function by disrupting membrane integrity by forming pores in host cells, eventually leading to cell death. The α-toxin forms a heptameric β-barrel pore and binds to the ADAM10 protease,, found on endothelial and epithelial cells, and this interaction is required for cytotoxicity **(Wilke and Wardenburg, 2010)**. In a murine sepsis model, α-toxin was required for virulence by impacting endothelial cell barrier function **(Powers *et al.*, 2012)**. The PSMs are a group of amphipathic α-helical cytolytic peptide toxins that attract, activate and subsequently lyse phagocytes such as neutrophils **(Wang *et al.*, 2007)**. PSMα-deficient MRSA displayed significantly reduced virulence in a murine sepsis model, while PSMβ-deficient MRSA killed similarly to wildtype (WT) **(Wang *et al.*, 2007)**. PSMs were thought to be genome-encoded however, PSM*-mec* is encoded on SCC*mec*, adjacent to the *mec* gene complex and the ability of PSM-*mec* to lyse neutrophils was lower than that of PSMα but higher than PSMβ **(Queck *et al.*, 2009)**. This demonstrates how some strains of MRSA evolve to acquire virulence factors by “toxin hitchhiking” on MGEs, which are important determinants of disease **(Queck *et al.*, 2009)**. Panton-Valentine leukocidin (PVL) and leukocidin AB (LukAB) are examples of two-component toxins. PVL binds to the C5aR and LukAB binds to the leukocyte intregrin CD11b **(Alonzo and Torres, 2014)**. Many USA300 and USA400 MRSA strains are PVL-positive and are often associated with severe skin infections and necrotising pneumonia **(Lina *et al.*, 1999; Gillet *et al.*, 2002)**. A well-known staphylococcal superantigen (sAg) is the toxic shock syndrome toxin (TSST) that is responsible for the cytokine storm and multi-organ failure associated with toxic shock syndrome. This is achieved by the crosslinking of major histocompatibility complex class II molecules on antigen-presenting cells (APCs) to T cell receptors (TCRs), which leads to overactivation of the immune system, and excess cytokine production **(Fleischer and Schrezenmeier, 1988; Stich *et al.*, 2010) (Fig. 1.1.3)**. Recently, it was shown that TSST interacts with TRBV12-3/12-4+ TCRs **(Shepherd *et al.*, 2023)**.

#### 1.1.3.3. Biofilm formation

Biofilms are sessile communities of bacteria that form on a surface, encased in a matrix of extracellular polymeric substance (EPS) that consists of many components including proteins, DNA and polysaccharides **(Donlan, 2002)**. *S. aureus* can establish biofilms during infections such as osteomyelitis on bones and endocarditis on heart valves as well as on inserted medical devices and this can result in chronic infection **(Kiedrowski and Horswill, 2011)**. Many *S. aureus* virulence factors have been associated with the development of biofilms, including the MSCRAMMs that are required for initial adhesion to a surface **(Foster *et al.*, 2014)**. For example, FnBPs are required for MRSA biofilm formation as *fnbpA/B* mutants display a diminished ability to form biofilms **(O'Neill *et al.*, 2008)**. The global regulator SarA positively regulates *fnbpA/B* gene expression, and as well as the Krebs cycle intermediate citrate, are required for biofilm formation **(Shanks *et al.*, 2008)**. Furthermore, the *S. aureus* surface protein G (SasG), a G5-E repeat family protein and thus a CWA, is also necessary for staphylococcal biofilm formation by forming long peritrichous fibrils and can do so independently of polysaccharide intercellular adhesin, which is also involved in biofilm formation **(Corrigan *et al.*, 2007)**. The SasG protein is comprised of a C-terminal domain that is anchored to the cell wall, multiple subunits of B domain and a singular N-terminal A domain. The B domains were revealed to be important in cell-cell interactions during the accumulation phase and this occurs upon cleavage (by an unknown protease) of the A domain, as SasG with recombinant B domain could not form biofilms **(Geoghegan *et al.*, 2010) (Fig. 1.1.3)**. Biofilm associated protein (Bap) on the other hand, is associated with both primary attachment of biofilms and cell-cell interactions **(Cucarella *et al.*, 2001)**.

#### 1.1.3.4. The regulation of *S. aureus* virulence factors

Virulence genes must be tightly regulated in order to survive and adapt to different environmental conditions. Thus, *S. aureus* encodes for multiple regulators of virulence, most of which are two-component systems (TCSs). TCSs consist of a membrane-bound histidine sensor kinase receptor and a response regulator: the histidine kinase recognises external signals leading to its autophosphorylation at the histidine residue **(Liu *et al.*, 2019)**. This then results in autophosphorylation of the response regulator by donation of the phosphoryl group to an aspartic acid residue on the response regulator **(Liu *et al.*, 2019)**. The accessory gene regulator (Agr) is a global virulence regulator that controls the expression of many virulence factors. Agr is involved in a quorum sensing system resulting in the upregulation of toxins and downregulation of adhesins in the post-exponential phase of growth **(Peng *et al.*, 1988; Recsei *et al.*, 1986)**.The *agr* locus consists of two divergent promoters P2 and P3 that produce the RNA transcripts RNAII and RNAIII respectively **(Morfeldt *et al.*, 1995)**. The *agr* locus consists of *agrA, agrB, agrC, agrD* and *hld*/δ-haemolysin. The P2 promoter controls the transcription of *agrA, agrB, agrC* and *agrD*: AgrA (response regulator) and AgrC (histidine kinase) comprise a TCS and AgrD encodes for an autoinducing peptide (AIP) precursor that is proteolytically processed by AgrB, a membrane bound peptidase. The P2 promoter also controls the RNAII transcript. The AgrAC TCS is responsible for detecting levels of AIP that are produced, leading to autophosphorylation of AgrA, which can control both the P2 promoter and the P3 promoter. The P3 promoter controls production of the RNAIII transcript, the major effector of the *agr* regulon, and once expressed, encodes for the δ-haemolysin or PSMγ, a cytolytic toxin **(Novick *et al.*, 1993; Janzon, Lofdahl and Arvidson, 1989)**. The control of P2 and P3 promoters by AgrA was thought to be its only purpose **(Queck *et al.*, 2008)**, however it is now known that the *agr* regulon regulates many virulence factors. AgrA controls the expression of the PSMs **(Peschel *et al.*, 2001)** and RNAIII positively upregulates α-toxin **(Morfeldt *et al.*, 1995)**.Interestingly, the PSM-*mec* transcript is a regulatory RNA similar to RNAIII and inhibits AgrA, which then inhibits the expression of the genomic PSMs **(Kaito *et al.*, 2011; Kaito *et al.*, 2013)**. However, this is not the case for every *S. aureus* strain **(Kaito *et al.*, 2013)**. Protein A is also negatively regulated by RNAIII – the ribosome-binding site and start codon are inhibited to prevent translation but also the mRNA itself can be targeted for degradation **(Huntzinger *et al.*, 2005)**. The repressor of toxins (Rot) is regulated by RNAIII in a similar fashion as protein A **(Geisinger *et al.*, 2006)**. Thus, the regulatory capacity of the *agr* locus is widespread, evidenced by its ability to regulate many different staphylococcal virulence factors.

The *S. aureus* exoprotein expression SaeS sensor kinase and SaeR response regulator constitute the SaeR/S TCS also regulates virulence factors and like the *agr* operon, has two promoters. The secreted protein SPIN is upregulated by SaeR/S, as well as α-toxin, Efb, Eap, SCIN, TSST and FnBPA/B **(de Jong *et al.*, 2017a; Liu, Yeo and Bae, 2016)**. SaeR/S-deficient *S. aureus* strains led to 95% survival of mice in a sepsis model, suggesting that the virulence factors regulated by this TCS are important *in vivo* **(Voyich *et al.*, 2009)**. The *sarA* operon produces three transcripts: *sarP1,* *sarP2* and *sarP3*. *sarP1* and *sarP2* are expressed during mid-exponential phase, while *sarP3* is expressed during late-exponential and stationary phases as well as during environmental stress **(Manna, Bayer and Cheung, 1998)**. Virulence factors regulated by SarA include protein A and PVL, but also RNAIII and the *agrABCD* operon **(Dunman *et al.*, 2001; Bronner *et al.*, 2000; Cheung, Bayer and Heinrichs, 1997)**.Furthermore, the alternative sigma factor B (σB) is responsible for modulating the Gram-positive stress responses e.g., heat and oxidative stresses **(Bischoff *et al.*, 2004; Hecker *et al.*, 2009)**. σB is necessary for the adaptation of *S. aureus* during chronic infections. While a *sigB* single mutant demonstrates higher expression of toxins and is thus more virulent, a *sigB/sarA* or *sigB*/*agr* double mutant was avirulent **(Tuchscherr *et al.*, 2015)**, demonstrating the complexity of the different regulons.

It is clear from these studies that regulation of the staphylococcal virulence factors is hugely complex as there is crosstalk between the different regulators. For example, induction of transcripts from the *sae* operon P1 promoter requires RNAIII **(Novick and Jiang, 2003)**. There is also overlap in genes that the regulators control e.g. both SarA and SaeR/S upregulate α-toxin while repressor of toxins (Rot) is responsible for its downregulation **(Bronner, Monteil and Prevost, 2004)**. Additionally, Protein A is upregulated by Rot, but as mentioned is downregulated by *agr* via RNAIII **(Said-Salim *et al.*, 2003; Huntzinger *et al.*, 2005)**. Thus, the regulation of the different staphylococcal virulence factors must be controlled appropriately as dysfunction in one aspect of the regulatory network may have detrimental effects.

## 1.2. The stringent response

*S. aureus* is a ubiquitous pathogen and its adaptability makes it excellent at inhabiting multiple niches, as demonstrated by the multiple infection types that *S. aureus* is the causative agent for. When colonising a new environment such as the host during infection, conditions may become unfavourable. Under nutrient limitation within the environment, bacteria induce a conserved stress pathway termed the stringent response, which is coordinated by the nucleotide alarmone and signalling molecules guanosine penta-/tetraphosphate ((p)ppGpp) **(Potrykus and Cashel, 2008; Irving, Choudhury and Corrigan, 2021) (Fig. 1.2)**. While widely conserved, 35 species (mainly endosymbionts) do not encode for (p)ppGpp synthetase proteins and therefore cannot mount a stringent response **(Atkinson, Tenson and Hauryliuk, 2011)**. (p)ppGpp is produced by the RelA/SpoT Homologue (RSH) protein family **(Mittenhuber, 2001; Atkinson, Tenson and Hauryliuk, 2011)**, and production of this alarmone results in major changes to both the transcriptome and proteome due to its interaction with several target effector molecules, highlighting the pleiotropic nature of the stringent response. Hyperphosphorylated nucleotides such as (p)ppGpp and (p)ppApp were first discovered approximately 50 years ago **(Cashel and Gallant, 1969; Oki *et al.*, 1976)**. In 1969, *Escherichia coli* deprived of amino acids were revealed to produce hyperphosphorylated nucleotides, termed “magic spots” **(Cashel and Gallant, 1969)**. This breakthrough paper allowed the commencement of research into how (p)ppGpp coordinates the stringent response. Since then, various other phosphorylated nucleotides have been identified for example pGpp, identified in *Enterococcus faecalis* and *Bacillus subtilis* **(Gaca et al., 2015; Yang et al., 2021)**.

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| **Figure 1.2. The structure of (pp)pGpp and RSH domain architecture. a)** Chemical structure of (p)ppGpp. The phosphate groups coloured red, blue and orange indicate whether the molecule is pppGpp, ppGpp or pGpp and corresponds to whether GTP, GDP or GMP are the backbone of the molecule. **b)** Long bifunctional RSH enzyme domain architecture. The N-terminal enzymatic domain consists of the HD domain (orange) and SYNTH domain (blue) and the C-terminal regulatory domain consists of a threonyl-tRNA synthetase GTPase and SpoT (TGS) motif (yellow), α-helical domain (grey), zinc finger domain with 3 conserved cysteine residues (ZFD/CC) (black) and an aspartokinase, chorismite mutase and TyrR/RNA recognition motif (ACT/RRM) (green). **(c)** Short alarmone synthetase consisting of only a SYNTH domain. **(d)** Short alarmone hydrolase consisting only of a hydrolase domain. |

The stringent response ultimately allows the switch from active growth to a dormant phenotype in order to promote bacterial survival during nutrient depletion. This is achieved by the downregulation of genes involved growth and metabolism and an upregulation of genes that allow adaptation to stress **(Irving, Choudhury and Corrigan, 2021)**. A consequence of these actions includes the upregulation of virulence **(Ronneau and Hallez, 2019)**, as bacteria endeavour to survive under harsh conditions, at the host’s expense. When conditions become favourable, bacteria can switch back to an active growth phenotype that can result in recurrent and chronic infections **(Gao *et al.*, 2010; Mwangi *et al.*, 2013; Geiger *et al.*, 2012)**.

### 1.2.1. Regulation of the RSH enzymes

#### 1.2.1.1. Synthesis and hydrolysis of (p)ppGpp

Enzymes from the RSH protein superfamily are responsible for the synthesis and hydrolysis of (p)ppGpp. RSH enzymes are grouped into three main classes, long RSH that are often bifunctional, with the ability to synthesise and hydrolyse (p)ppGpp and short RSH, subdivided into short alarmone synthetases (SASs) and short alarmone hydrolases (SAHs) **(Fig. 1.2)**. The first RSH discovered in *E. coli*, RelA, was named accordingly, as strains containing *relA* mutations displaying a ‘relaxed’ phenotype as opposed to ‘stringent’ **(Stent and Brenner, 1961)**. Adenosine triphosphate (ATP) and guanosine tri/diphosphate (GTP/GDP/GMP) molecules are necessary to produce one molecule of pppGpp/ppGpp/pGpp respectively **(Fig. 1.2)**. RSH enzymes catalyse the nucleophilic attack of the β-phosphate of ATP by the 3’-OH of the GTP/GDP/GMP ribose moiety, resulting in the transfer of a pyrophosphate group from ATP to GTP/GDP/GMP **(Sy and Lipmann, 1973)** thus producing a hyperphosphorylated guanosine nucleotide alarmone. Some RSH enzymes are also able to hydrolyse (p)ppGpp by removing a pyrophosphate (PPi) group, the δ and ε phosphates, to produce GTP and PPi **(Fig. 1.2)**. Basal levels of (p)ppGpp produced by *E. coli* amount to approximately 10-40 μM, while increasing to 800 μM by the end of exponential phase, with full induction of the stringent response resulting in concentrations between 1-2 mM **(Varik *et al.*, 2017; Zbornikova *et al.*, 2019; Haseltine and Block, 1973a; Cashel, 1975)**. It is important to note that (p)ppGpp is not only produced during the stringent response, and is produced at low concentrations during steady-state growth **(Imholz *et al.*, 2020)**.

#### 1.2.1.2. Long RSH

Long RSH enzymes have multiple domains in comparison to short RSH, consisting of an enzymatic N-terminal domain (NTD) and a regulatory C-terminal domain (CTD). The hydrolase and synthetase domains, HD and SYNTH domain respectively, comprise the NTD **(Fig 1.2)**. The HD domain is named according to conserved histidine and aspartate residues within the active site and its role in hydrolysing (p)ppGpp to PPi and GTP/GDP. The SYNTH domain however, synthesises (p)ppGpp, as described above. The CTD regulates the NTD and consists of four motifs: a threonyl-tRNA synthetase GTPase and SpoT (TGS) motif, α-helical domain, zinc finger domain with 3 conserved cysteine residues (ZFD/CC) and the aspartokinase, chorismite mutase and TyrR/RNA recognition motif (ACT/RRM) **(Steinchen and Bange, 2016)** **(Fig. 1.2)** however, the domain architecture of the CTD can differ depending on the species **(Atkinson, Tenson and Hauryliuk, 2011)**. The SYNTH and HD domains work in concert to regulate and maintain an optimal level of (p)ppGpp, as in the absence of a HD domain (p)ppGpp levels are uncontrolled, resulting in toxic accumulation **(Hogg *et al.*, 2004; Mechold *et al.*, 2002; Geiger *et al.*, 2010; Lee, Park and Seok, 2018)**. Thus, RSH proteins containing a HD domain are conditionally essential **(Takada *et al.*, 2020)**. Long RSH enzymes can be monofunctional or bifunctional. Most Proteobacteria express both a monofunctional and a bifunctional RSH while bacteria outside of this phylum tend to contain only one bifunctional RSH **(Atkinson, Tenson and Hauryliuk, 2011)**. *E. coli* contains the bifunctional SpoT and the monofunctional RelA*Ec*, which is thought to have arisen due to a gene duplication **(Mittenhuber, 2001)**. The ability of RelA*Ec* to only synthesise (p)ppGpp is due to loss of a conserved HDXXED motif within the active site of the hydrolase domain, rendering it non-functional **(Aravind and Koonin, 1998)**. It was recently discovered that the inactive HD domain in RelA*Ec*contains a 17-residue loop that regulates the SYNTH domain, and a mutation in the DNA sequence that encodes this loop abrogates SYNTH domain activity **(Sinha and Winther, 2021)**. In comparison, Rel*Sau* from *S. aureus* is bifunctional and is similar to SpoT in that they both contain an RXFD motif in close proximity to the GTP/GDP-binding pockets, while RelA contains an EXDD motif **(Sajish *et al.*, 2007)**. This may be responsible for the substrate specificity of Rel/SpoT for GTP vs RelA for GDP **(Sajish *et al.*, 2009)**.

(p)ppGpp production by Rel (Firmicutes) and RelA (Proteobacteria) is initiated by the detection of amino acid starvation. Bacteria sense the presence of uncharged tRNAs in the ribosomal aminoacyl ‘A’ site and long RSH enzymes are stimulated to produce (p)ppGpp when bound to a stalled ribosome **(Haseltine and Block, 1973a; Wendrich *et al.*, 2002)**. However, DarB from *B. subtilis,* which is regulated by cyclic-di-AMP (c-di-AMP), was shown to stimulate synthetase activity of Rel in a ribosome-independent manner during amino acid limitation **(Krüger *et al.*, 2021; Ainelo *et al.*, 2023)** revealing that additional stimulatory mechanisms exist. How bacteria sense ribosome-dependent amino acid starvation was unclear, until cryo-electron microscopy structures of RelA*Ec* complexed to stalled ribosomes were published **(Loveland *et al.*, 2016; Takada *et al.*, 2021; Brown *et al.*, 2016; Arenz *et al.*, 2016)**.When bound to the ribosome, (p)ppGpp synthesis is favoured as Rel/RelA is in an open conformation. In contrast, unbound Rel/RelA favours a closed conformation, serving as a way to regulate (p)ppGpp levels **(Arenz *et al.*, 2016; Gratani *et al.*, 2018)**. In the unbound state, the closed conformation allows the TGS/α-helical domains, that stimulate the HD domain and the ZFD/ACT/RRM domains, to inhibit the SYNTH domain by preventing the binding of ATP/GDP substrates **(Tamman *et al.*, 2022; Mechold *et al.*, 2002)**. Not only can the CTD negatively regulate the synthetase activity, but the TGS and ZFD domains are required for interaction of Rel/RelAwith the ribosome **(Gratani *et al.*, 2018; Kudrin *et al.*, 2018; Takada *et al.*, 2020)**. Upon binding to the ribosome e.g., in the event of amino acid limitation, Rel/RelA will adopt an open conformation to allow (p)ppGpp synthesis **(Arenz *et al.*, 2016)**. Stalled ribosomes as a result of the binding of uncharged tRNAs can be sensed by Rel/RelA **(Fig. 1.2.1.2)** however, while the Rel/RelA-ribosome association does not require uncharged tRNAs, it is beneficial to promote (p)ppGpp synthesis **(Loveland *et al.*, 2016; Takada *et al.*, 2021)**. The CTD motifs of Rel/RelA interact with the A-site where the uncharged tRNA is located, while the NTD faces away from the ribosome **(Arenz *et al.*, 2016; Brown *et al.*, 2016; Loveland *et al.*, 2016; Kudrin *et al.*, 2018)**.Furthermore, (p)ppGpp binding to Rel/RelA during amino acid limitation also stimulates further ppGpp production **(Shyp *et al.*, 2012)**. In contrast, (p)ppGpp production by SpoT is not induced upon amino acid limitation. The holo-acyl-carrier protein (ACP) binds to SpoT, but not Rel/RelA, via the TGS domain to signal fatty acid starvation and has also been implicated in signalling phosphate starvation **(Battesti and Bouveret, 2006; Germain *et al.*, 2019)**. ACP is acidic, which is optimal for binding to SpoT as it has a basic isoelectric point in comparison to Rel/RelA and may explain why Rel/RelA does not respond to fatty acid starvation **(Irving and Corrigan, 2018)**. Furthermore, SpoT activity is also induced upon iron and carbon limitation **(Lee, Park and Seok, 2018; Vinella *et al.*, 2005)**.

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| **Figure 1.2.1.2. Structure of RelA bound to the ribosome (Irving, Choudhury and Corrigan, 2021)**.RelAfrom *E. coli* (orange) bound to the ribosome (Protein Data Bank ID 5L3P). The A-site/RelA-bound (A/R)-tRNA is an uncharged tRNA that, when associated with the A site (dark green) of the ribosome (50S subunit (blue) and 30S subunit (light green)) will stimulate (p)ppGpp synthesis by Rel/RelA. The peptidyl-tRNA is shown in the P site of the ribosome (grey). The motifs within the RelA CTD interact with the A site while the NTD extends outward of the ribosome. |

#### 1.2.1.3. Short RSH - SAS

The SAS enzymes occur mainly within the Firmicutes and the first two identified, RelP and RelQ, were isolated from *Streptococcus mutans* **(Lemos et al., 2007)**. Since then, RelP and RelQ have been identified in other organisms such as *S. aureus* and *B. subtilis* **(Steinchen and Bange, 2016)**,as well as RelV from *Vibrio cholerae* **(Das et al., 2009)** and RelS and RelZ that are present in the Actinobacteria **(Ruwe, Kalinowski and Persicke, 2017)**. Unlike the long RSH proteins, SAS enzymes are comprised of a single SYNTH domain and lack a CTD **(Fig. 1.2)**. Interestingly, RelZ from *Corynebacterium glutamicum* and *Mycobacterium smegmatis* is the only SAS containing an additional domain, the RNase HII domain involved in resolving R loops, thus conferring the ability to reverse DNA damage **(Murdeshwar and Chatterji, 2012; Krishnan *et al.*, 2016)**. RelP and RelQ share 50% sequence similarity **(Steinchen *et al.*, 2018)** and are transcriptionally regulated as they are unable to sense stress via an accessory domain like Rel/RelA **(Irving and Corrigan, 2018; Irving, Choudhury and Corrigan, 2021)**. Transcription of RelP and RelQ is induced upon cell wall stress for example the exposure to cell wall-targeting antibiotics **(Geiger *et al.*, 2014; Abranches *et al.*, 2009)**, but also ethanol shock and alkaline shock **(Pando *et al.*, 2017; Nanamiya *et al.*, 2008)**. Moreover, RelP*Sau* and RelQ*Sau* are induced upon activation of the vancomycin-resistance associated sensor/regulator (VraS/R) TCS that is involved in cell wall stress **(Geiger *et al.*, 2014)**. Similar to RelA being positively regulated by ppGpp, RelQ*Bs* is regulated by pppGpp but not ppGpp **(Steinchen *et al.*, 2018)**. Once expressed, RelP and RelQ form homotetramers, creating two allosteric pppGpp binding sites **(Manav *et al.*, 2018; Steinchen *et al.*, 2018)**. Bioinformatic analyses have demonstrated that the SAS proteins have also been found in operons as toxin-antitoxin (TA) systems that deliver nucleotide second messengers to other bacteria as a mode of defence **(Jimmy *et al.*, 2020)**. Tas1, an SAS from *Pseudomonas aeruginosa*,produces the hyperphosphorylated adenosine ppApp that is delivered to recipient cells, depleting them of ATP and resulting in cell death **(Ahmad *et al.*, 2019)**.

#### 1.2.1.4. Short RSH – SAH

In comparison to SAS proteins, SAH proteins consist of a hydrolase domain **(Fig. 1.2)**. One SAH has been identified, a metazoan SpoT homologue (MESH1) in eukaryotes such as humans and *Drosophila melanogaster* with MESH1-deficient *D. melanogaster* displaying growth defects **(Sun *et al.*, 2010)**. MESH1 was also found in plant chloroplasts, but alongside a long RSH **(Tozawa and Nomura, 2011)**. Humans and *D. melanogaster* are not known to possess enzymes with a SYNTH domain **(Sun *et al.*, 2010)** and thus the function of MESH1 in these species in relation to (p)ppGpp is unknown. However, human MESH1 has been recently identified as an NADPH phosphatase and was involved in regulating iron accumulation-mediated programmed cell death (ferroptosis**) (Ding *et al.*, 2020)**. This suggests that MESH1 may have a broad substrate specificity **(Irving, Choudhury and Corrigan, 2021)**. While seven subgroups of possible SAH have been identified by bioinformatic analyses **(Atkinson, Tenson and Hauryliuk, 2011)**, an SAH has been characterised in *C. glutamicum*, named RelH**(Ruwe et al., 2018)**. Deletion of this gene did not cause major growth defects, and since other *Corynebacterium* species do not encode for the RelH protein, this suggests that it is not essential and may only be required under certain conditions **(Ruwe *et al.*, 2018)**.

#### 1.2.1.5. Other enzymes involved in (p)ppGpp metabolism

In addition to the long and short RSH enzymes, other enzymes are capable of (p)ppGpp turnover. In *E. coli* guanosine pentaphosphate phosphohydrolase (GppA) hydrolyses the 5’ γ-phosphate of pppGpp thereby producing ppGpp **(Keasling, Bertsch and Kornberg, 1993)**. As ppGpp is more potent of an effector in *E. coli* in comparison to pppGpp **(Mechold *et al.*, 2013)**, the activity of GppA must therefore allow higher cellular concentrations of ppGpp. Indeed, the pppGpp/ppGpp ratio increases in GppA-deficient bacteria **(Mechold *et al.*, 2013)**. Furthermore, in *B. subtilis* and *Bacillus anthracis* the nucleoside diphosphate linked moiety X (NuDix) hydrolase, named NahA, can further metabolise both pppGpp and ppGpp **(Yang *et al.*, 2021)**. Here, NahA was found to hydrolyse between the 5’ α- and β-phosphates of pppGpp and ppGpp, producing pGpp. The *nahA* mutant recovered from nutrient limitation much slower than WT, which was attributed to the increase in (p)ppGpp. pGpp was found to interact with enzymes in the purine biosynthetic pathway, but not the ribosome-associated GTPases that are known to bind to (p)ppGpp **(Yang *et al.*, 2021)**. NuDix hydrolases exist in other organisms: *E.coli* encodes for MutT, NudG and RppH **(Zhang *et al.*, 2018a; Gao *et al.*, 2020)** and *Thermus thermophilus* encodes for Ndx8, which hydrolyses (p)ppGpp to pGp **(Ooga *et al.*, 2009)**.

### 1.2.2. Physiological processes targeted by (p)ppGpp

(p)ppGpp functions as a signalling molecule to reprogram the transcriptional and translational machinery in order to make extensive metabolic changes that are pro-survival under nutrient limitation. Upregulated genes include those for the synthesis and transport of amino acids **(Geiger *et al.*, 2012)**. The downregulated genes include those involved in transcription, translation, DNA replication and other metabolic processes **(Sanchez-Vazquez *et al.*, 2019)**. This is done in order to initiate growth arrest, during which time bacteria can become tolerant to antibiotic treatment. The ability of (p)ppGpp to bind multiple effector proteins from a variety of metabolic pathways is owed to the conformational flexibility of (p)ppGpp **(Steinchen and Bange, 2016)**.

#### 1.2.2.1. Transcription

In *E. coli*, the transcription of approximately 750 genes was been altered as a consequence of (p)ppGpp accumulation **(Sanchez-Vazquez *et al.*, 2019)**. This is due to the interaction of (p)ppGpp with the RNA polymerase (RNAP), a well-known target of (p)ppGpp, with ppGpp more potent at altering metabolic processes than pppGpp **(Mechold *et al.*, 2013)**. During logarithmic growth, sigma factor σ70 binds to RNAP and promotes the transcription of genes required for cellular growth **(Dalebroux and Swanson, 2012; Österberg, del Peso-Santos and Shingler, 2011)**. Upon induction of the stringent response, (p)ppGpp and cofactor DnaK suppressor (DksA) directly bind to RNAP and prevent its binding to σ70-dependent promoters **(Österberg, del Peso-Santos and Shingler, 2011; Paul *et al.*, 2004) (Fig. 1.2.2.1)**. (p)ppGpp binds at two sites on RNAP: site one is present between the β and ω subunits of RNAP and site two occurs at the interface between DksA and RNAP **(Ross *et al.*, 2013; Ross *et al.*, 2016)**. The N-terminal MAR (methionine, alanine, arginine) motif located at site one is involved in the recognition and binding of (p)ppGpp and is present in Proteobacteria but not Firmicutes **(Hauryliuk *et al.*, 2015)**. The outcome of (p)ppGpp-mediated RNAP inhibition is the reduction in ribosomal RNA (rRNA) transcription by modulation of activity of the *rrn* promoters, ultimately resulting in decreased translation due to a decrease in the production of functional ribosomes.

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| **Figure 1.2.2.1. The effects of (p)ppGpp on bacterial transcription. a)** In Proteobacteria, the effect of (p)ppGpp on transcription occurs through interaction with RNAP – the binding of DksA, (p)ppGpp and σ70 prevents transcription with a major effect being the downregulation of ribosomal RNA (*rrn*) promoters. **bi)** In contrast in firmicutes, (p)ppGpp targets the GTP-synthesising enzymes in order decrease GTP levels which results in downregulation of the *rrn* promoters and leads to the upregulation of genes involved in adapting to stress. This is done in two ways, as described in **bii)** and **biii)**. **bii)** (p)ppGpp inhibita the GTP-synthesising enzymes that contribute to the *de novo* and salvage purine biosynthetic pathways. *De novo* GTP synthesis starts with phosphoribosyl pyrophosphate (PRPP) which is converted to 5-phosphoribosylamine (PRA) by PurF and this is converted to inosine 5’-phosphate (IMP). IMP dehydrogenase (GuaB) converts IMP to xanthosine 5’-phosphate (XMP) and GMP synthase (GuaA) converts XMP to GMP. GMP is converted to GDP by guanylate kinase (Gmk) and GDP is converted to GTP by nucleoside diphosphate kinase (Ndk). In contrast, the salvage pathway starts with nucleotides such as guanine. Guanine (GUA) and hypoxanthine (HPX) are similar in structure and therefore are both substrates of the enzyme HprT, which converts them to IMP and GMP respectively. These products can then be further metabolised in the *de novo* pathway. (p)ppGpp is represented by a yellow star. Enzymes are highlighted in blue. **biii)** Under nutrient-rich conditions, CodY is associated with DNA with GTP and BCAA as cofactors. Cellular GTP pools are high, and genes repressed by CodY are not being expressed. However, under nutrient limitation, the accumulation of (p)ppGpp decreases the GTP pool thus reducing the GTP available to act as a cofactor for CodY. CodY repression is then relieved, allowing the expression of genes involved in adapting to stress including amino acid biosynthetic genes and also genes related to virulence. |

In contrast, (p)ppGpp does not bind to RNAP in the Firmicutes, likely due to the RNAP ω-subunit lacking the MAR motif **(Hauryliuk *et al.*, 2015)**. Additionally, the Firmicutes do not encode for a homolog of DksA. Thus, changes to transcription upon induction of the stringent response are indirectly mediated by RNAP. Instead, a decrease in the cellular GTP pool is responsible: GTP is consumed as a substrate of (pp)pGpp production and (pp)pGpp interacts with and inhibits enzymes involved in GTP synthesis **(Kriel *et al.*, 2012; Corrigan *et al.*, 2016) (Fig. 1.2.2.1)**. Enzymes of the *de novo* and salvage purine biosynthetic pathways are inhibited by (pp)pGpp, including inosine monophosphate (IMP), amidophosphoribosyl transferase (PurF), hypoxanthine phosphoribosyltransferase (HprT) and the guanylate kinase (Gmk) **(Hochstadt-Ozer and Cashel, 1972; Anderson *et al.*, 2020; Kriel *et al.*, 2012)** resulting in a decrease in GTP production **(Fig. 1.2.2.1)**. Regulation of purine synthesis in order to alter cellular GTP levels seems to be exclusive to the Firmicutes despite the ability of (p)ppGpp produced by *E. coli* to bind PurF **(Wang *et al.*, 2019)**. Consequently, transcription within the Firmicutes is decreased as many genes, including the *rrn* operons, require GTP as an initiating nucleotide (iNTP), therefore reduction of cellular GTP pools decreases the transcription of these genes **(Krásný and Gourse, 2004; Krásný *et al.*, 2008)** while resulting in a concomitant increase in transcription of genes with ATP as the iNTP **(Krásný and Gourse, 2004; Krásný *et al.*, 2008)**. Furthermore, a global metabolic regulator and repressive transcription factor CodY represses the transcription of genes involved in amino acid biosynthesis and virulence genes. GTP and branched-chain amino acids (BCAA) are cofactors that mediate CodY repression **(Pohl *et al.*, 2009; Sonenshein, 2005; Majerczyk *et al.*, 2008) (Fig. 1.2.2.1)**. CodY functions by competing with RNAP or a transcriptional activator for binding to a promoter **(Belitsky, 2011)**.Many genes controlled by CodY are involved in flagellar production, sporulation and virulence and the virulence regulator *agr* is under the control of CodY **(Sonenshein, 2005; Majerczyk *et al.*, 2008)**. Cellular (p)ppGpp accumulation and concurrent GTP reduction results in CodY derepression, thus allowing the expression of genes necessary for stress adaptation **(Sonenshein, 2005)**. Analysis of the *S. aureus* transcriptome upon amino acid starvation revealed that 143 genes were upregulated due to CodY derepression, demonstrating that CodY has a significant role in gene regulation during the stringent response **(Geiger *et al.*, 2012)**. However, 161 genes were downregulated independently of CodY **(Geiger *et al.*, 2012)**, suggesting that CodY does not have a role in gene downregulation during the stringent response. (p)ppGpp-regulated genes found to be upregulated independently of CodY include *ftnA* and *dps* encoding for iron storage proteins, *katA* and *sodA* encoding for catalase and superoxide dismutase A and the *psmα1–4 and psmß1-2* operons encoding for the PSMs **(Horvatek *et al.*, 2020)**. These genes are involved in virulence **(Horvatek *et al.*, 2020; Fritsch *et al.*, 2020)**, demonstrating that genes not regulated by CodY are also involved in pathogenesis. Additionally, (p)ppGpp directly interacts with riboswitches as another method of regulating gene transcription. Riboswitches are non-coding regions of RNA that occur upstream of a gene and modulate their transcription. The *ykkc* class of riboswitches regulate BCAA-associated genes such as *ilvE* from *Thermosediminibacter oceani* upon (p)ppGpp binding, and these riboswitches are associated with glutamate synthase and ATP-binding cassette transporters **(Sherlock, Sudarsan and Breaker, 2018)**. This is corroborated by the fact that glutamate is upregulated during the stringent response in *E. coli* **(Imaizumi, Kojima and Matsui, 2006)**, indicating that a (p)ppGpp-binding riboswitch is responsible for increasing glutamate levels **(Sherlock, Sudarsan and Breaker, 2018)**.

#### 1.2.2.2. Translation

Unlike transcriptional regulation by (p)ppGpp that varies between different phyla, regulation of translation seems to be conserved in that similar proteins are targeted **(Milon *et al.*, 2006; Mitkevich *et al.*, 2010; Feng *et al.*, 2014)**. As well as downregulating transcription at the *rrn* promoters, (p)ppGpp also interferes with the maturation of 70S ribosomes by binding to and inhibiting the GTPases that are required for ribosomal assembly: RsgA, RbgA, Era and HflX **(Corrigan *et al.*, 2016; Persky *et al.*, 2009) (Fig. 1.2.2.2)**. RsgA and Era to bind to the 30S small ribosomal subunit, while RbgA and HflX bind the 50S subunit and are necessary for ribosome biogenesis **(Himeno *et al.*, 2004; Sayed, Matsuyama and Inouye, 1999; Uicker, Schaefer and Britton, 2006; Jain *et al.*, 2009)**. However, in *S. aureus*, RsgA and Era are not essential **(Campbell *et al.*, 2006; Wood *et al.*, 2019)** and in *B. subtilis* and *E. coli*, RbgA and Era respectively are essential **(Uicker, Schaefer and Britton, 2006; Sayed, Matsuyama and Inouye, 1999)** demonstrating that ribosome biogenesis differs between organisms and therefore the effect of (p)ppGpp on translation does also. An RsgA-deficient *S. aureus* strain demonstrated a slow-growth phenotype, and ribosome profile analysis revealed a decrease in 70S ribosomes and consequently more 50S and 30S subunits in comparison to WT **(Corrigan *et al.*, 2016)**. (p)ppGpp also mediates the dimerisation of 70S ribosomes to the inactive 100S ribosomes in order to halt translation **(Fig. 1.2.2.2)**. The transcription of hibernation factors: ribosome modulation factor (*rmf*); hibernation promoting factor (*hpf*); and ribosome-associated inhibitor (*raiA*), are activated by (p)ppGpp which promotes the formation of 100S ribosomes in *E. coli* **(Izutsu, Wada and Wada, 2001; Prossliner *et al.*, 2021; Song and Wood, 2020)**. These ribosomes can be split by the action of HflX in the event of nutrient repletion **(Zhang *et al.*, 2015; Basu and Yap, 2017)**. This phenomenon has also been reported in *B. subtilis,* where *yvyD*, a gene related to *hpf* due to 51% amino acid sequence similarity,was necessary for dimerisation but may not be the only effector of ribosome dimerisation **(Tagami *et al.*, 2012)**. Furthermore, the initiation, elongation and release factor GTPases that are required for translation are also inhibited by (p)ppGpp **(Fig. 1.2.2.2)**. Initiation factor 2 (IF2) required to recruit Met-tRNA to the 30S pre-initiation complex can no longer do so when bound to (p)ppGpp **(Milon *et al.*, 2006; Legault, Gros and Jeantet, 1972)**. Elongation factor Tu (EF-Tu) and elongation factor G (EF-G) are also inhibited by (p)ppGpp, which prevents binding of aminoacyl tRNAs to the ribosomal A-site and translocation of the polypeptide chain through the ribosome respectively **(Rojas *et al.*, 1984; Zhang *et al.*, 2018a)**. Lastly, recycling of release factors 1/2 (RF1/2) during translation termination by RF3 is inhibited due to (p)ppGpp binding **(Kihira *et al.*, 2012; Zhang *et al.*, 2018a)**. Therefore, inhibition of ribosomal biogenesis, the process of translation, as well as inactivation of functional ribosomes by (p)ppGpp has a negative effect on translation during the stringent response. As multiple facets of protein production are targeted by (p)ppGpp, this signifies the importance of downregulating cellular growth during nutrient limitation. However, while the levels of translation are decreased, it is not halted as translation is still required for cell viability. Recently, it has been suggested that translation during the stringent response is permissive and the specific mRNAs translated at this time are regulated by the 30S-bound IF2 **(Vinogradova *et al.*, 2020)**.

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| **Figure 1.2.2.2. The effects of (p)ppGpp on bacterial translation.** (p)ppGpp modulates different aspects of translation. By inhibiting the ribosome-associated GTPases RbgA and HflX (which associate with the 50S ribosomal subunit) and Era and RsgA (which associate with the 30S ribosomal subunit), (p)ppGpp can prevent maturation of the 70S ribosome. (p)ppGpp binds to factors involved in the process of translation by binding directly to them. (p)ppGpp-bound initiation factor IF2 to prevent addition of Met-tRNA, thus preventing the initiation of the translation. Elongation factors EF-Tu and EF-G are also inhibited by (p)ppGpp, preventing the binding of aminoacyl tRNAs to the A site of the ribosome and polypeptide chain translocation respectively. (p)ppGpp-bound release factor RF3 prevents recycling of RF1/2. (p)ppGpp promotes the formation of 100S ribosomes. (p)ppGpp upregulates transcription of the *rmf*, *hpf* and *raiA* genes that are necessary for the formation of 100S ribosomes (dimerised 70S ribosomes) which prevents translation. HflX acts to split the 100S to the 70S monomers when conditions become favourable. Green arrows indicate a positive interaction. |

### 1.2.3. The role of the stringent response during pathogenesis

During the stringent response, bacteria must upregulate genes that are associated with adaptation to stress and thus, there is a concurrent increase in the expression of virulence genes that contribute to survival under nutrient limitation. This is required in the context of infection, where the host will attempt to eliminate bacteria with an arsenal of defence mechanisms, and bacteria must adapt in order to evade the immune system and survive.

#### 1.2.3.1. Adhesion and invasion

Adherence to host cells and components is an integral process for successful colonisation and survival of a pathogen. Subsequent invasion is necessary for intracellular pathogens such as *E. coli, Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*. The DksA and (p)ppGpp-mediated activation of the transcriptional regulators *ler* and *pch* has resulted in upregulation of the locus of enterocyte effacement (LEE), resulting in an increased capacity of enterohaemorrhagic *E. coli* to adhere to intestinal epithelial cells (IECs) **(Nakanishi *et al.*, 2006)**. Enteropathogenic *E. coli* (EPEC)adherence to human epithelial cells *in vitro* has been attributed to RelA-mediated regulation of the *perABC* operon **(Spira, Ferreira and de Almeida, 2014)**. This operon is responsible for the expression of bundle-forming pilus and intimin which are necessary for the attachment of EPEC and the absence of RelA resulted in the inhibition of adherence **(Spira, Ferreira and de Almeida, 2014)**. In EPEC, LEE encodes for virulence factors such as a type III secretion system (T3SS) that delivers host-modulating effectors into cells, allowing the formation of actin pedestals: here bacteria are intimately attached to IECs, which aids survival **(Platenkamp and Mellies, 2018)**, demonstrating the importance of adhesion. Moreover, Rel- and HprT-deficient *L. monocytogenes* were less able to adhere to and were rapidly cleared by murine hosts during infection, with low detection of bacteria in the spleen and liver **(Taylor *et al.*, 2002)**. Rel deletion in *S. aureus* also led to decreased virulence in a murine haematogenic kidney abscess model and a decreased bacterial burden in the kidney in comparison to WT **(Geiger *et al.*, 2010)**. For some pathogens, adherence is a prerequisite for invasion. *S*. Typhimurium utilises a T3SS encoded on *Salmonella* pathogenicity island 1 (SPI-1) and a (p)ppGpp0 mutant demonstrated a decreased ability to invade IECs due to a lack of expression of key regulators *invF* and *hilA*  **(Pizarro-Cerdá and Tedin, 2004)**. Effectors of the T3SS allow the association of *S.* Typhimurium to the intestinal epithelium and the injection of effectors such as SopE and AvrA, required for modulating the host cytoskeleton and promoting invasion **(Lim *et al.*, 2014; Johnson *et al.*, 2017)**, thus emphasising the importance of SPI-1 for *S.* Typhimurium invasion. Additionally, a *Salmonella enterica* serovarTyphi (p)ppGpp0 mutant was less able to adhere to and invade IECs **(Dasgupta *et al.*, 2019)**. Fewer colonies of an *E. faecalis rel* mutant were recovered from rabbit heart valves in comparison to WT and this was attributed to a decreased ability to invade the cardiac endothelium rather than dissemination **(Colomer-Winter, 2017)**. Taken together, these studies demonstrate that (p)ppGpp has a role in regulating virulence factors, and its absence significantly decreases the virulence of many pathogens by affecting the key virulence determinants involved in adhesion and invasion.

#### 1.2.3.2. Immune evasion and biofilm formation

Many of the virulence factors encoded by pathogens have a role in evading the immune system in order to ensure survival. In *S. aureus,* the PSMs are required for neutrophil lysis **(Wang *et al.*, 2007)**, a prerequisite for dissemination. Induction of Relpost-phagocytosis by PMNs led to the upregulation of the PSMs, which increased survival of *S. aureus­* **(Geiger et al., 2012)**. Accordingly, *psm* and *rel* mutants demonstrated decreased survival post-phagocytosis **(Geiger et al., 2012)**, implicating the PSMs in intracellular survival within PMNs. PSMα and not PSMβ specifically, is necessary for phagosomal escape as Δ*psm*α mutants have reduced escape efficiencies while Δ*psmβ* escape efficiencies are comparable to WT **(Grosz *et al.*, 2014)**. Dasgupta and colleagues have demonstrated that (p)ppGpp is involved in capsular polysaccharide formation in *S.* Typhiand a (p)ppGpp0 mutant was susceptible to complement-mediated lysis **(Dasgupta *et al.*, 2019)**.

