Contents

List of TABLES

List of Figures

ACKNOWLEDGMENTS

Thank should go first to Allah whose mercy and blessing helped me and gave me the strength to complete this thesis.

I would like also to thank Professor Howard Foster for his kind supervision, advice and encouragement during my research and for his attention to details in the preparation of this thesis.

I am grateful to the academic staff in the department and the technicians as well as my colleagues in the department and all the staff of the school for their kindness and support.

Special thanks for my parents, wife, brothers and sisters and all my relatives as well as friends back home for their encouragements and prayers. I would like also to thank all the staff of the Saudi Cultural Bureau in London who smoothed the progress of my PhD study. Also, I would thank Dr. Muneer kashoqugi for his unlimited support and encouragements during my different levels of study and I would never forget to pray for him. My thanks also extended to Professor Idrees alturk who guided me to the microbiology world and will never forget his inspiring words and performance in my future career. Special thanks for Dr. Ali Kheyami for his unforgettable supports, advices and unlimited ambition that he gave it to me through my postgraduate studies in the UK. Last but not least, I would like to thanks the staff of the Community college in Al Madinah and the staff of training and scholarship in Taibah University for smoothing the processes during my PhD study.

DECLARATION

No portion of this work referred to in this report has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

List of abbreviations

ASD	Anti- Shine Dalgarno sequence
CcpA	carbon catabolite control protein A
CDW	Cell Dray Weight
cre-sites	catabolite-responsive element site
CTD	C-terminal regulatory domain
FAD	flavin adenine dinucleotide
FBP	fructose‑1,6-bisphosphate
Fur	ferric uptake regulator
Gmk	guanylate kinase
HPr	histidine containing protein
HprT	hypoxanthine phosphoribosyltransferase
iGTP	Initiating GTP (initiation of promotors transcription via GTP)
IMP	inosine monophosphate
kDa	Kilo Dalton
NTD	N-terminal enzymatic domain
PVL	Panton-Valentine leukocidin
SCC	staphylococcal cassette chromosome
SD	Shine-Dalgarno sequence
TCA	tricarboxylic acid

Abstract

Staphylococcus aureus is a major pathogen in both hospital and community settings and it causes infections ranging from mild skin and wound infections to life-threatening systemic illness and, together with the emergence of antibiotic resistance, has been a major cause of morbidity and mortality worldwide.

The stringent response, is a stress response that bacteria display to avoid death when subjected to amino-acid starvation. This phenomenon has been observed in different species among Gram positive and Gram negative bacteria but relatively few studies have observed the stress response in *Staphylococcus aureus.* The stringent response can be triggered by treatment with mupirocin, an antibiotic that mimics amino-acid starvation by inhibiting isoleucyl tRNA synthetase.

In this project *S. aureus* 8325-4 was exposed to sub-inhibitory concentrations of mupirocin (0.5 MIC = 0.25μg/ml^{-1}) to investigate the ability of this concentration to trigger the stringent response. The treatment with mupirocin was continued up to 24 h as previous studies only examined short periods of treatment. Growth was inhibited and the stringent response nucleotide ppGpp was detected 1 h after treatment which slowly decreased in concentration for up to 4 h combined with significant growth inhibition. However, ppGpp could not be detected at 12 or 24 h whereas growth resumed.

In addition, the effect of sub-inhibitory concentrations of mupirocin was observed on the TSST-1 producing *S. aureus* clinical strain (B49). Q-PCR showed up-regulation of *tst* gene, codes for TSST-1, and its regulator RNAIII transcription up to 8 h of exposure relative to controls, the toxin was not detected by Reverse Passive Latex Assay.

Further, RNA-seq analysis was used to observe the global transcriptional alterations caused by the stringent response in *S. aureus* at 1, 12 and 24 h. From the whole transcriptome profile, differentially expressed genes relative to control as well as from comparisons between treated cell time points were observed concentrating on 60 virulence genes and stress related genes that

were significantly increased through stringent response status (1 h). Although ppGpp was not detected at 12 h, cells were still under the influence of the stringent response. However, cell growth had resumed by 24 h which indicates recovery after exposure to sub-lethal concentrations of mupirocin.

The effect of the sub-inhibitory concentration of mupirocin on global gene expression in *S. aureus* is discussed in relation to global control of gene expression and clinical use. In addition, a scenario for *S. aureus* recovery from stringent response has been suggested here which might open doors for drug target determination in the future.

Chapter 1

General Introduction

1.1 *Staphylococcus aureus*

1.1.1 Microbiology background;

Staphylococcus aureus is a member of the family *Staphylococcaceae* and it is considered one of the most pathogenic organisms in the community and within hospitals. It is a Gram-positive coccus sized between 0.5-1 μm. The organism has several virulence factors that contribute in its survival and pathogenicity (Greenwood *et al*., 2002).

1.1.2 Clinical importance of *S. aureus*

Staphylococcus aureus is one of the most medically important pathogens as it can cause a wide spectrum of infections ranging from mild skin infections to life-threatening diseases including septicaemia, endocarditis, necrotizing pneumonia, cellulitis, impetigo, septic arthritis, septic shock and toxic shock syndrome. The bacterium is also found to be one of the most common causes of bloodstream, skin and soft tissue, lower respiratory tract infections and hospital acquired infection in different countries (Diekema *et al.*, 2001, Archer, 1998). *S. aureus* can be often acquired from the hospital environment causing hospital acquired infections (nosocomial infections) as well as from the community initiating community acquired infections. From 26% to 32% of the health individuals carry *S. aureus* in their internal nares (nasal carriage) and although they are not infected seriously with the bacterium, this carriage facilitates the spread of the organism in the community (Sivaraman *et al.*, 2009). However, in hospital acquired infections the increasing threat of *S. aureus* comes from the increasing acquisition and evolution of antibiotic resistance (Sivaraman *et al.*, 2009). Many strains of *S. aureus* have become resistant to the most commonly used antibiotics. For instance, the organism became resistant to penicillin shortly after its introduction into clinical use and currently >80% of both hospital and community isolates are penicillin resistant (Lowy, 2003). In order to treat these infections β-lactam antibiotics such as methicillin and

oxacillin were introduced but resistance to them also occurred and those strains were termed methicillin resistant *Staphylococcus aureus* (MRSA). These strains possess the *mecA* gene that encod PBP2A which has low affinity to B-lactam antibiotics and promote resistance (Fuda *et al.*, 2004) then limited choices such as vancomycin, inhibits cell wall synthesis via binding to the D-alaon on the peptide end, is applied to treat infections caused by these strains (Lowy, 2003). Strains with reduced vancomycin susceptibility have been reported and the *van* operon found to contain the genes responsible for this resistance by replacing vancomycin's target end of the pentapeptides (Showsh *et al.*, 2001).

This capability of *S. aureus* to acquire antibiotic resistance and the rising incidence of hospital and community acquired infections has increased the medical importance of the bacterium. For example, *S. aureus* was found to be the most frequently isolated pathogen from bloodstream infections skin and soft tissue infections, and pneumonia in the United States, Canada, Latin America, Europe, and the western pacific region between 1997- 1999 and notable increase in the methicillin resistance among hospital and community acquired *S. aureus* strains in the United States has been reported (Diekema *et al.*, 2001). Recent European surveillance indicates that MRSA pose a real challenge for the public health in England and Europe (Johnson, 2011).

Nasal carriage of *S. aureus* plays a crucial role in the development of *S. aureus* infections. For example, Lina and colleagues claimed that *S. aureus* nasal carriage can increase the risk factor for the surgical site infection and they suggested a direct link between nasal carriage and the development of staphylococcal infections (Lina *et al.*, 1999).