*In vitro*, studies have demonstrated the role of the stringent response for survival under conditions that can be found within phagocytes. For example, pathogens encounter oxidative stress after phagocytosis as a host immune defence mechanism. (p)ppGpp0 mutants are more sensitive to oxidative stress e.g. by hydrogen peroxide (H2O2) in multiple species including *S. aureus* and *E. faecalis* **(Horvatek et al., 2020; Yan et al., 2009; Holley et al., 2014; Wang et al., 2016)**, indicating a role for the stringent response in tolerating oxidative stress. Indeed, (p)ppGpp was revealed to be necessary for tolerance to endogenous oxidative stress and antibiotics in *S. aureus* and *P. aeruginosa* **(Fritsch *et al.*, 2020; Khakimova *et al.*, 2013; Martins, 2018)**. Stringent response-mediated regulation of the catalases *katA* and *katB*,was necessary for H2O2 tolerance **(Khakimova *et al.*, 2013)**. Furthermore, SOD has been implicated as a major factor in the development of tolerance in *P. aeruginosa* **(Martins et al., 2018)**- O2- produced endogenously and by immune cells, can be converted to diatomic oxygen and H2O2 by bacterial SodA/B as a mechanism of superoxide neutralisation. In *P. aeruginosa*, *sodB*, regulated by the stringent response, was shown to decrease cell membrane permeabilisation, thus hindering drug internalisation and emphasising the importance of the stringent response in the presence of oxidative stress **(Martins *et al.*, 2018)**. These reports suggest that tolerance of multiple pathogens to oxidative and antibiotic stress, could also occur within mammalian immune cells, where these stresses are prevalent.

In response to environmental stimuli such as nutrient limitation, bacteria are able to form biofilms and thus the stringent response has been implicated in biofilm formation. For instance, *E. faecalis* (p)ppGpp0 and single *codY* single mutants are less able to form biofilms in a catheter-associated urinary tract infection (CAUTI) model **(Colomer-Winter *et al.*, 2019)**. A *Bordetella pertussis* (p)ppGpp0 mutant displayed a biofilm formation defect and this was attributed to decreased expression of *fim2* and *bsp22* **(Sugisaki *et al.*, 2013)**. Fim2 and Bsp22 proteins are subunits of fimbriae and a T3SS respectively, that were postulated to be necessary for biofilm architecture **(Sugisaki *et al.*, 2013)**. (p)ppGpp also regulates type 1 fimbriae by upregulating transcription of the *fim* genes in uropathogenic *E. coli* (UPEC) **(Åberg, Shingler and Balsalobre, 2006)**. A UPEC (p)ppGpp0 mutant demonstrated reduced biofilm-forming ability due to decreased expression of type 1 fimbriae **(Åberg, Shingler and Balsalobre, 2006)**. Interestingly, an analysis of 118 UPEC clinical isolates revealed that strains with higher levels of basal (p)ppGpp were associated with the ability to form biofilms *in vitro* and an increased growth rate **(Karczewska *et al.*, 2023)**. Indeed, increased growth is not a typical consequence of the stringent response, and so the role of basal (p)ppGpp levels must be further studied in order to understand this phenotype **(Karczewska *et al.*, 2023)**. Upon sub-inhibitory concentrations of vancomycin, RelP*Sau* and RelQ*Sau* have been implicated in biofilm formation by an Agr- and CodY-independent mechanism **(Salzer *et al.*, 2020)**. This is evidenced by no biofilm formation when treated with vancomycin and in the absence of (p)ppGpp **(Salzer *et al.*, 2020)**. Though (p)ppGpp is implicated in biofilm formation as demonstrated, there are some species that are able to form biofilms even in the absence of (p)ppGpp. For example, a *Porphyromonas gingivalis* (p)ppGpp0 mutant was able to form biofilms with approximately twice the mass of a WT biofilm, however the number of dead cells in (p)ppGpp0 mutant biofilms was higher than WT **(Kim and Davey, 2020)**. Furthermore, for *Pseudomonas putida*, (p)ppGpp was required for biofilm dispersal rather than formation **(Diaz-Salazar *et al.*, 2017)**. As the stringent response is conserved across many bacterial species, it is plausible that the positive effect it has on virulence and pathogenesis is not universal, explaining why ppGpp-mediated biofilm formation differs across species.

#### 1.2.3.3. Antibiotic tolerance and persistence

Perhaps one of the most interesting roles of the stringent response is its involvement in the development of antibiotic tolerance that can result in bacterial persistence. The decreased growth associated with the stringent response is similar to the persistent phenotype and both phenomena can result in recalcitrant infections **(Gao *et al.*, 2010; Fauvart, de Groote and Jan, 2011)**. As bacteria are in a dormant-like state during the stringent response and metabolic processes are downregulated, this affects antibiotic efficacy, leading to tolerance and prolonged bacterial survival. For example, amino acid limitation was demonstrated to be a prerequisite of antibiotic tolerance to ampicillin and ofloxacin in *E. coli,* where the absence of a functional stringent response resulted in antibiotic sensitivity **(Fung et al., 2010)**. In *E. faecalis,* Δ*rel* strains produce (p)ppGpp via expression of RelQ which conferred vancomycin tolerance, suggesting that even low levels of (p)ppGpp are sufficient for a tolerance phenotype **(Abranches *et al.*, 2009)**. Similarly, as the presence of RelP*Sau* and RelQ*Sau* SAS enzymes were shown to confer tolerance to vancomycin, a double mutant in *S. aureus* could not withstand high doses of vancomycin in comparison to its WT counterpart **(Geiger *et al.*, 2014)**. These *in vitro* studies indicate the importance of the stringent response for the development of antibiotic tolerance that may lead to persistence. Persister strains can tolerate high concentrations of antibiotics and the contribution of the stringent response for persister formation has been demonstrated. The absence of RelA reduced the ability of *E. coli* to form persister cells tolerant to ampicillin and ofloxacin *in vitro* **(Amato and Brynildsen, 2015)**. Furthermore, single-cell analysis has demonstrated that mutations in the HipA kinase toxin prevents binding of the HipB antitoxin and has been identified in clinical *E. coli* isolates **(Schumacher *et al.*, 2015)**. This mutation led to ciprofloxacin tolerance and persister formation in a bladder cell culture model **(Schumacher *et al.*, 2015)**. Persister formation in *E. coli* can also occur in a toxin-antitoxin-independent manner with the formation of 100S ribosomes being implicated **(Song and Wood, 2020)**. In *S. aureus,* persister colonies within RAW 264.7 murine macrophages were induced upon exposure to oxacillin, clarithromycin and gentamicin and this phenomenon was reduced in a Rel-deficient strain **(Peyrusson *et al.*, 2020)**. The stringent response is also essential for the establishment of mycobacterial infections and has been implicated in persistence and long-term viability **(Dahl *et al.*, 2003; Primm *et al.*, 2000)**. Moreover, *S. aureus* persistent bacteraemia isolates have been revealed to upregulate the purine biosynthesis pathway, leading to increased GTP and increased (p)ppGpp **(Li *et al.*, 2020)**. It was demonstrated that PSM expression was upregulated as a result, as well as the ability to survive within and kill PMNs **(Li *et al.*, 2020)**. These studies highlight the different mechanisms by which the stringent response is important in the development of antibiotic tolerance and therefore persistence. Further studying this phenomenon is necessary to understand the negative effect that induction of the stringent response can have on patient treatment options and prognosis.

#### 1.2.3.4. Antibiotic resistance

The stringent response has also been associated with antibiotic resistance. Through whole genome sequencing of an MRSA strain, a *rel* mutation that introduced a stop codon after the NTD was identified in β-lactam resistant strains **(Mwangi *et al.*, 2013)**. As the CTD negatively regulates the NTD, uncontrolled (p)ppGpp production occurs in the absence of the CTD **(Gratani *et al.*, 2018)** and therefore this strain is likely to overexpress (p)ppGpp **(Mwangi *et al.*, 2013)**. Thus, elevated (p)ppGpp levels due to acquired *rel* mutations may lead to resistance. This has been demonstrated in a clinical setting where an MRSA isolate with an F128Y substitution mutation in the HD domain resulted in constitutive Rel synthetase activity **(Gao *et al.*, 2010)**. The *agr* locus was upregulated in this strain and ultimately resulted in the formation of small colony variants (SCVs). Due to increased synthetase activity, higher levels of (p)ppGpp were reported and SCV formation resulted in persistent and chronic infection and lengthy treatment **(Gao *et al.*, 2010)**. This study emphasises how (p)ppGpp overproduction can be beneficial for the survival of bacteria. As well as Rel*Sau*, RelQ*Sau* has also been implicated in β-lactam resistance, as the absence of RelQ has led to downregulation of *mecA* expression **(Bhawini *et al.*, 2019)**. Due to the propensity of *S. aureus* to rapidly evolve and become resistant to multiple antibiotics, it is important to understand how the stringent response impacts this. This is necessary for the development of more efficacious stringent response inhibitors.

### 1.2.4. Stringent response inhibitors

Research into the stringent response has resulted in the development of drug compounds designed to inhibit the effects of (p)ppGpp. After the initial discoveries of ppGpp analogues with anti-stringent response activity in both Gram-negative and Gram-positive bacteria **(Wexselblatt *et al.*, 2010)**, Relacin, a 2′-deoxyguanosine-based ppGpp analogue was synthesised based on the crystal structures of *Streptomyces equisimilis* long RSH Rel*Seq* **(Wexselblatt *et al.*, 2012)** **(Fig. 1.2.4b)**. Here, relacin was synthesised so that the 5′ and 3′ pyrophosphate groups of ppGpp are replaced with glycyl-glycine dipeptides and this structure was shown to bind to the Rel*Seq* GDP-binding domain via hydrophobic interactions and hydrogen bonds. Therefore, by competitively inhibiting GDP binding, relacin can reduce RelA/Rel-mediated (p)ppGpp production. While relacin is effective against RelA/Rel from *E. coli* and *B. subtilis in vitro,* studies at the cellular level revealed that there was no effect on survival of *E. coli* after drug treatment, which was suggested to be due to cell penetrability **(Wexselblatt *et al.*, 2012)**. However, relacin inhibited *B. subtilis* sporulation and biofilm formation **(Wexselblatt *et al.*, 2012).** Relacin is RelA/Rel-specific and does not inhibit SAS, perhaps due to low sequence identity **(Gaca *et al.*, 2015; Geiger *et al.*, 2014)**. Since then, relacin has been improved by replacing the glycyl-glycine dipeptide with glutamyl-glutamine, creating relacin-2d, which demonstrated higher efficacy than relacin **(Wexselblatt *et al.*, 2013)**. However, the millimolar concentrations required for the potency of this drug may not be appropriate in the clinical setting **(Hobbs and Boraston, 2019)**. By acetylating the amine group of the guanine moiety (compounds AC and AB) **(Fig. 1.2.4c)**, Syal and colleagues were able to demonstrate anti-mycobacterial activity through inhibition of biofilm formation and formation of an elongated cell phenotype resembling an *M. smegmatis* ∆*rel* mutant **(Syal *et al.*, 2017)**. In comparison to the millimolar range concentration required by relacin-2d, the efficacy of compounds AC and AB was observed at approximately 40 μM **(Syal *et al.*, 2017)**. Additionally, a nucleotide library of guanosine phosphonate compounds has been screened **(Fig. 1.2.4d)**– the most potent candidate was DR-M014 with an IC50value in the micromolar range **(Beljantseva *et al.*, 2017)**. These compounds were able to inhibit RelA*Ec* and one compound, DR-4250 inhibited RelQ*Ef*, however DR-M014 showed no activity against RelQ*Ef* and no activity was measured for either compound in *B. subtilis* **(Beljantseva *et al.*, 2017)**.

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| **Figure 1.2.4. Structures of stringent inhibitors.** The structures of **a)** ppGpp with the phosphate groups coloured blue and orange to indicate whether the molecule is ppGpp or pGpp; **b)** Relacin with the glycyl-glycine bridges replacing the 5’ and 3’ phosphate groups found in ppGpp; **c)** compound AB and compound AC showing acetylation of the ribose moiety in both compounds and benzoylation in compound AB; **d)** DR-M014 and DR-4520 with phosphonate groups highlighted in blue. |

AMPs have also been considered as stringent response inhibitors and are not designed with RSH enzymes as a target. A cationic anti-biofilm peptide 1018, thought to target (p)ppGpp for degradation, was shown to inhibit biofilm formation by many species including *E. coli*, MRSA, *P. aeruginosa* and *S.* Typhimurium **(de la Fuente-Núñez  *et al.*, 2014)**. Low concentrations of 1018 elicited biofilm dispersal and higher concentrations led to cell death. Furthermore, RelA/SpoT overproduction led to reduced susceptibility, while RelA/SpoT-deficient strains exhibited a phenotype similar to the presence of the 1018 peptide **(de la Fuente-Núñez  *et al.*, 2014)**. However, it was suggested that peptide 1018 does not specifically target (p)ppGpp, and that physicochemical properties such as hydrophobicity rather than direct interaction of the peptide with (p)ppGpp, are responsible for the results reported by de la Fuente-Nunez and colleagues **(Andreson, Tenson and Hauryliuk, 2016)**. Peptide 1018 has recently been modified to produce 1018M, a peptide that prevented the formation of MRSA biofilms and was more potent than peptide 1018 **(Jiale *et al.*, 2021)**. All three RSH gene transcripts were modulated upon exposure to the 1018M peptide in order to reduce MRSA biofilm formation **(Jiale *et al.*, 2021)**. Nevertheless, further research into the exact mechanism by which this peptide functions is required.

Lastly, as the stringent response is required for the long-term survival of *Mycobacterium tuberculosis* **(Primm et al., 2000)**, different vaccine candidates have been developed. The immunity of mice and guinea pigs to Rel*Mtb* via a DNA vaccine was revealed to enhance the activity of the first-line drug isoniazid as Rel*Mtb* is an antigen during mycobacterial infections **(Chuang *et al.*, 2020)**. The vaccine was enhanced by fusing Rel*Mtb* to a chemokine Macrophage Inflammatory Protein-3 α that targets antigens, in this case, Rel*Mtb* to dendritic cells and this method has been shown to improve immune responses **(Gordy *et al.*, 2016; Karanika *et al.*, 2022)**. Intramuscular injection of the MIP-3α/relMtb fusion and intranasal administration of relMtb also potentiated isoniazid activity **(Karanika et al., 2022)**, demonstrating how vaccines may be a useful adjunct to long-term therapies such as those associated with tuberculosis.

## 1.3. The role of phagocytes during *S. aureus* infection

Upon breach of host physical barriers through damage, injury or surgery, *S. aureus* can be exposed to and infect layers of the skin, deep tissues and even the bloodstream which allows the development of various infections. Here, bacteria will encounter the innate immune system, which is the first line of defence against pathogens. *S. aureus* was considered an extracellular pathogen until it was shown that this bacterium was able to survive and proliferate within neutrophils **(Gresham *et al.*, 2000)**, thus contradicting previous views. It is now known that *S. aureus* can be taken up by and invade a variety of cells including both professional and non-professional phagocytes **(Strobel *et al.*, 2016).** Professional phagocytes encompass cells such as neutrophils and macrophages, important components of the innate immune system, that are required to take up and destroy foreign material such as pathogens. *S. aureus* can tolerate the harsh environment of phagocytes, which allows survival and subsequent escape **(Horn et al., 2018; Kubica et al., 2008)**, similar to other intracellular bacteria such as *S.* Typhiand *L. monocytogenes*. In this section, the role of neutrophils and macrophages are described and their contribution in the defence against *S. aureus* will be discussed.

### 1.3.1. The functions of neutrophils and macrophages

Phagocytic cells were first reported in 1875 by William Osler where he visualised the uptake of carbon particles by alveolar macrophages **(Ambrose, 2006)**. This phenomenon was then extensively studied by Ilya Metchnikoff for which he shared a Nobel Prize with Paul Ehrlich **(Tauber, 2003)**. Pluripotent haematopoietic stem cells (HSCs) in the bone marrow differentiate into either the myeloid or lymphoid progenitor. Cells of the myeloid progenitor that contribute to the innate immune system include the granulocytes: basophils, eosinophils, mast cells and neutrophils, and the agranulocytes: monocytes/macrophages and dendritic cells **(De Kleer *et al.*, 2014)**. Of these cells, neutrophils, macrophages/monocytes, dendritic and mast cells comprise the professional phagocytes. These innate immune effector cells are able to actively detect and ingest pathogens in order to eliminate threats to the host and thus, form an important first line of defence.

#### 1.3.1.1. Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMNs) are the most abundant circulating phagocyte **(McGuinness, Kobayashi and DeLeo, 2016)**. Due to their abundance, neutrophils are typically the first immune cell that a pathogen will encounter. Neutrophils are produced in the bone marrow and subsequently enter the peripheral vasculature for approximately 6-12 hours **(Summers *et al.*, 2010)** before they are recruited to tissues when required. They are short-lived cells and it has been suggested that the neutrophil half-life is approximately 24 hours **(Lahoz-Beneytez *et al.*, 2016).** Once fully activated, neutrophils have three main functions: phagocytosis, degranulation and the production of neutrophil extracellular traps (NETs), termed NETosis **(Rosales, 2018; Mayadas, Cullere and Lowell, 2014; Papayannopoulos, 2018)**.

Following uptake of foreign material by phagocytosis, as neutrophils are granulocytes, they degranulate. Granules are membrane-bound compartments that contain various antimicrobial agents and are stored within neutrophils. The process of granules fusing with microbe-containing phagosomes is termed degranulation **(Hirsch and Cohn, 1960; Borregaard, Sorensen and Theilgaard-Wnchl, 2007)**. Granules come in different forms: azurophilic, specific and gelatinase. Each granule contains a mixture of proteins and degradative enzymes including but not limited to: AMPs, cathepsins, elastases and defensins, depending on the type of granule, however all granules contain lysozyme **(Hirschho and Weissman, 1965; Lollike *et al.*, 1995)**. Azurophilic granules are named as such due to their affinity to azure A, a dye component of the Romanowsky stain used to differentiate blood cells **(Theil, 2012)**. Azurophilic granules contain a variety of proteins but are the only granule type to contain the enzyme MPO **(Faurschou and Borregaard, 2003)**. Specific granules are known to contain high concentrations of the iron sequestration protein lactoferrin **(Masson, Heremans and Schonne, 1969)**, but low concentrations of gelatinase, while gelatinase granules display the opposite **(Bainton, Ullyot and Farquhar, 1971; Kjeldsen *et al.*, 1993; Borregaard *et al.*, 1995)**.

As part of its microbicidal arsenal, neutrophils are also able to form NETs. NETs consist of chromatin fibres including histones, modified with antimicrobial components such as MPO and neutrophil elastase (NE) found within the neutrophil, all of which are ejected from neutrophils via the process of NETosis **(Brinkmann *et al.*, 2004)**. This phenomenon was first observed in 1996 **(Takei *et al.*, 1996)** and further characterised in 2004 **(Brinkmann *et al.*, 2004)**. This process was termed NETosis, as Takei and colleagues reported neutrophil death post NET release, however it is now established that NET release does not always result in cell death. Accordingly, two types of NETosis have been described: classical/suicidal NETosis that results in a novel type of programmed cell death **(Fuchs *et al.*, 2007)**; and vital/non-lytic NETosis where neutrophil viability is retained **(Vorobjeva and Chernyak, 2020; Pilsczek *et al.*, 2010)**. Briefly, NETosis formation is initiated by the production of reactive oxygen species (ROS), and the signalling pathways that allow this, trigger MPO signalling pathways. MPO will activate NE, which is required to prevent phagocytosis and promote chromatin condensation **(Papayannopoulos *et al.*, 2010; Papayannopoulos, 2018)**, ultimately resulting in NETosis. The purpose of NETosis is to trap extracellular pathogens with web-like chromatin and subsequently eliminate bacteria using antimicrobial proteins/enzymes, and is postulated to occur in order to prevent dissemination **(Branzk *et al.*, 2014)**.

#### 1.3.1.2. Monocytes and Macrophages

Once produced in the bone marrow, monocytes, like neutrophils will enter the bloodstream and comprise approximately 3-8% of the circulating cell population, which increases in the event of inflammation **(Monie, 2017)**. Monocytes will circulate for approximately 24-48 hours and unless they are recruited to specific tissues where they differentiate into tissue-resident macrophages (TRM), they will die **(Davies *et al.*, 2013)**. However, not all TRM are differentiated from monocytes – some TRM are able to self-renew and this ability is owed to primitive haematopoietic progenitors of an embryonic origin that reside within specific tissues **(Ajami *et al.*, 2007; Sieweke and Allen, 2013; Hashimoto *et al.*, 2013)**. These macrophages are seeded to tissues during embryonic development and thus, challenge the previous notion that all TRM are bone marrow-derived **(Hashimoto *et al.*, 2013)**. Similar to neutrophils, bone marrow-derived macrophages (BMDMs) are short-lived and are able to differentiate from monocytes when necessary, while embryonically derived macrophages are long-lived and are able to self-maintain **(Bain *et al.*, 2014; Guilliams and Scott, 2017)**. Monocytes/embryonic progenitors that give rise to TRM undergo major transcriptional changes in order to adapt to, and function appropriately in their resident tissue **(Gautier *et al.*, 2012)**. The different types of TRM include Langerhans cells (skin), Kupffer cells (liver), microglia (brain), alveolar macrophages (lung alveoli) and red pulp macrophages (spleen). Macrophages are thus known for their plasticity, highlighted by their different functions depending on their resident tissue. While the transcriptional profile and cell surface markers of TRM differ between organs, the function of TRM/monocytes remains the same. Major functions of macrophages/monocytes include the development, maintenance and surveillance of tissues, as well as the ability to generate an immune response to invading pathogens and subsequently clear foreign material **(Italiani and Boraschi, 2014; Pidwill *et al.*, 2021)**.Tissue-specific functions of macrophages are important for tissue homeostasis. For example, red pulp macrophages and Kupffer cells are required for erythrocyte clearance and alveolar macrophages are required for surfactant clearance **(Davies *et al.*, 2013)**. Furthermore, cardiac macrophage-depleted mice displayed poor healing, implicating cardiac macrophages in wound healing following myocardial injury **(van Amerongen *et al.*, 2007)**. The maintenance and development roles of macrophages demonstrate that phagocytosis occurs for reasons beyond immunity.

Macrophages are often regarded as immune sentinels, as they actively phagocytose materials in the surrounding environment in order to identify possible threats **(Mills, 2012)**. Macrophages express multiple receptor types on their cell surface to do this, which aids the recognition of foreign bodies such as pathogens. These include pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). With monocytes circulating the bloodstream and TRMs traversing tissues and organs, these cells are well-equipped to deal with dead/dying cells and foreign materials that pose a threat **(Franken, Schiwon and Kurts, 2016)**. While macrophages are considered an effector of the innate immune system, their ability to present antigens on their surface and thus act as an APC, indicate that they have an important role in initiating the adaptive immune response **(Flannagan, Heit and Heinrichs, 2015)**.

Macrophage polarisation is a phenomenon that occurs following macrophage stimulation and allows the cell to carry out a specific function. M1 macrophages are primed to initiate a proinflammatory response e.g., in response to foreign bodies whereas, M2 macrophages are inclined towards tissue maintenance and repair **(Mills, 2012)**. For example, macrophages are activated to the M1 phenotype by the recognition of pathogen associated molecular patterns (PAMPs), while the M2 phenotype is activated upon detection of danger associated molecular patterns (DAMPs) **(Mills, 2012)**. Regulation of macrophage polarisation is therefore paramount, as if the appropriate macrophage response is not established, pathologies can occur. Endotoxin tolerance can occur, which erroneously favours the M2 phenotype instead of M1 **(Del *et al.*, 2009)**, rendering macrophages unable to respond to pathogens. The metabolism of M1 and M2 macrophages differ, resulting in these contrasting phenotypes. While non-polarised macrophages metabolise arginine, M1 macrophages metabolise nitric oxide (NO) and citrulline, and M2 macrophages metabolise ornithine and polyamines **(Mills *et al.*, 2000)**. NO acts to prevent cell proliferation, while ornithine does the opposite **(Wu and Morris, 1998; Mills, 2001)**, thus giving rise to the M1 and M2 phenotypes. M1 and M2 macrophages were named as such due to their ability to promote Th1 and Th2 responses **(Mills *et al.*, 2000; Italiani and Boraschi, 2014)**. This is the process by which naïve T cells become Th1 or Th2 cells, which is associated with proinflammatory and anti-inflammatory responses, respectively **(Berger, 2000)**. As T cells comprise part of the adaptive immune system and can be regulated by polarised macrophages, a further link between innate and adaptive immunity is illustrated.

### 1.3.2. How is *S. aureus* recognised by the innate immune system?

Upon infection and colonisation, pathogens such as *S. aureus* can be sensed by professional phagocytes. Staphylococcal cell wall components, as well as cell wall debris, can be recognised by phagocytes through binding to mammalian cell surface receptors such as PRRs. PRRs are able to bind to various microbial elements (PAMPs) that initiate complex signalling networks and thus, allow phagocytes to appropriately respond to pathogens and foreign bodies through immune cell activation and cytokine release. Here, TLRs and CLRs will be discussed, as these receptors are present on the surface of phagocytes. In comparison, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are intracellular proteins that interact with microbial components once they enter the cytosol.

#### 1.3.2.1. Toll-like receptors

TLRs, first described in the fruit fly *D. melanogaster*, were identified as the receptor family required for downstream signalling resulting in the production of AMPs **(Anderson, 2000)**. Due to this, *D. melanogaster* is naturally resistant to microbial infections. TLRs are transmembrane proteins consisting of an extracellular leucine-rich repeat domain, a single transmembrane helix and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain **(Botos, Segal and Davies, 2011)**. Since the discovery of TLRs, ten homologues have been identified in humans (TLR1-10) in comparison to the 12 possessed by mice. TLR2 recognises cell wall components such as peptidoglycan, lipoproteins and LTA **(Takeuchi *et al.*, 1999; Aliprantis *et al.*, 1999)**. TLR4 is required for the detection of lipopolysaccharide (LPS) **(Poltorak *et al.*, 1998)** and TLR5 responds to flagellin, the monomer that makes up flagella filaments **(Hayashi *et al.*, 2001)**. TLRs 3, 7, 8 and 9 are important for identifying various nucleic acids such as dsRNA (TLR3), ssRNA (TLR7/8) and ssDNA (TLR9) containing CpG motifs (commonly associated with foreign pathogenic nucleic acids) **(Alexopoulou *et al.*, 2001; Zhang *et al.*, 2018b; Bauer *et al.*, 2001)**. TLRs 1 and 6 act in concert with TLR2 to form heterodimers, while all other TLRs are able to respond to stimuli as homodimers **(Takeuchi *et al.*, 2002)**. TLRs are not solely associated with a specific pathogen, as they respond to structurally unrelated ligands. However, TLR2 is implicated in the host response to the presence of *S. aureus* **(Underhill *et al.*, 1999; Dziarski *et al.*, 2001)**. TLR2 can recognise staphylococcal components such as wall teichoic acids (WTA) and LTA and in the presence of PSMs, the TLR2/6 heterodimer can activate downstream signalling cascades **(Hajjar *et al.*, 2001)**. In the absence of TLR2, staphylococcal clearance in murine models is diminished. For example, in comparison to WT, TLR2-deficient mice are unable to clear *S. aureus* after subcutaneous mouse infection **(Hoebe *et al.*, 2005)**. Furthermore, TLR2-deficient mice were reported to have higher rates of murine nasal colonisation in comparison to the WT counterpart **(González-Zorn *et al.*, 2005)**. Following activation of TLR2, the intracellular TIR domain interacts with myeloid differentiation primary response 88 (Myd88), which initiates signalling cascades leading to the release of transcription factor NF-κB, a necessary component for the expression of proinflammatory mediators **(Fournier and Philpott, 2005)**.

#### 1.3.2.2. C-type lectin receptors

CLRs respond to carbohydrates on the cell surface of pathogens and can exist as both a transmembrane and cytosolic receptor **(Brown, Willment and Whitehead, 2018)**. Carbohydrate-binding is achieved through the C-type lectin-like domain of CLRs and conserved motifs within this domain allows binding to specific sugars e.g. the EPN (glutamic acid, proline, asparagine) motif allows binding to mannose, N-acetylglucosamine, L-fucose and L-glucose, while the QPD (glutamine, proline, aspartate) motif allows binding to N-acetylgalactosamine and galactose **(Zelensky and Gready, 2005)**. CLRs are a type of phagocytic PRR that, once bound to PAMPs, allows the encapsulation of pathogens within cytoplasmic vesicles, thus facilitating phagocytosis **(Freeman and Grinstein, 2014)**. Dectin-1 is a well-known CLR, found abundantly on dendritic cells and deficiencies of this protein diminishes the host ability to respond to fungal pathogens **(Ferwerda *et al.*, 2009)**. CLRs have mainly been associated with immunity against fungi and viruses, however recent studies have shown their role in immunity against *S. aureus*. Langerhans cells, a skin TRM, exclusively expresses the langerin CLR which enables recognition of the N-acetylglucosamine component of staphylococcal WTAs **(van Dalen *et al.*, 2019)**.

### 1.3.3. Phagocytosis

Once identified, for pathogens to be eliminated and to achieve immunity, they must be phagocytosed by professional phagocytes. This allows antigens from the pathogen to be processed and presented at the phagocyte cell surface, for recognition by adaptive immune cells such as T cells. Endocytosis is the uptake of extracellular material (including pathogens) by a cell and is governed by ligand-binding to cell-surface receptors, resulting in the internalisation of a membrane-bound vesicle. This is termed receptor-mediated endocytosis, and phagocytosis is a similar phenomenon that is also receptor-mediated. In contrast to endocytosis, phagocytosis allows the uptake of materials larger than 0.5 μm **(Aderem and Underhill, 1999)**. While receptor-mediated endocytosis requires the clathrin scaffold protein, phagocytosis is actin-dependent **(Swanson, 2008)**. Macrophages (depending on polarisation) phagocytose to remove dead/senescent cells for tissue maintenance **(Mills, 2012; Aderem and Underhill, 1999)** but also upon identification of a pathogen and the latter is also the case for neutrophils. Phagocytosis is thus, the ingestion of particles and cells. The process of phagocytosis comprises ligand-binding to specific receptors on the phagocyte cell surface, coordinated remodelling of the actin cytoskeleton and subsequent inward folding of the membrane to form a ‘cup’, that is closed to produce a membrane-bound compartment termed the phagosome **(Swanson, 2008)**.

In order for phagocytosis to occur, multiple receptor types that are expressed at the phagocyte cell surface, are required for interaction with e.g., a pathogen. These include scavenger, complement and Fc receptors. Scavenger receptors are a heterogeneous family of receptors with the ability to bind diverse ligands. There are eight classes of scavenger receptors (classes A, B and D-I) and members of each class are structurally varied **(Canton, Neculai and Grinstein, 2013)**. Scavenger receptor A (SR-A), a class A receptor, is involved in phagocytosis of *S. aureus* by the liver resident Kupffer cells. SR-A in conjunction with mannose-binding lectin (MBL) (member of the CLR family), which also increases expression of SR-A, resulted in augmented phagocytosis **(Ono *et al.*, 2006)**. Furthermore, the class B scavenger receptor CD36 is required to recognise LTA and CD36-deficient mice are more susceptible to *S. aureus* systemic infection **(Stuart *et al.*, 2005)**. However, while scavenger receptors are involved in phagocytosis, it is unclear whether these receptors are solely required to initiate phagocytosis. For example, it was shown that CD36 activates TLR2/6 signalling **(Stuart *et al.*, 2005)**, suggesting that other pathways are required for phagocytosis to occur.

The complement cascade is a complex component of the innate immune system consisting of numerous proteins that undergo proteolysis and was named due to its ability to ‘complement’ antibody-mediated killing. The complement cascade can be activated in three main ways: the classical, alternative and lectin pathways. The classical pathway is activated upon IgG/IgM antibody interaction with the first component of the cascade, C1q **(Nesargikar, Spiller and Chavez, 2012)**. The alternative pathway occurs when C3b, which is abundant in plasma, interacts with a pathogen/damaged cell **(Thurman and Holers, 2006)**. The lectin pathway involves the binding of collectins (MBL and ficolin) to carbohydrates found on foreign bodies and results in MBL-associated serine protease interacting with complement proteins and thus, activating the cascade **(Takahashi *et al.*, 2008)**. The complement cascade ultimately acts to form a membrane attack complex that lyses cells, opsonise foreign bodies to stimulate phagocytosis, or recruit phagocytes. The complement component iC3b protein can bind *S. aureus* as an opsonin and is recognised by complement receptors 3 and 4 (CR3/4) that are expressed on phagocytes leading to phagocytosis of *S. aureus* **(Lukácsi et al., 2017)***.*

The Fc receptors bind to the Fc region of antibodies. Pathogens opsonised with antibodies are more likely to be internalised by phagocytes, for example the Fcγ receptor binds to IgG that will mediate phagocytosis **(Mellman *et al.*, 1988)**. As *S. aureus* is a commensal organism, hosts will develop tolerance. This is exemplified by higher serum IgG levels in people colonised with *S. aureus* **(Kloppot *et al.*, 2015)**. Fc, complement and scavenger receptors will work in concert with other cell surface receptors such as TLRs to aid the process of phagocytosis.

### 1.3.4. Phagosomal maturation

Following uptake by professional phagocytes, pathogens such as *S. aureus* are sequestered within phagosomes. Phagosomal maturation is the process by which microbe-containing phagosomes undergo fission and fusion events with intracellular membrane-bound organelles containing microbicidal agents. This culminates in the formation of a mature phagolysosome and the unfavourable conditions within this compartment act to eliminate pathogens. Specifically, the phagosome will fuse with endosomes and lysosomes to form the phagolysosome and this pathway is often regarded as an endo-lysosomal pathway **(Vieira, Botelho and Grinstein, 2002)**.

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| **Figure 1.3.4. Phagosomal maturation.** Upon recognition of bacteria by cell surface receptors, phagocytosis occurs, forming a nascent phagosome. The most important effector is Rab5 which, once active, recruits Vps34 PI3K that allows the production of PI(3)P. The tether, EEA1 is subsequently recruited and associated with Rab5. EEA1 and SNARE mediate the fusion of the early endosome with the nascent phagosome to form an early phagosome. Mon1/Ccz-1 mediate the activation of Rab7 and deactivation of Rab5 by ultimately activating HOPS. Active Rab7 recruits RILP and ORPL1 which allows trafficking of the phagosome towards the Golgi apparatus where late endosomal fusion can occur. It is here that late phagosomes express LAMP1/2 (lysosomal markers). The SNARE complex comprised of VAMP7/8 and syntaxin 7/8 coordinate fusion of lysosomes to produce the phagolysosome. v-ATPase is abundant within the phagolysosome. |

#### 1.3.4.1. The early phagosome

Fusion of early endosomes with the nascent phagosome forms the early phagosome. The early endosome is an organelle responsible for sorting endocytosed materials and will mature into late endosomes and are mildly acidic (pH ~6.1) **(Grant and Donaldson, 2009; Luzio *et al.*, 2009; Maxfield and McGraw, 2004)**. The Rab5 GTPase in its active GTP-bound state is associated with the endosomal and phagosomal membranes **(Ullrich *et al.*, 1994; Bucci *et al.*, 1992; Flannagan, Jaumouille and Grinstein, 2012) (Fig. 1.3.4)**. The cues that lead to Rab5 activation and recruitment are unclear, however the contents of the phagosome play a role in the mechanism by which it is activated **(Bucci *et al.*, 1992; Pei *et al.*, 2014; Kitano *et al.*, 2008).** The function of Rab5 is to coordinate fusion of early endosomes to phagosomes and this is done by recruiting multiple effector proteins. Rab5 recruits the Vps34 [phosphatidylinositol](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/phosphatidylinositol) (PI) 3-kinase (PI3K) that enables production of phosphatidylinositol 3-phosphate (PI(3)P) in abundance, a regulator of the early endosome **(Christoforidis *et al.*, 1999a; Christoforidis *et al.*, 1999b)**. The early endosome antigen 1 (EEA1) is a molecular tether subsequently recruited due to PI(3)P signalling pathways **(McBride *et al.*, 1999)**. EEA1 associates with Rab5 and in conjunction with N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (receptors that mediate membrane fusion), allows fusion of the early endosome to the phagosome to produce an early phagosome **(Christoforidis *et al.*, 1999a; Christoforidis *et al.*, 1999b)**. The vacuolar-ATPase (V-ATPase) is present on early phagosomal membranes but at a lower concentration than observed as the phagosome matures **(Pauwels *et al.*, 2017)**.

#### 1.3.4.2. The late phagosome

The late phagosome is characterised by the presence of Rab7 and concomitant absence of Rab5 **(Fig. 1.3.4)**. The Mon1 protein (a Rab5 effector) complexes with Rab5 and recruits Ccz-1, which also associates with Rab5 **(Kinchen and Ravichandran, 2010)**. Ccz-1 tethers GDP-bound Rab7 and recruits it to the early phagosomal membrane, ultimately leading to Rab7 activation by homotypic fusion and protein sorting (HOPS) and Rab5 inactivation **(Kinchen and Ravichandran, 2010; Poteryaev *et al.*, 2010; Rink *et al.*, 2005)**. Rab7 recruits effector proteins Rab7-interacting lysosomal protein (RILP) and oxysterol-binding protein–related protein 1 (ORPL1) to the late phagosome **(Cantalupo *et al.*, 2001; Johansson *et al.*, 2007)**. These effectors coordinate the traffic of the phagosome centripetally in a microtubule-dependent manner, towards the Golgi apparatus that is necessary for future late endosomal and lysosomal fusion **(Harrison *et al.*, 2003)**. The membrane proteins lysosome-associated membrane proteins 1 and 2 (LAMP-1/2) are also required for Rab7 recruitment and killing of pathogens, as LAMP-deficient cells demonstrated a reduced ability to phagocytose *Neisseria gonorrhoeae* **(Huynh *et al.*, 2007; Binker *et al.*, 2007)**.

#### 1.3.4.3. The phagolysosome and its microbicidal mechanisms

Once taken up by a phagocyte, the phagolysosome represents the most microbicidal compartment that a pathogen will encounter **(Fig. 1.3.4.3)**. A SNARE complex, comprised of vesicle-associated membrane protein 7 and 8 (VAMP7/8) and syntaxin 7 and 8, coordinates the fusion of lysosomes to late phagosomes, thus producing the phagolysosome **(Flannagan, Jaumouille and Grinstein, 2012; Wade *et al.*, 2001) (Fig. 1.3.4)**. This SNARE complex is enriched on the surface of phagosomes and lysosomes **(Becken *et al.*, 2010)**. As phagosomal maturation occurs, the pH decreases with every compartment due to the presence of v-ATPase on endosomes and lysosomes **(Kinchen and Ravichandran, 2010)**. v-ATPase is a 14-subunit protein complex that actively increases the phagosomal pH by pumping protons **(Maxson and Grinstein, 2014)** and is the major component responsible for phagolysosomal low pH. The acidification of this compartment serves to eliminate pathogens and is also required for the function of cathepsins and AMPs such as LL-37. *S. aureus* growth at pH 4.5 is severely impeded *in vitro* and thus the low pH environment of the phagolysosome is not optimal for exponential growth **(Bore *et al.*, 2007)**. However, *S. aureus* has developed mechanisms to aid survival in the presence of low pH. The GraS sensor kinase of the GraXRS regulatory system is responsible for sensing low pH and expression of genes regulated by this system including *mprF* and the *dltABCD* operon have led to the development of AMP resistance **(Flannagan *et al.*, 2018)**. It was also shown that *S. aureus* replicated within the acidic phagolysosomal environment **(Flannagan *et al.*, 2018)**, concurrent with the notion that *S. aureus* is an intracellular pathogen and replication within phagocytes acts as a means of dissemination.

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| **Figure 1.3.4.3. The phagolysosome.** Within the phagolysosome, many microbicidal insults are present. v-ATPase lowers the phagolysosomal pH by providing an influx of H+ ions. The compartmental pH can also be altered, to a lesser extent, by itaconic acid, a Krebs cycle intermediate. Itaconic acid is produced by aconitase that is encoded for by the *IRG1* gene. Itaconic acid positively modulates the NRF2/HO1 signalling pathway, thereby inhibiting the inflammasome and reducing inflammation within the host. Itaconic acid acts on bacteria by inhibiting isocitrate lyase and therefore the glyoxylate shunt, an important shunt of the Krebs cycle that is important for bacterial growth. Superoxide (O2- ) and nitric oxide radicals (·NO) are generated by NADPH oxidase and iNOS respectively. O2- can be detoxified by converting to H2O2 by superoxide dismutase (SOD). H2O2 is converted to HOCl by myeloperoxidase (MPO). H2O2 can also react with staphylococcal ferrous iron (Fe2+) to produce the hydroxyl radical (**۰**OH) and hydroxide ion (OH-). Enzymes such as lysozyme act on the cell wall and hydrolyse the β(1-4) glycosidic linkages of peptidoglycan. The cathepsins are cysteine proteases and degradative enzymes that destroy biofilms but also positively modulate cytokine production. The cationic AMPs LL-37 and HNP1-4 cause cell lysis. Green arrows represent a positive interaction. |

Itaconic acid/itaconate is a weak organic acid and immunometabolite that was recently discovered to be produced by LPS-stimulated RAW 264.7 murine macrophages **(Strelko *et al.*, 2011)**. Within phagolysosomes, it can be found at physiological concentrations of approximately 55-120 pM **(Singh *et al.*, 2021)**. Itaconic acid is produced by the immune response gene 1 (*IRG1*), encoding for aconitate decarboxylase, responsible for converting aconitic acid to itaconic acid and therefore is a product of the Krebs cycle. Itaconic acid can exert its antimicrobial effects by inhibiting isocitrate lyase, a major component of the glyoxylate shunt that is an important pathway for optimal growth in bacteria, and has been associated with responding to ROS stress **(Zhu *et al.*, 2021; Ahn *et al.*, 2016; Lorenz and Fink, 2001) (Fig. 1.3.4.3)**. Itaconic acid also has immunomodulatory effects, giving the enzyme responsible for producing it, its name. For example, the charged conjugate base itaconate acts to reduce inflammation during *S. aureus* ocular infection by modulating NRF2/HO1 signalling and inhibiting the NLRP3 inflammasome **(Singh *et al.*, 2021).** A recent study has shown that itaconate inhibits glycolysis and the oxidative burst of murine neutrophils *in vitro* **(Tomlinson *et al.*, 2023)**, which are key components of neutrophil metabolism and killing respectively, and are integral processes during infection. Therefore, in these experiments, itaconate production by neutrophils was beneficial for *S. aureus* survival as the presence of itaconate diminished the ability of neutrophil to kill bacteria **(Tomlinson *et al.*, 2023)**. In contrast, in a murine *S. aureus* pneumonia model, WT mice and *Irg*-deficient mice showed no difference in inflammation despite a higher bacterial load in WT mice, suggesting that itaconate acts to control inflammation which is beneficial to the host **(Tomlinson *et al.*, 2023)**. As demonstrated, itaconate does have antimicrobial effects; however, during chronic airway infection, itaconate exposure selected for *S. aureus* strains that display decreased glycolysis that redirected carbohydrate metabolism to an increased production of extracellular polysaccharide instead, fuelling biofilm formation **(Tomlinson et al., 2021)**. From these studies, it is evident that itaconate has different effects on both *S. aureus* and the host depending on the tissue and type of infection and further studies must be conducting on this newly-identified antimicrobial metabolite.

The oxidative burst is the main source of ROS produced by the phagolysosome, which is one of the most important host defence mechanisms employed by professional phagocytes **(Dupre-Crochet, Erard and Nubetae, 2013)**.Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is a multi-subunit protein existing as seven isoforms, four of which are expressed in immune cells **(Fulton and Barman, 2016) (Fig. 1.3.4.3)**. The importance of NOX is established as impaired function and subunit assembly results in host susceptibility to infection **(Segal, Romani and Puccetti, 2009)**. Upon activation by proinflammatory stimuli e.g. bacterial cell wall components binding to and activating PRRs, the transmembrane and cytoplasmic subunits of NOX assemble **(Panday *et al.*, 2015)**. Consequently, electron transfer resulting in oxidation of NADPH and reduction of diatomic oxygen superoxide ions O2- are produced. The role of NOX2 with regards to *S. aureus* has been studied – NOX2-deficient BMDMs failed to clear *S. aureus* and was associated with improved staphylococcal survival and thus dissemination, demonstrating the important role of NOX2 **(Tosetti *et al.*, 2021)** .

While O2- is microbicidal, it is also deleterious to the host. O2- produced by NOX can be converted to the less toxic H2O2 by mammalian SOD **(Hyslop *et al.*, 1995) (Fig. 1.3.4.3)**. H2O2 and chloridecan then be converted to hypochlorous acid (HOCl) by MPO **(Winterbourn *et al.*, 2006)**, produced mainly by neutrophils (azurophilic granules) but also by macrophages. Production of these ROS acts to protect the host by damaging and killing pathogens. For example, HOCl has shown antimicrobial activity against *S. aureus* **(Abid, Maalej and Rouis, 2004)** and both HOCl and H2O2 have anti-biofilm activity **(Lineback *et al.*, 2018)**. H2O2 interacts with iron via Fenton chemistry, resulting in the production of toxic hydroxide (OH-) and hydroxyl radicals (·OH) **(Repine, Fox and Berger, 1981)**. More than one type of ROS is required for the elimination of *S. aureus*. O2- increases intracellular free iron by destruction of Fe-S clusters and iron liberated as a result of this can react with H2O2 via the Fenton reaction **(Keyer and Imlay, 1996)**. This is exemplified by a report of iron-overloaded *S. aureus* demonstrating susceptibility to killing by H2O2 **(Hoepelman *et al.*, 1990)**.However, *S. aureus* has an arsenal of mechanisms by which it can evade killing by ROS. *S. aureus* can adopt an SCV phenotype after prolonged exposure to H2O2, with SCVs displaying increased catalase activity and deficiency in use of the electron-transport chain **(Painter *et al.*, 2015)**. The antioxidant proteins such as catalase, alkylhydroperoxide reductase (AhpC) (both members of the PerR regulon), staphylococcal SodA and staphyloxanthin detoxify H2O2 andO2- respectively **(Cosgrove *et al.*, 2007; Karavolos *et al.*, 2003; Liu *et al.*, 2005)**. Furthermore, the SaeR/S two component system was involved in resistance to HOCl and H2O2 stress in a catalase-independent manner **(Guerra *et al.*, 2016)**. The quorum sensing response regulator AgrA has been implicated as an oxidant sensor due to a disulphide bond and may be involved in responding to H2O2  stress **(Sun *et al.*, 2012)**.

In addition to ROS, reactive nitrogen species (RNS) also play a role in the host defence. The inducible nitric oxide synthase (iNOS) produces nitric oxide radicals (·NO), which similar to ROS, goes on to damage cellular components and metabolites **(Serbina *et al.*, 2003) (Fig. 1.3.4.3)**. iNOS consists of an N-terminal oxygenase domain and a C-terminal reductase domain and the protein itself exists as a homodimer **(Flannagan, Jaumouille and Grinstein, 2012)**. Signals required to induce expression of iNOS are recognition of PAMPs via their cognate receptors and thus the production of proinflammatory cytokines such as interferon-γ (IFN-γ) **(Bogdan, Rollinghoff and Diefenbach, 2000)**. *In vitro* data has demonstrated that the nitric oxide donor spermine NONOate decreases the growth of JE2 *S. aureus* 2-fold **(Carvalho *et al.*, 2017)**, thus demonstrating its antimicrobial effects. While ROS are produced immediately due to their presence in the phagolysosome, RNS are produced later as signalling cascades such as those resulting in NF-κB activating are required for expression of iNOS **(Chen, Chen and Lin, 1999)**. This may indicate that the production of ROS is more important than RNS, especially given the importance of the oxidative burst **(Dupre-Crochet, Erard and Nubetae, 2013)** and since an iNOS-deficient murine infection of *S. aureus* demonstrated that iNOS contributed to, but was not required for pathogenesis **(Nathan and Shiloh, 2000)**.

Within the phagolysosome, a wide range of antimicrobial proteins and AMPs are present with varying functions that act in concert to kill invading pathogens, of which the key proteins and peptides will be discussed. Lysozyme targets peptidoglycan by hydrolysing β (1-4) glycosidic linkages, thus affecting cell wall integrity of bacteria **(Fig. 1.3.4.3)**. However, *S. aureus* is intrinsically resistant to lysozyme due to the presence of O-acetyltransferase A, which acetylates the N-acetylmuramyl residues of peptidoglycan, thus decreasing the ability of lysozyme to hydrolyse peptidoglycan **(Bera *et al.*, 2005)**. Furthermore, cathepsins are cysteine proteases found in lysosomes and thus the phagolysosome. Cathepsin G was shown to degrade *S. aureus* biofilms via neutrophil degranulation, which subsequently allowed neutrophil phagocytosis of bacteria **(Kavanaugh *et al.*, 2021)**. Cathepsins K and L play a role in *S. aureus* clearance by macrophages,with cathepsin K involved in interleukin-6 (IL-6) production **(Müller *et al.*, 2014)**. Other degradative enzymes include phosphatases, lipases and nucleases. Phagocytes also produce cationic AMPs. The LL-37 cathelicidin is the sole member of the human cathelicidin family of AMPs, a cationic peptide that functions by interacting with and penetrating the negatively charged bacterial cell membrane, culminating in bacterial cell lysis **(Kuroda *et al.*, 2015; Kahlenberg and Kaplan, 2013)**. In addition to interacting with and killing *S. aureus*, LL-37 has immunomodulatory effects. LL-37 can opsonise bacteria in order to increase the rate of phagocytosis **(Lishko *et al.*, 2016)** and was shown to aid intracellular killing of *S. aureus* with an increase in ROS production **(Tang *et al.*, 2015)**. Four of the six human α-defensins are found exclusively within neutrophils (human neutrophil α-defensin 1-4 (HNP1-4)), specifically the azurophilic granules. As mentioned previously, MprF and the *dltABCD* operon are implicated in the resistance to peptides such as daptomycin. Extra copies of the *dltABCD* operon rendered *S. aureus* resistant to HNP1-3 via membrane modification with LTA/WTA leading to an increase in net positive charge **(Peschel *et al.*, 1999)**. Similarly, MprF coordinates membrane lipid modification which enabled resistance to defensins **(Peschel *et al.*, 2001)**.

### 1.3.5. *S. aureus* escape from professional phagocytes

As demonstrated, the host immune system is equipped with a plethora of mechanisms to kill pathogens, from initial recognition to uptake into phagocytes and yet, *S. aureus* is able to evade killing at nearly every step. *S. aureus* is an intracellular pathogen and has been revealed to undergo clonal expansion resulting from a bottleneck within professional phagocytes **(McVicker *et al.*, 2014)**, therefore phagocytes can be used as a replication niche and thus, dissemination if able to escape. Phagosomal escape by *S. aureus* was initially described in bovine mammary epithelial (MAC-T) cells by inducing host cell apoptosis **(Bayles *et al.*, 1998)**. *S. aureus* replicates prior to host cell death and demonstrates membrane-blebbing, a characteristic of apoptosis/necrosis **(Flannagan, Heit and Heinrichs, 2016)**. Infection of THP-1 macrophages with *S. aureus* demonstrated incomplete phagolysosomal maturation and reduced acidification as a result **(Jubrail *et al.*, 2016)**. However, infected macrophages were unable to initiate apoptosis/necrosis as a defence mechanism, suggesting that *S. aureus* may also manipulate host programmed cell death **(Kubica *et al.*, 2008; Jubrail *et al.*, 2016)**. In contrast, infection of neutrophils with *S. aureus* led to cell death via programmed necrosis independent of the staphylococcal toxins **(Kobayashi *et al.*, 2010)**. *S. aureus* has survived for 3-4 days in MDM vacuoles before lysing cells and subsequently escaping, and contrary to the report by Kobayashi and colleagues, α-toxin was required forsurvival **(Kubica *et al.*, 2008)**. Thus, *S. aureus* utilises its virulence factors to escape host killing. *S. aureus* escape from neutrophils can be facilitated by the leukocidin AB toxin by forming pores in the cell membrane **(DuMont *et al.*, 2013b)**. The cytotoxic toxin PSMα is required for replication within and phagosomal escape from THP-1 macrophages **(Grosz *et al.*, 2014)**. Furthermore, the expression of the *agr* locus ultimately led to membrane permeabilisation that enabled endosomal escape from MAC-T **(Qazi *et al.*, 2001; Shompole *et al.*, 2003)**. The *agr* effector RNAIII encodes for δ-toxin, which is able to disrupt membranes in cooperation with β-toxin (staphylococcal sphingomyelinase) to coordinate endosomal escape from epithelial and endothelial cells **(Giese *et al.*, 2011)**. These studies emphasise the importance of the staphylococcal toxins for survival within both professional and non-professional phagocytes.

### 1.3.6. Non-professional phagocytes: human tonsillar epithelial cells

Unlike professional phagocytes, non-professional phagocytes are named as such due to their ability to phagocytose but this not being their main function **(Rabinovitch, 1995)**. These cells include epithelial and endothelial cells, among many. Epithelial cells also represent the first line of physical defence against pathogens and are categorised as sentinels. Stratified epithelium of the skin and squamous epithelia that protects multiple body organs represent the cell types that pathogens encounter before invasion of the host **(Günther and Seyfert, 2018)**. Thus, these cells are able to recognise PAMPs and DAMPs using PRRs. Like macrophages, epithelial cells also participate in the clearance of dying/dead cells, however they utilise efferocytosis (ingestion of apoptotic cells) or extrusion (the shedding of an apoptotic layer of cells) **(Freeman and Grinstein, 2016; Günther and Seyfert, 2018)**. Pathogens can also invade epithelial cells, particularly those that are intracellular such as *S. aureus* and *L. monocytogenes* **(Bayles et al., 1998; Ortega, Koslover and Theriot, 2019)**.

#### 1.3.6.1. Epithelial cell immune defence

As part of the host defence, the airway epithelium is lined with mucus that acts to protects against but can also trap pathogens **(Pietrocola *et al.*, 2017)**. The main defence mechanism by epithelial cells is the production of AMPs. Epithelial cells secrete AMPs such as the β-defensins, which have also demonstrated anti-staphylococcal activity *in vitro* **(Lehrer and Ganz, 1999; Chen *et al.*, 2005)**. Furthermore, LL-37 is also expressed in airway epithelia **(Bals *et al.*, 1998)**. However, pathogens are still able to invade epithelial cells. Invasion by *S. aureus* is mediated via the zipper mechanism, which utilises cell surface receptors for internalisation and is used by bacteria such as *S. aureus* and *L. monocytogenes* **(Veiga et al., 2007)**. Staphylococcal adhesins such as FnBPA/B interact with host cell surface α5β1 integrins to coordinate entry **(Sinha *et al.*, 1999; Sinha *et al.*, 2000).** Once internalised by epithelial cells, *S. aureus* was shown to be trafficked into phagolysosomes **(Lam *et al.*, 2010)**, highlighting the similarities in killing by professional and non-professional phagocytes.

#### 1.3.6.2. The role of *S. aureus* in recurrent tonsillitis

Human tonsillar epithelial (HTE) cells constitute multiple layers of stratified squamous epithelium and serve as a first line of defence in the human tonsils. The tonsils are a set of lymphoid organs of which there are four types: palatine, pharyngeal, lingual and tubual. The palatine tonsils are located in the lateral oropharynx, encountering inhaled and ingested pathogens **(Olofsson, Hellstrom and Hammarstrom, 1998)** and thus are thought to have immune functions, for example, tonsillar immune cells produce IgA **(Cantani *et al.*, 1986)**. However, inflammation of the palatine tonsils occurs frequently and is termed tonsilitis, an upper respiratory tract infection. While both bacteria and viruses are causative agents of tonsillitis, *S. aureus* is one of the predominant pathogens in addition to *Streptococcus pyogenes*,with approximately 57.7 % of cases attributed to the former while 20.2 % are attributed to the latter **(Zautner *et al.*, 2010; Cavalcanti *et al.*, 2019; Katkowska, Garbacz and Stromkowski, 2017; Kostic *et al.*, 2022)**. When antibiotic therapy is unsuccessful at treating recurrent tonsilitis as high rates of resistance to β-lactams have been reported **(Cavalcanti *et al.*, 2019)**, tonsillectomies are required **(Zautner *et al.*, 2010)**. Recurrent infections occur due to persistence of *S. aureus* within the tonsillar tissue **(Cavalcanti *et al.*, 2019)**, which may occur as a result of upper airway colonisation **(Fredheim *et al.*, 2015)**. The mechanisms utilised by *S. aureus* to cause recurrent tonsillitis are currently unclear, however α-toxin can affect intestinal epithelial barrier function by reducing the expression of proteins involved in tight junction formation **(Kwak *et al.*, 2012)**. PSMα is required for replication within and phagosomal escape from HeLa epithelial cells **(Grosz *et al.*, 2014)**. Furthermore, the cysteine protease staphopain was identified to facilitate host intracellular killing of epithelial cells and was required for mouse lung colonisation **(Stelzner *et al.*, 2021)**.

## 1.4. Zebrafish embryos as an animal model

The mammalian murine model of *S. aureus* infection is recognised for being one of the most beneficial models to staphylococcal infection research, due to the genetic, anatomical and physiological similarities between mice and humans **(Parker, 2017; Dow and Lowe, 2012)**. Many *S. aureus* infections have been studied in murine models, for example, sepsis **(Tarkowski *et al.*, 2001)**, nasal colonisation **(Xu *et al.*, 2015)** and endocarditis **(Gibson *et al.*, 2007)**. While the use of mammalian models has contributed immensely to infection research, there are many disadvantages including the necessity of large amounts of mammals for experiments **(Garcia-Lara et al., 2005)**. Importantly, though mice are similar to humans, they are still significantly different. Mice do not respond to *S. aureus* infections as humans do due to the preference and specificity of staphylococcal virulence factors to the human counterparts **(Parker, 2017)**. For example, the LukAB toxin interacts with the human CD11b subunit I domain of CR3 but binding was undetectable with regards to the murine counterpart **(DuMont *et al.*, 2013a)**. Furthermore, financial and ethical reasons and the need for higher throughput models has led to invertebrate models being utilised **(Garcia-Lara et al., 2005)**. For instance, approximately 60% of proteins found in humans can also be found in the fruit fly *D. melanogaster*. In comparison, zebrafish are a high-throughput vertebrate model containing >80% of disease-causing genes that occur in humans **(Howe *et al.*, 2013)** and are considered to be as good a model as mice as an infection model **(Buchan, Foster and Renshaw, 2019)**.

Zebrafish (*Danio rerio*) are a small freshwater tropical fish, around ~4 cm in length, that are native to South Asia. Their embryos develop rapidly and are approximately 1 mm in diameter, enabling researchers to visualise and exploit the different stages of development under basic light microscopy. Furthermore, as zebrafish embryos are transparent, they can easily be visualised under light/confocal microscopy, which is useful when utilising transgenic lines or fluorescent pathogens. Their small size as both embryos and adults makes them easy to maintain – this and their ability to reproduce quickly and in abundance, makes zebrafish suitable for high-throughput screening and infection studies.

Zebrafish embryos gain a functional innate immune system within the first two days of life: macrophages emerge in the bloodstream at 25 hours post fertilisation (hpf) and neutrophils at 30 hpf **(Herbomel, Thisse and Thisse, 1999; Lieschke *et al.*, 2001)**. Zebrafish also possess the complement system and are able to mount all three complement cascades **(Gongora, Figueroa and Klein, 1998; Traver *et al.*, 2003)**. As jawed vertebrates, the zebrafish adaptive immune system develops approximately 4-6 weeks post fertilisation and this is when the immune system becomes functionally mature **(Lam *et al.*, 2004; Howe *et al.*, 2013)**. Therefore, by using zebrafish embryos, the innate immune system can be studied discretely from the adaptive immune system. Pathogens injected into to fluid-filled compartments such as the circulation are mainly ingested by macrophages however, neutrophils are the primary phagocytes when pathogens are present on tissue surfaces **(Prajsnar *et al.*, 2008; Colucci-Guyon *et al.*, 2011)**, a feature that is shared with humans **(Renshaw and Trede, 2012)**. Zebrafish macrophages and neutrophils use the respiratory burst as a microbicidal mechanism **(Hermann *et al.*, 2004)** and can produce antibodies upon immunisation **(Lam *et al.*, 2004)**. This highlights similarities between the zebrafish and human immune systems that contributes to their usefulness as an infection model.

### 1.4.1. Advances in zebrafish transgenesis

Zebrafish are used to advance our knowledge of the vertebrate immune system. The zebrafish genome can be easily manipulated and is therefore, genetically tractable with its fully sequenced genome available for use. DNA manipulation/genome editing techniques have allowed the construction of transgenic zebrafish cell lines. The first transgenic line was constructed via the injection of restriction-enzyme linearised DNA into the one-cell stage zebrafish embryo – approximately 5% of injected fish after four months contained this foreign DNA **(Stuart, McMurray and Westerfield, 1988)**. Since then, other methods of creating transgenic lines have been developed. Retroviral vectors have been utilised and while success rates are often 100%, viral vectors are labour intensive to construct and can only carry small DNA sequences **(Lin *et al.*, 1994; Suster *et al.*, 2009)**. The Tol2 transposable element allows the insertion of specific genes flanked by Tol2 ends and can carry sequences up to 11 kb **(Kawakami, 2007; Urasaki, Morvan and Kawakami, 2006)**. Bacterial artificial chromosomes (BAC) have also been employed that make use of Tol2 ends and can carry up to 350 kb of DNA, which is necessary when promoter sequences are large **(Yang *et al.*, 2011; Bussmann and Schulte-Merker, 2011)**. Using methods such as these, fluorophores including green fluorescent protein (GFP) can be placed under the control of a promoter of interest in zebrafish and different cell types can be fluorescently tagged leading to the construction of many transgenic lines **(Choe *et al.*, 2021)**. Given that zebrafish embryos are transparent, visualising fluorescently tagged cells *in vivo* is a major advantage that this model poses over mammalian models. GFP has been expressed under the control of the MPO promoter, allowing neutrophil activity to be visually tracked *in vivo* in real-time and has provided insight into neutrophil-mediated inflammation **(Renshaw *et al.*, 2006; Mathias *et al.*, 2006)**. The promoter of macrophage expressed gene 1, a gene expressed exclusively by macrophages, has been used to visualise macrophages and has enabled their interactions with neutrophils to be tracked **(Ellett *et al.*, 2011)**. Lymphocytes have also been tagged by utilising genes involved in antibody production **(Jessen *et al.*, 1998)**.

### 1.4.2. Zebrafish embryo models of bacterial infection

Due to the many advantages of zebrafish, as well as the advent of tools such as transgenic lines, zebrafish embryos have been used to model various infection types from several organ systems and probe the role of the immune system. *Mycobacterium marinum* is a fish pathogen that causes tuberculosis and is used to understand how *M. tuberculosis* causes disease in humans. The use of a zebrafish embryo model for *M. marinum* systemic infection revealed the formation of macrophage aggregates akin to granulomas in the presence of only an innate immune system **(Davis *et al.*, 2002)**. In this report, the transparency of the zebrafish embryos allowed infection dynamics to be observed in real-time **(Davis *et al.*, 2002)**. Since then, further studies have demonstrated that granuloma formation served to aid dissemination rather than contain infection **(Volkman *et al.*, 2004)**. *Burkholderia cenocepacia,* a bacterium that causes opportunistic infections in cystic fibrosis patients, was shown in zebrafish embryos to use macrophages and not neutrophils as a niche for replication **(Mesureur *et al.*, 2017)**. This is an example of a study that utilised multiple transgenic cell lines with macrophages, neutrophils and key components of signalling pathways being fluorescently tagged **(Mesureur *et al.*, 2017)**. Another opportunistic pathogen of cystic fibrosis patients is *P. aeruginosa*, whose T3SS, as well as *lasR* and *mvfR* quorum sensing genes, have been necessary for virulence in zebrafish embryos **(Brannon *et al.*, 2009; Clatworthy *et al.*, 2009)**. The role of *Shigella flexneri* as a foodborne pathogen has also been investigated in zebrafish embryos. A T3SS-deficient strain was shown to be avirulent in zebrafish embryos, demonstrating the importance of these systems for virulence, as in *P. aeruginosa*  **(Mostowy *et al.*, 2013; Mesureur *et al.*, 2017)**. Furthermore, embryos infected with *S. flexneri* used neutrophils as scavengers to eliminate cells that were unable to kill bacteria **(Mostowy *et al.*, 2013)**. A model for systemic *S. aureus* infection was developed, which demonstrated the necessity of professional phagocytes for infection by myeloid cell depletion using morpholino oligonucleotides that transiently knockdown genes, and showed that macrophages engulfed more bacteria than neutrophils **(Prajsnar *et al.*, 2008).** The role of virulence regulators such as PerR was also demonstrated, as a *perR* mutant was attenuated *in vivo* **(Prajsnar et al., 2008)**. Furthermore, Prajsnar and colleagues also established a zebrafish embryo model of systemic infection within *E. faecalis*,whichrevealed that the extracellular polysaccharide antigen enabled evasion of phagocytosis and that the quorum sensing *fsr* regulon was responsible for tissue damage **(Prajsnar *et al.*, 2013)**.Additionally, an *E. faecalis atlA* mutant affected proper septum cleavage, leading to the formation of long chains rather than diplococci, and thus resulted in susceptibility to phagocytosis **(Salamaga *et al.*, 2017)**. These studies highlight how zebrafish infection by different bacteria has shed light on both how the immune system functions to eliminate invading pathogens, and how bacteria are able to evade the immune system. While murine models are considered the ‘gold standard’, zebrafish embryos are also able to give valuable insight into the pathologies of different infections that may be applicable in the clinical setting.

### 1.4.3. Humanised zebrafish models

As mentioned, a limitation of using an animal model is that the animal may not respond to infections as humans would, which can hinder the development of drugs and vaccines. This is usually due to bacteria being unable to interact with the animal counterparts of a human receptor. Thus, in order to circumvent these issues, humanised zebrafish models are being utilised. Cytokines are integral cell signalling proteins with immunomodulatory effects required for the establishment of a pro- or anti-inflammatory response. Conservation between cytokine genes from mice and humans differs, which has led to the development of humanised mouse models so that responses observed in mice are more representative of what would occur in humans **(Brocker *et al.*, 2010; Wunderlich *et al.*, 2010)**. Furthermore, mice are required to be immunocompromised in order to accept the xenograft and this may affect the reliability of the model **(Rajan *et al.*, 2020; Hamilton, Sabroe and Renshaw, 2018)**. As zebrafish have the added advantage of transparency and genetic tractability, zebrafish humanised models have also been established. The human haematopoietic cytokines GM-CSF, SCF, and SDF1α have been xenografted into zebrafish embryos and have been used to study patient-derived cancers **(Rajan *et al.*, 2020)**. Through xenografts, leukemogenesis has been modelled in zebrafish and has allowed the screening of multiple cancer therapeutic agents **(Rajan, Dellaire and Berman, 2016)**. An additional advantage over murine models is that generation of humanised zebrafish models has required 1-2 weeks in comparison to mice which required 3-6 weeks **(Rajan *et al.*, 2020; Corkery, Dellaire and Berman, 2011)**. Human HSCs have also been transplanted into zebrafish embryos, without myeloablative techniques, which has allowed the development and visualisation of these cells within zebrafish and can help to further our understanding of engraftment, an integral process of HSC transplants in humans **(Hamilton, Sabroe and Renshaw, 2018)**.

## 1.5. Aims and objectives

This study aims to understand why the stringent response is important for *S. aureus* pathogenicity *in vivo* within a zebrafish embryo infection model. As demonstrated, the stringent response pathway is responsible for regulating many virulence factors and has thus been involved in *S. aureus* pathogenesis. The staphylococcal stringent response has been implicated in persistence **(Li *et al.*, 2020; Peyrusson *et al.*, 2020)**, antibiotic resistance **(Gao *et al.*, 2010; Mwangi *et al.*, 2013; Bhawini *et al.*, 2019)**, antibiotic tolerance **(Geiger *et al.*, 2014)**, immune evasion **(Geiger *et al.*, 2012)**, biofilm formation **(Salzer *et al.*, 2020)**, tolerance to oxidative stress **(Fritsch *et al.*, 2020; Horvatek *et al.*, 2020)** and the development of murine pyelonephritis **(Geiger *et al.*, 2010)**. Thus, this study sought to perform a systematic analysis of the importance of the stringent response including the roles for RelP and RelQ, which are understudied *in vivo.* Firstly, this will be achieved by the construction of a toolbox containing various strains that can enable the investigation of the stringent response (chapter 3) including: the characterisation of a panel of (p)ppGpp synthetase deletion mutants, the construction of (p)ppGpp overexpression strains and the construction and characterisation of three (p)ppGpp probes. These include 1) a riboswitch-based molecular (p)ppGpp biosensor to directly report on intracellular (p)ppGpp levels, 2) a transcriptional dual promoter-reporter fusion for the indirect measurements of (p)ppGpp levels and 3) translational synthetase-GFP fusions to report on the levels of (p)ppGpp synthetase being expressed. The aim of constructing these probes was to elucidate when (p)ppGpp is produced during infection. However, only the translational synthetase-GFP were shown to be functional using the methods outlined in this study.

Secondly, stringent response mutants will be exposed to stresses found within the phagolysosome, a cytoplasmic body that is generated by phagocytes upon internalisation of pathogens (chapter 4). These stressors include low pH, ROS and AMPs. The stringent response has been implicated in tolerance to ROS in other *S. aureus* strains, so this will be recapitulated in the strains characterised in chapter 3. Accordingly, the *in vitro* tolerance of various *S. aureus* strains including the synthetase deletion mutants, (p)ppGpp overproduction strains, synthetase complement strains and a CodY-deficient strain to these stressors will be studied. This will enable further understanding of how the stringent response is required for survival within the phagolysosome.

Lastly, the virulence of these mutants will be investigated within a RAW 264.7 murine macrophage cell line, a human tonsillar epithelial cell line and within a systemic zebrafish embryo infection model. The absence and overproduction of (p)ppGpp will be examined, as well as the role of each synthetase. This will enable the effect of the stringent response on the ability of *S. aureus* to survive in mammalian cells *in vitro*, and kill zebrafish embryos to be studied. Induction of the stringent response is often associated with the concurrent increase in expression of virulence factors in various pathogens. Thus, it is important to study the effect that the stringent response has on MRSA pathogenesis as this pathogen is a causative agent of many infections and further research can enable the development of more efficacious stringent response inhibitors.

# Chapter 2: Materials and methods

## 2.1. Bacterial strains and growth conditions

All strains used in this study are listed in **Table 1**. *E. coli* strains were cultured in Luria Bertani broth (LB) (Fisher) and *S. aureus* strains in tryptic soy broth (TSB) (Becton, Dickinson and Company) or chemically defined medium (CDM) (recipe listed in **Table 2**) at 37˚C with aeration. Both broth and agar plates were supplemented with the appropriate antibiotics as indicated in the strain table.

|  |  |  |
| --- | --- | --- |
| Table 1. Bacterial strains | | |
| Strain | Relevant features | Reference |
|  | ***Escherichia coli* strains** |  |
| XL1-Blue | Cloning strain; TetR | Stratagene |
| RMC108 | pCN49 in in XL1-Blue; CarbR | (Charpentier *et al.*, 2004) |
| RMC115 | pCL55-iTET in XL1-Blue; iTET promoter in pCL55; CarbR | (Grundling and Schneewind, 2007) |
| RMC116 | pCL55-iTETr862 in XL1-Blue; iTETr862 promoter in pCL55; CarbR | (Corrigan *et al.*, 2013) |
| RMC139 | pALC2073 in XL1-Blue; CarbR | (Bateman *et al.*, 2001) |
| RMC468 | pCL55-iTETr862-*rel* in XL1-Blue; CarbR | Laboratory strain collection |
| RMC469 | pCL55-iTETr862-*relP* in XL1-Blue; CarbR | Laboratory strain collection |
| RMC470 | pCL55-iTETr862-*relQ* in XL1-Blue; CarbR | Laboratory strain collection |
| RMC546 | pALC2073-*relQ* in XL1-Blue; CarbR | Laboratory strain collection |
| RMC1762 | pCL55-P*ilvD*-sGFP-TT in XL1-Blue; CarbR | This study |
| RMC1763 | pCL55-P*rpsO*-dsRED in XL1-Blue; CarbR | This study |
| RMC1890 | pCL55iTET-Broccoli in XL1-Blue; CarbR | This study |
| RMC1891 | pCL55iTET-riboswitch-Broccoli XL1-Blue; CarbR | This study |
| RMC1894 | pCL55-iTET-*relSau-*NTD in XL1-Blue; CarbR | This study |
| RMC1895 | pCL55-iTET-*relSau-*NTD-his in XL1-Blue; CarbR | This study |
| RMC1896 | pCL55-iTETr862-*relSau-*NTD in XL1-Blue; CarbR | This study |
| RMC1897 | pCL55-iTETr862-*relSau-*NTD-his in XL1-Blue; CarbR | This study |
| RMC1898 | pCL55-iTET-*relSau-*NTD-∆HD in XL1-Blue; CarbR | This study |
| RMC1899 | pCL55-iTET-*relSau-*NTD-his-∆HD in XL1-Blue, CarbR | This study |
| RMC1901 | pCL55-iTETr862-*relSau-*NTD-∆HD in XL1-Blue; CarbR | This study |
| RMC1902 | pCL55-iTETr862-*relSau-*NTD-his-∆HD in XL1-Blue; CarbR | This study |
| RMC1920 | pRN12 in Top10F; CarbR | (de Jong *et al.*, 2017b) |
| RMC1937 | pRN12-Broccoli in XL1-Blue; CarbR | This study |
| RMC1938 | pRN12-riboswitch-Broccoli in XL1-Blue; CarbR | This study |
| RMC1942 | pRN12 (no mAmetrine) in XL1-Blue; CarbR | This study |
| RMC1965 | pCL55-PilvD-sGFP-TT-P*rpsO-*dsRED in XL1-Blue; CarbR | This study |
|  | ***Staphylococcus aureus* strains** |  |
| LAC\* | Erythromycin sensitive CA-MRSA LAC strain | (Boles *et al.*, 2010) |
| RMC202 | RN4220 | (Grundling and Schneewind, 2007) |
| RMC205 | pCL55 in RN4220; CamR | Laboratory strain collection |
| RMC206 | pCL55-iTETr862 in RN4220; CamR | Laboratory strain collection |
| RMC208 | RN4220 Δ*spa*; in-frame deletion of *spa* gene | (Grundling and Schneewind, 2007) |
| RMC448 | pGFP-F in RN4220; CamR | (Bose, Fey and Bayles, 2013) |
| RMC454 | pRFP-F RN4220; CamR | (Bose, Fey and Bayles, 2013) |
| RMC484 | pCL55-P*relP*-RelP-GFP in RN4220 Δ*spa*; CamR | Laboratory strain collection |
| RMC485 | pCL55-P*relQ*-RelQ-GFP in RN4220 Δ*spa*; CamR | Laboratory strain collection |
| RMC486 | pCL55-P*rel*-Rel-GFP in RN4220 Δ*spa*; CamR | Laboratory strain collection |
| RMC529 | pCL55-iTET-GFP in LAC\*; CamR | Laboratory strain collection |
| RMC880 | pKASBAR-GFP in SH1000; TetR | (Pollitt *et al.*, 2018) |
| RMC881 | pKASBAR-mCherry in SH1000; TetR | (Pollitt *et al.*, 2018) |
| RMC0898 | JE2 wildtype CA-MRSA USA300 strain LAC derivative, lacking plasmids p01 and p03. Erm sensitive | (Fey *et al.*, 2013) |
| RMC899 | JE2 Δ*relP* | (Carrilero *et al.*, 2023) |
| RMC901 | JE2 Δ*relQ* | (Carrilero *et al.*, 2023) |
| RMC902 | JE2 Δ*relP* Δ*relQ* | (Carrilero *et al.*, 2023) |
| RMC903 | JE2 Δ*relQ* Δ*relP* | (Carrilero *et al.*, 2023) |
| RMC904 | JE2 Δ*relP* Δ*relQ* Δ*relSau* | (Carrilero *et al.*, 2023) |
| RMC905 | JE2 Δ*relQ* Δ*relP* Δ*relSau* ((p)ppGpp0) | (Carrilero *et al.*, 2023) |
| RMC1783 | JE2 Δ*codY*; ErmR | (Fey *et al.*, 2013) |
| RMC1836 | pALC2073 in JE2; CamR | This study |
| RMC1837 | pALC2073 in JE2 (p)ppGpp0; CamR | This study |
| RMC1855 | pCL55-iTET-*relSau-*NTD-his-∆HD in JE2 wildtype; CamR | This study |
| RMC1856 | pCL55-iTETr862 in JE2; CamR | This study |
| RMC1857 | pCL55-iTETr862-*relSau-*NTD-his-∆HD in JE2 wildtype; CamR | This study |
| RMC1858 | pCL55-iTETr862 in JE2 (p)ppGpp0; CamR | This study |
| RMC1859 | pCL55-iTETr862-*relSau-*NTD-his-∆HD in JE2 (p)ppGpp0; CamR | This study |
| RMC1861 | pALC2073-*relQ* in JE2; CamR | Laboratory strain collection |
| RMC1862 | pALC2073-*rel* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1868 | pALC2073-*relP* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1869 | pALC2073-*relQ* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1870 | pCL55-iTETr862-*rel* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1871 | pCL55-iTETr862-*relP* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1872 | pCL55-iTETr862-*relQ* in JE2 (p)ppGpp0; CamR | Laboratory strain collection |
| RMC1892 | pCL55iTET-riboswitch-Broccoli in RN4220; CamR | This study |
| RMC1893 | pCL55iTET-Broccoli in RN4220; CamR | This study |
| RMC1903 | pCL55-iTET-*relSau-*NTD-∆HD in RN4220; CamR | This study |
| RMC1904 | pCL55-iTET-*relSau-*NTD-his-∆HD in RN4220; CamR | This study |
| RMC1905 | pCL55-iTETr862-*relSau-*NTD-∆HD in RN4220; CamR | This study |
| RMC1906 | pCL55-iTETr862-*relSau-*NTD-his-∆HD in RN4220; CamR | This study |
| RMC1907 | pCL55-iTET-*relSau-*NTD-∆HD in JE2 wildtype; CamR | This study |
| RMC1908 | pCL55-iTETr862-*relSau-*NTD-∆HD in JE2 wildtype; CamR | This study |
| RMC1909 | pCL55-iTETr862-*relSau-*NTD-∆HD in JE2 (p)ppGpp0; CamR | This study |
| RMC1910 | pCL55-P*ilvD*-sGFP-TT in RN4220 Δ*spa*; CamR | This study |
| RMC1911 | pCL55-P*rpsO*-dsRED in RN4220 Δ*spa*; CamR | This study |
| RMC1921 | pCL55-P*relP*-RelP-GFP in JE2; CamR | This study |
| RMC1922 | pCL55-P*relQ*-RelQ-GFP in JE2; CamR | This study |
| RMC1936 | pCL55-P*rel*-Rel-GFP in JE2; CamR | This study |
| RMC1939 | pRN12 (contains mAmetrine) in RN4220 Δ*spa*; CamR | This study |
| RMC1940 | pRN12-Broccoli in RN4220 Δ*spa*; CamR | This study |
| RMC1941 | pRN12-riboswitch-Broccoli in RN4220 Δ*spa*; CamR | This study |
| RMC1945 | pRN12 (no mAmetrine) in RN4220 Δ*spa*; CamR | This study |
| RMC1948 | pKASBAR-GFP in JE2; TetR | Laboratory strain collection |
| RMC1950 | pKASBAR-GFP in JE2 (p)ppGpp0; TetR | Laboratory strain collection |
| RMC1966 | pCL55-PilvD-sGFP-TT-P*rpsO-*dsRED in RN4220 Δ*spa*; CamR | This study |
| RMC2010 | pCL55-iTETr862-*rel* in JE2; CamR | This study |
| RMC2014 | (p)ppGpp0 ∆*codY*; ErmR | This study |
| RMC2021 | pCL55-iTETr862-*relP* in JE2; CamR | This study |
| RMC2022 | pCL55-iTETr862-*relQ* in JE2; CamR | This study |
| Antibiotics were used at the following concentrations: chloramphenicol (CamR) 7.5 and 10 μg/ml for integrated and multicopy plasmids respectively, carbenicillin (CarbR) 150 μg/ml, erythromycin (ErmR) 10 μg/ml, tetracycline (TetR) 2 μg/ml, Anhydrotetracycline (Atc) 50 ng/ml. | | |

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| --- | --- |
| Table 2. List of ingredients that comprise CDM. |  |
| Ingredients | g/l |
| Salts |  |
| KCl | 3.0 g |
| NaCl | 9.5 g |
| MgSO4 7H2O | 1.3 g |
| (NH4)2SO4 | 4.0 g |
| CaCl2 2H2O | 22 mg |
| KH2PO4 | 140 mg |
| FeSO4 7H­2O | 6 mg |
| MnSO4 4H2O | 10 mg |
| Carbon source | |
| Glucose | 5 g |
| Amino acids | |
| L-Arginine | 125 mg |
| L-Proline | 200 mg |
| L-Glutamine | 250 mg |
| L-Valine | 150 mg |
| L-Threonine | 150 mg |
| L-Phenylalanine | 150 mg |
| L-Leucine | 150 mg |
| L-Glycine | 50 mg |
| L-Serine | 30 mg |
| L-Aspartic acid | 90 mg |
| L-Lysine | 50 mg |
| L-Alanine | 60 mg |
| L-Tryptophan | 10 mg |
| L-Methionine | 10 mg |
| L-Histidine | 20 mg |
| L-Isoleucine | 30 mg |
| L-Tyrosine | 50 mg |
| L-Cystine | 80 mg |
| Vitamins | |
| Biotin | 0.1 mg |
| Thiamine | 2 mg |
| Nicotinic acid | 2 mg |
| Calcium pantothenate | 2 mg |
| Others |  |
| Citric acid | 6 mg |
| Thymine | 20 mg |
| Tris | 12.1 g |
| L-cysteine was the last reagent added to the media, the final solution was adjusted to pH 7.4 and subsequently filter sterilised using 0.22 µm filter | |

## 2.2. Mammalian tissue culture

RAW 264.7 murine macrophages (ATCC TIB-71) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) Low Glucose (Sigma) supplemented with 2 mM L-glutamine (Gibco) and 10% foetal bovine serum (FBS) (Merck) and were cultured at 37˚C in the presence of 5% CO2. RAW 264.7 macrophages were passaged into T75 flasks (Greiner Bio-One) until 70-80% confluence was achieved, and experiments were performed at passages 9-18.

## 2.3. Zebrafish strains and husbandry

Animal work was completed following the Animals (Scientific Procedures) Act 1986. London Wildtype zebrafish (*Danio rerio*) embryos were provided by the Bateson Centre aquaria, University of Sheffield and were maintained according to established standards**(Nüsslein-Volhard and Dahm, 2002)**. Adult fish were kept at 28.5˚C in a 14 hour/10 hour light/dark regime. Embryos were incubated at 28.5˚C in E3 medium (0.5 mM NaCl, 17 µM KCl, 33 µM CaCl2, 33 µM MgSO4, 0.00005% methylene blue).

## 2.4. Recombinant DNA manipulation

### 2.4.1. Polymerase chain reaction (PCR)

DNA fragments were amplified in a reaction mixture containing 2X Phusion High-Fidelity PCR Master Mix with HF Buffer (New England BioLabs), ~100 ng/μl template DNA and 10μM forward/reverse primers (Eurofins Genomics) **(Table 3)**. PCR conditions consisted of an initial denaturation step at 94˚C for 2 minutes, followed by 5 cycles of denaturation at 94˚C for 30 secs, annealing at 45˚C for 30 secs and elongation at 72˚C for X mins. Then, 30 cycles of denaturation at 94˚C for 30 secs, annealing at 53˚C for 30 secs and elongation at 72˚C for X mins. This is followed by a further elongation step at 72˚C for X mins and the samples were maintained at 16˚C. Elongation times were adjusted according to the size of the DNA fragment being amplified.

For *E. coli* colony PCR, single colonies were inoculated into a PCR mix including the reagents above and were run using the PCR conditions above.

For inverse PCR to mutate the hydrolase domain, the same PCR reagents and conditions were used as above except for the extension time. The PCR product was digested with DpnI (NEB) (20 U), to digest to digest methylated template DNA, for 1 hour at 37˚C and heat inactivated for 20 mins at 80˚C.

|  |  |  |  |
| --- | --- | --- | --- |
| TABLE 4. Primers and oligonucleotides used in this study | | | |
| Number | **Name** | **Sequence** | **Reference** |
| 006 | R-pCL55+insert | CACGTTTCCATTTATCTGTATACGGATC | Laboratory primer collection |
| 007 | F-pCL55+insert | AATTCCTCCTTTTTGTTGACACTCTATC | Laboratory primer collection |
| 078 | F-KpnI-RelQ*Sau* | GGGGGTACCGATATGTATACACCTCGTATC | Laboratory primer collection |
| 081 | F-KpnI-RelP*Sau* | GGGGGTACCCATCTCTATCAATTAAGCAC | Laboratory primer collection |
| 084 | F-KpnI-Rel*Sau* | GGGGGTACCGAGTTTAATCTCATACGACG | Laboratory primer collection |
| 094 | R-BamHI-RelP*Sau* | AATTGGATCCCCCTCTGTTATTTCAGAATG | Laboratory primer collection |
| 095 | F-NcoI-RelQ*Sau* | AATTCCATGGATGAATCAATGGGATCAGTTC | Laboratory primer collection |
| 096 | R-BamHI-RelQ*Sau* | AATTGGATCCCCATCATTTTCATGTTTTTTAGAACG | Laboratory primer collection |
| 122 | |  | | --- | | R-RelQ*Sau* | | |  | | --- | | CAATGATATAGTACTTGTTC | | Laboratory primer collection |
| 165 | F-AvrII-Rel*Sau* | AAACCTAGGCCTAAATCATTGTTTAAGGCG | Laboratory primer collection |
| 259 | R-KpnI-RelSau | GGGGGTACCTTCCAAACTCTTGTTAC | Laboratory primer  collection |
| 708 | R-Rel*Sau*-HD-mut | CGGTGTATCTTCAATTACAGAAGCCAAAAAACCTGCGACAATCG | Laboratory primer collection |
| 709 | F-Rel*Sau*-HD-mut | CGATTGTCGCAGGTTTTTTGGCTTCTGTAATTGAAGATACACCG | Laboratory primer collection |
| 772 | F-ilvD-EcoRI | AAAGAATTCAAAAATGATTATCCATTGTTCAATCG | This study |
| 773 | R-ilvD-sGFP-SOE | TTTGCTCCCGGGGTCGCTTCGCATAGTAAATTCCCC | This study |
| 774 | F-ilvD-sGFP-SOE | ATGCGAAGCGACCCCGGGAGCAAAGGAGAAGAACTT | This study |
| 775 | R-sGFP-TT-SOE | AATAGGCGCGCCTTATTTGTAGAGCTCATCCATGCC | This study |
| 776 | F-sGFP-TT-SOE | CTCTACAAATAAGGCGCGCCTATTCTAAATGCATAA | This study |
| 777 | R-TT-KpnI | GGGGGTACCTGTCACTTTGCTTGATATATGAG | This study |
| 778 | F-rpsO-BamHI | GGGGGATCCGAATATCTTTGACTTTGAGGATAATA | This study |
| 779 | R-rpsO-dsRED-SOE | TTCTGTATTATCTGAAATTGCCATAATCAATTTCCT | This study |
| 780 | F-rpsO-dsRED-SOE | ATGGCAATTTCAGATAATACAGAAGATGTTATTAAA | This study |
| 781 | R-dsRED-KpnI | GGGGGTACCTTATAAAAACAAATGATGACGACCTTCT | This study |
| 739 | R-SacII-Rel*Sau*-Nterm | AAACCGCGGTTAACTCTGTAAGTCATATTTTAAGG | This study |
| 740 | R-SacII-Rel*Sau*-Nterm-His | GGGCCGCGGTTAGTGATGGTGATGGTGATGACTCTGTAAGTCATATTTTAAGG | This study |
| 846 | F-EcoRV-broccoli | GGGGATATCTTGCCATGTGTATGTGGGAGACGG | This study |
| 847 | R-AvrII-broccoli | GGGCCTAGGCAAAAAACCCCTCAAGACCCG | This study |
| 848 | F-EcoRV-ppGpp-apt | GGGGATATCGGAAGTGTACCTTAGGGTTCCGG | This study |
| 849 | R-NheI-ppGpp-apt | GGGGCTAGCGGAACTTGCCGCTGGAGC | This study |
| 889 | F-pRN12insert | TTGCATGCCTGCAGGTCGACTCTA | This study |
| 890 | R-pRN12insert | TTATGCTTCCGGCTCGTATGTTGTGTGG | This study |
| 891 | F-KpnI-Broccoli | GGGGGTACCGATATCTTGCCATGTGTATGTGGGAGACGG | This study |
| 892 | R-EcoRI-Broccoli | GGGGAATTCCAAAAAACCCCTCAAGACCCGTTTAGA | This study |
| 893 | F-KpnI-ppgpp-apt | GGGGGTACCGATATCGGAAGTGTACCTTAGGGTTCCGG | This study |
| 894 | R-EcoRI-ppgpp-apt | GGGGAATTCCCTAGGCAAAAAACCCCTCAAGACCCG | This study |
| - | PU.1 morpholino | GATATACTGATACTCCATTGGTGGT | (Rhodes *et al.*, 2005) |
| Restriction sites are underlined. | | | |

### 2.4.2. Isolation of plasmid DNA

Plasmid DNA was isolated from 5 ml of the appropriate *E. coli* strain using the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific) as per manufacturer’s guidelines. Samples were eluted with 50 μl autoclaved distilled water and were stored at -20˚C.