In addition, *Staphylococcus aureus* is one of the most common bacteria that can cause either superficial or deep wound infection which might lead to either delay in wound healing or deterioration of wounds (Giacometti *et al.*, 2000). An infected wound may be characterized by increased or sustained pain, redness or swelling, pus discharge, unpleasant odour or non-

healing of the wound. Also, wound infection can occur as increasing erythema, oedema and continuous pain around the surgical site a few days after surgery (Janda *et al.*, 1997). Post-surgical infection is quite common and it is an important cause of morbidity and mortality (Heinzelmann *et al.*, 2002). It is also influenced by different factors such as disruption of tissue perfusion during the surgical operation, the state of hydration, nutrition and the patient medical conditions such as immune system condition and the presence of chronic diseases such as diabetes and the infecting organism's virulence properties (Heinzelmann *et al.*, 2002) There are different factors that can influence on the wound infection such as the wound depth, location and the organism that infects the wound (Bowler *et al.*, 2001). In addition, different factors can increase the possibility of *S. aureus* and other bacteria in general to gain access to a wound such as *via* airborne dispersal, direct contact with medical staff and equipment as well as endogenous transmission of the bacteria or self-contamination from patient's skin (Collier, 2004). In addition, toxic shock syndrome (TSS) disease can be associated with wound infections as a result of *S. aureus* toxin production. More details are discussed below.

S. aureus possesses a wide variety of virulence factors that are expressed at certain growth phases. For example, during the exponential phase where cells are dividing and growing at a constant rate (depending upon the composition of the growth medium as well as the conditions of incubation), virulence factors such as cell surface proteins are expressed. On the other hand, during the stationary phase where the population growth is reduced, because of either nutrient limitation or the effect of inhibitory metabolites, production of a range of virulence factors can be promoted, including toxins and lytic enzymes (Bronner *et al.* 2004). The major *S. aureus* virulence factors are described below (section 1.1.3) and details of their regulation are given in section 1.1.5.

1.1.3 *S.aureus* virulence factors

1.1.3.1 Cell surface proteins

Staphylococcus aureus can cause diseases through different mechanisms such as invasion and inflammation which are achieved *via* synthesis of extracellular molecules that facilitate bacterial adherence as well as promoting the bacterial evasion from host defense system (Kubica *et al.*, 2008, Archer, 1998). Cell wall associated virulence factors such as adhesion proteins and exopolysaccharides (adhesins) play a crucial role in this mechanism. *S. aureus* produces two groups of protein adhesins; the first group contains proteins that are covalently bound to the peptidoglycan, such as clumping factor (ClfA-B), protein A (Spa), fibronectin-binding proteins (FnBPs) and collagen adhesin (Cna) and their function is to assist the bacteria to adhere to different host extracellular matrices such as collagen, fibrinogen and fibronectin, as well as to promote evasion from host immune response (Götz, 2002, Foster and Höök, 1998, Kubica *et al.*, 2008).

The second group of adhesion proteins are secreted and partially bound to the cell wall, such as extracellular fibrinogen-binding protein (Efb), extracellular matrix protein (Emp) and extracellular adhesive protein (Eap). These adhesion proteins contribute in cell adhesion to host cell as well as modulating host immune defense (Foster and McDevitt, 1994, Kubica *et al.*, 2008). Expression of the surface binding proteins in *S. aureus* is tightly regulated in a growth-dependent manner and they are up-regulated in the exponential phase and repressed in the stationary phase of the cell growth. RNAIII which is a major virulence regulator in *S. aureus* seems to be responsible for the repression of many cell surface proteins during the l stationary phase either at transcriptional or translational levels (Novick *et al.*, 1993, Novick, 2003). For example, Otten and his colleagues claimed that, transcription of *fnb* and *spa* genes

is negatively controlled via RNAIII and the *fnb* seems more sensitive for RNAIII than *spa* gene (Saravia-Otten *et al.*, 1997).

a) Clumping factor

Clumping factor is a member of the *S. aureus* cell-wall binding proteins. It is a protein with a molecular weight of 21 kDa that cause cell aggregation in the presence of appropriate animal plasma (Hawiger *et al.*, 1982). This protein promotes cell binding to fibrinogen and it can be used to detect *S. aureus* (Kerrigan *et al.*, 2002) The cell aggregation that clumping factor causes seem to be an important factors to initiate *S. aureus* infections and it has been suggested that clumping factor can facilitate cell evasion from phagocytosis(Higgins *et al.*, 2006). The capability of clumping factor to bind to plasma and human platelets enhances *S. aureus* infections such as endocarditis and wound infection where the plasma and platelets are abundantly available for example, Moreillon and colleagues showed that in a rat endocarditis model, a clumping factor deficient strain of *S. aureus* (*ClfA2* mutant) was significantly less virulent than the wild-type strain (Moreillon *et al.*, 1995). Another study showed that *ClfA* promotes direct binding of *S. aureus* to specific platelet membrane receptor in human platelets (Siboo *et al.*, 2001). The *ClfA* and *Clf-B* genes encoder this protein and they are expressed in the early growth phase (McDevitt *et al.*, 1994, Ní Eidhin *et al.*, 1998).

b) Protein A

Protein A is one of the most important adhesin proteins. This 50 kDa protein covently binds to the peptidoglycan and comprises around 7% of the cell wall proteins and is responsible for non-specific agglutination in *S. aureus* (Romagnani *et al.*, 1982). Many strains of *S. aureus* produce protein A which makes it a reliable target for an identification test for *S. aureus*. In addition, protein A has the capability to attach to the Fc receptor of immunoglobulins IgG1, IgG2 and IgG4 (Lindmark *et al.*, 1983) which makes the attachment to specific receptors on the phagocytic cell membrane impossible which promotes the *S. aureus* evading the host

immune defenses (Romagnani *et al.*, 1982). The coding gene for protein A is *spa* and it is transcribed in the early exponential phase and suppressed by RNAIII during the stationary phase (Novick *et al.*, 1993, Novick, 2003)

1.1.3.2 Exotoxins;

Staphylococcus aureus produces wide range of exotoxins that can cause tissue damage and promote dissemination in the host's body and increases *S. aureus* virulence properties. These toxins include superantigens and cytotoxins. Most of these toxins produced at the stationary phase and seem to be tightly regulated in growth-dependent manner.

1.1.3.2.1 Superantigens

Superantigens are classes of toxins that can cause nonspecific T-cell activation, and a massive cytokine release which can lead to a life-threating condition. These toxins includes TSST-1 that causes Toxic Shock Syndrome (TSS) *S. aureus* enterotoxins (SEs) responsible for food poisoning, exfoliative toxins (ETs) that can cause tissue degradation and exfoliation during Staphylococcal Scalded Skin Syndrome(SSSS) (Proft and Fraser, 2003, Fraser and Proft, 2008)

a) Toxic shock syndrome toxin (TSST-1)

The toxic shock syndrome toxin produced by *S. aureus* can cause potentially fatal disease, toxic shock syndrome (TSS), with clinical features such as skin rash, fever and hypotension. This illness was originally described as a tampon-related infection in young healthy women (Shands *et al.*, 1980, McCormick *et al.*, 2001)**.** Currently, non-menstrual TSS cases are frequently reported as hospital and community acquired infection (Durand *et al.*, 2006). In addition, the toxin has the capability to increase the cytokine expression that might results in organ dysfunction, tissue damage and disseminated intravascular coagulation (McCormick *et al.*, 2001) The toxin is a single polypeptide with a molecular weight of 22 kDa that contains a

high percentage of hydrophobic amino acids. Predictive amino acid composition from the toxin nucleotides sequence shows that isoleucine is present in 8.7% of the total amino acids composition (Blomster-Hautamaa *et al.,* 1986). The gene that encodes TSST-1 is *tst* which is present in the bacterial chromosome and is carried on mobile pathogenicity islands SAP1, SAP2 or SAP3 that exist in 20% of *S. aureus* strains(Lindsay et al., 1998). Although these strains have the genetic ability to produce this toxin, fortunately TSS is quite rarely reported due to TSST-1 antibody expression in most individual bodies (Lindsay *et al.,* 1998, McCormick *et al.,* 2001).