### 2.4.3. Purification of digested DNA

Digested DNA was purified from using the GeneJET Gel Extraction Kit (ThermoFisher Scientific) as per manufacturer’s guidelines. Samples were eluted with 50 μl autoclaved distilled water and were stored at -20˚C.

### 2.4.4. Isolation of genomic DNA

The pellet from a 3 ml *S. aureus* culture was resuspended in 100 μl TSM (50 mM Tris pH 7.5, 0.5 M sucrose, 10 mM MgCl2) and lysed for 30 minutes at 37˚C with 5 μg of lysostaphin. 600 μl of Nuclei Lysis Solution (Promega) was added to the lysate and this was incubated at 80˚C for 10 minutes where samples were inverted after 5 minutes. 1.7 μg of RNase solution (Sigma) was added and this was further incubated at room temperature for 30 minutes. Cellular protein was precipitated using 200 μl Protein Precipitation Solution (Promega). After 5-minute incubation on ice, samples were pelleted and 600 μl of isopropanol was added to the supernatant to precipitate DNA. The sample was then washed with 600 μl ethanol and the DNA pellet was allowed to dry. DNA was rehydrated using 40 μl autoclaved distilled water. Samples were stored at -20˚C.

### 2.4.5. Digestion of DNA with restriction enzymes

Restriction digestions were set up as follows. DNA was combined with between 2-20 U restriction enzyme (NEB), 5 μl of 10X CutSmart Buffer (NEB) and was made up to 50 μl with autoclaved distilled water. Reactions were incubated at 37˚C for a minimum of 4 hours.

### 2.4.6. DNA fragment ligation

Target inserts and vectors were digested with restriction enzymes AvrII (2-20 U) and SacII (2-20 U) (NEB) and subsequently purified. Ligation reactions were set up resulting in a 3:1 insert: vector ratio. In a volume of 20 μl, inserts and vectors were incubated with 2 μl 10X Ligase Buffer (NEB), T4 DNA Ligase (400 U) and autoclaved distilled water. Reactions were incubated at room temperature overnight and reactions were heat-inactivated at 65˚C for 20 minutes.

### 2.4.7. Blunting of linearised plasmids

For the multicopy riboswitch-based molecular biosensor, the pRN12 multicopy plasmid that was utilised contained the mAmetrine fluorophore that needed to be removed by restriction digestion. Linearised plasmids were then blunted before ligation for the construction of a negative control. To do this, the NEB Quick Blunting™ Kit was used and the reaction was set up as follows; restriction digested pRN12 plasmid DNA was combined with 1 μl Quick Blunt enzyme, 2.5 μl of 10X Quick Blunt Buffer and dNTPs (final concentration 100 μM) and was made up to 25 μl with autoclaved distilled water. Reactions were incubated at 37˚C for 15 minutes after which the sample was heat inactivated at 70˚C for 10 minutes. Linearised and blunted plasmid DNA was then ligated using DNA ligase to circularise the plasmid.

### 2.4.8. Preparation and transformation of *E. coli* chemically competent cells

Chemically competent *E. coli* XL1-Blue were prepared by growing up 20 ml overnight culture of *E. coli* XL1-Blue in LB medium at 37˚C with aeration at 200 rpm. Cultures were diluted 1:100 into 1 L of PSI broth (0.5% (w/v) yeast extract, 2% (w/v) tryptone, adjusted to pH 7.4 using 0.1 M KOH with sterile MgSO4 (20 mM final concentration) added fresh before use) and grown to mid-exponential phase (OD600 0.5-0.7). Once the desired OD600 was reached, bacterial cells were cooled to 4˚C and maintained at this temperature. Cultures were then centrifuged at 6,000 × g for 10 minutes and bacterial pellets were resuspended in 200 ml ice-cold sterile transformation buffer I (TfbI) (30 mM CH3COOK, 100 mM RbCl, 10 mM CaCl2, 50 mM MnCl2, 15% glycerol, adjusted to pH 5.8 using 0.2 M acetic acid) and incubated on ice for 15 minutes. Bacteria were pelleted again at 6,000 × g for 10 minutes and resuspended in 25 ml ice-cold sterile TfbII (10 mM MOPS adjusted to pH 6.5 using KOH, 75 mM CaCl2, 10 mM RbCl, 15% glycerol) and incubated on ice for 15 minutes. Cultures were the aliquoted into 1.5 ml microcentrifuge tubes (500 μl per tube) and frozen using a ethanol/dry ice bath and subsequently stored at -80˚C.

For transformation, 100 μl of competent cells were incubated on ice with 12 μl of ligation product for 10 minutes, heat shocked for 45 seconds at 42˚C, and returned to ice for a further 5 minutes. To each reaction, 900 μl SOC media (0.5% yeast extract, 2% tryptone, 0.05% NaCl, 20 mM glucose, 2.5 mM KCl) was added before recovering at 37˚C for 1 hour. Cells were plated on the appropriate agar plates.

### 2.4.9. Preparation and electroporation of *S. aureus* electro-competent cells

Electrocompetent *S. aureus* RN4220 cells were prepared by back-diluting an overnight culture into 50 ml fresh TSB and incubating at 37˚C with aeration at 200 rpm for 3 hours. Cells were washed twice with 100 ml ice cold 0.5 M sucrose wash solution and then once with 50 ml. Cell pellets were resuspended in 1 ml wash solution and were stored by freezing using a ethanol/dry ice bath and subsequently kept at -80˚C.

For electroporation, 20 μl of the desired plasmid DNA was dialysed against autoclaved distilled water using a 0.025 μm filter (MF-Millipore) for 30 minutes. 100 μl competent cells and 15 μl dialysed DNA were added to an 0.1 cm electroporation cuvette (Bio-Rad) and the sample was electroporated under the following conditions: 100 Ω, 2.5 kV, 25 μF. Cells were recovered for 1 hour with BHI 0.5 M sucrose at 37˚C with aeration at 200 rpm and plated on the appropriate TSA plates.

### 2.4.10. Phage transduction into *S. aureus*

To prepare phage lysates, 5 ml of the appropriate *S. aureus* culture was back-diluted in fresh LB/TSB + 5 mM CaCl2 (1:50) and grown at 37˚C with aeration at 200 rpm for 3 hours. 100 μl of different phage dilutions (diluted in TMG buffer (10 mM Tris pH 7.5, 10 mM MgSO4, 0.1% gelatine) were added to 500 μl culture aliquots and was incubated at room temperature for 30 minutes. Subsequently, 5 ml top agar (0.8% Bacto-agar, 0.8% NaCl, 5 mM CaCl2) was added and each dilution was plated on the appropriate plates. The confluent plate was determined and overlaid with TMG buffer after which the top layer was scraped off and spun at 13,000 rpm for 10 mins. The supernatant/phage lysate was filter sterilised using a 0.22 μm filter and stored at 4˚C.

For the phage transduction, the pellet from a 5 ml culture of the appropriate *S. aureus* recipient strain was resuspended in 100 μl LB/TSB + 5 mM CaCl2 (5 X concentrated). 250 μl of concentrated culture and 400 μl of phage lysate were mixed and then incubated at 37˚C for 20 minutes. Ice cold 1 M sodium citrate was added to each sample to bring the overall sodium citrate concentration to 40 mM. Following this, the cells underwent three wash steps with ice cold 40 mM sodium citrate. The cells were then resuspended in 300 μl 40 mM sodium citrate. 100 μl and 200 μl were plated on plates containing 20 mM sodium citrate and the appropriate antibiotics.

## 2.5. Agarose gel electrophoresis

Appropriate volumes of DNA were mixed with 6X Purple Gel Loading Dye (NEB) and were loaded onto a 1% agarose gel (Oxoid) containing SYBR Safe DNA Gel Stain (ThermoFisher Scientific). The gels were run in 1 X TAE buffer (40 mM Tris base, 0.11% glacial acetic acid, 1 mM EDTA) at 110 V for 20-30 mins and were subsequently visualised.

## 2.6. Growth and survival curves

### 2.6.1. *S. aureus* growth curves

*S. aureus* overnight cultures were diluted to an OD600 of 0.05 in TSB or tris-maleate buffered TSB (TSB dissolved in 0.1 M tris and 0.1 M maleic acid (Sigma) adjusted to pH 4.5 and 7.5) and were supplemented with antibiotics when necessary. Diluted cultures were incubated at 37˚C for 8 hours and OD600 values were determined at hourly intervals.

### 2.6.2. Biolog Phenotype MicroArray PM10 plates

The *S. aureus* strains to be tested were streaked on TSA plates and grown overnight at 37°C. A single colony from each strain was re-streaked and grown again overnight. A sterile swab was used to inoculate 5 ml of IF-0a GN/GP Base inoculating fluid (1.2x) with the appropriate *S, aureus* culture, resulting in a suspension with 81% transmittance (or OD600 0.1). The 1X PM Inoculating Fluid was prepared by mixing 1.2X IF-10b GN/GP, 100X Dye mix H, 12X PM additive solution (2 mM MgCl2 6H2O, 1 mM CaCl2 2H2O, 0.005% yeast extract, 0.005% tween 80, 2.5 mM D-glucose, 5 mM sodium pyruvate and made up to 100 ml with autoclaved distilled water) and 13.64X bacterial cell culture made up in IF-0a GN/GP Base inoculating fluid and was made up to 12 ml. The 1X PM Inoculating Fluid was added to the Biolog Phenotype Microarray PM10 plate at 100 µl/well and growth was measured at OD590 for 20 hours at 37°C with aeration at 200 rpm in a Hidex Sense platereader

## 2.7. Mammalian cell infection

### 2.7.1. Preparation of *S. aureus* frozen stocks for macrophage infection

For the preparation of frozen *S. aureus* cultures, JE2 and (p)ppGpp0 overnight cultures were diluted to OD600 0.1 and grown to late stationary phase at 37˚C with aeration at 200 rpm in 25 ml TSB. Cells were washed three times in sterile phosphate-buffered saline (PBS) and resuspended in 5% BSA (dissolved in sterile PBS). Cultures were aliquoted into 1.5 ml microcentrifuge tubes (250 µl per tube) and stored at -80˚C. CFU/ml of the aliquots were determined by thawing the cultures two days post freezing, resuspending in 750 µl sterile PBS, serially diluting the culture and plating 100 µl onto TSA plates.

### 2.7.2. Macrophage infection assays

RAW 264.7 macrophages were seeded at 100,000 cells per well in a 24-well plate and were utilised once the cell density was at 200,000 cells per well. For RAW 264.7 macrophage infection, frozen cultures were thawed, resuspended in 750 µl of sterile PBS and the cultures were diluted to 1 x 106 CFU/ml in DMEM supplemented with 2 mM L-glutamine or both 2 mM L-glutamine and 10% FBS. These cultures were used to infect macrophages at an MOI of 5 and tissue culture plates were centrifuged at 277 x g for 2 minutes to sediment bacteria for synchronisation of infection. Infected macrophages were then incubated at 37˚C and 5% CO2 for 2-3.5 hours depending on the experiment. Extracellular bacteria in all wells were eliminated using 20 µg/ml lysostaphin for 30 minutes and later timepoints were maintained in supplemented DMEM containing 2 µg/ml lysostaphin until harvest of intracellular bacteria. At each timepoint, wells were washed thrice using sterile PBS to remove lysostaphin and cells were subsequently lysed using 250 µl 2% saponin by scraping with a pipette tip. Lysates were made up to 1 ml with 750 µl sterile PBS, serially diluted and plated onto TSA plates to determine the intracellular CFU/ml.

### 2.7.3. Human Tonsillar Epithelial cell infection assay

*S. aureus* JE2 and (p)ppGpp0 were either grown up to exponential phase (OD600 0.5) or overnight cultures adjusted to OD600 1 to achieve a stationary phase culture. Cultures from both growth phases were washed twice in sterile PBS prior to infection. HTE cells were infected at a multiplicity of infection (MOI) of 10 and incubated at 37˚C and with 5% CO2 for 1 hour 15 minutes, after which the infected cells were subject to 20 µg/ml lysostaphin treatment for a further 45 minutes to remove extracellular bacteria in all wells. Later timepoints were maintained in supplemented DMEM containing 2 µg/ml lysostaphin until harvest of intracellular bacteria. At the point of intracellular CFU/ml determination, HTE cells were washed six times in sterile PBS to remove lysostaphin and cells were lysed using 500 µl 0.05% trypsin in autoclaved distilled water and scraped using a pipette tip to liberate intracellular bacteria. Lysates were serially diluted and 100 µl were plated onto TSA plates.

## 2.8. Systemic infection of zebrafish embryos

### 2.8.1. Zebrafish embryo microinjections

For injection of exponential phase cultures, *S. aureus* overnightcultures were diluted to OD600 0.05 and grown to exponential phase (OD600 0.5) at 37˚C with aeration at 200 rpm with antibiotics if required. Once the cultures reached the correct OD600, they were washed twice in sterile and filtered PBS and resuspended in the appropriate volume of PBS to obtain the necessary dose. For stationary phase cultures, the appropriate overnight cultures were washed twice in sterile PBS and resuspended to OD600 3 which gives a dose of approximately 3000-4000 CFU/nl. At approximately 30 hours post fertilisation (hpf), London Wildtype zebrafish embryos were dechorionated using watchmaker forceps (Dumont) and anaesthetised by immersion in 0.02 % tricaine (0.4% (w/v) 3-amino benzoic acid ester (tricaine or MS322) in 20 mM Tris–HCl, adjusted to pH 7 and store at 4°C) in E3 (Pharmaq Ltd) to the E3 medium. The embryos were embedded in methylcellulose (Sigma-Aldrich) dissolved in E3 (3% w/v) on a glass slide. 1 nl of the appropriate *S. aureus* culture was injected into the circulation valley of the embryos using a pneumatic micropump (World Precision Instruments PV820) and a micromanipulator (WPI). Following injection, embryos were recovered in fresh E3 after 1 hour post infection (hpi), placed into individual wells of a 96-well plate. The inoculation dose was determined by injecting 2 nl of culture into 1 ml sterile PBS and plating 50 µl onto TSA plates. Embryos were maintained at 28.5˚C and monitored twice a day up to 93 hpi (5.2 dpf) and the number of dead embryos at each timepoint was recorded.

### 2.8.2. Microscopic analysis of zebrafish infection

*S. aureus*-injected embryos were mounted on a glass-bottom Fluorodish cell culture dish (World Precision Instruments) and immersed in E3 medium. Whole embryo images were taken at 24 hpi using a Leica DMi8 SPE-TCS confocal microscope using a HCX PL APO 2.5x/1, 10x objective.

### 2.8.3. Microinjection of morpholino-modified antisense oligonucleotides

Morpholino-modified antisense oligonucleotides against the Pu.1 transcription factor **(Rhodes *et al.*, 2005)** (Gene Tools) **(Table 4)**, encoded for by the *spi1b* gene, was injected into the yolk of one-cell stage zebrafish embryos at a concentration of 1 pmol and subsequently incubated at 28.5°C until injection with *S. aureus*. *S. aureus* cultures were injected into the circulation valley of zebrafish embryos at 30 hpf as per section 2.8.1. Embryos were maintained at 28.5˚C and monitored twice a day up to 72 hpi (5.2 dpf) and the number of dead embryos at each timepoint was recorded.

2.8.4. Growth of *S. aureus* in zebrafish embryos *in vivo*

In order to determine the number of bacteria within zebrafish embryos during infection, *S. aureus* cultures were injected into the circulation valley of zebrafish embryos at 30 hpf as per section 2.8.1. At each timepoint until 5.2 dpf, five live embryos and any dead embryos, as well as 200 µl of E3 medium were transferred to 0.5 ml screw cap microcentrifuge tubes (Alpha Lab) containing 1.4 mm ceramic beads (Qiagen). Each embryo was homogenised using a FastPrep-24™ 5G Homogenizer and homogenates were serially diluted and plated to determine the bacterial load of each embryo.

## 2.9. Antimicrobial assays

### 2.9.1. Growth in the presence of antimicrobial peptides

The appropriate *S. aureus* overnight cultures were washed once in 0.9% saline and diluted to OD600 0.05 in Roswell Park Memorial Institute (RPMI) supplemented with L-Glutamine, 25 mM HEPES and phenol red (Gibco). and were transferred to a 96-well plate at 200 μl/well. RPMI was buffered to pH 4.5 and 7.5 by adjusting the pH using 12 M HCl and 10 M NaOH and then filter sterilising using a 0.22 μm filter.  LL-37 cathelicidin (Apexbio, Stratech), dissolved in 25% acetic acid, was added at a final concentration of 12 μM and polymyxin B sulfate (Sigma), dissolved in autoclaved distilled water, was added at final concentrations of 32 and 64 μg/ml. Growth was measured in a Hidex Sense platereader at 37˚C with aeration at 200 rpm.

### 2.9.2. Tolerance assays

The appropriate *S. aureus* overnight cultures were diluted to OD600 0.05 and were grown to mid-exponential at 37˚C with aeration at 200 rpm and with antibiotics if required. Once the desired optical density (approximately OD600 0.35) was reached, the cultures were centrifuged at 4000 rpm for 10 minutes and subsequently washed twice in sterile PBS. Antimicrobial compounds (20 mM itaconic acid (Sigma), 100 mM H2O2 (Sigma) (from a 30% w/w stock) or 32 μM sodium hypochlorite/HOCl (Sigma) (from a stock containing 10-15% available chlorine)) were added to OD600 0.35 cultures and were incubated at 37˚C with aeration at 200 rpm and antibiotics if required. Samples were taken for CFU/ml determination at 0.5 or 1 hour after addition of each antimicrobial compound.

## 2.10. Fluorescence assays

### 2.10.1. Fluorescence measurements of riboswitch activation on a pCL55-iTET integrative vector

Overnight cultures of the appropriate RN4220 *S. aureus* strains containing the riboswitch-based molecular biosensor and the relevant controls were back-diluted to OD600 or 0.1 in CDM. *S. aureus* cultures were grown to mid-exponential phase (approximately OD600 0.3-0.4). The cultures were split into 2 separate cultures and were subsequently washed twice in CDM with leucine and valine (+L/V) or CDM without L/V (-L/V) to mimic amino acid starvation. Atc was added to all cultures at 100 ng/ml and were allowed to grow for one hour to induce expression from the plasmid or was added prior to fluorescence measurement. (5*Z*)-5-[(3,5-Difluoro-4-hydroxyphenyl)methylene]-3,5-dihydro-2-methyl-3-(2,2,2-trifluoroethyl)-4*H*-imidazol-4-one (DFHBI-1T) (Bio-Techne), the fluorogen that binds to Broccoli, was also added to cultures at a final concentration of 200 μM. Green fluorescence was measured at excitation and emission values of 485/520 nm every 30 minutes for 20 hours at 37˚C with aeration at 200 rpm using a Hidex Sense platereader.

### 2.10.2. Fluorescence measurements of riboswitch activation on the pRN12 multicopy vector

Overnight cultures of the appropriate RN4220 *S. aureus* strains containing the riboswitch-based molecular biosensor and the relevant controls were back-diluted to OD600 0.1 in CDM. *S. aureus* cultures were grown to mid-exponential phase (approximately OD600 0.5) after which, a 200 μl sample was taken. The cultures were split into 2 separate cultures, washed three times in CDM +L/V or CDM -L/V to mimic amino acid starvation and another 200 μl sample was taken. After the addition of DFHBI-1T at a final concentration of 200 μM, all 200 μl culture samples were incubated in the dark for 30 minutes at room temperature. Green fluorescence was subsequently measured at 485/520 nm at 15, 30 and 60 minutes at 37˚C with aeration at 200 rpm after the 30-minute incubation using a Hidex Sense platereader.

### 2.10.3. Fluorescence measurements of transcriptional activity of the dual promoter-reporter fusion

Overnight cultures of the appropriate RN4220 *S. aureus* strains containing the transcriptional dual promoter-reporter fusions and the relevant controls were back-diluted to OD600 or 0.1 in CDM. *S. aureus* cultures were grown to mid-exponential phase (approximately OD600 0.3-0.4) The cultures were split into 2 separate cultures and washed twice CDM +L/V or CDM -L/V or cultures were incubated with 0, 0.05 or 60 μg/ml mupirocin depending on the experiment to mimic amino acid starvation. Green fluorescence was subsequently measured at 485/520 nm and red fluorescence at 575/610 nm every 30 minutes for 20 hours at 37˚C with aeration at 200 rpm using a Hidex Sense platereader.

### 2.10.4. Fluorescence measurements of synthetase expression

Overnight cultures of the appropriate RN4220 *S. aureus* strains containing the translational synthetase-GFP fusions and the relevant controls were back-diluted to OD600 or 0.1 in CDM. *S. aureus* cultures were grown to mid-exponential phase (approximately OD600 0.3-0.4). Once exponential phase was reached, 60 μg/ml mupirocin, 5X MIC vancomycin (10 μg/ml) or 5X MIC ampicillin (640 μg/ml) was added to the synthetase-GFP cultures (mupirocin for Rel-GFP and vancomycin and ampicillin for RelP-GFP and RelQ-GFP). Green fluorescence was subsequently measured at 485/520 nm every 30 minutes for 15 hours at 37˚C with aeration at 200 rpm using a Hidex Sense platereader.

## 2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9.5.0 software. Normality of data was tested using the Shapiro-Wilk normality test in order to determine whether data was parametric or non-parametric. Statistical differences between conditions were assessed using Student’s t-tests, Mann-Whitney test, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons post-test or two-way ANOVA followed by Šídák's multiple comparisons post-test. Survival experiments were analysed using the Kaplan-Meier method and survival curves were compared using the log-rank (Mantel-Cox) test. *P* values are represented as follows, or as described in the figure legends; \* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* < 0.001; \*\*\*\* *P* < 0.0001.

# Chapter 3: Construction of a toolbox to investigate the importance of the staphylococcal stringent response *in vivo*

## 3.1 Introduction

During nutrient limitation the aim of the SR is to downregulate macromolecular processes to promote bacterial survival. In the process of promoting survival, the SR can have many effects including contributing to virulence, immune evasion and phagosomal escape **(Ronneau and Hallez, 2019)**. In *S. aureus*, virulence mechanisms that the SR has been associated with include the upregulation of cytolytic toxins such as the phenol-soluble modulins **(Geiger *et al.*, 2012)** and persistence in macrophages **(Peyrusson *et al.*, 2020).** These findings suggest that the SR has a role in promoting survival of *S. aureus* within the host. Furthermore, excess (p)ppGpp produced by an MRSA clinical isolate due to mutations within the hydrolase domain of the Rel synthetase has contributed to antibiotic tolerance and resistance, leading to recurrent infections **(Gao *et al.*, 2010)**. Upregulation of purine biosynthesis also led to the overproduction of (p)ppGpp, favouring persistent bacteraemia over resolving bacteraemia in an endovascular infection model **(Li *et al.*, 2020)**. Both of these studies suggest that higher levels of (p)ppGpp provides a survival advantage. Additionally, the SR has been implicated in resistance to β-lactam antibiotics **(Kim *et al.*, 2013; Mwangi *et al.*, 2013)** and specifically, the absence of the RelQ synthetase sensitises CA-MRSA to these antibiotics **(Bhawini *et al.*, 2019)**. These examples highlight the importance of the SR in *S. aureus* infection through the regulation of many different cellular processes.

Little is known about how and when (p)ppGpp is produced *in vivo*, as (p)ppGpp measurements are usually taken from cells grown *in vitro*. A study developed methods to detect (p)ppGpp levels using live-cell imaging in *E. coli* **(Sun et al., 2021)**, however as yet there is no method for studying this in *S. aureus*. As such, in order to understand how the SR affects *S. aureus* pathogenicity, a toolbox was designed to examine its importance within *in vivo* models. These tools include: (p)ppGpp synthetase deletion mutants that will allow the investigation of each synthetase during infection; a (p)ppGpp overproduction strain that will be used to examine whether surplus (p)ppGpp alters survival of *S. aureus*; fluorescent (p)ppGpp probes to detect (p)ppGpp production and synthetase-GFP fusions to examine the expression of the individual (p)ppGpp synthetases. These tools were used to study the SR within macrophage, tonsillar epithelial and zebrafish embryo infection models. This chapter explains the rationale behind the design of the toolbox and how with these tools, the impact of the SR and (p)ppGpp *in vivo* can be examined*.*

## 3.2. Characterisation of (p)ppGpp-synthetase mutants

*S. aureus* possesses three (p)ppGpp synthetases: the long RSH Rel, and two short RSH RelP and RelQ. In order to examine the importance of (p)ppGpp in *S. aureus* pathogenicity, we investigated a panel of synthetase-deletion mutants in a JE2 USA300 MRSA background. (p)ppGpp-synthetase single, double and triple mutants were obtained from Dr Ian Monk and are represented by the schematic in **Figure 3.2.1a**. Although it was known that these were markerless, in-frame mutations, the precise location of the deletion within each gene was unknown. Two isogenic versions of the double and triple mutants were constructed, however the order in which the synthetases were mutated highlights their difference and this is made apparent in the name of the strain. Previous research has indicated that (p)ppGpp-synthetase mutants should have the same growth pattern as wildtype strains under unstressed conditions **(Geiger *et al.*, 2014; Gratani *et al.*, 2018)**. Thus, growth curves were performed to ensure that the strains behaved as expected. In keeping with previous reports, growth curves of the mutants showed no phenotypic differences in comparison to wildtype JE2 in TSB **(Fig. 3.2.1b)**.

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| **Figure 3.2.1. *S. aureus* (p)ppGpp-synthetase mutant strains grow similarly in rich media. a)** Schematic representing the different synthetase deletions in comparison to JE2 WT. **b)** Growth of the *S. aureus* wildtype strain JE2, the single mutants Δ*relP* and Δ*relQ,* double mutants Δ*relPQ* andΔ*relQP* and triple mutants Δ*relPQA* andΔ*relQPA* in TSB medium (nutrient rich conditions) from a starting OD600 of 0.05. The growth curve was performed in triplicate from which mean values ± standard deviations were plotted. |

Providing the strains have been constructed correctly, the triple synthetase mutants should be susceptible to amino acid stress, which can be induced using the isoleucyl-tRNA synthetase inhibitor, mupirocin **(Cassels, Oliva and Knowles, 1995; Geiger *et al.*, 2010; Gratani *et al.*, 2018)**. To identify a concentration of mupirocin that would allow us to differentiate between wildtype and mutant strains, the (p)ppGpp-synthetase mutants were streaked on TSA plates containing increasing concentrations of mupirocin **(Fig. 3.2.2a)**. There was no growth of any strains on plates containing 0.5 μg/ml mupirocin indicating that this concentration is too high. However, all strains except Δ*relQPA* grew on the plate containing 0.05 μg/ml mupirocin. As the Δ*relPQA* strain is theoretically isogenic to Δ*relQPA* with the only difference being the order with which the synthetases were mutated, there should not have been a phenotypic difference between them. Therefore, it was suspected that the Δ*relPQA* strain was not a triple mutant, allowing its growth on the 0.05 μg/ml plate.

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| **Figure 3.2.2. Characterisation of the (p)ppGpp-synthetase deletion mutants.** **a)** (p)ppGpp synthetase deletion mutants were streaked on TSA plates containing mupirocin at concentrations 0 μg/ml, 0.05 μg/ml and 0.5 μg/ml. The experiment was repeated twice, with one representative shown. **b, c, d)** Primers flanking each synthetase, *rel***(b)**, *relP* **(c)** and *relQ* **(d)** were used to amplify each of the synthetase genes from genomic DNA. **e)** Domain architecture of *rel* with the HD (hydrolase domain) (blue), SYNTH (synthetase domain) (teal), ThrRS, GTPase and SpoT (TGS) (green), α (α-helical domain) (yellow), ZFD/CC (zinc finger domain/conserved cysteine) (peach) and ACT/RRM (aspartate kinase, chorismite and TyrA/RNA recognition motif) (pink). The red-outlined bar shows the 234 amino acid in-frame deletion which covers the HD and SYNTH domains, including two important catalytic motifs: DxxxxR and YxxxH. Primers RMC165 and RMC739/740 were used to amplify the N-terminal enzymatic domain and primers RMC084/259 were used to amplify *rel* for sequencing and PCR. PCR products were run on 1% agarose gels. Ladder sizes are shown. **f)** Schematic of *relP* showing the position of the SYNTH domain and the red-outlined bar shows the 29 amino acid in-frame deletion which includes the ExQIRT catalytic motif. Primers RMC081/94 were used to amplify *relP* for sequencing and PCR. **g)** Schematic of *relQ* showing the position of the SYNTH domain and the red-outlined bar shows the 29 amino acid in-frame deletion which includes the ExQIRT catalytic motif. Primers RMC078/122 were used to amplify *relQ* for sequencing and primers RMC95/96 were used to amplify *relQ* for PCR. **(h)** and **(i)**: Mutant strains were grown with 32P-labelled H3PO4 and the stringent response was induced with mupirocin administration (60 μg/ml). **(h)** shows the nucleotide extracts for JE2 WT, and the single mutants Δ*relP* andΔ*relQ* and **(i)** shows nucleotide extracts for JE2 WT, the double mutants Δ*relPQ* andΔ*relQP* and the triple mutants Δ*relPQA* andΔ*relQPA.* Nucleotide extracts were separated by spotting on PEI-cellulose TLC plate. Smaller nucleotides such as GTP travel further than larger nucleotides such as pppGpp. Nucleotide standards were run in parallel to aid in the identification of each spot (performed by RMC). |

To confirm whether the panel of strains contained the desired deletions, in particular Δ*relPQA,* each synthetase gene was examined by PCR from genomic DNA of all JE2 strains **(Fig. 3.2.2b, c and d).** Expected band sizes for wildtype genes were 1229 bp for *rel*, 920 bp for *relP* and 633 bp for *relQ*. However, PCR revealed that the Δ*relPQA* strain possessed a band corresponding to the wildtype *rel* gene in comparison to Δ*relQPA,* which had the expected mutant band size of 527 bp **(Fig. 3.2.2b)**. This suggests that *rel* is intact in Δ*relPQA* explaining why it was able to grow on 0.05 μg/ml plates in comparison to the correct triple mutant, Δ*relQPA* **(Fig 3.2.1d)**. Sequencing the synthetase genes using gene-flanking primers revealed that the (p)ppGpp-synthetase mutants were constructed by introducing in-frame deletions in each of the synthetases: *rel* (234 amino acids), *relP* (29 aa) and *relQ* (29 aa) **(Fig. 3.2.2e, f, g)**. The panel of synthetase mutants were designed to delete key residues in the synthetase domain that are involved in its catalytic activity, including DxxxxR, YxxxH and ExQIRT **(Steinchen and Bange, 2016)**

To confirm that these deletions led to a loss of (p)ppGpp production, (p)ppGpp levels were analysed by thin layer chromatography (TLC) where cellular nucleotide extracts from single, double and triple mutants treated with 60 μg/ml mupirocin were spotted and run on a TLC plate to separate nucleotides. This demonstrated that as expected, Δ*relQPA* did not produce (p)ppGpp but Δ*relPQA* did **(Fig. 3.2.2h and i)**. Thus, going forward, the Δ*relQPA* strain would be used for all future experiments and will be referred to as (p)ppGpp0 hereafter.

## 3.3. Construction and characterisation of a (p)ppGpp overproduction strain

To investigate the importance of (p)ppGpp in *S. aureus* pathogenicity, (p)ppGpp synthetase mutants and (p)ppGpp overproduction strains will be studied and compared within *in vivo* models. As (p)ppGpp overproduction has been implicated in antibiotic resistance in the hospital setting **(Gao *et al.*, 2010),** here, the construction and characterisation of a (p)ppGpp overproduction strain is described to understand the effect of this phenotype *in vivo*.

### 3.3.1. Cloning the (p)ppGpp enzymatic domain with and without a his-tag

A previous report described the construction of a (p)ppGpp overproduction strain achieved by plasmid-based expression of the NTD of Rel from *S. aureus*. This construct had a HD domain mutation that renders it non-functional and therefore unable to hydrolyse (p)ppGpp, and the lack of the CTD relieves the negative regulation that the CTD has on the NTD **(Gratani *et al.*, 2018).** Under stringent conditions, *S. aureus* mutants that lack the CTD of Relare found to be in a synthetase-OFF/hydrolase-ON state **(Gratani *et al.*, 2018)**, therefore, relieving the negative regulation of the CTD on the synthetase domain alone is not enough for (p)ppGpp overproduction. In order to construct a similar (p)ppGpp overproduction strain, the NTD of *rel* was amplified from LAC\* genomic DNA. The expected plasmid maps from cloning are summarised in **Figures 3.3.1a and 3.3.1b**. Two different reverse primers were used, without and with a hexa-histidine tag (RMC739 and 740 respectively) **(Fig. 3.3.1a)** and both reverse primers contained a SacII restriction site while the forward primer (RMC165) contained an AvrII restriction site. The expression vectors used, pCL55-iTET and pCL55-iTETr862 **(Corrigan *et al.*, 2015)** derived from pCL55 **(Lee, Buranen and Ye, 1991)**,integrate into the glycerol ester hydrolase (*geh*) lipase gene of *S. aureus* and expression is inducible with anhydrotetracycline (Atc)*.* The pCL55-iTETr862 vector has a tighter promoter in comparison to pCL55-iTET which has leaky expression; a leaky promoter will be beneficial to overproduce (p)ppGpp and so both were used. Both inserts and vectors were digested with AvrII and SacII, ligated and transformed into *E. coli* XL1 Blue. The resultant colonies were analysed by colony PCR using primers specific for the vector (RMC006 and RMC007) and positive clones for each construct were identified **(Fig. 3.3.1d)**.

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| **Figure 3.3.1.** **Cloning the (p)ppGpp enzymatic N-terminal domain into the integrative vectors pCL55-iTET and pCL55-iTETr862.** **(a)** and **(b)** The NTD (green) consisting of the HD domain (tan) and SD (peach), with and without a His-tag (pale yellow), was cloned into the vectors pCL55-iTET and pCL55-iTETr862 to produce four different plasmids. The vectors are integrative plasmids, integrating into the *S. aureus* *geh* gene via the L54a plasmid-encoded integrase (yellow) and expression of the N-terminal is achieved via the administration of anhydrotetracycline. Primers used for cloning the NTD (RMC165, 739 and 740) and the plasmid-specific primers used for confirmation of the clone (RMC006 and 007) are shown in black. NTD; N-terminal domain, HD; hydrolase domain (tan), SYNTH; synthetase domain (peach), CamR; chloramphenicol resistance in *S. aureus* (light blue), AmpR; ampicillin resistance in *E. coli* (grey), TetR; tetracycline repressor (lilac). **c)** Schematic of the HD domain from pCL55-iTET/pCL55-iTETr862 only, showing inverse PCR primers RMC708 and 709 used to introduce the HD domain mutation. WT DNA sequences are shown in black and the WT amino acid sequence is represented by the blue bar. The mutation introduced by the primers are shown in red and the mutated amino acid sequence is represented by the peach bar. **d)** Colony PCR products from transformants likely to carry the plasmid containing the correct insert. Primers specific for the vectors were used that amplified where the insert should be introduced. Lane 1; pCL55-iTET-NTD (1533 bp), lane 2; pCL55-iTET-NTD-his (1551 bp), lane 3; pCL55-iTETr862-NTD (1730 bp), lane 4; pCL55-iTETr862-NTD-his (1748 bp). PCR products were run on 1% agarose gels. Ladder sizes are shown. |

### 3.3.2. Mutating the hydrolase domain

Mutating the hydrolase (HD) domain of a strain with a CTD truncation allows the production of surplus (p)ppGpp **(Gratani *et al.*, 2018)**. To achieve this, pCL55-iTET/pCL55-iTETr862 containing the NTD with and without the his-tag, was subjected to inverse PCR. Primers RMC708 and 709 were used to introduce a substitution mutation within the HD domain **(Fig. 3.3.1c)**. The histidine and aspartate residues crucial for HD domain activity **(Aravind and Koonin, 1998)**, were substituted for alanine and serine **(Fig. 3.3.1c).** This plasmid DNA was transformed into *E. coli* XL1 Blue and the HD mutation was verified by sequencing, resulting in an overproduction plasmid expressing the NTD of *rel* with a non-functional HD domain.

### 3.3.3. Introducing the (p)ppGpp-overproducing construct into *S. aureus*

The vectors containing the mutated NTD were introduced into *S. aureus* to create the overproduction strains. To do this, plasmid DNA extracted from *E. coli* strains containing the overproduction plasmid expressing the NTD with and without his-tag and with a non-functional HD domain was electroporated into *S. aureus* RN4220. RN4220 is a restriction-deficient derivative of *S. aureus* NCTC 8325-4 that is useful for cloning **(Kreiswirth *et al.*, 1983).** DNA isolated from RN4220 can easily be transduced into other *S. aureus* strains as the DNA will not be recognised as foreign due to methylation in the absence of restriction enzymes **(Monk *et al.*, 2015)**. RN4220 strains containing the overproduction plasmids were streaked on tryptic soy agar (TSA) containing 1% egg yolk and 0.01% tellurite. The presence of egg yolk allows the identification lipase-positive/negative colonies, indicated by the presence or absence of an opaque zone of clearance respectively. Potassium tellurite (K2TeO3) is a toxic tellurium-based compound used as a selective agent in differential media such as Baird Parker **(Baird-Parker, 1962)**. Its presence allows for the identification of *S. aureus* as staphylococci are able to reduce tellurite to telluride, resulting in black colonies **(Fig. 3.3.2a)**.

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| **Figure 3.3.2. Mechanism and confirmation of the integration of the overproduction plasmids. a)** Schematic of an egg yolk tellurite plate.Overproduction strains in an RN4220 background were streaked on TSA containing 1% egg yolk and 0.01% potassium tellurite with lipase-positive RN4220 WT and lipase-negative RN4220 pCL55-iTET as controls. RN4220 with an intact *geh* gene will demonstrate a zone of clearance, indicating hydrolysis of lipids in the egg yolk. Strains with a pCL55-iTET/pCL55-iTETr862 insertion into the *geh* locus are unable to hydrolyse egg yolk lipids and therefore do not demonstrate a zone of clearance. Strains appear black due to the reduction of tellurite to telluride by *S. aureus.* **b)** The pCL55-iTET/pCL55-iTETr862 plasmids contain an L54a attP site upstream of the L54a integrase which allows recombination with the L54a attB site located at the 3’ end of the *geh* gene, resulting in the integration of pCL55-iTET/pCL55-iTETr862 into the bacterial chromosome.CamR; chloramphenicol resistance in *S. aureus* (light blue), AmpR; ampicillin resistance in *E. coli* (grey), TetR; tetracycline repressor (lilac), attP; bacteriophage attachment site, attB; bacterial attachment site, *geh*; glycerol ester hydrolase. **c)** Primers specific for each plasmid (RMC006/007) were used to conduct PCR on genomic DNA extracted from the (p)ppGpp overproduction strains and its controls. Lane 1; JE2 pCL55-iTET (315 bp), lane 2; JE2 pCL55-iTET-NTD-ΔHD-his (1551 bp), lane 3; JE2 pCL55-iTETr862 (512 bp), lane 4: JE2 pCL55-iTETr862-NTD-ΔHD-his (1730 bp), lane 5; (p)ppGpp0 pCL55-iTETr862 (512 bp) and lane 6; (p)ppGpp0 pCL55-iTETr862-NTD-ΔHD-his (1730 bp). PCR products were run on 1% agarose gels. Ladder sizes are shown. |

Both pCL55-iTET and pCL55-iTETr862 encode the L54a bacteriophage integrase which allows integration of the plasmid into the bacterial chromosome. This is achieved by site-specific recombination that occurs between the bacteriophage attachment site attP (located upstream of the integrase on the plasmid) and the bacterial attachment site attB which is located at the 3’ end of the *geh* lipase on the genome **(Lee and Iandolo, 1986)**. Overproduction strain colonies streaked on this agar demonstrated no zone of clearance, indicating that the plasmid had integrated into the *geh* gene, disrupting the gene and thus preventing lipid hydrolysis in comparison to controls **(Fig. 3.3.2a and 3.3.2b)**. For an overproduction strain, a WT background would be optimal as in addition to the chromosomal synthetases, the (p)ppGpp overproduction plasmid could allow for the highest levels of (p)ppGpp to be produced. Thus, phage 85 was used to transduce the integrated plasmids into the wildtype *S. aureus* strain JE2 and also the (p)ppGpp0 mutant background with integration confirmed as above **(Figure 3.3.2c)**. Transduction of pCL55-iTET-NTD-ΔHD (leaky plasmid containing the NTD only and a non-functional HD domain) into the (p)ppGpp0 mutant background was unsuccessful. This may be because of the leaky nature of the plasmid which may allow an overproduction of (p)ppGpp that is unfavourable for growth as the background strain does not contain a functional hydrolase domain.

### 3.3.4. Characterisation of the *S. aureus* (p)ppGpp-overproducing strains

To examine whether (p)ppGpp is being overproduced in these strains, growth curves were performed in the presence of the inducer Atc (100 ng/ml), which enables expression of pCL55-iTET plasmids. Here, there would be no difference between JE2 WT and the (p)ppGpp0 mutant in the presence of Atc. However, if (p)ppGpp is being produced upon induction of the overproduction strains, we would expect to see a reduction in OD600 over time due to the downregulation of growth that is characteristically associated with the induction of the SR. Interestingly, the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD, a (p)ppGpp0 mutant carrying an overproduction plasmid, shows a dramatic growth defect in comparison to the other strains in the presence of Atc **(Figure 3.3.3a)**. However, this is not the case for the WT strain containing the same plasmid (JE2 pCL55-iTETr862-NTD-his-ΔHD). When (p)ppGpp is produced in a ΔHD background, it becomes toxic as bacteria are unable to degrade excess (p)ppGpp. Thus, as (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD does not contain a functional HD domain on either the plasmid or the genome, (p)ppGpp accumulation becomes toxic, resulting in this characteristic decrease in OD600. There was no difference in growth between any of the strains in the WT background - this could be due to the functional hydrolase domain present on the genome controlling (p)ppGpp levels. An ideal (p)ppGpp overproduction strain would be constructed in a WT background in order to benefit from the chromosomal synthetases. However, as only the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD strain which does not contain any functional chromosomal synthetases displayed a growth defect upon induction of the plasmid, the ability of this strain to overproduce (p)ppGpp was further investigated.

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| **Figure 3.3.3. Characterisation of the (p)ppGpp overproduction strain. a)** Growth curves of the potential (p)ppGpp overproduction strains in the presence of 100 ng/ml anhydrotetracycline (Atc) with their controls. **b)** Growth curve of the *S. aureus* (p)ppGpp overproduction strain (p)ppGpp0 pCL55-iTETr862 –NTD-his-ΔHD vs its control (p)ppGpp0 pCL55-iTETr862 with (+) and without (-) Atc at 10 ng/ml. **c)** and **d)** are growth curves of the empty vector control and (p)ppGpp0 overproduction strain respectively grown with concentrations of Atc from 10 ng/ml (doubling dilutions). Both c) and d) show the same concentrations of Atc and so they share the same figure legend. All growth curves were set up in TSB medium from a starting OD600 of 0.05 for 8 hours. The proposed overproduction strain as well as the controls and synthetase-complemented null mutant strains were streaked on TSA containing 0.05 μg/ml mupirocin **(e)** and TSA containing 0.05 μg/ml mupirocin and 50 ng/ml Atc **(f)**. **g)** All strains containing proposed overproduction plasmids were analysed by TLC. Nucleotide standards (GTP, pppGpp and ppGpp) were run in parallel to aid in the identification of each spot as shown (**(c)** performed by RMC). Growth curves b) and c) were performed once while a) and d) were performed in triplicate from which mean values ± standard deviations were plotted. |

In order to titrate the levels of Atc to investigate if (p)ppGpp production can be controlled, the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD mutant was grown in the presence and absence of 10 ng/ml Atc **(Fig. 3.3.3b)** which showed results similar to **Figure 3.3.3a.** Furthermore, expression of the NTD from the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD mutant is tightly controlled as when grown in the absence of Atc, growth is similar to the empty vector control **(Fig. 3.3.3b)**. Thus, growth curves in the presence of Atc diluted from 10 ng/ml were performed in order to titrate the drug concentration using either the empty vector controls **(Fig. 3.3.3c)** or the overproduction strain **(Fig. 3.3.3d)** to determine a concentration that would allow (p)ppGpp overproduction *in vivo* without a dramatic growth defect. These results show that (p)ppGpp production from this plasmid is tightly regulated and requires very low levels of Atc to produce (p)ppGpp.