The expression of the *tst* occurs in the stationary growth phase and the expression at this phase is attributed to the effect of a network of global regulators such as the accessory gene regulator (*agr*) locus and Staphylococcal accessory regulator (*sarA*) locus. For example, the *tst* gene is positively regulated at the transcription level by the *agr* effector molecule RNAIII (Novick, 2003). RNAIII expression is tightly controlled in a growth-dependent manner, and the transcription of its promoter *P3* is activated by AgrA which is a responsive factor for the quorum sensing two components system in *S. aureus* (Novick, 2003, Arvidson *and* Tegmark, 2001). Also RNAIII transcription is decreased when the CodY protein is activated during the early exponential phase (Majerczyk *et al.,* 2008). In addition, the SarA DNA-binding protein regulates *tst* transcription directly by binding *tst* promoter and indirectly by positively regulating the RNAIII promoter *P3* which activates the *tst* transcription (Andrey *et al.,* 2010). Furthermore, the two-component system SrrAB (staphylococcal respiratory response) down regulates *tst* transcription and its effector RNAIII in low-oxygen level conditions. Also, SrrAB was reported to enhance the TSST-1 production in an aerobic environment (Yarwood *et al.,* 2001, Pragman *et al.,* 2004, Pragman *et al.,* 2007). Vojtov and colleagues (2002) have shown that TSST-1 exhibits repressive activity on exoprotein production, as well as its own gene *tst* at the transcription level (Vojtov *et al.,* 2002). Moreover, carbon catabolite control

protein A (CcpA), which is a transcription regulator that binds to a catabolite-responsive element site (cre-sites), has been found to repress *tst* transcription *via* binding the cre-site of the *tst* gene, leading to the negative transcription of the gene and the deletion of *CcpA* de-repressesd *tst* transcription and surprisingly down-regulates the RNAIII (Seidl *et al.*, 2008a, Miller and Bassler, 2001) which makes the *tst* regulation system quite complicated. Figure 1summarises some of the *tst* regulation network.

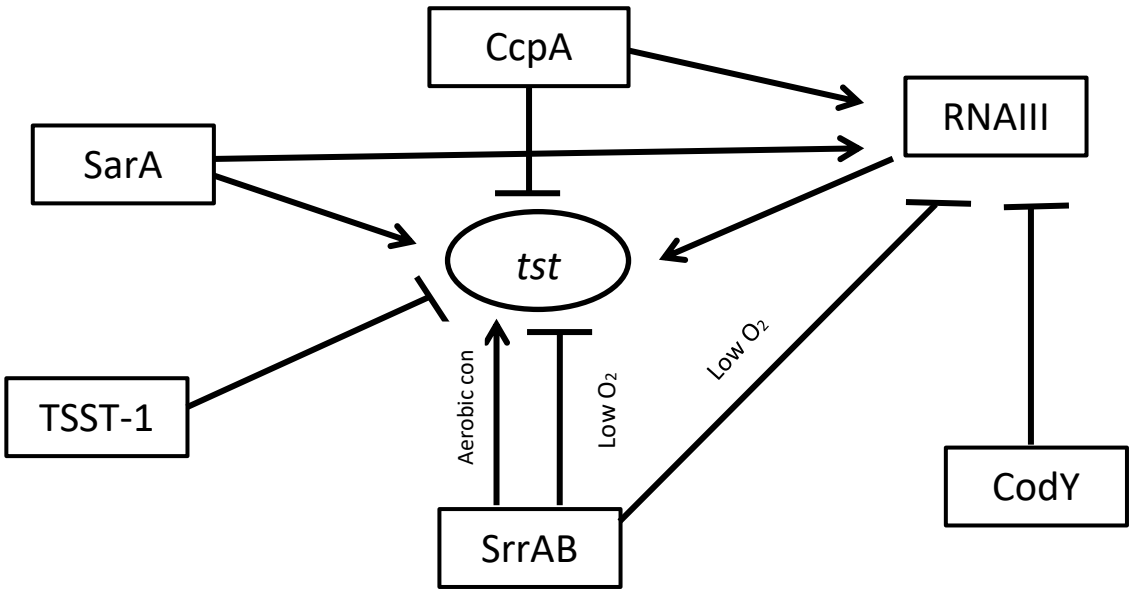


Figure 1 Regulation of *tst* transcription in *S.aureus*.

Transcription of *tst* is influenced by several regulatory factors. TSST-1 (toxic shock syndrome toxin-1), CcpA (Catabolite control protein A), SarA (Staphylococcal accessory regulator protein), SrrAB (Staphylococcal respiratory response protein). Arrows represent up-regulation, bars represent down-regulation.

b) Enterotoxins

Enterotoxins are a group of toxins that are produced by some staphylococci species including *S. aureus*. These toxins have a similar fundamental structure composed of single polypeptide chains with molecular weight from 27 to 34 kDa (Bhatia and Zahoor, 2007). *S. aureus* produces more than 21 different types of enterotoxin which are classified to groups on the

base of their antigenicity including enterotoxins A (SEA), B (SEB), C1 (SEC1), C2 (SEC2), C3 (SEC3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J (SElJ), K (SElK), L (SElL), M (SElM), N (SElN), O (SElO), P (SElP), Q (SElQ), R (SER), S (SES), T (SET), U (SElU), U2(SElU2) and V(SElV) (Argudín *et al.*, 2010). These toxins are heat stable and responsible for two thirds of the food-borne disease outbreaks which occurs as histological abnormalities in the gastrointestinal tract (Bhatia and Zahoor, 2007). In addition, these toxins can cause immunosuppression by reducing B and T cells and the coding genes for these toxins are located on accessory genetic elements, such as plasmids, pathogenicity islands (SaPIs), prophages, *S. aureus* genomic island vSa, or next to the staphylococcal cassette chromosome (SCC) elements (Marrack and Kappler, 1990, Schelin *et al.*, 2011). The maximum level of toxin's production can occur at the stationary phase of the cell growth and the *agr* locus as well as SarA seem to activate the transcription of some enterotoxin genes such as *seb, sec* and *sed* (Schelin *et al*., 2011)

c) Exfoliative toxins

Exfoliative toxin is one of the *S. aureus* exotoxins, protein 30 kDa, that associated with Staphylococcal Scalded Skin Syndrome (SSSS) or Ritter's disease which a disease affecting infants and characterized by the loss of superficial skin layer (Kapral and Miller, 1971, Bukowski *et al.*, 2010). The toxin(serine proteases) works by causing intra-epidermal splitting via cleaving the protein that is responsible for the cell-cell adhesion (Bukowski *et al.*, 2010). There are four different serotypes of the toxin (ETA, ETB, ETC and ETD) and the toxin present in 3-4% of *S. aureus* isolates (Bukowski *et al.*, 2010, Lina *et al.*, 1997). The ETs encoding genes are *eta* for ETA carried on phage, *etb* codes ETB located on plasmid and *etc* that responsible for ETC production and located on pathogenic island therefore these gene can potentially be transmitted to other cells (Lina *et al.*, 1997). Exfoliative toxins are produced through the late exponential phase or early stationary phase of cell growth and they tend to be

controlled in a growth –dependent manner as *agr* system showed positive regulation on *eta* and *etb* genes (Sheehan *et al.*, 1992)

1.1.3.2.2 Cytotoxins

S. aureus produces number of membrane damaging toxins and they can be classified into two groups; haemolysins includes (alpha, beta, delta, and gamma) and Panton-Valentine leukocidin.

Alpha-haemolysin (Alpha-toxin)

Many strains of *S. aureus* secrete an alpha toxin which shows toxic effects on a wide range of mammalian cells (Bernheimer and Schwartz, 1963). It has a single polypeptide chain with a 33 kDa molecular weight. The mature protein contains 293 residues and the toxin is capable of lysing rabbit erythrocytes with a high affinity, at least 100 times more so than other mammals and 1,000 times more than human erythrocytes (Bhakdi *et al.*, 1984). This toxin is one of the most potent membrane damaging toxins that *S. aureus* secretes as it can attach to its target cells and form pores leading to cellular contents leaking (Valeva *et al.*, 1997). The toxin is expressed at the late exponential phase or early stationary phase and its coding gene, *hla*, seems to positively regulate via *agr* also this gene was temperature-dependent as its maximum expression occurred at 42^{o}C and its transcription was detected in mid-exponential phase suggesting that *agr* was not the only regulator for this gene (Ohlsen *et al.*, 1997).

β-Haemolysin (Sphingomyelinase C)

β-haemolysin is secreted by *S. aureus* into the culture medium as a 330-amino-acid polypeptide with a predicted molecular weight of 39 kDa (Huseby *et al.*, 2007). β-haemolysin is produced in a high percentage of *S. aureus* strains particularly from animal isolates from bovine mastitis (Matsunaga *et al.*, 1993). The toxin is also named Sphingomyelinase C as it degrades sphingomyelin, a phospholipid that is abundant in mammalian cell membranes (Doery *et al.*, 1963, Low and Freer, 1977). In addition, its known as the hot-cold toxin

because of its unique activity on sheep blood agar plates as the toxin interacts with sheep red blood cells but does not lyse them at 37°C but if the red cells are then placed at 4°C the haemolytic activity of the toxin appears (Low and Freer, 1977). During *Staphylococcus* infections beta toxin seems to be involved in tissue damage and abscess formation and the toxin is encoded on *hlb* gene which is expressed in a growth-dependent manner as it's transcription occurs during the stationary phase and the *Sar* locus has been shown to positively regulates the transcription of this toxin (Cheung and Ying, 1994).

Delta-Haemolysin (Delta-Lysin, Delta-Toxin)

Delta haemolysin is an extracellular product composed of a 26 amino acid peptide with a molecular weight of around 3 kDa and is produced from many strains of *S. aureus* (Kantor *et al.*, 1972). Delta hemolysin is a potential exotoxin that exhibits cytotoxicity on wide range of different mammalian cells causing membrane damage. The coding gene for this toxin is *hld* and it is expressed in the stationary phase and the *agr* locus showed positive activation on this gene transcription (Recsei *et al.*, 1986).

Gamma-haemolysin and PV-Leukocidin

These toxins are composed of two non-associated secreted proteins, S and F. Gamma toxin is produced from most *S. aureus* strains whereas PV-Leukocidin has only been detected in 2-3% of isolates (Kuehnert *et al.*, 2006). These toxins can influences of the neutrophils and macrophages but gamma haemolysin is additionally capable to lyse a wide range of mammalian cells (Prevost *et al.*, 1995). Gamma toxin is transcribed from a single locus which contains three genes *hlgA, hlgB and hlgC* which has high similarities to locus *lukR* that encodes leukocidinR (Kamio *et al.*, 1993). The PV-Leukocidin is a crucial virulence factor in necrotizing diseases that might cause life threating infection in few days (Lina *et al.*, 1999). The toxin induces pores formation in the membranes of the susceptible cells leading to leak of

the cell content and causing skin and soft tissue infections such as necrotic lesions and necrotic haemorrhagic pneumonia (Kaneko and Kamio, 2004, Gillet *et al.*, 2002). PV-Leukocidin is encoded on a prophage (Φ-PVL) which is a genetic material from a bacteriophage that infects *Staphylococcus aureus* and makes it highly virulent (Melles *et al.*, 2006).

1.1.3.3 Extracellular enzymes

In addition to the exotoxin *S. aureus* shows the ability to produce a wide range of extracellular enzymes that can enhance different infections. Most of the extracellular enzymes are produced in the post-exponential phase such as protease, lipase and others.

a) Proteases

S. aureus can secrete 10 different types of proteolytic enzymes including metalloprotease, V8 or SspA serine protease, two cysteine proteases (staphopain A (SspA) and staphopain B (SspB)), and six serine-like proteases that are SspA homologues (SplA- F). The *spl* operon is transcribed at the stationary phase of cell growth and has been shown to be positively regulated via the *agr* system and its present in 64% of isolated *S. aureus* and deletion mutant of this operon showed attenuated virulence in comparison to the parent cell (Reed *et al.*, 2001). For example, *sspABC* and *sspBC* mutations showed reduced virulence in a murine skin abscess model (Shaw *et al.*, 2004) and metalloprotease has been shown to promote the cell survival in phagocytes during infection (Kubica *et al.*, 2008). In addition, Dunman and colleagues suggested a link between the proteoolytic activity and amino acid limitation through the stringent responses (Anderson *et al.*, 2006).

b) Staphylocoagulase

Coagulase production can be used to distinguish between pathogenic and nonpathogenic strains of *S. aureus*. Coagulase is an enzyme that promotes the conversion of fibrinogen to fibrin clots which can form a protective coating against phagocytosis around cells in the infected area in the host body. *S. aureus* produces coagulase as an extracellular product or it can be bound to the cell wall and growth condition of the cell can influence the production level of this enzyme (McDevitt *et al.*, 1992, Engels *et al.*, 1978). In addition, coagulase is expressed in the exponential growth phase in contrast to other extracellular proteins in *S. aureus* that are produced during the stationary phase and it seems to be down-regulated via the *agr* system effector regulator RNAIII during stationary growth phase. For example, in wild type cells coagulase has been expressed at the exponential growth phase whereas in the *agr* deletion mutant coagulase was expressed constantly during stationary growth phase which indicated the negative regulation of *agr* locus on the coagulase production (Lebeau *et al.*, 1994).

c) Lipase

Lipase (glycerol ester hydrolase) is an enzyme that catalyses the hydrolyse of lipids. Early observation showed that 99% of human strains of staphylococci and 75% of staphylococcal strains derived from animals exhibited lipolytic activity (Elek, 1959). Lipase is one of the extracellular enzymes that *S. aureus* produces either to establish colonization or to facilitate the cell survival from host defenses (Hu *et al.*, 2012). High level of lipase production was exhibited by *S. aureus* strains that caused deep infections such as septicaemia and pyomyositis (Rollof et al., 1987). Also, lipase has been suggested to promote *S. aureus* survival during the host defense response by inactivating the bactericidal lipids and to promote *S. aureus* persistence in mammalian skin and thus increase the pathogenic potential

of the organism(Shryock *et al.*, 1992, Rosenstein and Götz, 2000). In addition, lipase in *S. aureus* is produced at the stationary growth phase and seems to be expressed positively under the *agr* locus regulation and its coding genes found to be up-regulated during the stringent response in *S. aureus*. (Bronner *et al.*, 2004, Anderson *et al.*, 2006).

d) Catalase

Catalase is an enzyme that promotes the decomposition of toxic hydrogen peroxide (H_2O_2) to oxygen and water (Hampton *et al.*, 1996). All *Staphylococcus* species produce this enzyme and it can be used to differentiate between *Staphylococci* and *Streptococci* as the latter are unable to produce catalase. The coding gene for catalase is *katA* in *S. aureus* and it is under the positive regulation of ferric uptake regulator (Fur), a central regulator of iron homeostasis, and the transcription of this gene is induced at increasing level of iron during growth in *S. aureus* (Cosgrove *et al.*, 2007). This enzyme is important to protect the cells from the accumulated toxic hydrogen peroxide during growth or following phagocytosis, which can enhance S. *aureus* pathogenicity (Imlay, 2003, Hampton *et al.*, 1996)

e) Nuclease

Nuclease is one of the *S. aureus* enzymatic productions which shows capability to cleave either double or single stranded DNA or RNA generating 3′-nucleotides dinucleotide and thermo stable phosphates (Anfinsen, 1968). Nuclease was first discovered by Cunningham et al in 1956 and its production is a unique marker that can be used directly to detect *S. aureus* in clinical and food specimens (Cunningham *et al.*, 1956, Alarcon *et al.*, 2006). The enzyme has different forms nuclease I, II and III and is heat-stable and is sometimes referred to as thermonuclease (Tucker *et al.*, 1978). Nuclease is an important virulence factors in *S. aureus* as it plays a role in hydrolysing the DNA and RNA in the host cells causing tissue destruction. Also the enzyme is found to promote the evasion from the host immune response during lung

infection (Foster, 2005). Nuclease is secreted during stationary growth phase and thought to be under *agr* positive regulation (Smeltzer *et al.*, 1993). However, recent work by Olson and colleagues (2013) showed that *nuc*, the coding gene for nuclease, is under the control of the SaeRS two-component system and they claimed that nuclease is a conserved virulence factor among Gram positive cocci (Olson *et al.*, 2013).