The phenotype of the overproduction strain (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD in the presence of Atc and mupirocin was investigated along with (p)ppGpp0 strains complemented with each synthetase that were already available in the laboratory. These complement strains were investigated in order to compare the (p)ppGpp production by each synthetase in comparison to the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD strain. As expected, the (p)ppGpp0 pCL55-iTETr862 strain was unable to grow on media containing mupirocin in comparison to JE2 pCL55-iTETr862 **(Fig 3.3.3e)**. When the null mutant is complemented with full-length *rel*, growth is restored suggesting that even in the absence of the inducer Atc, *rel* is being expressed. This is expected as expression from pCL55-iTETr862 is leaky and Rel is induced upon exposure to mupirocin. However, the presence of *relP, relQ* and the overproduction plasmid does not restore growth, in keeping with previous knowledge that RelP and RelQ synthetases are not induced by amino acid stress. In the presence of Atc to induce expression on the pCL55-iTETr862 in addition to mupirocin, the presence of *rel* and *relP* did restore growth however expression of *relQ* and the resulting (p)ppGpp was not enough to allow growth on this media **(Fig. 3.3.3f).** Importantly, there was no difference in growth of (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD in the presence of mupirocin +/- Atc, and the growth was not comparable to when the null mutant was complemented with either *rel* or *relP*. This suggests that the overproduction strain is unable to produce enough (p)ppGpp to restore growth when under amino acid stress in the presence of mupirocin despite being induced with Atc, indicating that the construct is not fully functional.

To examine if the overproduction strain (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD was able to overproduce (p)ppGpp, (p)ppGpp levels of all strains containing overproduction plasmids and their controls were analysed by TLC **(Fig. 3.3.3g)**. Similar levels of pppGpp and ppGpp were detected across all strains in comparison to a JE2 WT control in the presence of mupirocin, demonstrating that the overproduction strain (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD did not overproduce detectable (p)ppGpp despite displaying a growth defect in the presence of Atc using this method **(Fig. 3.3.3a)**. These results corroborate the phenotype observed in **Figure 3.3.3f**.

### 3.3.5. Characterisation and construction of alternative (p)ppGpp overproduction strains

Due to the lack of detectable (p)ppGpp overproduction, existing laboratory strains that may have the ability to overproduce (p)ppGpp were investigated. The future applications for (p)ppGpp overproduction strains in this study include work both *in vivo* and *in vitro*, thus, strains containing integrative plasmids and multicopy plasmids were explored. Zebrafish embryos are generally infected with strains that do not contain multicopy plasmids due to the difficulties of maintaining the plasmid over the course of infection (4 days). Therefore, while integrative vectors are preferred, (p)ppGpp production from multicopy plasmids were also examined.

In this regard, the tetracycline-inducible pALC2073 multicopy plasmid was utilised. The (p)ppGpp0-pALC2073-*rel*, -*relP* and -*relQ* strains were available in the laboratory strain collection and were grown on solid media in the presence of mupirocin and the inducer, Atc. Administration of mupirocin leads to amino acid stress and Atc induces expression of the pALC2073 plasmid. In the presence of mupirocin, the (p)ppGpp0-pALC2073-*rel* strain grew well while the (p)ppGpp0-pALC2073-*relP* strain appeared to grow faintly and (p)ppGpp0-pALC2073-*relQ* grew similarly to the (p)ppGpp0 mutant suggesting that expression of *relQ* is not induced upon exposure to mupirocin **(Fig. 3.3.4a)**. These results imply that *rel* and *relP* are expressed in the absence of inducer, albeit at different levels. When in the presence of mupirocin and Atc, only the (p)ppGpp0-pALC2073-*relQ* was able to complement **(Fig. 3.3.4b)**, which suggested that this strain may overproduce (p)ppGpp.**Figure 3.3.4c** reveals that while the pALC2073-*rel* plasmid was able to produce pppGpp, it is clear that the pALC2073-*relQ* plasmid is able to overproduce (p)ppGpp in comparison to the (p)ppGpp0 mutant and the other complement strains. As the pALC2073-*rel* plasmid contains a HD domain, this may explain why this plasmid is unable to overproduce (p)ppGpp in the same way as pALC2073-*relQ*. Therefore, the pALC2073-*relQ* plasmid was introduced into a WT background to overproduce (p)ppGpp.

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| **Figure 3.3.4. Characterisation and construction of alternative (p)ppGpp overproduction strains.** JE2 pALC2073 and (p)ppGpp0 pALC2073 empty vector strains (controls) and (p)ppGpp0 pALC2073 complemented with *rel, relP* and *relQ* were streaked on TSA plates containing 0.05 μg/ml mupirocin **(a)** and TSA containing 0.05 μg/ml mupirocin and 50 ng/ml Atc **(b)**. **c)** These strains were then analysed by TLC. Nucleotide standards (GTP, pppGpp and ppGpp) were run in parallel to aid in the identification of each spot. Performed by RMC. **d)** Primers specific for pCL55-iTETr862 (RMC006/007) were used to conduct PCR on genomic DNA extracted from a lipase-negative clone. Lane 1; JE2 pCL55-iTET (315 bp), lane 2; positive clone of JE2 pCL55-iTETr862-*rel* (2741 bp). **e)** Positive clones of JE2 pCL55-iTETr862-*relP* andJE2 pCL55-iTETr862-*relQ.* Lane 1; JE2 pCL55-iTET (315 bp), lane 2-3; JE2 pCL55-iTETr862-*relP* (1211 bp), lane 4-5 ; JE2 pCL55-iTETr862-*relQ* (1161 bp). PCR products were run on 1% agarose gels. Ladder sizes are shown. |

For *in vivo* experiments, *rel*, *relP* and *relQ* were cloned into the integrative pCL55-iTETr862 vectors that utilise a tetracycline-inducible promoter. The advantage of this is that antibiotics are not required for plasmid maintenance and there is no prospect of plasmid loss during the course of infection as it is present on the genome. To construct these strains, pCL55-iTETr862-*rel*, pCL55-iTETr862-*relP*, pCL55-iTETr862-*relQ*, were transduced into JE2 WT using phage 85. PCR amplification of genomic DNA extracted from these clones confirmed the presence of the plasmid **(Figure 3.3.4e and f)**.

## 3.4. Construction of fluorescent (p)ppGpp probes

Current, well-established methods of (p)ppGpp detection include high-performance liquid chromatography (HPLC) **(Varik *et al.*, 2017; Jin *et al.*, 2018; Bokinsky *et al.*, 2013)** and separating radiolabelled cellular nucleotide extracts by TLC **(Geiger *et al.*, 2010; Geiger *et al.*, 2014; Zhang *et al.*, 2018a)**. These methods allow direct and sensitive measurement of (p)ppGpp however they are time-consuming, cannot measure (p)ppGpp levels in real-time and the use of radiolabelled phosphate is unsafe. Since then, the advent of tools to measure (p)ppGpp levels directly or indirectly has allowed (p)ppGpp detection in a variety of ways.

Compounds that are selective for (p)ppGpp exist as a method of (p)ppGpp detection. A fluorescent chemosensor, PyDPA, a molecule that fluoresces upon dimerisation and binding to ppGpp, was developed and has been useful for measuring ppGpp levels **(Rhee *et al.*, 2008; Li *et al.*, 2020)**. PyDPA is more specific and sensitive to (p)ppGpp than other nucleotides such as ATP and GTP **(Rhee *et al.*, 2008; Conti, Minneci and Sattin, 2019)**. Additionally, nanoclusters **(Zhang *et al.*, 2013)**, nanoparticles **(Chen *et al.*, 2018)** and europium quantum dots **(Rong *et al.*, 2020)** with the ability to detect (p)ppGpp and subsequently fluoresce have also been developed. However, as with HPLC and TLC, these methods only allow for measurements to be taken *in vitro*.

Promoter-reporter fusions have also been employed **(Gawin *et al.*, 2019; Bartoli  *et al.*, 2020; Goormaghtigh *et al.*, 2018; Pokhilko, 2017)**, which can be used both *in vitro* and *in vivo.* However, these methods measure transcriptional activity, which is not representative of the (p)ppGpp concentration within a bacterium. At present, there are no (p)ppGpp probes for the measurement of (p)ppGpp levels in *S. aureus* *in vivo* in real-time.These types of probes would be beneficial as they can allude to how (p)ppGpp levels change over time. Recently however, (p)ppGpp detection using live-cell imaging in *E. coli* has been achieved using a riboswitch-based molecular biosensor, allowing real-time detection **(Sherlock, Sudarsan and Breaker, 2018)**. Due to the gap in the field with regards to (p)ppGpp detection in *S. aureus* *in vivo*, a variety of *S. aureus*-specific (p)ppGpp probes to measure both synthetase productivity and (p)ppGpp levels were developed. These include a riboswitch-aptamer molecular biosensor, a promoter-reporter fusion and translational synthetase-GFP fusions.

### 3.4.1. Construction of a riboswitch-Broccoli molecular (p)ppGpp biosensor

Riboswitches are non-coding regions of mRNA that interact with many different ligands, including (p)ppGpp **(Irving, Choudhury and Corrigan, 2021)** which specifically binds to the *ykkc* (subtype 2a) class of riboswitches **(Sherlock, Sudarsan and Breaker, 2018)**. Binding of ligands to riboswitches controls the transcription of downstream genes. Genes regulated by the *ykkc* riboswitches encode for various proteins including efflux pumps that may translocate guanidine **(Barrick *et al.*, 2004; Nelson *et al.*, 2017)**, however the (p)ppGpp-specific *ykkc* subtype 2a riboswitches are associated with genes involved in, but not limited to, BCAA biosynthesis.

Recent studies have used riboswitches fused to RNA fluorophore-binding sequences (referred to as RNA aptamers hereafter) as molecular biosensors to measure levels of c-di-AMP and c-di-GMP **(Kellenberger *et al.*, 2015; Wang, Wilson and Hammond, 2016)** and (p)ppGpp **(Sherlock, Sudarsan and Breaker, 2018; Sun *et al.*, 2021)**. RNA aptamers are sequences of RNA that, when complexed with their cognate fluorogen, generate a fluorescent output **(Paige, Wu and Jaffrey, 2011) (Figure 3.4.1a)**. Many types of fluorogenic dye exist that exhibit at least 1000-fold increase in fluorescence in the presence of RNA aptamers in comparison to in the absence **(Cawte, Unrau and Rueda, 2020; Suss, Motiei and Margulies, 2021).** For example, RNA aptamers Broccoli and Spinach complex with a GFP mimic (DFHBI-1T) **(Figure 3.4.1d)**, while Mango will complex with thiazole orange **(Paige, Wu and Jaffrey, 2011; Suss, Motiei and Margulies, 2021; Filonov *et al.*, 2014)**. RNA aptamers are used to fluorescently tag RNA motifs such as riboswitches, similar to how protein fluorophores such as GFP are used to tag proteins. In this instance, using an RNA aptamer has some advantages as they have low background fluorescence as opposed to GFP, are more resistant to photobleaching than GFP and they do not require translation **(Ouellet, 2016; Paige, Wu and Jaffrey, 2011)**. As the SR is associated with a decrease in translation, utilising an RNA aptamer means that the production of fluorescence does not depend on protein expression and therefore, this not an issue that might otherwise be encountered if a protein fluorophore were used. Thus, the *ykkc* subtype 2a riboswitch motif **(Figure 3.4.1b, Table 4)** controlling the BCAA-associated gene *ilvE* from the Gram-negative *T. oceani* **(Sherlock, Sudarsan and Breaker, 2018)** as well as the RNA aptamer, Broccoli, were utilised in this study for the construction of a riboswitch-Broccoli molecular (p)ppGpp biosensor **(Figures 3.4.1c, Table 5)**.

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| **Figure 3.4.1. Components of the riboswitch-Broccoli molecular (p)ppGpp biosensor. a)** Schematic of an RNA aptamer fused to a riboswitch. In the absence of (p)ppGpp and DFHBI-1T there is no fluorescence. In the presence of (p)ppGpp and DFHBI-1T, (p)ppGpp binds to the riboswitch and DFHBI-1T will bind to the aptamer, resulting in fluorescence. **b)** Predicted secondary structure of the *ykkc* subtype 2a riboswitch. **c)** Predicted secondary structure of the F30-Broccoli fluorophore with the F30 scaffold (orange), the Broccoli fluorophore sequence (green) and the transducer sequence highlighted by the blue circle. The scaffold contains two different arms for two different fluorophore insertion points. RNA centroid secondary structures were computed using the Vienna RNA Websuite **(Gruber et al., 2008, Lorenz et al., 2011)**. **d)** Structure of DFHBI-1T. DFHBI-1T was drawn using ChemDraw Prime. |

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| Table 4. RNA sequences for the construction of a riboswitch-Broccoli biosensor. | | |
| **Name** | **Sequence (5’-3’)** | **Notes** |
| *ykkc* subtype 2a riboswitch from *T. oceani*  (RNA motif) | GGAAGUGUACCUUAGGGUUCCGGCCAUAAGGCGUCAGCGACCGAGCGGUACAAUCCGGGGAAACCCGGAACACCGUGAGCAUAAAAGGCUCCAGCGGCAAGUUCC | RNA sequence of the *ykkc* subtype 2a riboswitch from *T. oceani* **(Sun *et al.*, 2021)*.*** |
| F30-Broccoli  (RNA aptamer) | UUGCCAUGUGUAUGUGGGAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCCCACAUACUCUGAUGAUCCUUCGGGAUCAUUCAUGGCAA | RNA sequence of the F30-Broccoli fluorophore showing the F30 scaffold (yellow) and the Broccoli sequence (green). The Broccoli sequence contains a transducer domain highlighted in blue **(Filonov *et al.*, 2014; Filonov *et al.*, 2015)** |
| F30-Broccoli + T7 terminator | TTGCCATGTGTATGTGGGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTCCCACATACTCTGATGATCCTTCGGGATCATTCATGGCAAGCTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG | DNA sequence of the F30-Broccoli fluorophore showing the F30 scaffold (yellow) and the Broccoli sequence (green) and transducer domain (dark blue) **(Filonov *et al.*, 2014; Filonov *et al.*, 2015)** and the T7 terminator (light blue). |
| Riboswitch-Broccoli | GGAAGTGTACCTTAGGGTTCCGGCCATAAGGCGTCAGCGACCGAGCGGTACAATGCTGTCGAGTAGAGTGTGGGCTCGCAAGAGACGGTCGGGTCCAGCAACACCGTGAGCATAAAAGGCTCCAGCGGCAAGTTCC | DNA sequence of the riboswitch-Broccoli construct containing the riboswitch (pink), Broccoli fluorophore (green) and S2 transducer sequences (red) **(Sun *et al.*, 2021)** |
| Riboswitch-Broccoli + T7 terminator | GGAAGTGTACCTTAGGGTTCCGGCCATAAGGCGTCAGCGACCGAGCGGTACAATGCTGTCGAGTAGAGTGTGGGCTCGCAAGAGACGGTCGGGTCCAGCAACACCGTGAGCATAAAAGGCTCCAGCGGCAAGTTCCGCTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG | DNA sequence of the final riboswitch-Broccoli + T7 terminator construct containing the riboswitch (pink), Broccoli fluorophore (green) and S2 transducer sequences (red) and T7 terminator (light blue). |

The riboswitch-Broccoli molecular (p)ppGpp biosensor and the positive control was cloned into the integrative vector pCL55-iTET with a tetracycline-inducible promoter **(Fig. 3.4.2a and b)**. RNA aptamers (Broccoli) fused to RNA motifs (*ykkc* riboswitch) must be designed so that once transcribed, the resulting conformation allows efficient binding of a cognate fluorogen (DFHBI-1T) to Broccoli, but also for binding of the RNA motif to its ligand e.g. the *ykkc* riboswitch to (p)ppGpp. RNA scaffolds are often employed in order to maintain the stability of the resulting RNA structure. The F30 scaffold was used to create the F30-Broccoli sequence that would be cloned into an *S. aureus* expression vector to construct the positive control **(Table 5)**. This aptamer was modified from its predecessor, F29, to not only be more structurally stable but also resistant to degradation in mammalian cells as F29 was shown to be targeted for cleavage **(Shu *et al.*, 2014; Filonov *et al.*, 2015)**. The final sequence of the F30-Broccoli aptamer used for the positive control is shown in **Table 5** which includes a T7 terminator sequence at the 3’ end to ensure transcriptional termination which acts to prevent the formation of read-through transcripts. In order to construct the Broccoli positive control, primers RMC846/847 were used to amplify the sequence for the F30-Broccoli aptamer and the T7 terminator **(Table 5)** from a synthesised single-stranded oligonucleotide in order to produce a double-stranded 154 bp product with a 5’ EcoRV restriction site and a 3’ AvrII restriction site **(Fig. 3.4.2a)**. Both the amplified Broccoli/T7 terminator sequence and the integrative vector pCL55-iTET were digested with EcoRV and AvrII, ligated and transformed into *E. coli* XL1 Blue. The resulting plasmid, pCL55-iTET-Broccoli (positive control) was electroporated into RN4220 ∆*spa* and the successful insertion of the plasmid into the *geh* lipase gene is shown in **Figure 3.4.2c**.

The riboswitch-Broccoli molecular (p)ppGpp biosensor itself was designed to contain the (p)ppGpp-binding *ykkc* subtype 2a riboswitch from *T. oceani*, the Broccoli aptamer and two 2-nucleotide length transducers (named S2 accordingly) **(Table 5)** **(Sun *et al.*, 2021)**. This molecular (p)ppGpp biosensor was shown to demonstrate the highest fluorescence levels in the presence of (p)ppGpp in comparison to other sensors with different length transducer sequences and was therefore utilised in this study **(Sun *et al.*, 2021)**. The synthesised riboswitch-Broccoli oligonucleotide **(Table 5)** was amplified by PCR with primers RMC848/849 to produce a 148 bp double-stranded product and was digested with EcoRV and NheI along with the pCL55-iTET-Broccoli– this digestion liberates the Broccoli fluorophore from pCL55-iTET-Broccoli but retains the T7 terminator **(Fig. 3.4.2b)**. The backbone containing only the T7 terminator was gel-extracted, and both the backbone and the riboswitch-Broccoli insert were digested with EcoRV and NheI, ligated and transformed into *E. coli* XL1 Blue. This plasmid was extracted from *E. coli* and electroporated into RN4220 ∆*spa* and plasmid insertion was confirmed in lipase-negative clones by PCR **(Fig. 3.4.2d)**.

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| **Figure 3.4.2. Construction of the Broccoli positive control and the riboswitch-Broccoli molecular (p)ppGpp biosensor using an integrative plasmid.** **a)** Plasmid map of pCL55-iTET-riboswitch-Broccoli showing the riboswitch (pink), Broccoli (green) and the T7 terminator (orange). **b)** Plasmid map of pCL55-iTET-Broccoli showing Broccoli (green) and the T7 terminator (orange). Integrase (yellow), CamR; chloramphenicol resistance in *S. aureus* (blue), AmpR; ampicillin resistance in *E. coli* (grey), TetR; tetracycline repressor (lilac). **c)** Primers specific for pCL55-iTET(RMC006/007) were used to conduct PCR on genomic DNA extracted from lipase-negative clones of RN4220 pCL55-iTET-Broccoli (477 bp) (lanes 1-3). **d)** Primers RMC006/007 were used to conduct PCR on genomic DNA extracted from lipase-negative clones of RN4220 pCL55-iTET-riboswitch-Broccoli (446 bp) (lanes 1-4). PCR products were run on 1% agarose gels. Ladder sizes are shown. |

### 3.4.2. Characterisation of the riboswitch-Broccoli molecular (p)ppGpp biosensor

#### 3.4.2.1. *In vitro* characterisation

To confirm whether the resulting RNA secondary structure for the riboswitch-Broccoli molecular (p)ppGpp biosensor is folded correctly upon transcription, and is able to fluoresce upon ppGpp binding, an *in vitro* transcription assay was performed by RMC. RNA was transcribed from the synthesised single-stranded DNA in **Table 5** (Riboswitch-Broccoli) and was incubated in a buffer with DFHBI-1T and various nucleotides (ppGpp, GTP, GDP, ATP) in order to determine its selectivity. **Figure 3.4.3a** shows that after approximately 15 minutes incubation, the fluorescence increases substantially in the presence of ppGpp while this is not the case for the other nucleotide conditions. This suggests that the riboswitch-Broccoli aptamer is able to form the desired secondary structure once transcribed and bind to (p)ppGpp, demonstrating its selectivity for ppGpp.

#### 3.4.2.2. Live-cell characterisation – integrative vector

After confirming the selectivity of the riboswitch-Broccoli molecular (p)ppGpp biosensor *in vitro*, the next step was to test whether the construct can fold correctly and fluoresce within a live bacterial cell. *S. aureus* containing the riboswitch-Broccoli construct, the positive (Broccoli-containing) and negative (empty vector) controls were grown in CDM to early exponential phase (~OD600 0.3-0.4) Cultures were then washed twice with complete CDM or CDM excluding leucine and valine (CDM-L/V). The absence of leucine and valine induces the SR due to amino acid starvation as these amino acids are essential for *S. aureus* growth **(Lincoln, Leigh and Jones, 1995)**. After the addition of DFHBI-1T and Atc 100 ng/ml to induce plasmid expression via the tetracycline-inducible promoter, fluorescence was measured at 485/520 nm for 20 hours **(Fig. 3.4.3b).** In the absence of L/V (open symbols), fluorescence levels are higher than when L/V are present (closed symbols). A difference was observed between the presence and absence of L/V with the negative control which is unexpected as this construct does not contain a riboswitch-fluorophore aptamer. While the positive control RN4220 pCL55-iTET-Broccoli had the highest fluorescence levels, the biosensor itself did not fluoresce in a similar manner and in fact, demonstrated lower levels of fluorescence than the negative control.

To ensure that transcription of the plasmids was indeed being induced, Atc (100 ng/ml) was added during growth of the strains instead of after the cultures had been grown. Once ~OD600 0.3-0.4 had been reached, cultures were washed in either complete CDM or CDM-L/V and after the addition of Atc (100 ng/ml) and DFHBI-1T, fluorescence was measured for 20 hours. **Figure 3.4.3c** shows the same trend as in **Figure 3.4.3b** in that strains in the absence of L/V display higher levels of fluorescence than in the presence of L/V, but there was no difference in the amount of fluorescence of the strain containing the riboswitch-Broccoli construct in comparison to its controls.

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| **Figure 3.4.3. *In vitro* and *in vivo* characterisation of the riboswitch-Broccoli molecular (p)ppGpp biosensor using an integrative plasmid.** **a)** *In vitro* fluorescence activation of the riboswitch-Broccoli biosensor by various nucleotides. Nucleotides ppGpp (red), GTP (light blue), GDP (purple) and ATP (orange) were added at a final concentration of 100 μM in a buffer containing 1 M HEPES pH 7.5, 2 M KCl, 1 M MgCl2 and 100 μM DFHBI-1T and fluorescence was measured for 2 hours at 5-minute intervals. H2O control did not include nucleotide and blank did not include DFHBI-1T. Performed by RMC. **b)** Fluorescence curve of the riboswitch-Broccoli biosensor post amino acid stress. The negative control (RN4220 pCL55-iTET) (black), positive control (RN4220 pCL55-iTET-Broccoli) (pink) and biosensor-containing strain (RN4220 pCL55-iTET-riboswitch-Broccoli) (teal) were grown to ~OD600 0.3-0.4 in CDM. Cultures were split, washed twice in either complete CDM (closed symbols) or CDM-L/V (open symbols) and then induced for 1 hour with Atc 100 ng/ml. DFHBI-1T (200 μM) was added and cultures were incubated at 37 ˚C for 20 hours, with fluorescence measurements taken every 30 minutes. **c)** Fluorescence curve of the riboswitch-Broccoli biosensor post amino acid stress. The negative control (RN4220 pCL55-iTET) (black), positive control (RN4220 pCL55-iTET-Broccoli) (pink) and biosensor-containing strain (RN4220 pCL55-iTET-riboswitch-Broccoli) (teal) were grown to ~OD600 0.3-0.4 in CDM in the presence of Atc 100 ng/ml. Cultures were split, washed twice in either complete CDM (closed symbols) or CDM-L/V (open symbols) with Atc 100 ng/ml, DFHBI-1T (200 μM) was added and cultures were incubated at 37 ˚C for 20 hours, with fluorescence measurements taken every 30 minutes. Fluorescence (at 485/520 nm) and OD600 were measured using a Hidex Sense Platereader. All experiments were performed once. |

### 3.4.3. Construction of a riboswitch-Broccoli molecular (p)ppGpp biosensor using a multicopy vector

As the riboswitch-Broccoli molecular (p)ppGpp biosensor in pCL55-iTET did not fluoresce, the design of the construct was reconsidered. pCL55-iTET is an integrative vector and thus, each bacterium will contain one copy of the plasmid, which may explain why low levels of fluorescence were observed. Accordingly, the riboswitch-Broccoli aptamer and the T7 terminator was amplified from the integrative vector and inserted into the multicopy vector pRN12 **(de Jong *et al.*, 2017b)**. This plasmid contains a constitutive promoter p*sarA*. SarA is the staphylococcal accessory regulator, a DNA-binding protein that regulates the expression of virulence factors **(Morrison *et al.*, 2012).** The advantage of a constitutive promoter is that an inducer is not necessary for transcription.

To construct the positive control and the biosensor, the Broccoli aptamer and T7 promoter, as well as the riboswitch-Broccoli aptamer were PCR-amplified from pCL55-iTET-Broccoli (primers RMC891/892) and pCL55-iTET-riboswitch-Broccoli (primers 893/894) respectively. These primers introduced a 5’ KpnI site and a 3’ EcoRI site into both products. These products were ligated to the pRN12 backbone that had been digested with KpnI and EcoRI and transformed into *E. coli* XL1 Blue. A positive colony PCR product was identified, and the plasmid was then electroporated into RN4220 Δ*spa* with PCR conducted to confirm the presence of the correct plasmid **(Figure 3.4.4c).** To construct the negative control, the pRN12 backbone was transformed into *E. coli* XL1 Blue to form the negative control. pRN12 was also electroporated into RN4220 Δ*spa* and the presence was confirmed via PCR on isolated plasmid DNA **(Figure 3.4.4d)**.

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| **Figure 3.4.4. Construction of the Broccoli positive control and the riboswitch-Broccoli molecular (p)ppGpp biosensor using an multicopy plasmid.** Plasmid maps of **a)** pRN12-Broccoli with the Broccoli fluorophore under the control of p*sarA* and **b)** pRN12-riboswitch-Broccoli with the riboswitch-Broccoli aptamer under the control of p*sarA*. p*sarA*; orange, mAmetrine; light green, Broccoli; green, riboswitch; peach, T7 terminator; light blue, CamR; chloramphenicol resistance in *S. aureus* (blue), AmpR; ampicillin resistance in *E. coli* (grey). **c)** PCR of plasmid DNA isolated from RN4220 pRN12-Broccoli (lanes 1-4) (543 bp) and RN4220 pRN12-riboswitch-Broccoli (lanes 5-7) (580 bp) to confirm presence of the plasmid. d) PCR on plasmid DNA isolated from RN4220 pRN12 (no mAmetrine) using primers RMC889/890 to confirm its presence. PCR products were run on 1% agarose gels. Ladder sizes are shown. |

#### 3.4.3.1 Live-cell characterisation – multicopy vector

To assess whether the multicopy biosensor construct was functional, a similar fluorescence assay was conducted as with the integrative vector biosensor. The biosensor-containing *S. aureus* strain and its relevant controls were grown in CDM in the presence of chloramphenicol to maintain the plasmid to mid-exponential phase and the culture was subject to two washes in complete CDM or CDM-L/V. DFHBI-1T was added to samples taken before and after washing in CDM-L/V and the cultures were incubated at room temperature for 30 minutes in the absence of light as DFHBI-1T is light-sensitive. Fluorescence measurements were taken post incubation as demonstrated in **Figure 3.4.5.** This protocol was developed to mimic a fluorescence microscopy protocol taken from **(Sun *et al.*, 2021)** where cells were imaged before and after being washed in minimal media. However here, while the positive control pRN12-Broccoli displayed higher levels of fluorescence, the riboswitch-Broccoli biosensor fluoresced similar to the negative control. The data from experiments conducted with both the integrative vector and multicopy vector suggests that while the Broccoli positive control may fluoresce, albeit at a lower level than expected, the biosensor construct itself does not using these methods, for reasons that have yet to be elucidated.

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| **Figure 3.4.5. The riboswitch-Broccoli molecular (p)ppGpp biosensor (multicopy plasmid) does not fluoresce in response to amino acid stress.** The negative control (RN4220 pRN12) (black), positive control (RN4220 pRN12-Broccoli) (pink) and biosensor (RN4220 pRN12-riboswitch-Broccoli) (teal) were grown to ~OD600 0.3-0.4 in CDM. Samples were taken before (closed symbols) and after (open symbols) washes in CDM-L/V (open symbols). DFHBI-1T (200 μM) was added and cultures were incubated at room temperature for 30 minutes with fluorescence measurements (485/520 nm) taken at 0, 15, 30 and 60 minutes post incubation using a Hidex Sense Platereader. This experiment was performed once. |

### 3.4.4. Construction of a dual promoter-reporter fusion

As transcriptional-reporter fusions have been constructed to measure (p)ppGpp levels via fluorescence previously **(Gawin *et al.*, 2019; Goormaghtigh *et al.*, 2018; Bartoli  *et al.*, 2020)**, a transcriptional-reporter fusion using the promoters of genes that are known to be regulated by (p)ppGpp, was developed. The promoters of *S. aureus* genes *ilvD* and *rpsO* were used to design a dual reporter plasmid **(Fig. 3.4.6a)***.* The *ilvD* gene encodes for a dihydroxy-acid dehydratase involved in branched-chain amino acid (BCAA) synthesis **(Horvatek *et al.*, 2020)** that is necessary during the SR when nutrients are depleted. In contrast, *rpsO* encodes for a 30S ribosomal protein S15 **(Horvatek *et al.*, 2020)** and it is well known that cellular translation decreases resulting in decreased growth during stressful conditions. Therefore when (p)ppGpp accumulation occurs, ribosomal proteins such as *rpsO* are classically downregulated due to the repression of ribosomal RNA promoters. The *ilvD* and *rpsO* promoters were fused to sGFP and dsRED respectively so that changes in fluorescence can be observed. This would couple promoter activity to fluorescence, which would serve as an indirect measure of intracellular (p)ppGpp levels.

Neither one of the promoter-reporter fusions alone may be sufficient to act as an output for (p)ppGpp detection individually. The benefit of using two different promoter-reporter fusions individually and using promoters opposingly regulated by (p)ppGpp, is that it will give a broader perspective of (p)ppGpp’s regulatory capacity as the increase (*ilvD*) and concurrent decrease (*rpsO*) in promoter activity in the presence of (p)ppGpp can be observed. To this end, the *ilvD* and *rpsO* promoters were amplified from *S. aureus* LAC\* genomic DNA and codon-optimised fluorophores sGFP and dsRED were amplified from RN4220 pGFP and RN4220 pRFP respectively. The *ilvD* and sGFP fragments were spliced to produce *ilvD*-sGFP.

As shown in **Figure 3.4.6a**, the fluorophores were designed to be oriented in a convergent manner as expression levels are higher when genes are convergent rather than tandem/divergent. This is due to the latter two types of expression creating supercoils during transcription **(Yeung *et al.*, 2017)**. With convergent expression, a transcriptional terminator (TT) is required downstream of the reporters to control transcription of each fluorophore, preventing read-through transcripts **(Cox, Dunlop and Elowitz, 2010)**.Thus, TT was amplified from an *E. coli* strain containing the pCN49 plasmid which contains a TT and was spliced onto the *ilvD*-sGFP fragment to produce *ilvD*-sGFP-TT**.** Similarly, to construct the *rpsO*-dsRED fragment, *rpsO* and dsRED were spliced.

The *ilvD­*-sGFP-TT and *rpsO*-dsRED fragments were designed such that they were flanked by EcoRI/KpnI and KpnI/BamHI restriction sites respectively **(Fig. 3.4.6a)**. These fragments and the integrative vector pCL55 were digested with their respective restriction enzymes, ligated to produce pCL55-P*ilvD*-sGFP-TT and pCL55-P*rpsO*-dsRED and transformed successfully into *E. coli* XL1-Blue.

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| **Figure 3.4.6. Construction of the dual promoter-reporter fusion plasmids. a)** Plasmid maps of the individual promoter-reporter fusions and the dual promoter-reporter fusion. The *ilvD* (light green) and *rpsO* (peach) promoters were translationally fused to the fluorophores sGFP (green) and dsRED (red) respectively by SOE PCR. A transcriptional terminator (TT) (blue) was also spliced on to the *ilvD*-sGFP fragment. Each promoter-reporter insert was cloned into pCL55 to make pCL55-P*ilvD*-sGFP-TT and pCL55-P*rpsO*-dsRED. pCL55-P*rpsO*-dsRED was digested with KpnI and BamHI to remove the insert for ligation into pCL55-P*ilvD*-sGFP-TT to make pCL55-P*ilvD*-sGFP-TT-P*rpsO*-dsRED. Plasmid-specific primers RMC006/007 were used to conduct PCR on genomic DNA extracted from lipase-negative clones of **b)** RN4220 pCL55-P*rpsO*-dsRED(1273 bp), **c)** RN4220 pCL55-P*ilvD*-sGFP-TT (1800 bp) and **d)** RN4220 pCL55-P*ilvD*-sGFP-TT-P*rpsO*-dsRED (2785 bp) to confirm integration into the lipase gene. PCR products were run on 1% agarose gels. Ladder sizes are shown. |

To produce the final dual promoter-reporter fusion, the *rpsO*-dsRED fragment was isolated from its parent backbone by digestion with BamHI and KpnI and the pCL55-P*ilvD*-sGFP-TT vector was also digested with the same restriction enzymes. Subsequent ligation and transformation enabled the construction of the dual promoter-reporter fusion pCL55-P*ilvD*-sGFP-TT-P*rpsO*-dsRED in *E. coli* XL1-Blue. Additionally, the presence of dsRED was clear as colonies appeared red on nutrient-rich media as expected as *rpsO* is constitutively expressed. The dual promoter-reporter plasmid as well as the individual promoter-reporter plasmids were isolated from *E. coli* and introduced to RN4220 Δ*spa* by electroporation. Genomic DNA from the RN4220 strains were extracted and plasmid insertion was confirmed via PCR **(Fig. 3.4.6b-d).**

#### 3.4.4.1. Characterisation of the dual promoter-reporter fusion

To examine whether there are transcriptional changes associated with amino acid stress, GFP and RFP fluorescence were measured over time. During amino acid stress and thus, the induction of the stringent response, the BCAA-associated *ilvD* gene is expected to be upregulated and the ribosomal *rpsO* is expected to be downregulated, therefore an increase in GFP fluorescence and a decrease in RFP fluorescence is anticipated. The dual promoter-reporter fusion and the relevant controls were exposed to complete CDM and CDM-L/V and both GFP and RFP fluorescence were measured. **Figure 3.4.7a** shows that the dual reporter exhibits an increase in green fluorescence over time in the absence of L/V after an initial decrease which could suggest that *ilvD* transcription is turned on after 5 hours. However, the negative control RN4220 pCL55 displays comparable fluorescence to the dual reporter and the same trend of lower fluorescence being observed for -L/V in comparison to +L/V is followed. In contrast, red fluorescence was similar for the dual reporter, regardless of the presence of L/V **(Fig. 3.4.7b)** which may be due to the 27-hour half-life of dsRED.

Mupirocin was then used as an alternative amino acid starvation inducer to determine whether a stronger transcriptional response could be observed. **Figure 3.4.7c** shows that the strains did not produce notable green fluorescence as demonstrated by negative values. Unexpectedly, the dual reporter showed increasing levels of red fluorescence over time in the absence of mupirocin and in the presence, levels increased until approximately 2.5 hours, after which it plateaued **(Fig. 3.4.7d)**. From this transcriptional analysis, it is undetermined whether the dual promoter-reporter fusion is able to respond to amino acid stress. This could be due to various reasons, for example, whether all promoter elements are present to allow for transcription in the presence of stress.

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| **Figure 3.4.7. Transcriptional activity of the dual promoter-reporter fusion during amino acid stress. a)** and **b)** show fluorescence over time for strains exposed to CDM +/- L/V. Dual promoter-reporter fusions and the relevant controls were grown to ~OD600 0.3-0.4 in CDM before splitting cultures, washing twice in complete CDM or CDM-L/V and measuring fluorescence over time. **a)** Green fluorescence of the dual promoter-reporter fusion in RN4220 (dual reporter; black), negative control (RN4220 pCL55; blue), the individual sGFP fusion positive control (pCL55-P*ilvD*-sGFP-TT; green) and the original sGFP positive control (RN4220 GFP; lime green) in complete CDM (+) and CDM-L/V (-) over 20 hours measured at 485/520 nm. **b)** Red fluorescence of the dual promoter-reporter fusion in RN4220 (dual reporter; black), negative control (RN4220 pCL55; blue), the individual dsRED fusion positive control (pCL55-P*rpsO*-dsRED; red), the original dsRED positive control (RN4220 RFP; peach) and the SH1000 positive control (SH1000 RFP; wine) in complete CDM (+) and CDM-L/V (-) over 20 hours measured at 575/610 nm. **c)** and **d)** show fluorescence over time for strains exposed to mupirocin. Dual promoter-reporter fusions and the relevant controls were grown to ~OD600 0.3-0.4 in CDM before adding mupirocin and measuring fluorescence over time. **c)** Green fluorescence of the dual promoter-reporter fusion in RN4220 (dual reporter; black), negative control (RN4220 pCL55; blue), the individual sGFP fusion positive control (pCL55-P*ilvD*-sGFP-TT; green) and the SH1000 positive control (SH1000 GFP; pastel green) in the presence of mupirocin 0 μg/ml (closed symbols) or 0.5 μg/ml (open symbols) over 20 hours measured at 485/520 nm. **d)** Red fluorescence of the dual promoter-reporter fusion in RN4220 (dual reporter; black), negative control (RN4220 pCL55; blue), the individual dsRED fusion positive control (pCL55-P*rpsO*-dsRED; red) and the SH1000 positive control (SH1000 RFP; wine) in the presence of mupirocin 0 μg/ml (closed symbols) or 0.5 μg/ml (open symbols) over 20 hours measured at 575/610 nm. n=1 for each experiment. |

### 3.4.5. Construction and characterisation of synthetase-reporter fusions

The dual promoter-reporter fusion biosensor employs an indirect measure of (p)ppGpp production as promoter activity is detected by a fluorescence output. The change in promoter activity determines how transcription is modified with the induction of the stringent response. In contrast, synthetase-reporter translational fusions act to investigate the expression of the synthetases rather than just transcription, so changes in fluorescence provide a more accurate insight into the necessity of each synthetase under a given condition.

To this end, synthetase-GFP fusions were constructed. Phage transduction was employed to insert plasmids pCL55-P*rel*-*rel*-GFP, pCL55-P*relP*-*relP*-GFP, pCL55-P*relQ*-*relQ*-GFP into the JE2 WT chromosome and insertions were confirmed via PCR **(Fig. 3.4.8a and b). Figure 3.4.8c** illustrates the architecture of the synthetase-GFP fusions in the integrative vector pCL55. (p)ppGpp production by Rel is post-translationally regulated by stalled ribosomes and therefore amino acid stress **(Haseltine and Block, 1973b)**, while *relP* and *relQ* are transcriptionally induced upon administration of cell wall-targeting antibiotics **(Geiger *et al.*, 2014)** among other stresses. Accordingly, the synthetase-GFP fusions were exposed to these stresses and the level of fluorescence was measured. In the presence of mupirocin, the *rel*-GFP synthetase fusion exhibited higher levels of fluorescence than the negative control **(Figure 3.4.8d)**. Both vancomycin and ampicillin (cell wall-targeting antibiotics) were able to induce expression of RelPand RelQ as demonstrated in **Figures 3.4.8e and d**, however ampicillin led to a more substantial increase in fluorescence. These results suggest that in response to the relevant stressors, the synthetase-GFP fusions are expressed and therefore fluoresce, providing insight into the levels of (p)ppGpp. These synthetase-GFP fusions can now be used to investigate when each synthetase is expressed, and therefore important, *in vivo*.

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| **Figure 3.4.8. Construction and characterisation of the synthetase-GFP fusions.** Primers specific for each plasmid (RMC006/087) were used to conduct PCR on genomic DNA extracted from **a)** JE2 pCL55-P*rel*-*rel*-GFP (lanes 1 and 3) (3665 bp) and **b)** JE2 pCL55-P*relP*-*relP*-GFP (lanes 1 and 2) (1946 bp) and JE2 pCL55-P*relQ*-*relQ*-GFP (lanes 3 and 4) (2208 bp). **c)** Plasmid map schematic of the synthetase-GFP fusions with P*x* representing the synthetase promoter, *X* representing the synthetase gene and GFP (green). The synthetase-GFP fusions JE2 pCL55-P*rel*-*rel*-GFP (green), JE2 pCL55-P*relP*-*relP*-GFP (blue), JE2 pCL55-P*relQ*-*relQ*-GFP (purple) and the negative control JE2 pCL55-iTET (black) were grown to ~OD600 0.3-0.4 in CDM before adding mupirocin (60 μg/ml) to the *rel*-GFP fusion **(d)** and vancomycin at 5X MIC (10 μg/ml) **(e)** and ampicillin at 5X MIC (640 μg/ml) **(f)** to the *relP/Q-*GFP fusions and measuring the fluorescence at 485/520 nm for 15 hours. PCR products were run on 1% agarose gels. Ladder sizes are shown. |

## 3.5. Discussion

This chapter outlines the construction and characterisation of genetic tools that are necessary to investigate the importance of (p)ppGpp and the SR in *S. aureus* pathogenicity. These include (p)ppGpp synthetase-deletion mutants, (p)ppGpp overproduction strains and a range of (p)ppGpp probes: riboswitch-based molecular (p)ppGpp biosensors, transcriptional promoter-reporter fusions and translational synthetase-reporter fusions.

The synthetase-deletion mutants were successfully characterised. A method to distinguish between WT and the (p)ppGpp0 was established (growth in the presence of 0.05 μg/ml mupirocin) which is an easy method of strain determination. The synthetase genes from the (p)ppGpp0 mutant were sequenced in order to verify the size and nature of the deletions which revealed the catalytic motifs that were absent **(Fig. 3.2.2e-g)**. Here, all deletions were silent and in-frame which decreases the likelihood of the mutation affecting downstream genes. Rel was thought to be essential as *rel* deletion leads to lethality **(Gentry *et al.*, 2000)**. Excess (p)ppGpp production leads to toxic accumulation and thus, *rel* deletion in a WT background is lethal due to lack of a functional hydrolase domain, and uncontrolled surplus (p)ppGpp produced by RelP and RelQ when in the absence of Rel **(Takada *et al.*, 2020; Gratani *et al.*, 2018)**.Therefore, in order to construct a (p)ppGpp0 mutant, *relP and relQ* must be deleted first to remove the essentiality of the HD domain, after which, *rel* can be deleted. (p)ppGpp0 mutants are able to grow similarly to wildtype in rich media **(Fig. 3.2.1b)** so the absence of (p)ppGpp does not affect growth until grown in nutrient-limited media due to bacteria being unable to mount a stringent response **(Gratani *et al.*, 2018)**. This highlights the importance of regulating cellular (p)ppGpp levels as both the absence of (p)ppGpp and toxic accumulation can have detrimental effects on growth. The deleted regions of the synthetases of the (p)ppGpp0 mutant utilised in this study was achieved by allelic exchange, deleting *relQ* first, then *relP* and *rel.* Producing a (p)ppGpp0 mutant with silent, in-frame deletions, is advantageous in comparison to marked deletions as the introduction of an antibiotic resistant cassette may lead to polar effects, affecting downstream genes **(Ishikawa and Hori, 2013)**. Additionally, the deletion of multiple genes is limited when using antibiotics due to the antibiotic markers available and due to the ability of *S. aureus* to be multi-drug resistant **(Kato and Sugai, 2011).**

In this chapter, the overproduction strain was initially designed to overproduce (p)ppGpp by taking advantage of the fact that truncating the CTD of Rel relieves the negative regulatory effects that the CTD has on the NTD **(Gratani *et al.*, 2018)**. Gratani and colleagues demonstrated that this then led to (p)ppGpp overproduction when the expression of the NTD was induced on an inducible plasmid **(Gratani *et al.*, 2018)**. In this study, as described in section 3.3, the same construct was produced and confirmed by sequencing. As reported by Gratani and colleagues, the construct displayed a growth defect in rich media and this phenotype was also observed in **Figure 3.3.3a**. Moreover Northern blot analysis revealed that induction of (p)ppGpp synthesis caused a decrease in ribosomal *rpsL* transcripts and an increase in amino acid transporter transcripts **(Gratani *et al.*, 2018)**, suggesting that (p)ppGpp is in fact being overproduced by this construct. While a growth defect shown in **Figure 3.3.3a-d** suggested that the (p)ppGpp0 pCL55-iTETr862-NTD-his-ΔHD overproduction strain did overproduce (p)ppGpp, phenotypic analysis and analysis of nucleotide pools by TLC confirmed that this was not the case. It could be that the amount of (p)ppGpp produced was below the limit of detection using this method **(Fig. 3.3.3e-g)**. Perhaps measuring (p)ppGpp production via TLC is not a sensitive enough method for this strain and using HPLC instead would be a more accurate method of for nucleotide detection. Thus, characterisation of existing laboratory strains and construction of WT strains with an additional copy of each synthetase has led to the creation of a panel of (p)ppGpp overproduction strains that can be used for both *in vitro* and *in vivo* studies **(Fig. 3.3.4)** including JE2 pALC2073-*relQ* and JE2 pCL55-iTETr862-*rel/relP/relQ***.**

(p)ppGpp overproduction has previously been documented to occur naturally in a clinical MRSA isolate with an F128Y amino acid substitution leading to an impairment of the HD domain **(Gao *et al.*, 2010)**, which contributed to the persistence of this strain.In contrast, initially, this chapter detailed the production of a (p)ppGpp overproduction construct cloned into an inducible plasmid. This method of (p)ppGpp overproduction was chosen rather thanmimicking the F128Y mutation from the clinical isolate because but this type of mutation would not only require allelic exchange, which is a lengthy process, but a resistance cassette cannot be utilised as the mutation is a single amino acid substitution. A lack of an antibiotic resistance marker would add additional difficulties to identifying mutated strains during the process of strain construction. Additionally, in comparison to the construct designed by Gratani and colleagues, (p)ppGpp production from an F128Y substitution strain cannot be controlled, however, (p)ppGpp overproduction from this strain would be more representative of what has occurred naturally.