1.1.3.4 Biofilms

Forming a biofilm is one of the important mechanisms that *S. aureus* uses for causing disease. Biofilm is a complex aggregation of bacteria encapsulated by an extracellular matrix attached to a surface and exhibiting increased resistance to antimicrobial agents, environment stress and immunological defense (Götz, 2002). In the biofilm, bacteria change their lifestyle from a unicellular state to an adherent multicellular state by secreting binding molecules such as polysaccharide intercellular adhesin (PIA) (Götz, 2002) This microbial community range from a monolayer of single cells to a multilayered thick mucoid structure of cells. *S. aureus* is able to form biofilms on biotic and abiotic surfaces and biofilms protect the cells from the host immune response, and also from antimicrobial agents which make such infections difficult to treat (Götz, 2002, Stewart *and* William Costerton, 2001)

In general, *S. aureus* possesses a wide range of virulence factors that are encoded by different genes and their regulation is influenced by several factors either at transcriptional level or at post-transcriptional level. In the following section the mechanism of gene expression and DNA replication are briefly highlighted.

1.1.4 Brief review of DNA replication and gene expression in bacteria

The central dogma of molecular biology describes the flow of the genetic information to produce nucleic acids and proteins that are needed to maintain the living activities throughout

DNA replication, gene transcription and protein synthesis as summarised in figure 2. The mechanisms of these processes are briefly described below.

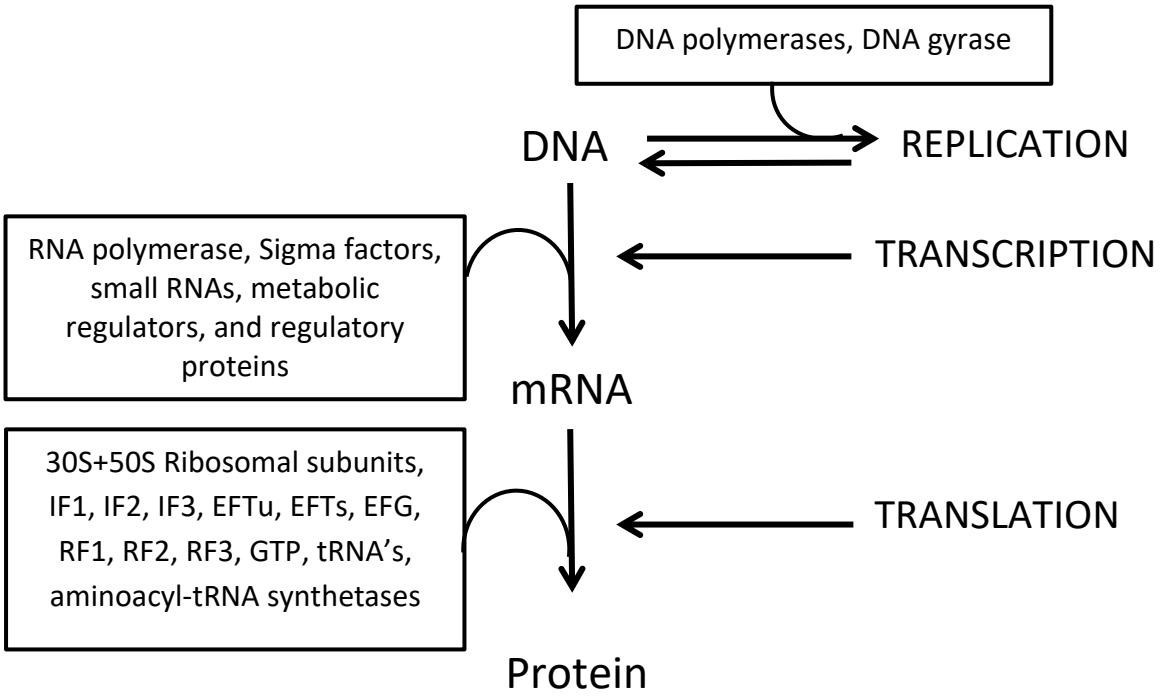


Figure 2 The central dogma of molecular biology.

1.1.4.1 DNA replication

The genetic information contained in a bacterial cell is found in one or more circular or linear chromosomes (a single circular chromosome in *S. aureus*) which comprise a double strand of deoxyribonucleic acid (DNA). Each strand contains nucleotides that are composed of nitrogen bases of guanine (G), adenine (A), thymine (T), or cytosine (C) and a phosphate group as well as the sugar deoxyribose. These nucleotides are joined together in a chain via covalent bonds between the sugar and the phosphate of the adjacent nucleotide. The nitrogen base in the first strand attaches to other bases in the second strand via hydrogen bonds according to the base pairing rules of A with T (or U in RNA) and C with G. The two strand are antiparallel to one

another, and one strand runs from the 5` end in the direction of the 3' end whereas the other runs from the 3' end to the 5' end direction (Marinas, 1992).

DNA replication is a process that allows a bacterial cell to pass an identical copy of its genome to a daughter cell. The replication is achieved through three steps: initiation, elongation and termination. In the bacterial chromosome, DNA replication starts at a specific sequence of nucleotides called the origin. The initiation protein (DnaA) and ATP bind to this sequence to create what is called a replication bubble by breaking the hydrogen bonds between adenine (A) and thymine (T) (Messer, 2002).

The replication bubble attracts the enzyme helicase which unwinds the two strands while it moves down the DNA molecule using ATP as an energy source to break the hydrogenbonds between the nitrogen bases (LeBowitz and McMacken, 1986). In addition, single strand binding proteins bind to the separate strands to prevent them from re-annealing (Meyer and Laine, 1990).

The elongation step begins when the helicase recruits DNA primase which is an enzyme that synthesises an RNA primer which contains a 3` hydroxyl group on the DNA template, The DNA polymerase III then commences to synthesise the new strand by adding complementary bases to the template strand bases one by one in the 5` to 3` direction (Baker and Bell, 1998). The new complementary strand that is synthesised in the same direction of the replication fork is named the leading strand. The other new strand is named the lagging strand and it is synthesised in fragments (Okazaki fragments) as it elongates in the opposite direction to the replication fork. Then the RNA primer at the one end of the fragment is cleaved by RNase H, and another enzyme, polymerase I, fills the gaps between the Okazaki fragments by adding DNA nucleotides after that DNA ligase closes the remaining nick on the strand to make it one continuous DNA strand (Baker and Bell, 1998).

Replication termination is achieved by inhibition of the replication fork when a protein called Tus binds to the terminus region which contains several DNA replication terminator sites that subsequently fuse the replication fork and terminates the replication process (Bussiere and Bastia, 1999).

1.1.4.2 Transcription

Transcription is a process where the information content of DNA is transferred to a specific nucleotides sequence, messenger RNA (mRNA), to be used for protein synthesis in the translation phase. The transcription is achieved in three steps: initiation, elongation and termination.

Initiation takes place when RNA polymerase and sigma factors, specific proteins which bind to gene to initiate transcription by binding to the promoter, a specific sequence that does not code for protein in the DNA (Gross *et al.*, 1998, Reznikoff *et al.*, 1985). After that, this initiation complex melts the DNA double strand by breaking the hydrogen bounds between G-C and A-T to make the template strand accessible for the RNA polymerase to begin the elongation. In the elongation step, the RNA polymerase moves along the template DNA strand to synthesise a complementary RNA sequence (mRNA) from the 5' toward the 3' until a terminator sequence is reached (Borukhov *et al.*, 2005). Two mechanisms of the termination event are known. The first, self-termination, is the most common mechanism. This occurs when the RNA polymerase encounters a particular sequence of bases, inverted repeats, such as CCGGGGAAAA in the transcribed DNA strand that forms a hairpin loop which is the destabilising structure for the RNA polymerase. The second termination mechanism depends on the termination enzyme which binds to the newly synthesised mRNA at the 5', and when the RNA polymerase encounters the termination sequence, the enzyme moves towards the 3'of the mRNA to bind RNA polymerase which results in dissociating the RNA polymerase from the DNA template (Nudler and Gottesman, 2002). After completing the transcription process, the newly synthesised mRNA and

the RNA polymerase are released. However, not all the base sequence in the mRNA will be translated, as the translation initiation might begin many nucleotides downstream of the 5' of the mRNA.

1.1.4.3 Translation

Translation in bacteria is the process by which the messenger RNA (mRNA) is translated into protein. Translation is achieved through three different phases: initiation, elongation and termination, using different components that are necessary for this operation namely mRNA, tRNA, small 30S ribosomal subunit, and large 50S ribosomal subunit, aminoacyl tRNA synthetases and initiation, elongation and termination factors and GTP as a source of energy (Gualerzi and Pon, 1990).

Translation is initiated when mRNA binds to a free 30S ribosomal subunit. This binding is facilitated by hydrogen bonding between the 16S RNA component of the 30S subunit and the ribosome binding site of the mRNA. Initiation factors (IF-1, IF-2 and IF-3) are involved as IF-3 binds to the 30S subunit and prevents premature association of 30S and 50S subunits, IF2 binds with special initiator tRNA charged with formylmethionine ($tRNA^{fMet}$) and IF-1 stimulates the activity of IF-2 and IF-3 (Antoun *et al.*, 2003, Malys and McCarthy, 2011, Kozak, 1999).

There are three sites in the ribosome (A, P and E). Each of these is accommodated to a specific process during translation. The A-site is the point of entry for the charged tRNA (except for the first aminoacyl $tRNA^{fMet}$ that enters at the P site). The P-site is the central site where the peptidyl tRNA is formed in the ribosome. The E-site is where uncharged tRNA exits the ribosome after it donates its amino acid to the growing peptide chain (Ramakrishnan, 2002).

The selection of an initiation site relies on the interaction between the 30S subunit and the mRNA template, as the 30S subunit binds to the mRNA template at a purine-rich region (the Shine-Dalgarno sequence) upstream of the AUG initiation codon. The Shine-Dalgarno sequence is complementary to a pyrimidine rich region on the 16S rRNA component on the 30S subunit (anti Shine-Dalgarno sequence). As a result, a double stranded RNA structure binds the mRNA to the ribosome which places $tRNA^{fMet}$ in the P site of the ribosome (Malys and McCarthy, 2011, Shine and Dalgarno, 1974)

This is followed by translation elongation by which amino acids are added to the carboxyl end of a growing chain of amino acids to form a nascent peptide. There are three main processes in translation elongation. The first step is bringing new aminoacylated tRNA into line as well as releasing uncharged tRNA from the E-site in the ribosome. This step is facilitated by EF-Tu that binds with GTP to convey the next aminoacylated tRNA into the A-site of the 50S ribosome. As a result, GTP is hydrolysed to GDP which subsequently reduces the affinity of the EF-Tu for the ribosome which diffuses away, leaving room for EF-G-GTP (Ramakrishnan, 2002).

The second step is forming a new peptide bond to elongate the polypeptide. After A-site filling, peptidyl transferease catalyses bond formation between the amino acid in the P-site with the new amino acid that is carried in the A-site, to form the first peptide bond (Rodnina and Wintermeyer, 2003).

The final step of elongation is shifting the tRNAs from the P and A sites to E and P sites respectively, as well as the translocation which moves the 30S ribosome subunit to the next codon on the mRNA. This process is catalysed by elongation factor EF-G which hydrolyses GTP to yield the energy needed for this step. The ribosome continues to synthesise the peptide coded in the mRNA until it reaches one of the stop codons UAG, UGA and UAA that

begins the translation termination phase (Ramakrishnan, 2002, Dinos *et al.*, 2005, Rodnina *et al.*, 1997).

When the ribosome reaches a stop codon, no tRNA can enter the A-site of the ribosome and the tRNA that carries the polypeptide is hold in the P-site. Release factors (RF1, RF2 and RF3) act at this stage to cleave the polypeptide from the tRNA, as well as to dissociate the 70S ribosome from the mRNA. Ribosomal subunits 30S and 50S are recycled to initiate the translation of another mRNA (Ramakrishnan, 2002, Nakamura and Ito, 2003).

1.1.4.4 Regulation of transcription

There are several mechanisms for the control of transcription for instance, a group of small proteins called sigma factors facilitate the initiation of transcription. The functions of sigma factors are to recognize the promoter sequence, to position the RNAP on the target promoter and to promote the unwinding of the DNA duplex near the transcription start point (Browning and Busby, 2004). An example for sigma factor is sig70 (σ^{70}) in *E.coli* which promotes RNAP to bind to genes that are involved in functions that facilitate cell growth. In *S. aureus* sigB (σB) activates more than 200 genes that involved in different functions include signalling pathways, cell envelope biosynthesis and *S. aureus* pathogenesis (Bischoff *et al.*, 2004).

Repressor proteins also can affect gene transcription by binding to a non-coding sequence in the operon, the operator, and prevent the RNA polymerase from proceeding through the functional genes in the operon leading to impeded transcription of these genes. An example of this mechanism is the repression of the tryptophan (*Trp*) operon in *E.coli* during the abundant availability of tryptophan in the environment (Shimizu *et al.*, 1973, Zubay *et al.*, 1972).

In addition, inducer molecules can promote gene transcription via binding then deactivating specific repressor proteins that are responsible for the impeded transcription of the operon. For instance, allolactose deactivates repressor protein for the lactose operon (*lac* operon) by

binding to it and changing the protein shape in a way that prevents the repressor protein binding to its target sequence on the operon, thus in the presence of lactose, the RNAP can bind to the promoter and transcribe the genes that *E.coli* cell needs to synthesise enzymes that responsible for lactose metabolism (Eron *and* Block, 1971).

Small non-coding RNAs are also involved in transcription regulation in bacteria (Storz *et al.*, 2006). For example, in *E.coli, a* 6S RNA is found to inhibits the transcription of sig70 (σ^{70})-dependent promoters via obstructing promoter recognition and activation of the sigma (S)-dependent promoter during nutrient deprivation (Storz *et al.*, 2006, Trotochaud and Wassarman, 2004).

Furthermore, forming secondary structure via base pairing binding between small RNAs and its target mRNA results in transcription termination which is another mechanism that small RNAs exhibits to regulate transcription (Storz *et al.*, 2006). A model for this mechanism is shown in staphylococcal plasmid pT181 which contains replication genes that encode replication protein C, essential for the plasmid replication, and the small noncoding RNAs either of RNAI or RNAII bind to the replication protein C mRNA and form secondary structures that lead to transcription termination (Brantl and Wagner, 2000, Storz *et al.*, 2006).

In addition, cell density can regulate transcription in bacteria through two component systems or quorum-sensing systems. This is a mechanism whereby many bacteria can coordinate particular gene expression in response to their population density in the environment. The system often consists of a membrane sensor (histidine kinase) and a cytoplasmic response regulator. The histidine kinase senses and responds to external stimuli (autoinducer), even if the stimuli do not penetrate the cytoplasm. In response to the stimuli, the conserved histidine is autophosphorylated. The phosphorous group is then transferred to the response regulator, which either activates or represses the transcription of the target gene (Dziejman and

Mekalanos, 1995). The two-component system plays a crucial role in controlling the metabolism, and regulating the virulence factors in a wide range of Gram negative bacterial species such as *E.coli, Salmonella typhimurium,* and *Haemophilus influenzae,* as well as Gram positive bacteria such as *Streptococcus pyogenes, Enterococcus faecalis, Streptococcus mutans, Clostridium perfringens and Staphylococcus aureus* (Miller and Bassler, 2001).