Another focus of this chapter was to design and construct various (p)ppGpp probes to gain insight into how the production of (p)ppGpp changes during nutrient limitation. As current gold-standard methods to measure (p)ppGpp include HPLC and TLC which can be costly, the riboswitch-based molecular biosensor, the promoter-reporter fusion and the synthetase-GFP fusions were constructed. Methods such as HPLC/TLC also measure (p)ppGpp concentrations *in vitro*, requiring cell lysates to measure cellular (p)ppGpp levels, rather than in real-time within bacteria. Whilst the probes created were confirmed to contain the expected sequences which was examined by sequencing, only the synthetase-reporter fusions responded to their respective stressors as highlighted by increased fluorescence in comparison to controls **(Fig. 3.4.8d-f).**

In this study, the Broccoli RNA aptamer was fused to the *ykkc* riboswitch which, in the presence of (p)ppGpp and DFHBI-1T, should fluoresce. Riboswitches have been utilised as molecular biosensors for their ability to bind small ligands such as nucleotides including cyclic-di-AMP and cyclic-di-GMP **(Syed *et al.*, 2020; Kellenberger *et al.*, 2015; Wang, Wilson and Hammond, 2016)** but also (p)ppGpp **(Sun *et al.*, 2021; Sherlock, Sudarsan and Breaker, 2018)**. While riboswitches have been used in *S. aureus* to measure levels of cyclic-di-AMP **(Syed *et al.*, 2020)**, this has not been achieved for (p)ppGpp. This could be because of the recent characterisation of the *ykkc* riboswitches **(Sherlock, Sudarsan and Breaker, 2018)**, that has led to the successful use of a riboswitch-based molecular biosensor in *E. coli* **(Sun et al., 2021)**. Therefore, the need for a riboswitch-based method of quantifying *S. aureus* cellular (p)ppGpp levels is necessary given its role in virulence and pathogenesis.

Many types of RNA aptamers exist including Spinach and Mango however, the Broccoli RNA aptamer was utilised as the construct was available as reported by Sun and colleagues. The RNA aptamer Spinach was initially used to tag RNAs **(Paige, Wu and Jaffrey, 2011)** until the development of Spinach2 which is more fluorescent than Spinach and folds more efficiently **(Strack, Disney and Jaffrey, 2013)**. Broccoli was then identified as an RNA tag that was superior to Spinach2, with multiple improvements including increased thermostability as well as an increased brightness during fluorescence **(Filonov *et al.*, 2014)**. While studies have identified other RNA tags such as Mango for use in mammalian cells **(Autour *et al.*, 2018; Cawte, Unrau and Rueda, 2020)**, green RNA tags such as Spinach and Broccoli have been used mainly for measuring nucleotide levels within bacteria.

*In vitro* transcription assays revealed that the riboswitch-Broccoli molecular (p)ppGpp biosensor was functional **(Fig. 3.4.3a)**, however this was not the case when fluorescence was measured in live cells. This then brings into question the functionality of the riboswitch-Broccoli aptamer within bacteria. It is important to note that the *ykkc* subtype 2a riboswitch sequence was taken from *T. oceani,* a Gram-negative and thermophilic organism isolated from deep sea sediment. The *T. oceani* riboswitch may not function in *S. aureus* as it would in its native species, which may explain the results observed in **Figures 3.4.3b**-**d**. For example, Gratani and colleagues showed that under nutrient-rich conditions, Rel*Sau*-NTD favoured hydrolase activity in *S. aureus* however when expressed in non-native *E. coli,* synthetase activity was favoured. Similarly, as the NTD of Rel behaved differently when expressed in *S. aureus* and *E. coli*, it may be that heterologous expression of the *ykkc* riboswitch in *S. aureus* may results in the riboswitch motif not functioning in the way that it would in its native organism. Sun and colleagues used this riboswitch-based biosensor in *E. coli,* a Gram-negative organism, which may explain why (p)ppGpp production was detected, while this was not the case in *S. aureus*. DFHBI-1T **(Fig. 3.4.1e)** is a small compound and has previously been used to detect (p)ppGpp in Gram-negative *E. coli* **(Sun *et al.*, 2021)** and Gram positive *S. aureus* **(Syed *et al.*, 2020)** and *L. monocytogenes* **(Kellenberger et al., 2015)** and so it is unlikely that the compound was unable to pass the cell wall and membrane, so low fluorescence levels are perhaps not attributed to this.

Sun and colleaguesdetected (p)ppGpp using the riboswitch-based molecular biosensor using fluorescence microscopy by imaging live cells before and after amino acid stress. A platereader was used in this study **(Fig. 3.4.2-3.4.5)** however, microscopy would be a superior method of visualisation in comparison to a platereader as OD600 readings do not need to be taken for normalisation, and if the constructs are functional, fluorescence can easily be seen at a single-cell level. Fluorescence microscopy is also more sensitive for the detection of the different reporters and may be more suitable than a platereader. For the riboswitch-Broccoli molecular biosensor, imaging cells before and after nutrient-limitation will allow the detection of changes in fluorescence. For the dual reporter biosensor, timelapse microscopy could allow visualisation of transcriptional changes over time by measuring changes in fluorescence of the promoter-reporter fusion. This could reveal when each promoter is upregulated.

The dual promoter-reporter fusion was designed to use two different fluorophores controlled by promoters that respond to (p)ppGpp in order to monitor changes in cellular (p)ppGpp levels. The constitutive *rpsO* promoter was functional on the pCL55 vector as positive clones containing the dual promoter-reporter fusion appeared as red colonies. Generally, the fluorescence assays conducted were unable to show the expected changes in fluorescence under nutrient limitation **(Fig. 3.4.7)**. As the *ilvD* and *rpsO* promoters were both some of the most highly up- and downregulated promoters respectively during the staphylococcal stringent response **(Geiger *et al.*, 2012; Horvatek *et al.*, 2020)**, these promoters were expected to respond accordingly to stress. As the stringent response decreases translation as part of the characteristic growth defect phenotype, the *rpsO* promoter is expected to be downregulated at this time. However, as the *rpsO* promoter is constitutively active until the induction of the stringent response, the decrease in *rpsO* transcription does not reduce the amount of dsRED already expressed within the cell and therefore, red fluorescence will likely stay constant. The half-life of the red fluorophore used, dsRED.T3, has not yet been established however, the half-life of dsRED1 is reported as approximately 4.6 days **(Verkhusha *et al.*, 2003)**. This suggests that the red fluorescence levels may stay constant, rather than decrease in the time period that was investigated, indicating that this fluorophore is not suitable for detecting transient changes in promoter activation. Furthermore, the dual promoter-reporter fusion was designed so that the fluorophores were expressed in a convergent manner which ultimately avoids DNA supercoils in comparison to tandem/divergent expression **(Yeung *et al.*, 2017)**. To prevent read-through transcripts as a result of convergent expression, TTs were employed which act to properly terminate the transcription of each fluorophore. Previous studies have utilised multiple TTs in a transcriptional fusion that employed three fluorophores **(Gawin *et al.*, 2019; Cox, Dunlop and Elowitz, 2010).** These reports incorporated three or more TTs between fluorophores while this study only utilised one **(Fig. 3.4.6a)**. It may be that transcriptional termination requires more than one TT. This construct may have also benefited from a multicopy vector instead of an integrative vector. An integrative vector was used for stability as the plasmid does not require maintenance using antibiotics, which is important to consider for use *in vivo* within zebrafish embryos. However, a multicopy vector would maximise fluorescence levels in comparison due to multiple copies being present. It is unclear at this point why the dual reporter biosensor does not fluoresce, but this is likely due to the design of the construct.

In summary, this chapter has detailed the rationale, characterisation and production of tools that can be used to investigate the importance of the stringent response in the pathogenicity of *S. aureus*. (p)ppGpp synthetase-deletion mutants have been characterised and (p)ppGpp overproduction strains were constructed for use within infection models. The construction of various (p)ppGpp probes was attempted, however not all were successful. While the (p)ppGpp synthetase-GFP translational fusions demonstrated synthetase activity upon induction, the expected fluorescence levels were not detected from the riboswitch-based molecular (p)ppGpp biosensor and the dual reporter transcriptional promoter-reporter fusions. This highlights the challenges of constructing molecular biosensors. Further work is required in order to develop (p)ppGpp probes in Gram-positive organisms.

# Chapter 4: The role of (p)ppGpp for survival of *S. aureus in vitro*

## 4.1. Introduction

*S. aureus* was long thought to be an extracellular pathogen **(Finlay and Cossart, 1997)** until it was revealed that staphylococci are in fact able to survive within the intracellular milieu of PMNs as well as extracellularly within the body **(Gresham *et al.*, 2000)**. Whilst intracellular, within both professional and non-professional phagocytes, staphylococci are met by various insults within the phagolysosome including but not limited to low pH, reactive oxygen/nitrogen species and antimicrobial peptides provided by the host immune system **(Horn *et al.*, 2018)**. Immune cells and their killing mechanisms act to eliminate bacteria to prevent infection and dissemination however, *S. aureus* amongst other pathogens, are able to resist killing and survive using their own defence mechanisms. For example, acidification of *S. aureus-*containing phagolysosomes is detected by the GraXRS regulatory system which results in the development of resistance to AMPs **(Flannagan *et al.*, 2018)**. The production of toxins also contribute to intracellular survival including cytolysins such as the PSMs that lyse many cell types including PMNs **(Cheung *et al.*, 2014)**. Additionally, *S. aureus* produces enzymes such as AhpC, and catalase **(Cosgrove *et al.*, 2007)** and superoxide dismutase **(Karavolos *et al.*, 2003)** for peroxide/H2O2 and superoxide detoxification respectively. Using these defence mechanisms, bacteria that are able to survive and persist within the hostile intracellular environment until conditions become favourable, can subsequently multiply and escape, leading to dissemination and worsening of infection **(Kubica *et al.*, 2008)**.

As intracellular bacteria are exposed to many stressors as well as nutritional limitation, the stringent response is likely to be one of the pathways required for survival within professional phagocytes. For example, the contribution of the stringent response for persister formation has been demonstrated as the absence of RelA reduced the ability of *E. coli* to form ampicillin and ofloxacin persisters *in vitro* **(Amato and Brynildsen, 2015).** Furthermore, many stringent response-mediated molecular mechanisms that lead to persistence have been studied including toxin-antitoxin systems **(Schumacher *et al.*, 2015)** and dimerisation of ribosomes **(Song and Wood, 2020)**. The only known study implicating the role of the stringent response for survival of *S. aureus* within macrophages was conducted by Peyrusson and colleagues, who found that the load of antibiotic-induced persister colonies were reduced in a *rel*-negative background and an additional decrease in load was observed in the absence of both *rel* and *codY.* This study suggested that the stringent response was one of the pathways involved in the development of persistence. Additionally, *in vitro* work has shown that the stringent response is necessary for the upregulation of PSMs in a Rel-dependent manner **(Geiger *et al.*, 2012)** with Rel also implicated in responding to oxidative stress **(Horvatek *et al.*, 2020)**. Thus, the stringent response, in particular Rel, is important for survival of bacteria within macrophages and PMNs, with persistence being the most studied phenomena. As phagocytes such as neutrophils and macrophages are the first line of defence against infection and can be an intracellular niche for the growth of *S. aureus*, it is necessary to further investigate the role of the staphylococcal stringent response in pathogenesis and survival. This chapter investigates how the stringent response is important for *S. aureus* survival by examining tolerance in the presence of stressors that mimic the conditions within a phagolysosome using *in vitro* tolerance assays.

## 4.2. *In vitro* investigation of the importance of the stringent response under conditions that mimic the phagolysosome

Upon internalisation by phagocytosis, *S. aureus* are encapsulated in a phagosome which matures and fuses with a lysosome **(Pidwill *et al.*, 2021)**. This fusion process allows the formation of a cytoplasmic body, named the phagolysosome, containing many antimicrobial components including: v-ATPase to lower pH, AMPs, antimicrobial metabolites, various degradative enzymes and ROS-producing enzymes **(Pidwill *et al.*, 2021).** These host defence mechanisms target different components of the bacterial cell, allowing for its destruction and subsequent antigen presentation at the surface of various immune cells **(Poirier and Av-Gay, 2015)**.

### 4.2.1. The effect of antimicrobial peptides on the viability of *S. aureus* stringent response mutants

AMPs are important components of the host immune defence against phagocytosed pathogens and their main function is to damage microbial cell membranes. This is achieved by their net positive charge that enables interactions with the negatively charged cell membrane and usually culminates in either membrane disruption or cell lysis **(Pidwill *et al.*, 2021)**. A role for the stringent response in the defence against AMPs has been examined in *E. coli* O157:H7, where in response to exposure to amphibian-derived AMPs, there was an increase in the transcription of the long RSH, *spoT* **(Scotti et al., 2022)***.* It was suggested that AMPs interact with and degrade (p)ppGpp **(de la Fuente-Nunez *et al.*, 2014)**, so investigating the role of other AMPs with regards to the stringent response and *S. aureus* could yield interesting results. As the amphibian-derived AMPs are cationic, similar to those found in humans, the contribution of the stringent response to resistance to AMPs was investigated.

Accordingly, the stringent response mutants ∆*relQP*, (p)ppGpp0 and JE2 *codY*::Tnwere exposed to the cationic AMPs, LL-37 and polymyxin B. RPMI medium was used for these experiments so that growth conditions were more representative of the host environment in comparison to rich media such as TSB, as RPMI is formulated for the culture of, and primarily used for, human lymphocytes. LL-37 is the sole member of the human cathelicidin family of AMPs, a cationic peptide that functions by interacting with and penetrating the negatively charged bacterial cell membrane, culminating in bacterial cell lysis **(Kuroda *et al.*, 2015; Kahlenberg and Kaplan, 2013)**. In *B. subtilis*, it has been suggested that RelP can be induced upon administration of LL-37 **(Pietiäinen *et al.*, 2005)**, therefore the effect of the LL-37 on *S. aureus* WT and (p)ppGpp0 survival were evaluated. LL-37 cathelicidin functions optimally at low pH, and thus RPMI was buffered to pH 5.5 but was also used at pH 7.5. At pH 5.5, a concentration of 12 μM **(Chen *et al.*, 2005)** prevented growth of all strains while the absence of LL-37 allowed growth **(Fig. 4.2.1a)**. In contrast, the microbicidal effects of LL-37 were abrogated in the presence of RPMI buffered to pH 7.5 which has previously been reported **(Chen *et al.*, 2005) (Fig. 4.2.1b)**. Here, differences between WT and the (p)ppGpp0 mutant were observed, however as this neutral pH is not the appropriate condition with which LL-37 should function, these results were disregarded **(Fig. 4.2.1b)**. As the trend in OD600 for both 0 and 12 μM is similar at neutral pH, it is likely that this is due to growth of *S. aureus* strains in RPMI and this growth defect has been reported **(Carrilero *et al.*, 2023)**. These results confirm that LL-37 functions optimally at low pH and suggests that 12 μM is too great a concentration to elucidate the role of the stringent response in the survival of *S. aureus* in the presence of LL-37.

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| **Figure 4.2.1. Antimicrobial activity of 12 μM LL-37 and 32-64μg/ml polymyxin B against *S. aureus* reveals no difference in growth between WT, stringent response mutants and *codY*::Tn*.*** *S. aureus* strains JE2 WT, Δ*relQP,* (p)ppGpp0and *codY*::Tnwere grown in buffered RPMI 1640 medium from a starting OD600 of 0.05 and endpoint OD600 measurements were taken at 24 hours. Growth in the presence of 0 and 12 μM LL-37 at pH 5.5 **(a)** and pH 7.5 **(b)** and 0, 32 and 64 μg/ml polymyxin B at pH 5.5 **(c)** and pH 7.5 **(d)**. Experiments with LL-37 and polymyxin B were performed in duplicate from which mean values ± standard deviations were plotted. |

*S. aureus* was also exposed to polymyxin B at acidic and neutral pH. Polymyxin B is a polypeptide antibiotic in the same antibiotic family as colistin (polymyxin E) – both are produced by soil bacterium *Paenibacillus polymyxa*. Polymyxins target both the outer and inner membrane of Gram-negative bacteria **(Poirel, Jayol and Nordmann, 2017; Sabnis *et al.*, 2021)**. The α,γ-diaminobutyric acid of polymyxin B interacts with lipid A of LPS, leading to LPS destabilisation and subsequent cell membrane permeabilisation, resulting in cell lysis **(Poirel, Jayol and Nordmann, 2017)**. While polymyxins are known to classically target Gram-negative bacteria and not Gram-positive bacteria due to interactions with LPS, the antimicrobial activity of polymyxin B has been demonstrated against *S. aureus* **(Boyen *et al.*, 2012; Yoshida and Hiramatsu, 1993; Chang *et al.*, 2015).** Furthermore, inhibition of ATP synthase sensitises *S. aureus* to the polymyxins **(Liu *et al.*, 2020; Vestergaard *et al.*, 2017).** As polymyxins have a similar structure to cationic AMPs such as LL-37 **(Poirel, Jayol and Nordmann, 2017)** and have previously been efficacious against *S. aureus*, the involvement of the stringent response for defence against polymyxins was investigated. Unlike LL-37, polymyxin B is efficacious at neutral pH and does not require low pH to exert its antimicrobial effects, as demonstrated by growth at 0, 32 and 64 μg/ml **(Fig. 4.4.1c)**. However, at pH 7.5, both 32 and 64 μg/ml polymyxin B inhibited growth for all strains equally **(Fig. 4.4.1d),** suggesting that both concentrations of polymyxin B are not able to aid in distinguishing whether the stringent response has a role in evading killing by polymyxins, and that concentrations between 0 and 32 μg/ml must be further investigated.

### 4.2.2. The effect of low pH on the viability of *S. aureus* stringent response mutants

The decrease in pH associated with the maturation of the phagolysosome is required as part of the array of insults used by professional phagocytes to combat intracellular bacteria. The main contributor to this low pH is the proton-pumping v-ATPase, present on the membrane of endosomes and lysosomes and therefore, phagolysosomes, and is responsible for maintaining an adequate proton gradient **(Sun-Wada *et al.*, 2009; Westman and Grinstein, 2021)**. Acidic pH also functions to enhance the efficacy of the LL-37 cathelicidin, as demonstrated in **Figure 4.4.1a and b**, and the cathepsin proteases **(Pidwill *et al.*, 2021)**. While low pH is classically associated with being a host defence, *S. aureus* is known to utilise the gradual decrease in pH as a cue to upregulate virulence e.g. the resistance to AMPs **(Flannagan *et al.*, 2018).** Here, the GraXRS regulatory system is a sensor of low pH, allowing the evasion of *S. aureus* killingwithin an acidified phagolysosome, allowing replication *in vivo* **(Flannagan, Heit and Heinrichs, 2016; Flannagan *et al.*, 2018)**.

A previous study has demonstrated that ppGpp aids the dimerisation of transcription factor SlyA in *S.* Typhimurium, which ultimately leads to resistance to low pH **(Zhao *et al.*, 2008)**, however another study has opposed this view by demonstrating that SlyA dimerisation was not affected by ppGpp levels **(Bartoli  *et al.*, 2020)**. Furthermore, *Helicobacter pylori*, which was previously thought to lack the ability to mount a stringent response **(Scoarughi, Cimmino and Donini, 1999)**, produced (p)ppGpp upon exposure to low pH and was also associated with decreased viability in comparison to WT **(Wells and Gaynor, 2006; Mouery *et al.*, 2006)**. In *E. coli*, the lysine decarboxylase LdcI, which eventually acts to increase pH, directly binds to (p)ppGpp in response to a decrease in extracellular pH **(Kanjee *et al.*, 2011)**. In addition to this, DksA binds to *E. coli* RNAP in a pH-dependent manner, which led to the suggestion that DksA could be a pH sensor **(Furman *et al.*, 2015)**.While studies have shown that Gram-negative bacteria mount an stringent response to acid stress, it is unclear whether the stringent response responds directly to acid stress in Gram-positive bacteria based on the lack of evidence.

Thus, to understand whether the stringent response has a role in dealing with acid stress in *S. aureus*, WT, the (p)ppGpp0 mutant and *codY*::Tnwere grown in the presence of TSB buffered to pH 4.5 and 7.5 with the weak acid maleic acid (tris-maleate buffer) **(Flannagan *et al.*, 2018) (Fig. 4.2.2)**. At pH 4.5, the (p)ppGpp0 mutant demonstrated a modest but significant growth defect, while *codY*::Tndisplayed a slight growth defect in comparison to WT with significant differences observed only during mid-late exponential phase **(Fig. 4.2.2a and b)**. In comparison, at pH 7.5, both the WT and the (p)ppGpp0 mutant grew similarly **(Fig. 4.2.2d)** as shown in **(Fig. 3.2.1b).** Differences were only observed between WT and *codY*::Tnat 4 and 6 hours, with growth being restored to WT levels during stationary phase **(Fig. 4.2.2e)**. There were no differences in growth between the (p)ppGpp0 mutant and *codY*::Tnat either pH **(Fig. 4.2.2c and f)**. To further examine the effect of low pH on these strains, the Biolog Phenotype MicroArray PM10 plate (which uses glutaric acid to lower pH and triethanolamine to raise the pH) was utilised to identify any differences in growth between WT and the (p)ppGpp0 mutant between pH 3.5 and 7 **(Fig. 4.2.3)**. As demonstrated by the plate map, the PM10 plate consists of 96 wells containing lyophilised reagents that once resuspended with bacterial culture, cultures with different pH. While 96 different conditions were tested, there was no observable phenotypic differences between the JE2 and (p)ppGpp0 mutant-inoculated platesexcept for well G4 (data not shown). However, as the pH values of 3-7 were of interest in this study, only those were considered. **Figures 4.2.4a and b** demonstrate both WT and the (p)ppGpp0 mutant separately, which seem to grow similarly at each pH studied, with growth ceasing at pH values below 5. This is confirmed when growth at pH 5 and 5.5 are considered in isolation as the cultures grown at these pH values displayed growth defects (not as extensive as pH 4.5 and below), but no difference between the strains was detected **(Fig. 4.2.4c)**. While a growth defect was detected when the WT and the (p)ppGpp0 mutant were exposed to acidic conditions in **Figure 4.2.2**, this was not evident in **Figure 4.2.4** using the Biolog Phenotype MicroArray PM10 plate. This may be due to differences in methodology between the experiments, as well as because the growth curve using the Biolog Phenotype MicroArray PM10 plate was performed once only.

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| **Figure 4.2.2*.* The (p)ppGpp0 mutant displays a slight growth defect during acid stress.** Growth of the *S. aureus* JE2 WT, (p)ppGpp0 and *codY*::Tnin TSB medium buffered to pH 4.5 **(a)** and 7.5 **(b)** in 0.1 M tris-maleate from a starting OD600 of 0.05 for 20 hours in a Hidex Sense platereader. Growth curves in **(a-c)** and **(d-f)** were performed in duplicate with mean values ± standard deviations plotted. Statistical analysis was performed using two-way ANOVA with Šídák's multiple comparisons test and mean values ± standard deviations were plotted. \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* < 0.001, \*\*\*\* *P* < 0.0001. |

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| **Fig. 4.2.3. The Biolog Phenotype MicroArray PM10 plate map.** Each well in the plate contained chemical compounds that allowed the interrogation of different pH effects, which when reconstituted with 1X inoculating fluid, gave rise to the condition that each well is labelled. |

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| **Figure 4.2.4. Growth of JE2 and the (p)ppGpp0 mutant under different pH in a Biolog Phenotype MicroArray PM10 plate.** Biolog Phenotype MicroArray PM10 plates were used to grow **(a)** JE2 WT and **(b)** the (p)ppGpp0mutant in the presence of pH ranging from 3.5 to 7 which employed glutaric acid to lower the pH and triethanolamine to raise the pH. **c)** represents the pH 5 and 5.5 growth curves from **a)** and **b)** to visualise any differences more clearly. The inoculating fluid was made up to 12 ml with 1.2X IF-10b, 100X Dye mix H, 12X PM additive solution, ddH2O and 13.64X bacterial culture (13.64X bacterial culture had an OD600 value of 0.1/81% transmittance) and was added at 100 μl/well. Growth was measured at OD590 for 20 hours in a Hidex Sense platereader. Growth curves were performed once. |

The growth of WT, the (p)ppGpp0 mutant and *codY*::Tnunder acidic conditions was investigated over a 20-hour period in **Figure 4.2.2** and **4.2.4.** Next, the tolerance of WT and the (p)ppGpp0 mutant to itaconic acid was studied to elucidate whether there was a requirement for (p)ppGpp for survival.Itaconic acid, a weak organic acid, is produced by immune response gene 1 (*IRG1*) which encodes for aconitate decarboxylase, an enzyme that converts aconitic acid to itaconic acid. It can be found in phagolysosomes and exists at physiological concentrations of approximately 55-120 pM **(Singh *et al.*, 2021)**. Studies have shown that itaconic acid can exert its antimicrobial effects by inhibiting isocitrate lyase, a major component of the glyoxylate shunt which is an important pathway for optimal growth in bacteria, and has been associated with responding to ROS stress **(Zhu *et al.*, 2021; Ahn *et al.*, 2016; Lorenz and Fink, 2001)**. Itaconic acid also has immunomodulatory effects. For example, the charged conjugate base itaconate acts to reduce inflammation during ocular infection by modulating NRF2/HO1 signalling and inhibiting the NLRP3 inflammasome **(Singh *et al.*, 2021).** Thus, the involvement of the stringent response in *S. aureus* tolerance to itaconic acid was investigated.

As *Legionella pneumophila* is reported to be susceptible to 5 mM itaconic acid *in vitro*, the tolerance of *S. aureus* was trialled at 5, 10, 15, 20 and 30 mM. WT *S. aureus* was grownto early exponential phase, washed in PBS and exposed to the aforementioned concentrations of itaconic acid, after which samples were taken for CFU/ml determination. While 5 and 10 mM itaconic acid did not demonstrate bactericidal activity with regards to *S. aureus*, 20 mM was the optimal concentration to visualise the difference in killing between WT and the (p)ppGpp0 mutant and 30 mM was deemed too high a concentration (data not shown).Thus, the WT and (p)ppGpp0 mutant were exposed to 20 mM itaconic acid for 1 hour and the CFU/ml was determined **(Fig. 4.2.5).** Fewer (p)ppGpp0 colonies were consistently recovered and thus, percentage bacterial survival was reduced in comparison to WT, suggesting that the absence of the stringent response decreases tolerance of *S. aureus* to itaconic acid. As itaconic acid is a weak acid, its ability to decrease the pH was examined. **Table 5** shows that after the addition of 20 mM itaconic acid to PBS containing *S. aureus*, the pH decreases from 6 to 4 and that this change in pH is constant for 1 hour after addition of the acid. Both the WT and (p)ppGpp0 mutant were also exposed to PBS only, which confirmed that killing over the course of the assay was due to itaconic acid as CFU/ml were constant from T=0 to T=1 hour (data not shown). As previously mentioned, itaconic acid has immunomodulatory functions as well as being able to decrease in pH. To determine whether microbicidal activity was due to the decrease in pH of bacterial culture or by another mechanism, *S. aureus* was exposed to 20 mM itaconic acid that was neutralised to pH 7.5. **Figure 4.2.6** demonstrates that the neutralisation of itaconic acid abrogates its ability to kill *S. aureus*, while itaconic acid at pH 2.15 shows substantial killing by 30 minutes. Therefore, the decrease in pH that is achieved by itaconic acid is necessary for its killing activity in this experiment. The immunomodulatory effects of itaconic acid could not be investigated here as this experiment was performed *in vitro* and *in vivo* studies are required to further understand how itaconic acid affects the host.

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| **Figure 4.2.5. Tolerance of the (p)ppGpp0 mutant to itaconic acid stress is reduced in comparison to WT.** JE2 WT (black) and (p)ppGpp0 (pink) were grown to early exponential phase (approximately OD600 0.35) in tryptic soy broth (TSB) and were subsequently washed twice in 1 x PBS. Itaconic acid was added at a concentration of 20 mM and the cultures were incubated at 37 ˚C for 1 hour after which samples were taken for CFU/ml determination. Percentage bacterial survival with mean and standard deviation plotted. Statistical analysis performed using Mann-Whitney test. \*\* *P <* 0.01. |

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| **Table 5. pH of bacterial culture used for tolerance assays in the presence of 20 mM itaconic acid and neutralised itaconic acid.** | |
| **Condition** | **pH** |
| Culture in PBS | 6 |
| Itaconic acid stock solution | 2.15 |
| Culture in PBS + itaconic acid | 4 |
| Culture in PBS + itaconic acid after 1 hr incubation | 4 |
| Neutralised itaconic acid stock | 7.5 |
| Culture + neutralised itaconic acid | 7 |
| Culture + neutralised itaconic acid after 1 hr incubation | 7 |

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| **Figure 4.2.6. The antimicrobial effects of itaconic acid are due to its ability to decrease the pH of bacterial culture.** JE2 WT and (p)ppGpp0 were grown to early exponential phase (approximately OD600 0.35) in tryptic soy broth (TSB) and were subsequently washed twice in 1 x PBS. 20 mM itaconic acid/itaconate was added and the cultures were incubated at 37 ˚C for 0.5 hours with samples taken at the specified timepoints. |

### 4.2.3. Tolerance of the JE2 WT and (p)ppGpp0 mutant to reactive oxygen species

The phagolysosome contains many sources of ROS that are responsible for oxidative stress, which act to eliminate phagocytosed pathogens by damaging cellular components such as DNA, RNA and lipids **(Fasnacht and Polacek, 2021)**. The phagolysosomal membrane contains the multi-subunit NADPH oxidase, an enzyme responsible for producing superoxide anions (O2-) that ultimately results in the oxidative burst. Superoxide anions can be converted to H2O2 by SOD and H2O2 can be converted to both HOCl and OH- by MPO via the Fenton reaction **(Pidwill *et al.*, 2021).** H2O2 can be found at physiological concentrations of approximately 2-4 μM in professional phagocytes **(Winterbourn *et al.*, 2006)**, while HOCl can be found at higher concentrations of 20-400 μM **(King, Jefferson and Thomas, 1997)**. The oxidative burst produced by the phagolysosome is one of the most important host defence mechanisms employed by professional phagocytes **(Dupre-Crochet, Erard and Nubetae, 2013)** and plays an integral role in *S. aureus* clearance **(Surewaard *et al.*, 2016)**.

An MSSA (p)ppGpp0 mutant has previously exhibited susceptibility to H2O2 by incubating *S. aureus* with different concentrations of H2O2,and measuring growth over time via growth curves **(Horvatek *et al.*, 2020)**. Furthermore, an MRSA (p)ppGpp0 mutant in the stationary growth phase *S. aureus* was shown to display a reduced tolerance to HOCl **(Fritsch *et al.*, 2020)**. These studies demonstrate the involvement of the stringent response in surviving ROS stress within the phagolysosome. To investigate this in a JE2 MRSA background, *S. aureus* WT and the (p)ppGpp0 mutant during the exponential growth phase were exposed to H2O2 and HOCl and tolerance was quantified via percentage bacterial survival. Similar to itaconic acid, the (p)ppGpp0 mutant demonstrated a reduced tolerance to both ROS stressors **(Fig. 4.2.7)**. The tolerance of H2O2 was examined during the stationary growth phase, however an inhibitory concentration of H2O2 could not be determined – exponential phase *S. aureus* was inhibited by 100 mM H2O2 however stationary phase bacteria were able to withstand concentrations of 1 M (data not shown). Additionally, while exponential phase *S. aureus* were susceptible to HOCl at 32 μM, stationary phase bacteria were inhibited at concentration approximately 10 times this value (data not shown). In keeping with previous results, the (p)ppGpp0 mutant is more susceptible to H2O2 and HOCl, further emphasising that the stringent response plays a role in dealing with ROS stress.

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| **Figure 4.2.7. Tolerance of the (p)ppGpp0 mutant to reactive oxygen species found within professional phagocytes is reduced in comparison to WT.** JE2 WT (black) and (p)ppGpp0 (pink) were grown to early exponential phase (approximately OD600 0.35) in TSB and were subsequently washed twice in 1 x PBS. **(a)** Hydrogen peroxide (H2O2)was added at 100 mM and **(b)** hypochlorous acid (HOCl) at 32 μM and the cultures were incubated at 37 ˚C for 1 hour after which samples were taken for CFU/ml determination. Percentage bacterial survival with mean and standard deviation plotted. Statistical analysis performed using Mann-Whitney test. *\* P <* 0.05, \*\* *P <* 0.01. |

### 4.2.4. The effect of (p)ppGpp overproduction on the tolerance of JE2 WT and (p)ppGpp0 *in vitro*

Mutations resulting in the overproduction of (p)ppGpp have previously been identified in a clinical *S. aureus* strain isolated from a patient with a persistent infection that was not responding to antibiotic therapy **(Gao *et al.*, 2010)**. This highlights the benefits of (p)ppGpp overproduction for the survival of *S. aureus*. As (p)ppGpp acts to aid bacteria in surviving stresses such as nutrient limitation, excess (p)ppGpp may serve to provide enhanced protection that may explain why acquiring an HD domain mutation is favourable during persistence. However, toxic accumulation is also a possibility which may be detrimental for survival of *S. aureus*. Thus, investigating how (p)ppGpp overproduction affects the tolerance of *S. aureus* to itaconic acid, H2O2 and HOCl was of interest. Here, the JE2 pALC2073-*relQ* (p)ppGpp overproduction strain was utilised. For all stressors, the overproduction of (p)ppGpp led to an increase in tolerance in comparison to the JE2 empty vector strain, with a more pronounced phenotype observed when exposed to itaconic acid and HOCl **(Fig. 4.2.8)**. These results indicate that surplus (p)ppGpp has a protective effect *in vitro* and contributes to the tolerance of *S. aureus* to itaconic acid, H2O2 and HOCl*.*

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| **Figure 4.2.8. Tolerance of *S. aureus* to stressors found within professional phagocytes is increased in the presence of surplus (p)ppGpp.** JE2 pALC2073 (black) and JE2 pALC2073-*relQ* (orange) were grown to early exponential phase (approximately OD600 0.35) in TSB with chloramphenicol (10 μg/ml) and Atc (50 ng/ml) which were used to maintain the plasmid and induce expression of the plasmid respectively. Cultures were subsequentlywashed twice in 1 x PBS and exposed to 20 mM itaconic acid **(a)**, 100 mM H2O2 **(b)**, and 32 μM HOCl **(c)** in the presence of chloramphenicol (10 μg/ml) and Atc (50 ng/ml). After introduction of each stressor, the cultures were incubated at 37˚C for 30 minutes after which samples were taken for CFU/ml determination. Percentage bacterial survival with mean and standard deviation are plotted. Statistical analysis performed using Mann-Whitney test. *\* P <* 0.05, \*\* *P <* 0.01. |

### 4.2.5. Determining the contribution of each synthetase to acid and ROS stress

The (p)ppGpp synthetases are induced upon different stresses and this can differ between species. In *E. coli*,RelA has multiple promoters P1-4, from which transcription occurs and these promoters respond to different stresses. For example, while transcription from *relA*P1 is constitutive, transcription from *relA*P2 can be induced upon carbon limitation amongst others **(Nakagawa, Oshima and Mori, 2006)**. The bifunctional synthetase SpoT from *E. coli* interacts with uncharged ACP which serves as a sensor of cellular fatty acid levels **(Battesti and Bouveret, 2006)**. In contrast, fewer studies have been conducted on signals that trigger Rel*Sau* however,it is well known that Rel responds to amino acid limitation by interacting with stalled ribosomes, as the absence of Rel renders bacteriaunable to grow in the presence of the isoleucyl-tRNA synthetase inhibitor mupirocin **(Cassels, Oliva and Knowles, 1995)**. More recently however, the c-di-GMP-binding protein DarB was shown to interact with and activate Rel **(Ainelo *et al.*, 2023)**, thus providing an alternative method of Rel activation other than amino acid starvation conditions only. Studies also demonstrate that expression of RelP and RelQ can be induced upon stresses such as cell wall-targeting antibiotics **(Abranches *et al.*, 2009; Geiger *et al.*, 2014)**, alkaline shock **(Abranches *et al.*, 2009; Nanamiya *et al.*, 2008)**, ethanol shock **(Pando *et al.*, 2017)** and cationic AMPs **(Pietiäinen *et al.*, 2005)** but it is unclear which synthetases are induced upon acid and ROS stress. To elucidate this, the (p)ppGpp0 mutant complemented with *rel* and *relP* in pCL55-iTETr862, an Atc-inducible integrative vector (part of the laboratory strain collection), were utilised to identify if a particular synthetase was responsible for producing (p)ppGpp for a particular stress. For the following experiments, only itaconic acid and one ROS stressor (H2O2) were utilised. In the presence of itaconic acid, percentage survival of the (p)ppGpp0 mutant complemented with *rel* showed no difference in growth in comparison to the (p)ppGpp0 mutant **(Fig. 4.2.9a)**. In contrast, complementing *relP* seemed to increase percentage survival in comparison to the (p)ppGpp0 mutant **(Fig. 4.2.9a)**, and therefore tolerance, suggesting that the (p)ppGpp produced by RelP and not Rel, or the presence of the RelP protein itself, is important for acid stress. As RelP must form a tetramer to function as a synthetase, and (p)ppGpp can allosterically bind to and increase (p)ppGpp production by RelP **(Manav *et al.*, 2018; Steinchen *et al.*, 2018)**, it could be this translational regulation that allows RelP to increase tolerance of *S. aureus.* When these strains were exposed to H2O2, the presence of *rel* and *relP* only demonstrated a tolerance phenotype similar to WT **(Fig. 4.2.9b)**. Therefore, it may be that both synthetases are involved in responding to ROS stress, but that not one synthetase is more advantageous than the other.

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| **Figure 4.2.9. (p)ppGpp produced by Rel and RelP are sufficient to tolerate oxidative stress however only RelP is required to tolerate acid stress.** JE2 pCL55-iTETr862 (black), (p)ppGpp0pCL55-iTETr862 (pink) and (p)ppGpp0 pCL55-iTETr862-*rel* (lilac) and (p)ppGpp0 pCL55-iTETr862-*relP* (blue) were grown to early exponential phase (approximately OD600 0.35) in tryptic soy broth (TSB) with Atc (50 ng/ml) to induce expression of the plasmid. Cultures were subsequently washed twice in 1 x PBS. **(a)** 20 mM itaconic acid and **(b)** 100 mM H2O2 was added to *S. aureus* cultures as well as Atc (50 ng/ml). After introduction of each stressor, cultures were incubated at 37 ˚C for 30 minutes after which samples were taken for CFU/ml determination. Percentage bacterial survival was calculated with mean and standard deviation plotted. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparisons test. \* *P* < 0.05, \*\* *P* < 0.01. |

### 4.2.6. The effect of a *codY* deletion on the tolerance of JE2 WT and (p)ppGpp0

The CodY transcription factor represses genes related to stress including nitrogen and amino acid metabolism, as well as some virulence-associated genes **(Pohl *et al.*, 2009; Sonenshein, 2005)**, under nutrient-rich conditions. In *S. aureus*,this repression requires GTP and BCAA as cofactors. During the stringent response, as (p)ppGpp levels rise, cellular GTP pools deplete, leading to the derepression of CodY and thus the expression of these stress-related genes in order to cope with the change in environment **(Majerczyk *et al.*, 2008)**. A study has shown that upon amino acid starvation in *S. aureus*, 143 genes were upregulated due to CodY derepression, however 161 genes were downregulated independently of CodY, suggesting that CodY is mainly involved in the upregulation of genes during the stringent response and not downregulation **(Geiger *et al.*, 2012)**. As CodY derepression upregulates genes involved in amino acid metabolism, it may be that the (p)ppGpp produced by Rel relieves CodY as these genes are necessary during amino acid limitation. Consequently, it was hypothesised that the absence of CodY may affect how *S. aureus* respond to these stresses. To examine this, the laboratory strain JE2 *codY*::Tnwas subject to the tolerance assay in the presence of itaconic acid and H2O2 to understand whether the genes repressed by CodY can aid survival in the presence of these stresses. As this strain lacks CodY, the genes that CodY represses are being expressed. In comparison to WT and the (p)ppGpp0 mutant, the *codY*::Tnmutant in the presence of both itaconic acid and H2O2were considerably more tolerant **(Fig. 4.4.7)**, suggesting that the expression of genes repressed by CodY are beneficial for the survival of *S. aureus.* Since an increased tolerance phenotype was observed in the absence of CodY, a (p)ppGpp0 background lacking CodY was also constructed by phage transduction to study the affect. In this instance, a (p)ppGpp0 *codY*::Tnstrain restored survival in the presence of both itaconic acid and H2O2 in comparison to the (p)ppGpp0 mutant, and to a level equivalent to the JE2 *codY*::Tnmutant **(Fig. 4.4.7)**. These results describe a role for CodY in the tolerance of *S. aureus* *in vitro* and indicate that it is the repression of CodY in the (p)ppGpp0 mutant strain that renders it susceptible to itaconic acid and H2O2.

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| **Figure 4.2.10. The absence of CodY restores tolerance of the (p)ppGpp0nullmutant to WT levels in the presence of itaconic acid and H2O2 *in vitro.*** JE2 WT (black), (p)ppGpp0(pink) and JE2 *codY*::Tn(teal) and (p)ppGpp0 *codY*::Tn(purple) were grown to early exponential phase (approximately OD600 0.35) in tryptic soy broth (TSB) and were subsequently washed twice in 1x PBS. **(a)** Itaconic acid was added at 20 mM and **(b)** H2O2 at 100 mM and the cultures were incubated at 37 ˚C for 30 minutes after which samples were taken for CFU/ml determination. Percentage bacterial survival with mean and standard deviation plotted. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. \* *P* < 0.05, \*\* *P* < 0.01, \*\*\*\* *P* < 0.0001. |

## 4.3. Discussion

This chapter outlines the contribution of the stringent response for the survival of *S. aureus* when exposed to conditions that can be found within both professional and non-professional phagocytes. Therefore, to understand the mechanisms by which the staphylococcalstringent response may be involved, stringent response mutants were exposed to stresses that occur within the phagolysosome such as AMPs, acid and ROS stress and their viability under these stresses were investigated.