Moreover, small nucleotides such as ppGpp can show another mechanism that alters transcription in response to environmental conditions particularly nutrient limitation. For example, in *E.coli* ppGpp is synthesised during amino acid limitation and this nucleotide binds directly to the RNAP with cofactors, DksA, to regulate the transcription of some genes by destabilizing open complexes at their promoters (Chatterji and Kumar Ojha, 2001). This mechanism is described in detail in section 1.3

1.1.4.5 Translation control

Several factors can contribute and affect the translation processes in bacteria. One of these factors is mRNA sequence and structure which plays essential role in its interaction efficiency with the translation machinery (Laalami *et al.*, 1996). For instance, in *E.coli* different elements in the mRNA such as the cognate initiation codon for the interaction with fMet-tRNA and the complementary sequence of SD to the ASD in 16S rRNA might lead to increasing expression of the mRNA(Shine and Dalgarno, 1974).

In addition, forming a secondary structure in the ribosome binding site (RBS) of the mRNA via *cis*-acting elements reduces the efficiency of translation as it sequesters the formation of the translation complexes (mRNA, 30S and fMet-tRNA (Schlax and Worhunsky, 2003). Similarly, temperature changes might cause secondary structures for the mRNA and effect its translation) (Schlax and Worhunsky, 2003).

Furthermore, the intracellular level of the GTP pool can a play a substantial role in translation regulation as it contributes in each step of translation. For example, translation initiation factor IF2 is important to form the 30S initiation complex and IF2 activation relies on the GTP abundance, also hydrolyse of the GTP promotes this initiation factor to release from the 70S complex which permits elongation and IF2 recycling (Luchin *et al.*, 1999). Additionally, EF-Tu-GTP binding facilitates transporting the next charged tRNA into the A-site of the 30S subunit and GTP is the energy source for the movement of the 30S subunit along the mRNA (translocation) as well as shifting the tRNA in the P and A site of the 30S subunit to E and P sites (Laalami *et al.*, 1996). GTPs contributions in the translation processes is summarised in figure 3.

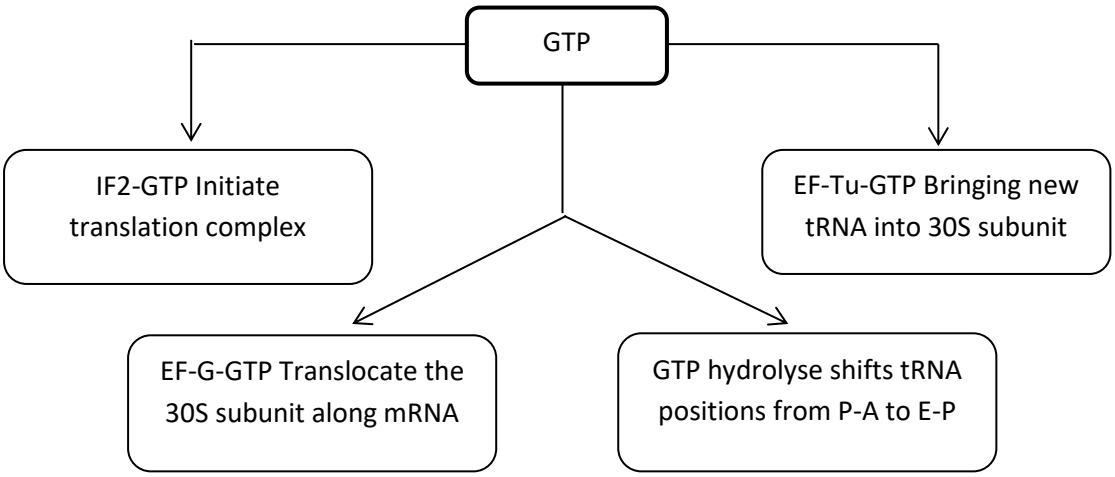


Figure 3 The involvement of GTP in the translation processes

In general, many factors can effect gene expression in bacteria such as growth phase, metabolic composition, cell density as well as the environmental conditions surrounding the cells. In *S. aureus* the effect mechanisms of these factors are achieved through different regulatory response systems like accessory gene regulators (*agr*) and global transcriptional regulators such as Staphylococcal accessory regulator (*sar*) and others. These regulators systems play a crucial role in *S. aureus* pathogenicity, by either up-regulating or down-regulating these genes as will be highlighted below.

1.1.5 Regulation of virulence factors expression in *S.aureus*

1.1.5.1 Accessory gene regulator system (*agr*)

S.aureus regulates some of its virulence genes by a well characterized two-component system, Agr, a quorum sensing system that plays a fundamental role in virulence factor synthesis during growth. This system was thought to be responsible for regulating the production of a few pathogenic enzymes and toxins, but subsequent studies revealed that the *agr* system regulates more than 28 genes that encode pathogenicity enzymes and toxins in *S.aureus.* For instance, *agr* system seems to exert a repressive effect on cell surface proteins such as protein A, fibronectin binding proteins A-B during the post-exponential phase of growth. Conversely, *agr* induces transcription of exotoxins including enterotoxin A, B, C and E as well as range of different enzymes including lipase, staphylokinase and V8 serine protease (Ji *et al.*, 1995).

The *agr* system consists of two adjacent promoters, promoter *P2* and *P3*. *P2* control four genes, *agrABCD* (Figure 4). *AgrB* and *agrD* together constitute a quorum-sensing system. The two-component system contains *agrA* which is a response regulator, and *agrC*, a transmembrane protein that is phosphorylated upon binding of autoinducing peptides (AIP). *AgrB* and *agrD* are involved in modifying and exporting AIP which binds to agrC (for autophosphorylation) in order to transfer a phosphate group to AgrA. The phosphorylation of AgrA leads to transcription activation for P2 and P3, and the synthesis of RNAII and RNAIII respectively (Ji *et al.*, 1995, Arvidson and Tegmark, 2001). The functioning of the *agr* system is illustrated in Figure 4. RNAII regulates the agrABCD structural proteins, whereas RNAIII is an effector regulator for the *agr* system. This occurs in the post-exponential phase of the growth as a result of the accumulation of AIP (Arvidson and Tegmark, 2001, Novick *et al.*, 1995a).

RNAIII, which is one of the largest regulatory RNAs, up-regulates the transcription of secreted protein genes such as *tst* (TSST-1), *hla* (α-toxin), *hld* (δ-toxin) (Novick, 2003, Morfeldt *et al.*, 1995) and down-regulates genes that are involved in cell surface protein production such as *spa* (protein A) and *fnbA, fnbB* (fibronectin binding protein A, B) via *agr* system (Novick *et al.*, 1993, Huntzinger *et al.*, 2005).

In addition, the RNAIII regulatory effect can occur at both the transcriptional and post-transcriptional levels. For instance, base pairing between RNAIII and *spa* mRNA can occur and prevent the formation of the ribosomal initiation complex which consequently inhibits the translation of the protein. In addition RNAIII can work as an antisense RNA by pairing to the staphylocoagulase (*coa)* mRNA which promotes the endoribonuclease III to recognize the repressed mRNA, and initiate degradation activity on the mRNA (Huntzinger *et al.*, 2005, Chevalier *et al.*, 2010).

In general, the two-component system *agr* plays an important role in the infection processes. For instance, in some conditions such as the presence of bacteria in the blood stream, where bacterial numbers are inadequate to establish an infection, the *agr* system seems to be repressed (low level of RNAIII). This enables a high expression of cell surface proteins and the adhesive phenotype of the bacterial cells occurs. However, in other infections such as abscesses, where the density of the bacteria is high, the *agr* system is activated and abundant RNAIII exists, resulting in increasing levels of toxins and enzyme production leading to tissue damage(Arvidson and Tegmark, 2001).