To study how the stringent response is important for *S. aureus* survival in the harsh conditions of the phagolysosomal environment, stringent response mutants were exposed to various stressors. At the concentrations of AMPs (LL-37 and polymyxin B) used in this study, differences in growth were not observed between the mutants and WT **(Fig. 4.2.1)** due to unsuitable concentrations of AMP used. This therefore did not allow the role of the stringent response to be probed. Further optimisation is required to titrate the concentrations of AMP required to detect a difference, especially as an increase in *spoT* and *relP* transcription has been reported in the presence of AMPs **(Scotti *et al.*, 2022; Pietiäinen *et al.*, 2005)**. It is interesting that polymyxins such as polymyxin B have antimicrobial effects again *S. aureus* as they specifically target LPS which, *S. aureus* lacks, and this phenomenon should be further investigated to understand the mechanism of action in the absence of LPS. While polymyxin B did lead to a decrease in growth at both 32 and 64 μg/ml *in vitro,* polymyxins such as colistin do not have any effect against Gram-positive bacteria like *S. aureus* at clinically relevant concentrations **(Betts *et al.*, 2016; Sabnis *et al.*, 2021)**. It may be that the staphylococcal stringent response does not contribute to the survival of *S. aureus* to the AMPs examined, however further studies are required to determine the optimal concentration of the AMPs in order to confirm this.

Phagosome maturation is accompanied by acidification which acts to kill phagocytosed bacteria. The low pH is also required for the function of cathepsin proteases and LL-37 cathelicidin. Growing *S. aureus* in pH 4.5 tris maleate-buffered rich media showed a slight growth defect of the (p)ppGpp0 mutant in comparison to WT **(Fig. 4.2.2a)**. This may have been because although the low pH was unsuitable for growth of the mutant, the nutrients in TSB may have made the media too rich to perceive a bigger difference. In comparison, the growth curves performed using Biolog Phenotype MicroArray PM10 plate did not reveal any differences in growth between the WT and (p)ppGpp0 mutant **(Fig. 4.2.4)**. However, this was only performed once and thus must be repeated in order to ascertain whether a difference in growth can be observed at low pH. At both neutral and acidic pH in rich media, the *codY*::Tnmutant displayed a slight growth defect comparison to WT during exponential phase **(Fig. 4.2.2b and e)**, suggesting that when measuring growth under low pH, and therefore the ability of *S. aureus* to resist acid stress, the absence of CodY is not beneficial. The tolerance of the (p)ppGpp0 mutant to itaconic acid was decreased in comparison to WT **(Fig. 4.2.5)**. Tolerance of *S. aureus* to itaconic acid was measured in PBS, which lacks the nutrients for *S. aureus* to grow and therefore perhaps enabled a greater distinction between WT and the (p)ppGpp0 mutant than when buffered rich media was used to measure resistance to acid stress in **Figure 4.2.2a**. It should be noted that tolerance assays conducted with itaconic acid would often show variation between biological repeats, thus requiring many repeats. As a result, the Shapiro-Wilk test was performed into order to determine the normality of the data set before a statistical test was performed. Also, even though the ability of itaconic acid to decrease pH was determined to be its microbicidal characteristic in this assay, this does not take into account the contribution of the immunomodulatory effects of itaconic acid, and this can only be investigated within the host. Furthermore, a decrease in tolerance of the (p)ppGpp0 mutant was also observed in the presence of ROS such as H2O2 and HOCl. Therefore, the stringent response has a role in aiding the survival of *S. aureus* under itaconic acid, H2O2 and HOCl stress.

As the absence of (p)ppGpp decreases the ability of *S. aureus* to tolerate these stressors, it was then found that the overproduction of (p)ppGpp functions to increase tolerance**.** Gao and colleagues had demonstrated that (p)ppGpp overproduction *in vivo* led to persistence and provides a survival advantage **(Gao *et al.*, 2010)**. The *in vitro* experiments in **Figure 4.2.8** reveal that (p)ppGpp overproduction can result in increased tolerance, also suggesting that surplus (p)ppGpp is beneficial for viability under stress as shown *in vivo* **(Gao *et al.*, 2010)**. Other stringent response mutants have arisen in a clinical setting, for example, another HD domain mutation was shown to partially induce the stringent response in a case of *E. faecium* persistent bacteraemia **(Honsa *et al.*, 2017)**. Honsa and colleagues also revealed that this clinical isolate was tolerant to multiple antibiotics. These cases highlight how the uncontrolled stringent response can have an impact on patient outcomes.

The (p)ppGpp synthetases are induced upon various stresses as previously mentioned. Here, the presence of RelP was able to increase tolerance to itaconic acid beyond WT levels, while Rel was unable **(Fig. 4.2.9a)**. For H2O2, neither synthetase was solely responsible for coping with the ROS stress suggesting that they both may be involved or the production of (p)ppGpp alone is sufficient, regardless of its source **(Fig. 4.2.9b)**. Further studies could include investigating the effect of complementing *relQ* in a (p)ppGpp0 background, as a previous study has shown that after the uptake of *S. aureus* by macrophages, *relP* and *relQ* transcription were highly upregulated within 30 minutes with *rel* transcribed at a lower level **(Peyrusson *et al.*, 2020)**. These data coincide with what was observed in this study, suggesting that Rel is not the synthetase activated upon acid stress, implying that acid stress may induce RelP and also possibly RelQ in a pathway that is independent of Rel. It is possible that since RelP and not Rel, is able to complement upon induction with Atc, RelP may physically interact with a downstream effector.

Furthermore, it would be interesting to use itaconic acid, H2O2 and HOCl in combination. All three stressors were used at concentrations higher than they occur physiologically e.g. 100 mM H2O2 was necessary for inhibition of *S. aureus* however phagolysosomal concentrations are reported to be approximately 2-4 μM **(Winterbourn *et al.*, 2006)**.Using these stressors in combination may be more representative of what naturally occurs *in vivo* and may also require lower concentrations. For example, the glyoxylate shunt, an important metabolic pathway in bacteria, is involved in coping with ROS stress. As itaconic acid inhibits isocitrate lyase, an important component of the glyoxylate shunt **(Ahn *et al.*, 2016; Lorenz and Fink, 2001)**, quantifying the tolerance of *S. aureus* using itaconic acid and a source of ROS in combination may yield interesting results. In this chapter, the effect of the stringent response on survival of *S. aureus* in the presence of ROS were studied. Reactive nitrogen species were also investigated in the form of spermine NONOate, a nitric oxide donor, however growth inhibition was not observed at the concentrations used (data not shown).

Based on the literature, the effect of itaconic acid on *S. aureus* tolerance has not been studied. However, an MSSA(p)ppGpp0 mutant was unable to grow in the presence of 3.2 mM H2O2 over 20 hours and was more susceptible to killing via a killing assay in comparison to WT **(Horvatek *et al.*, 2020)**.The killing assay used by **Horvatek *et al*.,** required 80 mMH2O2 for inhibition, however in this study, 100 mM H2O2 was required. Additionally, a study that quantified the tolerance of MRSA to HOCl showed that stationary phase bacteria required 3.5 mM HOCl to demonstrate inhibition of *S. aureus* **(Fritsch et al., 2020)***.* In this study, it is shown that *S. aureus* in the exponential growth phase was inhibited at 32 μM HOCl **(Fig. 4.2.7b),** and during stationary phase, a HOCl concentration approximately ten times this amount was required (data not shown) which corroborates the concentrations reported by Fritsch and colleagues**.** These results highlight the importance of studying MRSA as this is clinically relevant, but also the different growth phases as well. It is important to stress that the tolerance assay using itaconic acid, H2O2 and HOCl does not reflect the conditions in a phagolysosome accurately as the physiological concentrations of these stressors are found at much lower that what was used in this study. The stringent response is more likely to be important for initial survival while bacteria are establishing infection in a new environment.

The CodY regulon is known to negatively regulate many genes during nutrient-rich conditions. During the SR, this repression is relieved, leading to the expression of these genes which are usually related to amino acid metabolism and GTP synthesis. During nutrient limitation, the derepression of CodY therefore acts to increase the uptake of amino acids when the environmental amino acid concentrations are limited. CodY derepression also allows the expression of virulence related genes, linking metabolism to virulence **(Ye *et al.*, 2021; Waters *et al.*, 2016; Roux *et al.*, 2014)**. Inducing the expression of virulence genes during these unfavourable conditions may allow bacteria to increase their likelihood of survival. For example, CodY indirectly affects expression of multiple virulence genes by controlling *agr* and can affect expression of the pore-forming α-toxin **(Waters *et al.*, 2016; Majerczyk *et al.*, 2010).** The absence of CodY in both WT and the (p)ppGpp0 mutant backgrounds led to increased tolerances resulting in minimal killing over time that was particularly evident in the presence of H2O2 **(Fig. 4.2.10b)**. This suggests that the expression of these previously repressed genes is beneficial under acid and ROS stress.

When grown in the presence of acid stress (tris maleate buffer) the *codY*::Tnmutant demonstrated a growth defect **(Fig 4.2.2b and e)**. However, the tolerance of the *codY*::Tnmutant to itaconic acid was increased in comparison to WT and therefore showed an increased ability to survive **(Fig. 4.2.10)**. The differences in these results may be due to fundamental differences in the methods used for these experiments i.e., measuring growth vs survival. It may be interesting to measure growth in the presence of itaconic acid and perform a tolerance assay in the presence of maleic acid to understand whether each respective acid has the same effect or not.

In conclusion, *in vitro* tolerance assays with itaconic acid, H2O2 and HOCl has demonstrated that the absence of a functional stringent response reduces tolerance to these stressors. (p)ppGpp overproduction on the other hand has a protective effect that results in an increased tolerance. The (p)ppGpp produced by RelP is shown to be important under acid stress, while neither Rel nor RelP synthetases were solely necessary for ROS stress. Lastly, the expression of genes repressed by CodY are beneficial under stress conditions by significantly increasing tolerance in a WT background, and by restoring virulence in the (p)ppGpp0 mutant. These data provide further insight into how the stringent response allows *S. aureus* to adapt to the different stresses found within the phagolysosome. As the stringent response is important for many aspects of infection such as persistence and tolerance, it is necessary to study these interactions to aid the development of stringent response inhibitors.

# Chapter 5: The role of (p)ppGpp within macrophage and zebrafish embryo infection models

## 5.1. Introduction

Zebrafish (*Danio rerio*) are a well-established animal model for various types of disease and infection. Despite being non-mammalian, zebrafish are jawed vertebrates with high genetic homology to humans- more than 80% of disease-associated genes have a human counterpart **(Howe *et al.*, 2013)**. Furthermore, advantages over mammalian models include its rapid embryonic development, genetic manipulability and its transparency at the embryonic stages which allows for live imaging of embryos **(Teame *et al.*, 2019)**. With a functional innate immune system by 30 hpf **(Herbomel, Thisse and Thisse, 1999; Lieschke *et al.*, 2001)**, zebrafish embryos are also useful for studying host-pathogen interactions, as demonstrated by the numerous infection models that exist. For example, Prajsnar and colleagues developed a systemic infection model to study the infection dynamics of *S. aureus* and *E. faecalis* within the zebrafish circulatory system **(Prajsnar *et al.*, 2008; Prajsnar *et al.*, 2013)**. Studies such as these have informed us about how pathogens interact with the host. Following antibiotic treatment, *S. aureus* have been revealed to undergo clonal expansion resulting from a bottleneck within professional phagocytes **(McVicker *et al.*, 2014)**. As *S. aureus* can replicate within macrophages, escape and subsequent dissemination can also occur. This highlights how the zebrafish embryo model is valuable and explains the impact it has on furthering our knowledge of host-pathogen interactions and disease pathogenesis.

The stringent response contributes to the survival of *S. aureus* and other pathogens within professional phagocytes. As immune cells constitute the host defence, it is also important to consider the impact of the stringent response for the survival of pathogens *in vivo*. For example, an *S.* Typhimurium (p)ppGpp0 mutant was unable to replicate in mouse spleens after five days of infection in comparison to WT **(Pizarro-Cerdá and Tedin, 2004)**. The stringent response is also essential for the establishment of mycobacterial infections and has been implicated in long-term viability **(Dahl *et al.*, 2003).** The absence of (p)ppGpp has affected the ability of *E. faecalis* to form biofilms in CAUTI **(Colomer-Winter *et al.*, 2019)**. Furthermore, an *S. aureus* *rel* mutant could not form cutaneous lesions in mice as well as WT **(Mansour *et al.*, 2016)** and was unable to cause pyelonephritis in mice **(Geiger *et al.*, 2010).** These reports give an insight into how the stringent response is important for *in vivo* survival and virulence and emphasises the need for further research to elucidate the mechanisms by which the stringent response is involved.

This chapter aims to investigate how the stringent response is important for *S. aureus* host-pathogen interactions within mammalian cell culture models and a systemic infection model using zebrafish embryos. RAW 264.7 murine macrophages and neutrophils isolated from human blood were the intended models however due to limited access to the University of Sheffield Medical School during the COVID-19 pandemic, neutrophil experiments could not be undertaken. Instead, the role of the stringent response for oral infections such as tonsillitis were studied in a human tonsillar epithelial (HTE) model. By investigating the killing capacity of different stringent response mutants, the involvement of the stringent response was examined by studying the absence of (p)ppGpp, (p)ppGpp overproduction, the role of each synthetase, and the role of the transcriptional repressor CodY.

## 5.2. The absence of a functional stringent response does not affect the killing of *S. aureus* by RAW 264.7 macrophages

As the stringent response has previously been implicated in the development of persistence in murine macrophages, the ability of professional phagocytes to kill *S. aureus* with and without a functional stringent response was investigated. The murine macrophage cell line RAW 264.7, was utilised. This immortalised cell line was isolated from BALB/c mice with Abelson murine leukaemia virus-induced tumour **(Taciak *et al.*, 2018)**. RAW 264.7 macrophages were infected with *S. aureus* WT and (p)ppGpp0 mutant for 2.5 hours in serum-free DMEM containing 2 mM L-glutamine, at a multiplicity of infection (MOI) of 5, after which intracellular bacterial counts were determined up to 4.5 hours post infection (hpi). **Figure 5.2.1** shows that internalisation of *S. aureus* is around 4000-6000 CFU/ml, contrary to previous investigations which report higher MRSA internalisation at approximately 105 CFU/ml **(Cohen *et al.*, 2018)**. Over time, the intracellular counts of the (p)ppGpp0 mutant seem to stay constant while WT counts varied between the 30-minute increments suggesting variation **(Fig. 5.2.1).** As bacterial internalisation by RAW 264.7 macrophages was not as high as expected, steps were taken to increase uptake to ensure that intracellular counts were representative of *in vivo* infection.

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| **Figure 5.2.1. WT and the (p)ppGpp0 mutant are killed similarly by RAW 264.7 murine macrophages.** RAW 246.7 murine macrophages were challenged with *S. aureus* (WT (black) and the (p)ppGpp0 mutant (pink)) at an MOI of 5 (1 x 106 CFU per 200,000 cells) and incubated for 4.5 hours at 37˚C with 5 % CO2. RAW 264.7 macrophages were infected for 2 hours, after which extracellular bacteria were eliminated using 20 μg/ml lysostaphin (Lss) for 30 minutes. For later timepoints, macrophages were incubated with 2 μg/ml lysostaphin until at the specified timepoints, intracellular CFU/ml were determined by lysing macrophages with 2 % saponin. Experiment was performed once. |
| To examine bacterial uptake in more detail, intracellular and extracellular counts of bacteria in the absence of serum were investigated after different incubation periods. Macrophages were infected for 0.5 and 2.5 hours and the *S. aureus* CFU/ml within macrophages and in the surrounding medium was determined **(Fig. 5.2.2a and b)**. At both timepoints there were more bacteria extracellular than intracellular, but by 2.5 hours the CFU/ml within macrophages increases by approximately 78% for both WT and (p)ppGpp0 from 0.5 hours **(Fig. 5.2.2c)**, consistent with an increase in uptake of *S. aureus* over time. The presence of foetal bovine serum (FBS) in cell culture media serves to opsonise bacteria in order to facilitate their uptake by phagocytosis. **Figure 5.2.1** represents a preliminary optimisation experiment where FBS was a component of the culture media but not the infection media. Consequently, to increase the uptake of bacteria, FBS was added at a final concentration of 10% to DMEM that also contained L-glutamine. In addition to this, after inoculating the media with bacteria and adding the culture to the macrophages, the tissue culture plates were centrifuged at 277 x G for 2 minutes to sediment the bacteria and allow for synchronisation of infection **(Flannagan *et al.*, 2018). Figure 5.2.2d** shows the intracellular and extracellular counts at 2 and 3.5 hpi after incorporating these modifications. In comparison to **Figure 5.2.2a, Figure 5.2.2d** demonstrates slightly higher intracellular count with WT counts increasing from 4.7 x 104 to 1.7 x 105 CFU/ml and (p)ppGpp0 counts increasing from 1 x 105 to 2.7 x 105 CFU/ml. |

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| **Figure 5.2.2. Optimisation of *S. aureus* uptake by RAW 264.7 murine macrophages.** RAW 246.7 murine macrophages were challenged with *S. aureus* WT and the (p)ppGpp0mutant at an MOI of 5 (1 x 106 CFU per 200,000 cells) and incubated for the specified timepoints **a)** 0.5 hours and **b)** 2.5 hours at 37˚C with 5 % CO2. Serum-free DMEM supplemented with 2 mM L-glutamine was used for the experiment represented by **a)-c)**. Both extracellular (grey bars) and intracellular (chequered bars) CFU/ml were determined by sampling the extracellular media to measure bacteria that had not been internalised and by lysing the macrophages to recover bacteria that had been internalised using 2 % saponin respectively. **c)** demonstrates the percentage change between 0.5 and 2 hours. **d)** The intracellular and extracellular counts of WT and the (p)ppGpp0mutant were performed as in **a)-c)** but using DMEM supplemented with 10 % FBS and 2 mM L-glutamine instead and prior to incubation, tissue culture plates were centrifuged for 2 minutes at 277 x G to sediment bacteria. Experiments represented by **a)-d)** were performed once. |

The macrophage killing assay was then repeated but including longer timepoints of 6, 10 and 24 hpi to investigate long-term killing. At 2.5 hpi, the mean intracellular counts of WT were 2.61 x105 CFU/ml and for (p)ppGpp0 the mean counts were 1.4 x 105 CFU/ml **(Figure 5.2.3a)** as opposed to the counts of 103 in **Figure 5.2.1**. The increase in intracellular WT CFU/ml after addition of FBS and centrifugation for infection synchronisation demonstrates the importance of these factors for increasing the number of bacteria internalised. While the CFU/ml increases slightly between 2.5 and 6 hpi for WT, the general trend indicates that bacteria are being killed over time between 0 and 24 hours **(Figure 5.2.3a)**. CFU/ml and percentage of inoculum graphs show that fewer intracellular (p)ppGpp0 bacteria are recovered from macrophages in comparison to WT **(Figure 5.2.3a and b)**, however the percentage change of *S. aureus* from 2.5 hours suggests that while the WT and (p)ppGpp0 counts differ, the rate at which both strains are being killed are the same **(Figure 5.2.3c)**. Thus, in this cell culture model, it seems that the absence of the stringent response does not impact killing of *S. aureus* by RAW 264.7 macrophages over time, even though there is a significant difference in the number of bacteria are 8 hpi **(Fig. 5.2.3b)**.

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| **Figure 5.2.3. WT and the (p)ppGpp0 mutant are killed similarly by RAW 264.7 murine macrophages. a)-c)** RAW 246.7 murine macrophages were challenged with *S. aureus* (WT (black) and the (p)ppGpp0 mutant (pink)) at an MOI of 5 (1 x 106 CFU per 200,000 cells) and incubated for 24 hours in DMEM supplemented with 10 % FBS and 2 mM L-glutamine at 37˚C with 5 % CO2. RAW 264.7 macrophages were infected for 2 hours (end of infection period represented by first black arrow), after which extracellular bacteria were eliminated using 20 μg/ml lysostaphin (Lss) for 30 minutes (end of lysostaphin treatment represented by second black arrow). For later timepoints, macrophages were incubated with 2 μg/ml lysostaphin until at the specified timepoints, intracellular CFU/ml were determined by lysing macrophages with 2 % saponin. **a)** is presented as percentage of CFU/ml, **b)** is presented as percentage of inoculum and **c)** is presented as percentage change from 2 hours. The experiment was performed in triplicate with mean values ± standard deviations plotted. Statistical analysis was performed using two-way analysis of variance (ANOVA) with Šídák's multiple comparisons post-test \* *P <* 0.05. |
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## 5.3. Investigating the expression of (p)ppGpp synthetases in the presence of stressors and during macrophage infection

It is well known that the (p)ppGpp synthetases are induced upon different stresses e.g. Rel is induced upon amino acid limitation **(Cassels, Oliva and Knowles, 1995)** while RelP and RelQ are induced upon cell wall stress for example administration of antibiotics amongst other stresses **(Abranches *et al.*, 2009; Geiger *et al.*, 2014)**. However, it is unclear when each synthetase is induced in the presence of a particular stressor. Identifying when each synthetase is expressed during infection may give insight into when each stressor within the phagolysosome affects the bacteria most, and thus when the expression of a particular synthetase is necessary. To determine when the expression of each synthetase occurs, synthetase-GFP fusions (bacteria only) were analysed by flow cytometry **(Fig. 5.3.1a-f)** and GFP fluorescence was measured at two hours post antibiotic administration. **Figure 3.4.8** shows that the fluorescence detected from each synthetase-GFP fusion was not very high when using a platereader, however a flow cytometer may be more sensitive than a platereader as the cell population is gated to remove debris and so this method was tested. The flow cytometry plots for the pCL55 empty vector control show that in both the presence and absence of 60 μg/ml mupirocin (5X MIC), which induces expressed of Rel by mimicking amino acid starvation, there is no change in green fluorescence, as expected as the empty vector control does not express GFP **(Fig. 5.3.1a)**. The green fluorescence counts for the positive control JE2-GFP, is increased in comparison with a peak at 104 indicating a fluorescent population, but again, there is no difference in fluorescence when mupirocin is present or absent **(Fig. 5.3.1b)**. The Rel-GFP synthetase fusion (JE2 pCL55-P*rel­*-*rel*-GFP) however, fluoresces similar to the empty vector control in the presence and absence of mupirocin **(Fig. 5.3.1a and c)**. These results are validated by the mean fluorescence intensity values in **Figure 5.3.1d-f** which demonstrates that the empty vector and Rel-GFP synthetase fusion fluoresces similarly, while the JE2 GFP positive control exhibits much higher fluorescence. RelP-GFP and RelQ-GFP synthetase fusions were also examined in the presence of 640 μg/ml ampicillin (5X MIC) however, these experiments yielded the same results as the Rel-GFP fusion, in that no change in fluorescence was observed after two hours incubation with ampicillin data not shown). The flow cytometry data suggests that upon antibiotic stress, the synthetase-GFP fusions do not fluoresce at two hours using flow cytometry.

To further examine the expression of Rel and to determine the importance of this synthetase during infection, primary human monocyte-derived macrophages (MDMs) infected with the Rel-GFP synthetase fusion were visualised by microscopy. Bacteria were stained red with NHS ester to allow discrimination of individual bacteria. Preliminary experiments performed by Dr Lucy Urwin have demonstrated that in comparison to WT, fewer (p)ppGpp0 mutant colonies were recovered from MDMs at 6 hpi. These results demonstrate that the stringent response is necessary at this 6 hpi, thus, the macrophages were imaged at this timepoint to determine whether Rel was being expressed while in the presence of the stressors found in a phagolysosome. The Alexa Fluor 555 channel shows intracellular *S. aureus* however, the GFP channel reveals autofluorescence of the MDMs rather than fluorescent bacteria **(Fig. 5.3.1g)**. Two foci are highlighted by the white arrow however, these are likely to be artefacts given the amount of autofluorescence exhibited by the MDMs **(Fig. 5.3.1g)**. The RelP-GFP and RelQ-GFP synthetase fusions were subject to the same analyses and it was revealed that these synthetase do not fluoresce within MDMs at 6 hpi either (data not shown). These results suggest that at 6 hpi, fluorescence of the synthetase-GFP fusions was not detectable using the detection systems available.

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| **Figure 5.3.1. The Rel-GFP synthetase fusions do not fluoresce in the presence of mupirocin or intracellularly within MDMs.** pCL55 empty vector **a)** the JE2 GFP positive control **b)** and the Rel-GFP synthetase fusion (P*rel*-*rel*-GFP) **c)** were incubated in the presence or absence of 60 μg/ml mupirocin. Samples were collected before (0 hrs) and after (2 hrs) addition of mupirocin and GFP expression was quantified by flow cytometry. Flow cytometry plots in **a)-c)** represent samples taken after 2 hrs. **d)-f)** Median fluorescence intensities (MFI) values data from flow cytometric analyses were plotted for pCL55 empty vector, JE2 GFP and P*rel*-*rel*-GFP. **g)** Primary human monocyte-derived macrophages (MDMs) were infected with P*rel*-*rel*-GFP at MOI 20, using bacteria pre-stained with Alex Fluor 555 NHS Ester (red). Infected cells were fixed and visualised by microscopy at 6 hpi using a Nikon Ti-E inverted microscope with Nikon 60X Plan Apochromat objective. Scale bars represent 50 μm. All experiments performed by Dr Lucy Urwin. |

## 5.4. The stringent response may be important in establishing oral infection

Tonsilitis is the inflammation of tonsils, most frequently the palatine tonsils, and can be caused by both bacteria and viruses. *S. aureus* is one of the predominant causative agents of recurrent tonsilitis in addition to *Streptococcus pyogenes*,with studies showing that approximately 57.7 % of cases are attributed to the former while 20.2 % are attributed to the latter **(Zautner *et al.*, 2010; Cavalcanti *et al.*, 2019; Katkowska, Garbacz and Stromkowski, 2017; Kostic *et al.*, 2022)**. Recurrent infections are due to persistence of *S. aureus* within the tonsillar tissue **(Cavalcanti *et al.*, 2019)** and therefore, there may be a role of the stringent response in the colonisation of this niche and the development of persistence, as the stringent response has been previously implicated for *S. aureus* persistence in RAW 264.7 macrophages **(Peyrusson *et al.*, 2020)**.

Consequently, to investigate the role of the staphylococcal stringent response in oral infection, an HTE cell line was employed. As epithelial cells are non-professional phagocytes (cells that can phagocytose, but phagocytosis is not their main function), invasion, rather than ability to phagocytose, of HTE cells by *S. aureus* was measured. HTE cells were challenged with *S. aureus* WT and the (p)ppGpp0 mutant strains that had been grown to both stationary **(Fig. 5.4.1a-c)** and exponential **(Fig. 5.4.1d-f)** growth phases and intracellular counts were determined. Here, both growth phases were investigated as different virulence factors are expressed depending on the phase. Additionally, depending on the type of and location of infection, bacteria will be in different growth states. Nutritional requirements differ during different growth phases also, for example, cellular (p)ppGpp levels are usually higher in stationary phase cultures due to a lack of nutrients and so the effect of the stringent response is particularly relevant at this phase. Interestingly, for both stationary **(Fig. 5.4.1a-c)** and exponential phases **(Fig. 5.4.1d-f)**, there was an increase in CFU/ml by 8 hpi. For stationary phase bacteria **(Fig. 5.4.1a-c)**, both WT and the (p)ppGpp0 mutant demonstrated growth by 8 hpi, however the (p)ppGpp0 mutant grew to a lesser extent, suggesting either a growth defect or a virulence defect. Exponential phase bacteria show the same trend as WT, however the (p)ppGpp0 mutant is unable to grow over time, evidenced by small changes in CFU/ml up to 24 hpi **(Fig. 5.4.1d-f)**. The (p)ppGpp0 mutant in exponential phase does not grow to the same extent as when in stationary phase, which indicates that growth phase is important during infection as well as a functional stringent response.

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| **Figure 5.4.1. WT displays enhanced replication in comparison to the (p)ppGpp0 mutant in non-professional phagocytes during both stationary and exponential growth phases.** Human tonsillar epithelial (HTE) cells were challenged with stationary and exponential phase *S. aureus* (WT (black) and the (p)ppGpp0 mutant (pink)) at an MOI of 10 (5 x 105 CFU per 50,000 cells) incubated for 24 hours at 37˚C with 5 % CO2. **a)-c)** show invasion of HTE cells by stationary phase bacteria while **d)-f)** show invasion THE cells by exponential phase bacteria. HTE cells were infected for 1 hour 15 mins (end of infection period represented by first black arrow), after which extracellular bacteria were eliminated using 20 μg/ml lysostaphin (Lss) for 45 minutes (end of lysostaphin treatment represented by second black arrow). For later timepoints, HTE cells were incubated with 2 μg/ml lysostaphin until at the specified timepoints, intracellular CFU/ml were determined by lysing HTE cells with 0.05% trypsin in dH2O. **a)** and **d)** are presented as percentage of CFU/ml, **b)** and **e)** are presented as percentage of inoculum and **c)** and **f)** are presented as percentage change from 2 hours. The experiments represented by **a)-c)** were performed once and the experiments represented by **d)-f)** were performed in duplicate with mean values ± standard deviations plotted. HTE cell culture and infection was performed by Henna Khalid and intracellular CFU determination was performed by NRC. |
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## 5.5. Investigating the importance of (p)ppGpp for *S. aureus* survival and virulence during systemic infection

### 5.5.1. Optimisation of the dose of *S. aureus* required to kill 50% of embryos

In order to produce a systemic infection, London Wildtype zebrafish embryos were injected at 30 hpf into the circulation valley, a protocol developed by Prajsnar and colleagues **(Prajsnar *et al.*, 2008)**. The circulation valley/duct of Cuvier is a collection of blood vessels that span the zebrafish embryo laterally. Injecting laterally is possible but difficult as the yolk sac can easily be punctured in the process of injection and any embryos injected in the yolk sac must be discarded. Therefore, injecting into the returning circulation where the circulation valley culminates, can allow for an easier injection as well as systemic infection **(Fig. 5.5.1a).** Fluorescent *S. aureus* (JE2-GFP and (p)ppGpp0-GFP)were injected into the returning circulation and embryos were imaged 24 hpi. Bacterial clusters were observed throughout the embryo, confirming that injection into the circulation valley leads to systemic infection **(Fig. 5.5.2)**.

A dose of *S. aureus* JE2 WT USA300 MRSA that led to 50% killing of zebrafish embryos was first determined. Previous data from the laboratory has demonstrated that approximately 550 CFU/embryo of LAC\* USA300 MRSA were required to elicit 50% killing of embryos. To this end, 1 nl of JE2 WT cultures at concentrations of 750 and 550 CFU/nl were injected into zebrafish embryos and survival was monitored for up to 93 hpi **(Fig. 5.5.1b).** Only 30% of embryos were killed when injected with 750 CFU and approximately 20% with 550 CFU. Due to the 550 CFU dose not achieving the same percentage killing as seen previously with LAC\*, the differences between JE2 and LAC\* were examined. JE2 was constructed from LAC\* by curing two plasmids, p01 and p03. p01 is a 3.1 kb cryptic plasmid, while p03 is a 27 kb plasmid that confers erythromycin resistance **(Fey *et al.*, 2013)**, and with JE2 cured of both of these plasmids, it may be that 550 CFU/nl was not a high enough dose to kill 50% of embryos. This could suggest that the absence of these two plasmids impacts virulence but further investigation is required to confirm whether this hypothesis is true or not.

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| **Figure 5.5.1. Optimisation of zebrafish embryo systemic injections. a)** Lateral view of a 30 hpf London Wildtype zebrafish embryo. The location of the injection site, the circulation valley (blue circle) is highlighted, as well as the needle. The schematic below details the anatomical features of the zebrafish embryo including the eye, forebrain, notochord. The circulation valley (including the duct of Cuvier) is represented by black arrows and spans the lateral orientation of the yolk (as well as the cells of the hatching gland) and culminates in the area anterior to the yolk sac. The injection site is highlighted (blue circle) – 1 nl of bacterial culture is injected into the circulation valley beneath the cells of the hatching gland, where the duct of Cuvier culminates **b)** Survival of zebrafish embryos injected with *S. aureus* (WT) at intended doses of 750 CFU/nl (red) and 550 CFU (pink) (actual doses are shown in parentheses) at 30 hpf into the circulation valley. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. The negative control was performed using PBS (brown). Experiment performed once. |
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### 5.5.2. The effect of (p)ppGpp on the establishment of systemic infection in zebrafish embryos

Previous research has shown a role for the stringent response in the establishment of infection in both Gram-positive and Gram-negative bacteria, as well as the acid-fast mycobacteria **(Geiger *et al.*, 2010; Dasgupta *et al.*, 2019; Primm *et al.*, 2000)**. To investigate whether the stringent response is required to establish systemic infection within zebrafish embryos, JE2 WT and the (p)ppGpp0 mutant were injected into the circulation valley **(Fig. 5.5.3).** Different doses were trialled and after optimisation, a dose of approximately 3000-4000 CFU of JE2 was required to achieve 50% killing. *S. aureus* in both exponential **(Fig. 5.5.3a)** and stationary **(Fig. 5.5.3b)** growth phases were investigated. The (p)ppGpp0 mutant from both growth phases killed fewer embryos in comparison to WT, suggesting a role for the stringent response in the pathogenesis of *S. aureus*. This also highlights that even though there are differences in genes expressed during the stationary and exponential growth phases, (p)ppGpp is required for the establishment of infection in this model regardless of growth phase.

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| **Figure 5.5.3. Systemic infection of zebrafish embryos with *S. aureus* (p)ppGpp0 mutant results in reduced killing of embryos in comparison to WT when using both stationary and exponential phase cultures.** Survival of zebrafish embryos injected with *S. aureus* ((p)ppGpp0(pink) versus WT (black)) in exponential phase **(a)** and stationary phase **(b)** at doses of 3000-4000 CFU/nl at 30 hpf into the circulation valley. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: a) (p)ppGpp0versus WT, \*\*\*\* *P* < 0.0001, b) (p)ppGpp0versus WT, \*\* *P* = 0.0048. Experiments were performed in quadruplicate for (a) and in triplicate (b). |
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### 5.5.3. Determining whether the (p)ppGpp0 mutant attenuation *in vivo* is due to a growth or virulence defect

To examine whether attenuation of the (p)ppGpp0 mutant was due to a growth or virulence defect, WT and the (p)ppGpp0 mutant were injected into zebrafish embryos and at each timepoint, five live fish and any dead fish were homogenised, and the CFU/embryo was determined **(Fig. 5.5.4).** At 21 hpi, bacterial loads range from 105 to 106 for both WT and the (p)ppGpp0 mutant, demonstrating that both *S. aureus* strains were able to replicate similarly within the embryos from the inoculum (103), which suggests that the (p)ppGpp0 mutant is not attenuated due to a replication defect. Additionally, the (p)ppGpp0 mutant was isolated from embryos at numbers higher than the inoculum at timepoints up to 69 hpi, suggesting that the (p)ppGpp0 mutant was able to replicate later on during infection as well **(Fig. 5.5.4b)**. When infected embryos were isolated for homogenisation, embryos infected with the WT were more often found dead in comparison to embryos infected with the (p)ppGpp0 mutant as demonstrated by a higher number of closed circles, concurrent with the survival curves in **Figure 5.5.3**. However not all dead embryos were included in the dataset as many embryos infected with WT had disintegrated substantially, which may lead to *S. aureus* growing within the dead embryo, resulting in unreliable CFU/embryo measurements. Additionally, the (p)ppGpp0 mutant-infected embryos were able to survive with a load of bacteria between 101 to 104, while this was not entirely the case with WT-infected embryos. This could imply that the attenuated (p)ppGpp0 mutant can allow embryos to survive at these loads while embryos infected with JE2 at the same bacterial load may not.

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| **Figure 5.5.4. WT and the (p)ppGpp0 mutant replicate similarly *in vivo* in a systemic zebrafish embryo infection model.** Growth of *S. aureus* WT **(a)** and (p)ppGpp0**(b)** in zebrafish embryos after injection of 3000-4000 CFU into the circulation at 30 hpf. Five live embryos and any dead embryos were taken at the specified timepoints for CFU/embryo determination. Open circles represent live embryos and closed circles represent dead embryos. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. Experiment was performed in triplicate with one representative shown. |

## 5.6. Control of the stringent response mutant within zebrafish embryos is myeloid cell-dependent

The importance of professional phagocytes and the mechanism by which they exert their antimicrobial effects have been previously discussed- stringent response mutants are susceptible to components of the phagolysosome, as shown in chapter four. Taking this into account, understanding how *S. aureus* interacts with the zebrafish innate immune system during infection is important and therefore the effect of myeloid cell depletion was studied. In order to achieve this, morpholino-modified antisense oligonucleotides were employed. These are short sequences of DNA consisting of one of the four bases affixed to carbon one of a methylenemorpholine ring, forming a monomer. Monomers are linked by phosphorodiamidate bonds to form the oligonucleotide **(Summerton and Weller, 1997).** Morpholinos are used to transiently knockdown genes. The Pu.1 morpholino facilitates the knockdown of the Pu.1/Spi1b transcription factor by steric block of translation of the Pu.1/Spi1b mRNA. Pu.1/Spi1b is a lineage determinant and an integral component in the differentiation of pluripotent haematopoietic stem cells into cells of the myeloid lineage **(Burda, Laslo and Stopka, 2010)**. While the Pu.1 morpholino designed by Rhodes and colleagues that was used in this study was said to target the AUG translational start site, the morpholino actually binds to exon 3 as shown in **Figure 5.6.1a**. However, translation of Pu.1/Spi1b in zebrafish embryos is still inhibited despite the binding site being 170 bp downstream of the translational start site as the expression of *pu.1/spi1b* mRNA is abolished upon morpholino administration **(Rhodes *et al.*, 2005)**.Thus, the knockdown of Pu.1 in zebrafish embryos results in the delayed appearance of cells such as macrophages and neutrophils until 48 and 36 hpf **(Herbomel, Thisse and Thisse, 2001; Renshaw *et al.*, 2006)** instead of the typical 25 and 30 hpf respectively **(Herbomel, Thisse and Thisse, 1999; Lieschke *et al.*, 2001)**. At the one-cell stage of embryonic development (approximately 0.2 hpf), 1 pmol of the Pu.1 morpholino was injected into the yolk sac of the embryos and this was followed by injection of *S. aureus* at 30 hpf into the circulation valley **(Fig. 5.6.1b)**. From **Figure 5.5.2** it is evident that the (p)ppGpp0 mutant is attenuated in comparison to WT. However, in **Figure 5.6.1b,** depletion of myeloid cells using the Pu.1 morpholino restores virulence of the (p)ppGpp0 mutant to WT levels, suggesting that (p)ppGpp is required for the survival within myeloid cells and that these cells are necessary for controlling infection at this infectious dose.

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| **Figure 5.6.1. Knockdown of Pu.1 transcription factor mRNA restores virulence to wildtype levels in the *S. aureus* (p)ppGpp0 mutant. a**) Schematic of the location of morpholino binding in the pu.1/spi1b mRNA. The Pu.1 morpholino designed by Rhodes and colleagues binds within exon 3 of the *pu.1/spib1* mRNA. **b)** Survival of PU.1 knockdown zebrafish embryos injected with *S. aureus*, comparing WT (black), (p)ppGpp0 (pink), JE2 Δ*codY* (teal) and (p)ppGpp0 Δ*codY* (purple) at doses of 3000-4000 CFU at 30 hpf into the duct of Cuvier. 1 pmol of Pu.1 morpholino was injected into the yolk of one-cell stage embryos. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: (p)ppGpp0 vs WT, ns *P* = 0.5275. The experiment was performed in triplicate. |
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## 5.7. The effect of (p)ppGpp overproduction *in vivo*

This study has demonstrated the protective effect of (p)ppGpp overproduction *in vitro* **(Fig. 4.2.8)** and therefore this was also studied *in vivo* to examine how (p)ppGpp overproduction affects *S. aureus* survival within zebrafish embryos. The (p)ppGpp overproduction strain JE2 pCL55-iTETr862-*rel* was injected into zebrafish embryos alongside the empty vector controls **(Fig. 5.7.1a).** While the (p)ppGpp0 mutant displayed the characteristic attenuation of virulence leading to fewer embryos being killed in comparison to WT, the (p)ppGpp overproduction strain killed significantly fewer embryos than WT **(Fig. 5.7.1a)**. These results are in contrast to the *in vitro* data, suggesting that (p)ppGpp overproduction is not beneficial for the survival of *S. aureus in vivo.* Likewise, Gao and colleagues found that a HD domain mutation that led to a permanently activated stringent response in a clinical isolate, when mimicked in the laboratory, was also attenuated in a *Galleria mellonella* model, corroborating the data in **Figure 5.7.1a**. (p)ppGpp overproduction from RelP and RelQ were also examined, and similar results were observed as with the Rel-overproduction strain **(Fig. 5.7.1b).** This highlights the importance of regulating levels of (p)ppGpp within bacteria, and that while (p)ppGpp overproduction may increase tolerance *in vitro*, this is not the case *in vivo.*

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| **Figure 5.7.1. (p)ppGpp overproduction in systemic zebrafish embryo infection model attenuates *S. aureus* virulence.** Survival of zebrafish embryos injected with *S.* aureus, comparing WT (JE2 pCL55-iTETr862) (black), (p)ppGpp-null mutant ((p)ppGpp0 pCL55-iTETr862) (pink), the Rel (p)ppGpp overproduction strain (JE2 pCL55-iTETr862-*rel*) (purple) **(a)** and the RelP/Q (p)ppGpp overproduction strains (JE2 pCL55-iTETr862-*relP* (blue) and JE2 pCL55-iTETr862-*relQ* (orange)) **(b)** at doses of 3000-4000 CFU at 30 hpf into the circulation. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: **a)** (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862, \*\* *P* = 0.0011; JE2 pCL55-iTETr862-*rel* vs JE2 pCL55-iTETr862, \* *P* = 0.0399; (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862-*rel,* ns *P =* 0.2637. **b)** (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862, \*\*\* *P* = 0.0006; JE2 pCL55-iTETr862-*relP* vs JE2 pCL55-iTETr862, ns *P* = 0.0992; JE2 pCL55-iTETr862-*relQ* vs JE2 pCL55-iTETr862, ns *P* = 0.0550; (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862-*relP,* ns *P =* 0.0562; (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862-*relQ,* ns *P =* 0.1224. In both **(a)** and **(b)**, experiments were performed in triplicate. |
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## 5.8. Determination of the (p)ppGpp synthetase required for *S. aureus* systemic infection

As Rel from *S. aureus* is required for virulence in a murine haematogenic kidney abscess model **(Geiger *et al.*, 2010)** and a cutaneous abscess model **(Mansour *et al.*, 2016)**, the absence of Rel was first investigated. JE2 WT, the (p)ppGpp0 triple mutant and ∆*relQP* were injected into embryos. The survival curves demonstrated that the ∆*relQP* mutant was able to kill similarly to JE2 WT **(Fig. 5.8.1a)**, indicating that the presence of *rel* only is sufficient for virulence during infection. To further understand the importance of Rel, the existing laboratory strains LAC\* WT and LAC\* *relsyn* mutant, a mutant lacking three conserved amino acids (amino acids Y308, Q309 and S310) within the SYNTH domain rendering it non-functional, were studied. There was no difference in killing observed between embryos injected with LAC\* WT and LAC\* *relsyn*. This could suggest that (p)ppGpp produced by RelP and RelQ is sufficient to compensate for the absence of Rel **(Fig. 5.8.1b).** Additionally, **Figure 5.5.1b** demonstrated that JE2 WT does not kill 50% of infected embryos when injected with 550 CFU, as LAC\* has previously been shown to do. In **Figure 5.8.1b**, the dose required of LAC\* strains was 1500 CFU/nl, measuring at approximately half the dose required for JE2 strains, confirming that LAC\* is more virulent than JE2 and the two plasmids it harbours in comparison to JE2 may indeed confer virulence.

In order to further study the role of each synthetase during infection, *rel* was first complemented in a (p)ppGpp0 mutant background, which resulted in killing similar to WT **(Fig. 5.8.1c).** This is comparable to the killing observed by ∆*relQP* in **Figure 5.8.1a**, confirming the importance of Rel *in vivo* as shown previously **(Geiger *et al.*, 2010; Mansour *et al.*, 2016)**. In contrast, complementing *relP* did not restore virulence and its ability to kill zebrafish embryos was indistinguishable from the (p)ppGpp0 mutant **(Fig. 5.8.1d)**, suggesting that the (p)ppGpp produced by RelP is not enough to compensate in the absence of both Rel and RelQ. In summary, the presence of Rel alone is sufficient for virulence during the systemic infection of zebrafish. RelP and RelQ together are also sufficient but the expression of RelP alone is not. It may be that all three (p)ppGpp synthetases are involved during infection, but Rel could have a greater role. In order to elucidate this, further research including complementing RelQ, is necessary.

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| **Figure 5.8.1. (p)ppGpp produced by Rel is important for *S. aureus* virulence in a systemic zebrafish embryo infection model.** Survival of zebrafish embryos injected with *S. aureus* at 30 hpf into the circulation. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. **a)** Injection of JE2 WT (black), (p)ppGpp0 (pink) and ∆*relQP* (green) (dose 3000-4000 CFU). **b)** Injection of LAC\* WT (black) and LAC\* *relsyn* (orange) (dose 1500 CFU). **c)** Injection of JE2 pCL55-iTETr862 (black), (p)ppGpp0 pCL55-iTETr862 (pink) and (p)ppGpp0 pCL55-iTETr862-*rel* (purple) (dose 3000-4000 CFU). **d)** Injection of JE2 pCL55-iTETr862 (black), (p)ppGpp0 pCL55-iTETr862 (pink) and (p)ppGpp0 pCL55-iTETr862-*relP* (blue) (dose 3000-4000 CFU). **a)** Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: (p)ppGpp0 vs JE2 WT, \*\* *P* = 0.0027; ∆*relQP* vs JE2 WT, ns *P* = 0.5358; (p)ppGpp0 vs ∆*relQP,* \*\*\*\* *P <* 0.0001. **b)** Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: LAC\* WT vs LAC\* *relsyn*, ns *P* = 0.1499. **c)** Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862, \*\*\* *P* = 0.0002; (p)ppGpp0 pCL55-iTETr862-*rel* vs JE2 pCL55-iTETr862, ns *P* = 0.7305; (p)ppGpp0 pCL55-iTETr862 vs (p)ppGpp0 pCL55-iTETr862-*rel,* \*\*\* *P =* 0.0007. **d)** Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: (p)ppGpp0 pCL55-iTETr862 vs JE2 pCL55-iTETr862, \*\* *P* = 0.0073; (p)ppGpp0 pCL55-iTETr862-*relP* vs JE2 pCL55-iTETr862, \*\* *P =* 0.0096; (p)ppGpp0 pCL55-iTETr862 vs (p)ppGpp0 pCL55-iTETr862-*relP,* ns *P* = 0.9704. In **(a)**, **(c)** and **(d)**, experiments were performed in triplicate, while **(b)** was performed in quadruplicate. |
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## 5.9. Investigating the effect of deleting CodY on *S. aureus* virulence

The transcriptional repressor CodY regulates many genes including those involved in amino acid metabolism, uptake and transport and also many virulence genes **(Pohl *et al.*, 2009).** Virulence genes include the virulence regulator *saeS* and quorum-sensing regulator *agrA,* capsular polysaccharide biosynthetic genes, fibrinogen-binding protein *efb,* fibronectin-binding protein *fnBPA* and toxin such as the α-haemolysin and TSST-1 **(Majerczyk *et al.*, 2010; Pohl *et al.*, 2009)***.* Chapter four details how the absence of CodY significantly increased the tolerance of *S. aureus* to itaconic acid and ROS stress. It also restored tolerance of the (p)ppGpp0 mutant to levels that surpass WT. Previous *in vivo* studies in a murine necrotising pneumonia model and a murine dermonecrosis model has shown that deletion of *codY* in an MRSA background led to hypervirulence, demonstrated by increased percent mortality and increased area of dermonecrosis respectively **(Montgomery *et al.*, 2012)**. Therefore, the role of CodY during systemic infection was investigated as an increase in cellular (p)ppGpp levels due to induction of the stringent response results in CodY derepression and thus, the expression of the virulence genes mentioned above. However, injection of the JE2 *codY*::Tnand (p)ppGpp0 *codY*::Tnmutants resulted no difference in virulence with regards to the respective controls **(Fig. 5.9.1)**, suggesting that the absence of CodY is not beneficial for survival of *S. aureus in vivo* in a zebrafish embryo systemic infection model.

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| **Figure 5.9.1. Deletion of *codY* transcriptional repressor does not affect virulence of *S. aureus* in a systemic zebrafish embryo infection model*.*** Survival of zebrafish embryos injected with *S. aureus*, comparing WT (black), (p)ppGpp0 (pink), JE2 *codY*::Tn(teal) and (p)ppGpp0 *codY*::Tn(purple) at doses of 3000-4000 CFU at 30 hpf into the circulation. Survival was monitored until 93 hpi when the embryos reached 5.2 days post fertilisation. Pairwise comparisons (log-rank (Mantel-Cox) test) were as follows: (p)ppGpp0 vs WT, \* *P* = 0.0481; JE2 Δ*codY* vs WT, ns *P* = 0.6612; (p)ppGpp0 Δ*codY* vs (p)ppGpp0*,* ns *P =* 0.5074; (p)ppGpp0 vs JE2 Δ*codY,* ns *P =* 0.1391; (p)ppGpp0 Δ*codY* vs WT \* *P =* 0.0108. The experiment was performed in triplicate. |
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## 5.10. Discussion

This chapter aimed to understand how the stringent response is involved in *S. aureus* survival and virulence during infection of professional and non-professional phagocytes and systemic infection in a zebrafish embryo model. While no difference in killing between WT and the (p)ppGpp0 mutant was observed in RAW 264.7 macrophages, the ability of the (p)ppGpp0 mutant to invade HTE cells seemed to be decreased in comparison to WT. Furthermore, it was observed that the synthetase-GFP fusions did not fluoresce during flow cytometry and microscopy analysis and thus fluorescence was not detected intracellularly within MDMs. The stringent response was found to contribute to virulence as the (p)ppGpp0 mutant was attenuated and its ability to kill zebrafish embryos was reduced in comparison to WT. Attenuation of the (p)ppGpp0 mutant appears to be due to a virulence defect rather than a growth defect as the (p)ppGpp0 mutant was able to replicate *in vivo* as well as WT. While the absence of (p)ppGpp hindered *S. aureus* virulence, (p)ppGpp overproduction was also detrimental for *S. aureus* survival *in vivo*. Furthermore, the impact of Rel for *S. aureus* virulence has been demonstrated in various animal models and here it is shown that (p)ppGpp produced by Rel, controlled by a non-native promoter, and not RelP is sufficient during a zebrafish embryo systemic infection model. Interestingly, the absence of CodY did not corroborate the *in vitro* tolerance assay findings in **Figure 4.2.10**, as the *codY*::Tnmutants did not display higher killing or virulence in comparison to WT. These findings give an insight as to how the stringent response is implicated for *S. aureus* survival and virulence during infection and demonstrates that the stringent response is an important regulatory pathway that contributes to *S. aureus* virulence.

RAW 264.7 murine macrophages are a well-established cell culture model used to make predictions about how human monocytes/macrophages may interact with pathogens. For over 40 years, this cell line has been deemed suitable to study these interactions due to its similarity to human macrophages e.g., the ability to perform phagocytosis, pinocytosis and the production of NO in response to stimulation by LPS **(Taciak *et al.*, 2018)**.As RAW 264.7 macrophages were derived from leukaemic lymphoid cells, they are immortalised which poses an advantage over primary cells isolated from human blood donors as they can be easily propagated. Additionally, acquiring human blood donors can be difficult as the reliability of donors, amount of blood donated, and the number of cells vary from donor to donor. As the stringent response has previously been implicated as a pathway involved in the development of persistence within macrophages **(Peyrusson *et al.*, 2020)**, a difference in killing of the (p)ppGpp0 mutant in comparison to WT was expected, however this study did not yield this result demonstrated by equivalent rates of killing by both strains **(Fig. 5.2.3c)**.

For this assay, FBS was used to increase the number of bacteria taken up by the RAW 264.7 macrophages by opsonisation, however as FBS is heat-inactivated and so complement proteins and antibodies will no longer be functional, it may have been beneficial to also stimulate the macrophages prior to infection. Common stimulators include LPS and IFN-γ, and RAW 264.7 macrophages are known to produce NO in response to activation by LPS **(Taciak *et al.*, 2018)**. LPS-mediated stimulation results in an increase in the amount of Fcγ receptor-mediated killing by macrophages, and therefore a lack of stimulation may decrease the phagocytic ability of macrophages **(Palermo *et al.*, 1997; Rubel *et al.*, 1999)**. Macrophages activated due to LPS stimulation should phagocytose more bacteria and this may aid in distinguishing whether the stringent response is necessary for survival as higher intracellular CFU/ml may give more reliable results. Furthermore, stimulating the macrophages will allow for an infection model that is more similar to *in vivo* conditions. Additionally, initial uptake of *S. aureus* could have been improved by trialling higher MOIs.

For future experiments, it may be interesting to trial other cell lines such as J774A.1 or human cell lines such as THP-1. While an established cell line can be more reliable, it may be more useful to trial MDMs isolated from human peripheral blood as results from these experiments may be more representative of what actually occurs during infection, despite the caveat of increased variability as previously mentioned. Furthermore, immortalised cell lines are more likely to acquire changes to their genotype/phenotype as a result of multiple passages **(Geraghty *et al.*, 2014)**. In this study, RAW 264.7 macrophages were used at passage numbers up to 18, which is a suitable passage number as the maximum has been reported as between 18 and 30 **(Taciak *et al.*, 2018)**. Macrophages used at higher passage numbers are subject to changes in growth and morphology, amongst other characteristics, and this therefore may introduce variability **(Taciak *et al.*, 2018)**. As different experiments were conducted using cells that had been passaged a different number of times, this may increase the likelihood of variability. Since these experiments were performed, killing assays using MDMs isolated from human blood have been conducted by Dr Lucy Urwin from our laboratory group. These results show that the (p)ppGpp0 mutant is killed at a significantly higher rate than WT, reiterating that the use of primary cells is more appropriate to elucidate the role of the stringent response during infection.

Flow cytometric and microscopic analyses of the synthetase-GFP fusions revealed that the Rel, RelP and RelQ synthetase fusions do not fluoresce either after incubation with a stress after 2 hours or during infection at 6 hpi, as demonstrated by microscopy **(Fig. 5.3.1g)**. As it is well known that the synthetases are induced upon different stresses, it may just be that induction is not enough to produce an adequate level of fluorescence. It may be that the construct itself could be improved. For example, other fluorophores could be trialled such as superfolder-GFP or enhanced-GFP which are engineered to fluoresce brighter than traditional GFP due to specific mutations **(Shaner, Patterson and Davidson, 2011)**. Moreover, the issues regarding visualising fluorescence may also be due to the sequence of the native promoter and coding sequence used. Promoter regions vary in size, and it may be that an insufficient amount of the promoter region was utilised for the construct. Furthermore, autofluorescence was detected during microscopy and autofluorescence of mammalian cells is an issue that is frequently encountered **(Aubin, 1979; Surre *et al.*, 2018; Kozlova *et al.*, 2020)**. It is possible that the synthetase-GFP fusions were unable to fluoresce at a higher level than autofluorescence and so steps could be taken to reduce this. For example, using specific media such as a non-phenol red-containing RPMI that may reduce background fluorescence.

As experiments on human neutrophils were not possible due to the COVID-19 pandemic, a HTE cell line was utilised as *S. aureus* can invade non-professional phagocytes such as these, which also enabled the study of how the stringent response is important for recurrent tonsilitis. HTE cells represent a physical barrier, a first line of defence for the tonsillar lymphoid tissue **(Guenther and Seyfert, 2018).** Thus, epithelial cells are able to kill bacteria similarly to professional phagocytes as they also form phagosomal compartments **(Lam *et al.*, 2010)**.By infecting these cells with both stationary and exponential phase bacteria, the (p)ppGpp0 mutant was observed to survive less well after invasion than WT, particularly at 8 hpi. This could mean that the (p)ppGpp0 mutant is unable to replicate as well or is more susceptible to intracellular killing. Exponential phase cells were able to invade at and grow to higher CFU/ml than stationary phase cells for both WT and the (p)ppGpp0 mutant **(Fig. 5.4.1d-f)** emphasising the importance of growth phase for infection. This coincides with previous research where *S. aureus* in the exponential growth phase had an enhanced invading capacity in comparison to stationary phase *S. aureus* **(Ji, Yang and Ji, 2020)***.*

In the absence of (p)ppGpp, bacteria are unable to mount a stringent response in response to stresses such as nutrient limitation. Many studies have reported that *rel/relA* or (p)ppGpp0 mutants demonstrate a decrease in virulence and pathogenesis *in vivo*, implicating the stringent response in the establishment of infection **(Nakanishi *et al.*, 2006; Taylor *et al.*, 2002; Pizarro-Cerdá and Tedin, 2004; Geiger *et al.*, 2010)**. Similarly, this study reveals that (p)ppGpp is required during systemic infection of zebrafish embryos and this was demonstrated by attenuation of virulence **(Fig. 5.5.2)**. Long-term examination of *S. aureus* survival within zebrafish embryos e.g., measuring embryo survival at timepoints beyond 5.2 dpf, may help to determine whether WT *S. aureus* are able to persist in comparison to the (p)ppGpp0 mutant, and the basis behind this phenomenon.

In this study, a difference in virulence between JE2 and LAC\* USA300 strains was observed, which could be due to the lack of p01 and p03 plasmids in JE2. This suggests that these plasmids may encode virulence factors that significantly impact the ability of *S. aureus* LAC\* to kill zebrafish embryos and this should be further studied to examine whether this is the case. This dose of LAC\* (1500 CFU) was also higher than what was reported in preliminary experiments (550 CFU). Though the dose of JE2 had to be increased to 3000 CFU in order to reach 50% killing of zebrafish embryos, other studies have achieved approximately 70% killing with a lower dose of 1500 CFU and this same dose was also required for SH1000 which is MSSA in comparison to MRSA **(Connolly *et al.*, 2017)**. These data indicate differences between laboratory strains which may require alteration of doses in order to achieve 50% killing.

Bacterial burden experiments demonstrated that the (p)ppGpp0 mutant was able to grow as well as WT within zebrafish embryos despite demonstrating attenuated virulence by survival curve analysis **(Fig. 5.5.3)**. Similar results have been shown previously also, where abscess formation by an *S. aureus* *rel* mutant was diminished, but the CFU/abscess was similar to WT **(Mansour *et al.*, 2016)**. Furthermore, an *M. tuberculosis rel* mutant was able to grow as well as WT in a mouse model while being less able to form granulomas, and it was only after five weeks of infection that a difference in CFU was detected **(Dahl *et al.*, 2003)**. However, fewer colonies of an MSSA *rel*syn mutant was recovered from murine kidneys in comparison to WT and was also associated with reduced formation of abscesses **(Geiger *et al.*, 2010)**. This suggests that the absence of Rel affects growth *in vivo*, however Geiger and colleagues did not evaluate the killing capacity of WT vs the *rel*syn mutant and differences may be due to the fact that MSSA was used and not MRSA. While some experiments corroborate what was observed in zebrafish embryos and suggests that the stringent response mutants exhibit a virulence defect rather than a growth defect, this experiment presented many caveats. As five live embryos and any dead embryos were selected from the population of injected zebrafish embryos, only certain dead embryos could be isolated for homogenisation, thus introducing a potential for bias. Most dead zebrafish embryos had disintegrated substantially by the time samples were to be taken, which could give *S. aureus* an opportunity to replicate within the embryo remains. Due to this, only moderately disintegrated embryos were selected for CFU determination in order to avoid unreliable CFU counts that are an overestimation of the actual number of *S. aureus* per embryo. In order to overcome this, it may be beneficial to take samples at earlier timepoints before 21 hpi, to allow sampling of embryos closer to the time of death to minimise decomposition.

Overproduction of (p)ppGpp *in vivo* did not have a protective effect on *S. aureus*, suggesting that the increase in (p)ppGpp levels was not concurrent with the ability of *S. aureus* to kill zebrafish embryos **(Fig. 5.7.1)**. As mentioned, (p)ppGpp overproduction has naturally occurred in a clinical isolate and when the resulting HD domain mutation was introduced into a laboratory strain, virulence was attenuated in a *G. mellonella* model **(Gao *et al.*, 2010)**, similar to what was seen here using the zebrafish embryo systemic infection model. This brings to light the importance of maintaining optimum levels of (p)ppGpp within a cell as overproduction of (p)ppGpp can lead to toxic accumulation, possibly impacting growth. The reduced growth that (p)ppGpp-overproducing bacteria may experience can have beneficial effects in the long-run. The (p)ppGpp overproduction strains in this study were unable to kill zebrafish embryos as well as WT, so long-term infection may lead to persistence as demonstrated in other reports **(Peyrusson *et al.*, 2020; Li *et al.*, 2020)**.The ability to persist could allow *S. aureus* to survive under harsh conditions and possibly disseminate, leading to the worsening of infection.

The long, bifunctional RSH Rel has been deemed an important synthetase in many different organisms and *in vivo* models **(Geiger *et al.*, 2010; Mansour *et al.*, 2016; Pizarro-Cerdá and Tedin, 2004; Dahl *et al.*, 2003)** and therefore its importance was investigated in this study. However, in these reports, bacterial burden from organs/abscesses were measured and were used to make conclusions on bacterial virulence. In comparison, this study directly demonstrated animal (zebrafish) survival as a result of infection, and not just bacterial burden. This is important to note, as how a pathogen grows is not always indicative of its pathogenic ability, as demonstrated in **Figure 5.5.4**. Infecting zebrafish embryos with the ∆*relQP* mutant revealed that killing by this strain is similar to WT **(Fig. 5.8.1a)**, suggesting that Rel is the only synthetase required for virulence. However, injection of LAC\* *relsyn* resulted in killing similar to WT **(Fig. 5.8.1b)**, despite not containing a functional copy of *rel.* This implies that RelP and RelQ could be compensating for the absence of Rel, and that it is not the synthetase that is important *in vivo*, but the (p)ppGpp that is produced. When *rel,* but not *relP* was complemented in a (p)ppGpp0 background, virulence was restored, adding further evidence to support the sufficiency of Rel **(Fig. 5.8.1c and d)**. In order for this to be confirmed, complement experiments need to be conducted *in vivo* using RelQ to determine whether or not this synthetase can restore virulence. As *relP* cannot complement *in vivo,* it must not produce enough (p)ppGpp alone. Therefore, it is necessary to determine whether *relQ* can as this can shed light as to why there was no difference in killing between LAC\* WT and LAC\* *relsyn*. It may be that RelQ can produce enough (p)ppGpp to make up for the absence of Rel in LAC\* *relsyn* but this is speculation. A caveat to complementing the synthetases on an inducible plasmid is that the amount of synthetase expressed is likely not physiological as induction occurs artificially. Complementing the synthetases on a vector whilst using the native promoter may be a superior method of assessing the importance of each synthetase as the amount produced will be more representative of what might occur during infection.

Infection of zebrafish embryos with the JE2 *codY*::Tnand (p)ppGpp0 *codY*::Tnled to similar killing as embryos infected with the respective controls. This was surprising considering Montgomery and colleagues showed that a USA300 strain lacking CodY exacerbated infection in both murine pneumonia and skin infection, leading to an increase in percent mortality **(Montgomery *et al.*, 2012)**. Accordingly, they also showed that virulence-associated genes directly/indirectly regulated by CodY were upregulated in the ∆*codY* mutant, such as *saeR* and *RNAIII* andthe toxins *hla* and *lukF-PV* suggesting that these are upregulated *in vivo* and may be responsible for the observed hypervirulence **(Montgomery *et al.*, 2012)**. In contrast, an MSSA ∆*codY* mutant did not exhibit hypervirulence in a haematogenous kidney abscess model and was indistinguishable from WT with regards to virulence **(Geiger *et al.*, 2010)**, similar to what was seen here in a systemic zebrafish embryo infection model. Taken together, CodY appears to have different functions upon induction of the stringent response depending on the organism which may be due to variation in the CodY regulon across species. However, it is also important to note that the stringent response affects metabolism and virulence independent of CodY.

In order to study the importance of CodY and its role during the stringent response, a *codY*::Tnmutant was used in this study to observe the phenotype in its absence. However, the constitutive derepression of CodY that is achieved by artificial ∆*codY* mutants is not representative of a natural infection and therefore, having these previously repressed genes constantly expressed may not be beneficial. For example, the genes repressed by CodY are derepressed in a temporal manner. During nutrient limitation, genes related to overcoming this limitation will be expressed first e.g., BCAA synthetic genes, while virulence genes such as toxins, are expressed last **(Waters *et al.*, 2016; Brinsmade, 2017)**. As the stringent response may be transient (depending on nutrient availability), and therefore (p)ppGpp-mediated CodY derepression could also be transient, it could be that at different points during infection, CodY derepression may not be necessary. This demonstrates the link between nutrient availability and virulence and could indicate that constant derepression of CodY may not be beneficial *in vivo*. Furthermore, another caveat to using a *codY*::Tn mutant is that during infection, the WT strain may also derepress CodY and if this is the case, then both WT and the *codY*::Tn mutant may be expressing genes of the CodY regulon which would mean that survival of embryos infected with both strains may be indistinguishable.

As mentioned in chapter four, the tolerance of both JE2 *codY*::Tn and (p)ppGpp0 *codY*::Tn mutants to H2O2 and itaconic acid was increased in comparison to their controls, but this was not observed *in vivo*. The *in vitro* tolerance assays spanned only 30 minutes while in the presence of high and physiologically irrelevant concentrations of each stressor. In contrast, zebrafish infection was monitored up to 93 hpi and the environment that *S. aureus* encounters is much more complex. This may justify why these results were inconsistent with the survival curves. Furthermore, the effect of *S. aureus* on zebrafish embryo survival was only measured up to 5.2 dpf, the point at which the embryos become protected by law. While both JE2 *codY*::Tn and (p)ppGpp0 *codY*::Tn were not more virulent than WT and (p)ppGpp0, it may be interesting to investigate the long-term host-pathogen interactions with these strains as it may be that the absence of CodY favours survival rather than virulence and so long-term investigations may elucidate this.

In summary, the (p)ppGpp0 mutant was inhibited in a HTE cell culture model, demonstrating the importance of the stringent response within non-professional phagocytes. While a difference between WT and the (p)ppGpp0 mutant was not observed in RAW 264.7 macrophages, preliminary data by others in the laboratory using MDMs has shown that the (p)ppGpp0 mutant can be killed more easily. This indicates that the cell types used during experiments is an important consideration and that some cell types may be more suitable than others. The synthetase-GFP fusions were unable to shed light on the expression of each synthetase during infection, which demonstrates the further work that is necessary to elucidate this, for example the redesigning of the construct. For the first time, the contribution of the stringent responsefor *S. aureus* virulence has been demonstrated in a zebrafish embryo systemic infection model. Both the absence and overproduction of (p)ppGpp results in a virulence defect, which stresses the importance of maintaining optimal levels of cellular (p)ppGpp for survival. However, overproduction of (p)ppGpp can also be beneficial with regards to long-term survival of *S. aureus* *in vivo*. Furthermore, the role of Rel and RelP has been revealed, with Rel considered to be the key synthetase for *S. aureus* virulence. Lastly, while the role of CodY during infection is not entirely clear based on previous studies, here, the absence of CodY is not advantageous for *S. aureus in vivo* and does not upregulate virulence. It is important to study these aspects of the stringent response during infection in order to gain a better understanding of the mechanisms by which (p)ppGpp impacts both survival and virulence during nutrient limitation.

# Chapter 6: Discussion

The stringent response is a conserved bacterial reaction to nutrient limitation that aids adaptation to stress. Environmental conditions can become adverse upon host colonisation and thus, the stringent response, mediated by the signalling molecule (p)ppGpp, acts to modify the transcriptome and proteome in order to maximise the chances of bacterial survival **(Sanchez-Vazquez *et al.*, 2019)**. This is often associated with a concurrent increase in virulence that favours survival **(Dalebroux *et al.*, 2010)**. The involvement of the stringent response in biofilm formation **(Sugisaki *et al.*, 2013; Salzer *et al.*, 2020)**, antibiotic tolerance **(Fung *et al.*, 2010; Abranches *et al.*, 2009; Geiger *et al.*, 2014)** and persistence **(Amato and Brynildsen, 2015; Schumacher *et al.*, 2015; Dahl *et al.*, 2003)** has also been demonstrated. Previous reports have implicated the stringent response in the pathogenicity of *S. aureus* **(Geiger *et al.*, 2010; Geiger *et al.*, 2012; Geiger *et al.*, 2014; Peyrusson *et al.*, 2020; Li *et al.*, 2020; Salzer *et al.*, 2020; Gao *et al.*, 2010; Mwangi *et al.*, 2013)** and thus, the aim of this study was to comprehensively understand why the stringent response is important for *S. aureus* pathogenicity and virulence. This was to be achieved by constructing and characterising strains that would allow the investigation of the absence of (p)ppGpp, the overproduction of (p)ppGpp, as well as how (p)ppGpp levels change during infection with the use of (p)ppGpp probes in *S. aureus*. Infection of RAW 264.7 macrophages and HTE cells with *S. aureus* and exposing *S. aureus* to phagolysosomal stresses would further the current understanding of how the stringent response aids intracellular survival. Finally, the use of zebrafish embryos to model *S. aureus* systemic infection would allow investigation of the role of the stringent response *in vivo*. This is important to study as MRSA bacteraemia is a major concern **(Turner *et al.*, 2019)** and the stringent response has been implicated in its persistence **(Li *et al.*, 2020)** and contributed to difficulties with patient treatment **(Gao *et al.*, 2010)**.

Chapter 3 details the construction and characterisation of tools to allow the investigation of the stringent response *in vitro* and *in vivo*. A panel of (p)ppGpp synthetase-deletion mutants provided by Dr Ian Monk were characterised and by sequencing it was revealed that the Rel, RelP and RelQ in the double and triple (p)ppGpp synthetase mutants were inactivated by silent in-frame deletions **(Fig. 3.2.2)**. These mutants were used in chapters 4 and 5 to examine how the absence of (p)ppGpp affects survival and virulence of *S. aureus.* Furthermore, as (p)ppGpp overproduction in a clinical isolate resulted in antibiotic resistance due to a HD domain mutation (uncontrolled (p)ppGpp production) **(Gao *et al.*, 2010)**, the construction of an overproduction strain was attempted. This strain was based on work by Gratani and colleagues and encoded for the Rel (p)ppGpp synthetase lacking the CTD and with a HD domain mutation on an integrative plasmid (pCL55-iTETr862) where expression can be controlled using the inducer Atc **(Fig 3.3.1)**. However, this strain, whilst displaying a growth defect in the presence of Atc, (**Fig. 3.3.3a, b and d)** was shown not to produce any more (p)ppGpp than the controls when analysed by TLC **(Fig. 3.3.3g)**. Therefore, alternative (p)ppGpp overproduction strains were constructed on both integrative (pCL55-iTETr862) and multicopy (pALC2073) vectors for *in vivo* and *in vitro* use respectively. Here, additional copies of the (p)ppGpp synthetases were introduced into JE2 WT and expression was also controlled via Atc **(Fig. 3.3.4)**.

The construction of (p)ppGpp probes to report on (p)ppGpp levels both directly and indirectly, was largely unsuccessful. Riboswitch-based molecular biosensors fused to RNA-aptamers, such as Broccoli and Spinach, have previously been utilised to report on cellular nucleotide levels **(Kellenberger *et al.*, 2015; Sherlock, Sudarsan and Breaker, 2018; Sun *et al.*, 2021)**. As these biosensors have been employed in *S. aureus* to measure c-di-AMP levels**(Syed et al., 2020)**, it was also attempted in this study. Using the construct described by Sun and colleagues, constructs were designed and characterised **(Sun *et al.*, 2021)**. Sun and colleagues designed a biosensor consisting of the *ykkc* riboswitch fused to the RNA aptamer, Broccoli. Here, the researchers used fluorescence microscopy to observe fluorescence generated by this biosensor (an indicator of (p)ppGpp production) within live *E. coli* and in response to amino acid starvation **(Sun *et al.*, 2021)**. Consequently, this study utilised the same biosensor construct to investigate when (p)ppGpp is produced in *S. aureus.* While *in vitro* transcription assays confirmed the functionality of the construct **(Fig. 3.4.3a)**, fluorescence measurements of constructs on both single copy integrative vectors and multicopy vectors within live *S. aureus* revealed that fluorescence was not detectable using the methods employed in this study **(Fig. 3.4.3 and 3.4.5)**. Ultimately, the most likely explanation for this is that the *ykkc* riboswitch, taken from the Gram-negative *T. oceani*, cannot be stably expressed in a heterologous manner within Gram-positive bacteria such as *S. aureus.* However, it is also possible that the methods used to detect fluorescence levels produced by the riboswitch-based molecular biosensor are not sensitive enough and thus, fluorescence microscopy may be a better alternative method.

Similarly, the transcriptional dual promoter-reporter fusion was unable to shed light on the transcriptional activity of promoters of genes that have been reported to be highly up- (*ilvD*) and downregulated (*rpsO*) upon induction of the stringent response **(Geiger *et al.*, 2012)**. Promoter-reporter fusions have previously been employed to investigate different aspects of the stringent response **(Bartoli  *et al.*, 2020; Goormaghtigh *et al.*, 2018; Pokhilko, 2017; Pathania *et al.*, 2021)**. Bartoli and colleagues demonstrated that by utilizing a transcriptional promoter-GFP fusion using the promoters of genes controlled by the SlyA virulence regulator, that SlyA is not directly affected by (p)ppGpp **(Bartoli  *et al.*, 2020)** as previously reported **(Zhao *et al.*, 2008)**. Using this construct, it was shown that transcription from these promoters was detected even in the absence of (p)ppGpp, revealing that the effects of (p)ppGpp on SlyA may not be direct in *E. coli* **(Bartoli  *et al.*, 2020)**. Furthermore, Goormaghtigh and colleagues used TA transcriptional fusions to demonstrate that *E. coli* does not utilise TA systems regulated by (p)ppGpp during the development of persistence **(Goormaghtigh *et al.*, 2018)**.

As in this study, the *ilvD* promoter (upregulated by (p)ppGpp) was previously used as a component of a p*ilvD-lacZ* fusion that was used to measure its transcriptional activity **(Pathania *et al.*, 2021)**. Here, upon exposure to mupirocin, an increase in fluorescence was observed suggesting that *ilvD* transcription is upregulatedin response to the increase in (p)ppGpp. Also, higher fluorescence was observed during stationary in comparison to exponential phase **(Pathania *et al.*, 2021)**. Similar to Pathania and colleagues, this study sought to understand whether (p)ppGpp production could be detected by measuring the activity of promoters that are up- and downregulated by (p)ppGpp. This was done using a single construct containing promoters that are opposingly regulated by (p)ppGpp and are fused to fluorophores (dual promoter-reporter fusion). In contrast to the controls, the dual promoter-reporter fusions did not demonstrate an increase in green fluorescence (signifying an increase in *ilvD* transcription) and a decrease in red fluorescence (signifying a decrease in *rpsO* transcription) **(Fig. 3.4.7)**. As the stringent response is transient depending on nutrient availability, it may be that the half-life of the fluorophores utilised in this study were too long to accurately report the change in promoter transcription upon exposure with amino acid starvation. Additionally, changes in promoter activity may not have been represented accurately if the promoter elements were not all included in the initial design of the construct.

While sequencing confirmed that all (p)ppGpp probe construct sequences were as intended, only the translational synthetase-GFP fusions were shown to express the synthetases, as measured by an increase in fluorescence output, in response to antibiotic stress *in vitro* **(Fig. 3.4.8)**. The experiments in **Figure 3.4.8** demonstrated that within an hour of administration of the appropriate antibiotic, fluorescence of the synthetase-GFP fusions increased. The level of fluorescence detected using a platereader was not high, particularly in the case of the Rel-GFP fusion **(Fig. 3.4.8d)**. As flow cytometry may be a more sensitive method of measuring fluorescence, this method was used to determine whether fluorescence could be detected at two hours post antibiotic administration **(Fig 5.3.1a-f)**. However, no fluorescence was detected at 2 hours *in vitro* or at 6 hpi when measuring fluorescence within MDMs via microscopy **(Fig. 5.3.1g)**. Optimisation of the construct design may be required to understand why fluorescence was not detected within mammalian cells. For example, ensuring the presence of an adequate amount of promoter region which can be done by taking a larger region than what was taken. Pathania and colleagues used a 515 bp fragment of the *ilvD* promoter plus the first five amino acids of the *ilvD* gene **(Pathania *et al.*, 2021)**, while this study used a 489 bp fragment including the first four amino acids. As promoter regions vary in size, it may be useful to trial different sized promoter regions and investigate any differences in fluorescence measured.

Of the tools developed and characterised in chapter 3, only the (p)ppGpp-synthetase deletion mutants and the (p)ppGpp overproduction strains were successfully used for *in vitro* and *in vivo* analysis. Previous studies investigating the ability of immune cells to kill stringent response mutants in *S. aureus* were performed using human neutrophils **(Geiger *et al.*, 2012)** and as far as is known, these studies have not been conducted in cell lines. As cell lines are easy to propagate, are generally reliable and the results produced can be consistent between repeats, this study utilised the RAW 264.7 macrophage cell line. It was demonstrated that there was no difference in killing of WT and the (p)ppGpp0 mutant by the RAW 264.7 macrophage cell line **(Fig. 5.2.3)** indicating that it may be better to use human cell lines or macrophages derived from human blood donors instead. Thus, since these experiments, others in the laboratory have demonstrated that human MDMs are a better model as a difference in killing was observed between WT and the (p)ppGpp0 mutant, thus reiterating the suitability of using human cells to study the stringent response. As the monocytes from which the macrophages were derived were isolated from human blood, the results from these experiments are more representative of human infection than when using RAW 264.7 macrophages, as pathogens do not always respond to murine receptors as they do human receptors **(Parker, 2017)**.

In contrast to the results observed in RAW 264.7 macrophages, host-pathogen interactions were also studied using HTE cells. It is important to understand how *S. aureus* interacts with non-professional phagocytes such as epithelial cells as they are the first line of defence as they constitute the physical barrier of epithelial cells such as skin and mucosa. *S. aureus* are known to frequently colonise the external tonsillar epithelium as well as the internal tonsillar tissue, which is suggested to result in recurrent tonsillar infections **(Zautner *et al.*, 2010)**. When both stationary and exponential phase *S. aureus* invaded the HTE cells, fewer (p)ppGpp0 mutant colonies were recovered which could suggest that they are unable to replicate or are susceptible to intracellular killing **(Fig. 5.4.1)**. *In vitro* data revealed that the (p)ppGpp0 mutant was more susceptible to acid and ROS stress, which may also be the case in the phagolysosome within HTE cells **(Fig. 4.2.5 and 4.2.7)**. However, future work for example time-lapse microscopy, could identify whether the (p)ppGpp0 mutant is able to grow as well as WT intracellularly within HTE cells. Studies have shown the importance of the stringent response for invasion in Gram-negative bacteria **(Pizarro-Cerdá and Tedin, 2004; Lim *et al.*, 2014; Johnson *et al.*, 2017; Dasgupta *et al.*, 2019)** and Gram-positive bacteria **(Colomer-Winter *et al.*, 2019)**. As the presence of a functional stringent response leads to the upregulation of virulence factors in *S. aureus* as described in section 1.2.3, this can increase the ability of *S. aureus* to invade during nutrient limitation, in order to find a more nutrient-rich environment. Furthermore, it was revealed that WT exponential phase *S. aureus* were better at replicating, demonstrated by higher CFU counts at 8 hpi **(Fig. 5.4.1e and f)**. *S. aureus* may be in different phases of growth during infection, and this can depend on the type of infection and the location. *S. aureus* has been suggested to initiate infection during the lag phase of growth and then grow rapidly during the exponential phase, where many virulence factors are expressed to enable bacteria to adjust to and exploit the host environment **(Harris, Foster and Richards, 2002)**. These include adhesins such as the FnBPs **(Pöehlmann-Dietze *et al.*, 2000)**,among many others, that are involved in adhesion and internalisation by many different mammalian cell types **(Sinha *et al.*, 1999)**. The different genes expressed during the different phases of growth explain why exponential phase cells fare better than stationary phase cells during initial infection of HTE cells, especially as bacteria usually initiate stationary phase growth when the population density is high, and the environmental nutrients are low. Future work to further understand the difference in virulence between the growth phases can include RNA-Seq to understand how the transcriptome of bacteria in the exponential and stationary growth phases differ. Furthermore, qPCR and ELISA analysis of the HTE cells after infection with stationary and exponential phase bacteria can shed light on whether a particular growth phase elicits a greater proinflammatory response in comparison to the other.

As mentioned, the (p)ppGpp0 mutant was unable to tolerate the phagolysosomal stresses as well as WT *in vitro* **(Fig. 4.2.5 and 4.2.7)** and *in vivo* within zebrafish embryos the (p)ppGpp0 mutant was attenuated in comparison to WT **(Fig. 5.5.3)**. The bacterial burden experiments demonstrated that this was not due to a replication defect**(Fig. 5.5.4)**, suggesting that the absence of (p)ppGpp affects virulence of *S. aureus*, which has been demonstrated by many studies **(Colomer-Winter *et al.*, 2019; Dasgupta *et al.*, 2019; Sugisaki *et al.*, 2013; Åberg, Shingler and Balsalobre, 2006)**. (p)ppGpp produced by the stringent response has multiple targets, affecting many subcellular processes to regulate metabolism and virulence and therefore has an effect on the different stages of infection. For example, adhesion of EHEC to intestinal epithelial cells is mediated by a T3SS regulated by (p)ppGpp **(Nakanishi *et al.*, 2006)** and an *S.* Typhimurium (p)ppGpp0 mutant showed a decreased ability to invade the intestinal epithelium **(Pizarro-Cerdá and Tedin, 2004)**. The staphylococcal stringent responsehowever has been implicated in antibiotic persistence **(Peyrusson *et al.*, 2020; Li *et al.*, 2020)**,indicating that the stringent response is important for intracellular survival during infection. The cytotoxic PSMs were revealed to be upregulated following phagocytosis of *S. aureus* **(Geiger et al., 2012)***,* and given their function of lysing immune cells, it is clear that the role of PSMs are to aid escape from professional phagocytes. Li and colleagues provide additional evidence of this by reporting increased expression of PSMs in persistent bacteraemia isolates that overproduce (p)ppGpp and that this increased expression also led to protection from hNP-1 **(Li *et al.*, 2020)**. These findings suggest an important role of the stringent response for escape and dissemination of *S. aureus* and so the absence of (p)ppGpp and thus, the inability to regulate PSMs may contribute to the decrease in killing of zebrafish embryos by the (p)ppGpp0 mutant during systemic infection.

Overproduction of (p)ppGpp *in vitro* increased the tolerance of *S. aureus* to acid and ROS stress **(Fig. 4.4.5)**, suggesting that (p)ppGpp overproduction aids survival. However, within zebrafish embryos, (p)ppGpp overproduction did not have a protective effect on *S. aureus* and did not increase the virulence of *S. aureus* **(Fig. 5.7.1)**, similar to what has been reported in an MRSA clinical isolate **(Gao *et al.*, 2010)**. This highlights the requirement of cellular (p)ppGpp regulation during infection and the need for optimal (p)ppGpp levels, as both concentrations that are too little or too high, negatively impact *S. aureus* virulence. It would be interesting to study embryos infected with WT and the overproduction strains past 5.2 dpf in order examine the survival of these embryos and whether (p)ppGpp overproduction aids long-term survival of bacteria and initiates the development of persistence. Furthermore, transcriptional analysis of bacteria isolated from infected embryos may provide insight into how (p)ppGpp overproduction in any surviving bacteria is having an effect on virulence.

**Figure 4.4.6** revealed that complementation with RelP but not Rel confers tolerance to itaconic acid stress, while complementation with either Rel or RelP helped tolerance of *S. aureus* to H2O2. This highlights how each synthetase has different roles with regards to surviving different stresses. As RelQ was not tested in this study, it is currently unknown what role it has, if any, during systemic infection. While RelP complementation was not sufficient to restore virulence during systemic infection **(Fig. 5.8.1d)**, this does not negate its role and contribution during infection. The oxidative burst within the phagolysosome has been deemed one of the most important components of host defence **(Dupre-Crochet, Erard and Nubetae, 2013)**. Therefore, given the importance of Rel during infection and its contribution to the tolerance to H2O2, it can be speculated that due to this, Rel is sufficient for virulence *in vivo*. These results corroborate previous studies that have demonstrated a role of the stringent response for promoting tolerance to ROS. This has been attributed to (p)ppGpp-mediated regulation of the catalase and SOD enzymes in *P. aeruginosa* **(Khakimova *et al.*, 2013; Martins *et al.*, 2018)**, which could suggest a similar mechanism of tolerance in *S. aureus*, which could be further studied by transcriptomic analysis of the staphylococcal *katA* and *sodA* genes. Furthermore, *S. aureus* is known for replicating within the acidic conditions of the phagolysosome **(Flannagan, Heit and Heinrichs, 2016; Flannagan *et al.*, 2018)**, which may explain why responding to acid stress may not be as integral as responding to oxidative stress, further clarifying the necessity of Rel during infection. However, as there was no difference in killing of embryos infected with LAC\* WT and LAC\* *relsyn*, the role of RelP and RelQ in the absence of Rel must be further investigated. As mentioned, the role of RelQ alone was not examined and so complementing RelQ both *in vitro* and *in vivo* may shed light on its contribution to survival and virulence. It is also important to note that Rel and RelP were complemented on a tetracycline-inducible plasmid and therefore, it is unknown if the expression of these synthetases was similar to native expression.

In chapter four, it was revealed that both the JE2 *codY*::Tnand (p)ppGpp0 *codY*::Tn mutants were better able to withstand exposure to itaconic acid and H2O2 and displayed enhanced survival *in vitro* in comparison to WT and the (p)ppGpp0 mutant **(Fig. 4.2.10)**. Interestingly, this was not the case *in vivo* where neither JE2 *codY*::Tn or the (p)ppGpp0 *codY*::Tnmutant displayed hypervirulence in comparison to their controls **(Fig. 5.9.1)**. It is noteworthy that virulence is not increased in the absence of CodY given that this transcription factor negatively regulates many virulence-associated genes for example, those involved in flagellar production and sporulation and also the virulence regulator *agr* **(Sonenshein, 2005; Majerczyk *et al.*, 2008)**. However, ∆*codY* mutants in other Gram-positive bacteria have not displayed hypervirulence either. For example, in *E. faecalis* the absence of CodY resulted in a decreased ability to colonise bladders in a murine CAUTI model, but when a *codY* deletion was introduced to a (p)ppGpp0 mutant, virulence was restored **(Colomer-Winter *et al.*, 2019)**, which was not the case in the current study. In a murine neonatal subcutaneous and an adult intravenous infection model, a *Streptococcus agalactiae* ∆*codY* mutant was attenuated in comparison to WT and was therefore less able to kill mice **(Pellegrini *et al.*, 2022)**. Additionally, the CAMP factor pore-forming toxin *cfb*, a virulence factor, is positively regulated by CodY and therefore in a∆*codY* mutant, there was downregulation of this toxin **(Pellegrini *et al.*, 2022)**. In *Streptococcus suis*, a∆*codY* mutant showed decreased adherence to human epidermoid cancer cells and was less virulent when injected into mice intraperitoneally, while a (p)ppGpp0 ∆*codY* mutant showed even lower levels of adherence and was completely avirulent **(Zhu *et al.*, 2019).** qRT-PCR analysis of specific genes revealed that some virulence genes were downregulated in the *S. suis* ∆*codY* mutant including *fbps* (fibrinogen-binding protein), *arcA* (arginine deiminase) and *sao* (surface antigen one) **(Zhu *et al.*, 2019)**. These studies in *Streptococcus* spp. demonstrate that the absence of CodY may also lead to downregulation of virulence-associated genes, which may explain the attenuated virulence of the ∆*codY* mutant. This could therefore be possible in other pathogens such as *S. aureus* and may explain the results observed in zebrafish embryos.

It is still unclear why some studies report hypervirulent ∆*codY* mutants while others report attenuation. This is mostly likely due to differences in species, strain and the infection models tested. It is evident that the (p)ppGpp and CodY regulatory networks are complex and intertwined, and therefore it may be necessary to investigate the expression of the CodY regulon from bacteria isolated from infected embryos to explain the phenotype observed in the systemic infection of zebrafish embryos. This is necessary to understand what virulence-associated genes are being upregulated in the absence of CodY and by what mechanism e.g., via another regulator.

In conclusion, this study has provided insight into how the stringent response is important for *S. aureus* pathogenesis. By examining the effect of phagolysosomal stresses on *S. aureus* stringent response mutants, the role of (p)ppGpp for tolerating these stresses has been shown, demonstrating the contribution of the stringent response for intracellular survival. For the first time, the function of the stringent response during systemic infection has been investigated within a zebrafish embryo model and has revealed the requirement of (p)ppGpp for full virulence. The role of Rel and RelP both *in vitro* and *in vivo* was shown, and this has shed light on how Rel and RelP may be responsible for tolerating acid and ROS stress, which has not been extensively studied in *S. aureus*. Overproduction of (p)ppGpp conferred tolerance to stress conditions found within phagocytes *in vitro* but not *in vivo*, reiterating the importance of maintaining optimal levels of (p)ppGpp within bacteria. Finally, deletion of CodY confers tolerance *in vitro* but not *in vivo*, indicating that CodY repression is important for *S. aureus* *in vivo* within a zebrafish embryo systemic infection model*.* These studies will allow further understanding into the complexities of the (p)ppGpp regulatory pathways, which can inform the development of more effective stringent response inhibitors for the treatment of infections caused by strains such as MRSA.

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