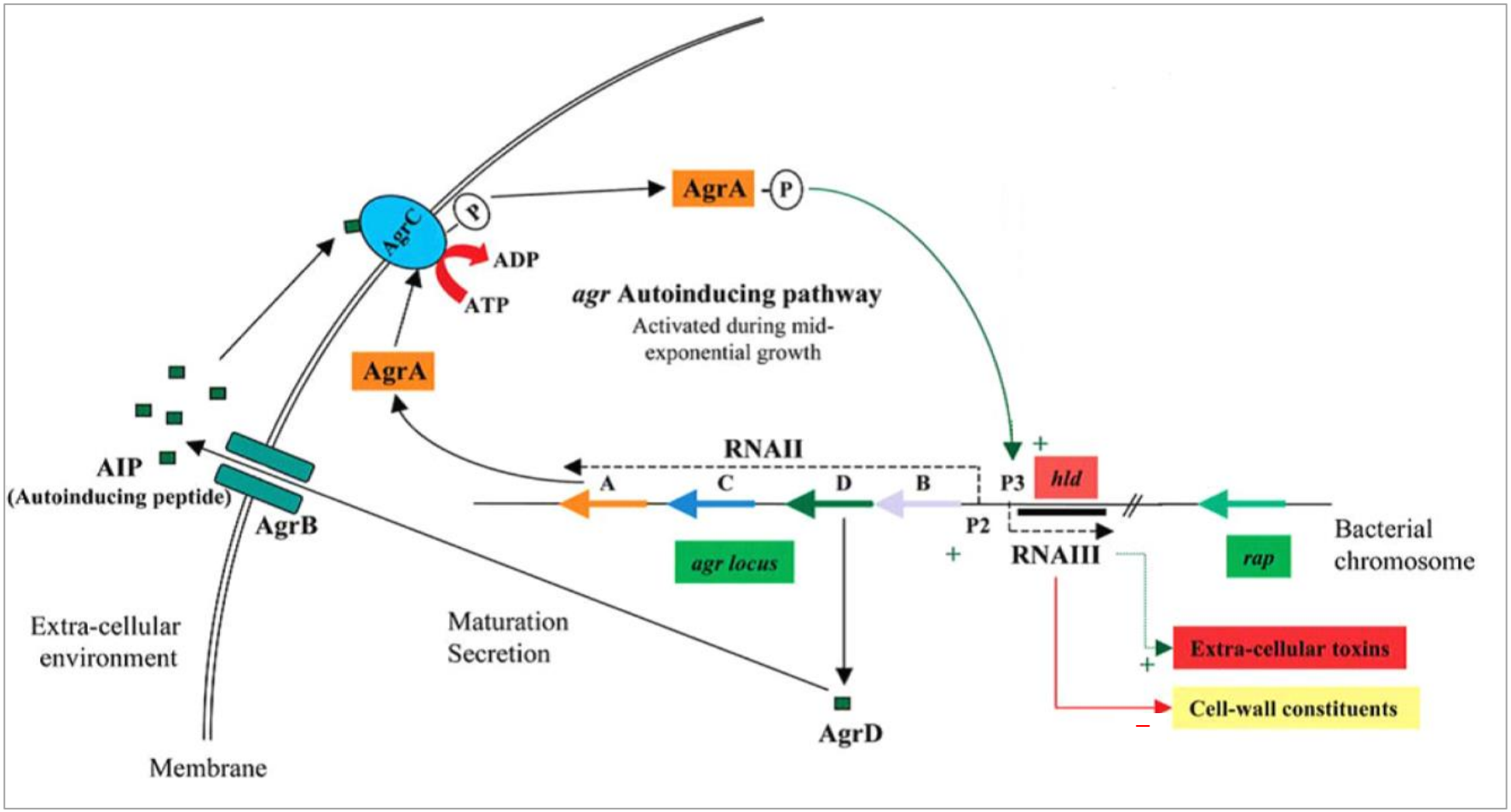


Figure 4 Quorum-sensing system (two-component system) agr in *S. aureus*.

AgrBC modify and export Autouinducing peptide (AIP). AgrAC are responsive molecules that involved in P2 and P3 transcription activation resulting in RNAII and RNA III synthesis. Modified from (Bronner *et al.* 2004).

1.1.5.2 *Staphylococcal* accessory element (*sae*)

In addition to the *agr* regulation system, the staphylococcus accessory element (*sae*) is another two-component system that contributes to *S.aureus* virulence factor regulation. It consists of two transcribed genes *SaeR* and *SaeS* (Bronner *et al.*, 2004).

This regulatory system affects the expression of many virulence factors in *S.aureus* that are involved in adhesion and immune evasion such as protein A and coagulase as well as alpha and beta haemolysin genes (*hla, hlb*). For example, SaeR is found to be a positive regulator for haemolysin toxins (α, β, and γ) and a negative regulator for *spa*. The Sae regulation system seems to work independently of other regulation systems such as agr and SarA (Dziejman and Mekalanos, 1995).

1.1.5.3 *Staphylococcal* respiratory response (srrAB)

The locus of the two-component system SrrAB consists of two genes that overlap in 20 nucleotides *srrA* and *srrB.* This encodes the responsive regulator protein and *srrAB,* that encodes the histidine kinase (Bronner *et al.*, 2004). SrrAB is a pleiotropic virulence factor regulator in *S.aureus* that can link between the respiratory metabolism, toxin production and environmental signals. The transcription of this regulator is more evident in anaerobic and microaerobic conditions than in aerobic conditions. This two-component system affects the transcription of several virulence factors and regulators such as RNAIII, protein A and TSST-1 through direct binding to their promoters. For example, ssrAB shows a negative effect on RNAIII and protein a transcription in aerobic conditions. Interestingly, ssrAB promotes TSST-1 production in aerobic conditions but not in anaerobic or microaerobic conditions and functions independently from the agr system (Pragman and Schlievert, 2004).

1.1.5.4 Sar family regulatory proteins

Beside two-component signal transduction systems, the global transcriptional regulator *Staphylococcal* accessory regulator (*sar*) also regulates the virulence factors in *S. aureus*. SarA was described in 1992 and later found to be essential for the transcription of *agr RNAII* and *RNAIII,* as it can bind *agrP2* and *agrP3* in the *agr* system (Arvidson and Tegmark, 2001).

Expression of the *SarA* gene is growth phase dependent, and its product is a DNA-binding protein which is expressed from three promoters (*sarP1, sarP2* and *sarP3*). *sarP1* and *sarP2* expression occurs during the early exponential phase of the growth, and are sigma A (sigma factor that directs the transcription of RNA during the growth phase) dependent, while *sarP3* is expressed in the post-exponential phase and is sigma B (sigma factor produced during the stationary phase) dependent (Bronner *et al.*, 2004).

SarA contributes to the regulation of several virulence factors in *S.aureus*, such as up-regulation of TSST-1 and α-toxin and down-regulation of expression of collagen binding protein and proteases. SarA regulation activity can be either by direct binding to the DNA, or indirectly via other regulatory systems such as agr. In addition, mutations of the SarA have been shown to attenuate the *S. aureus* virulence significantly (Bronner *et al.*, 2004, Rechtin *et al.*, 1999, Cheung *et al.*, 2008).

A subsequent study on the Sar locus has revealed another virulence factor regulator called sarS or sarH1. This regulator can bind to *P3* in *agr* and has homology to sarA (Cheung *et al.*, 2001). Moreover, another regulator, sarR which has been found to show ability to repress the expression of sarA promoters and additional regulators have been discovered such as sarT that seems to repress its adjacent promoter sarU as well as contribute with SarA to repress *hla* transcription and sarU which is a positive regulator for RNAIII. Although abundant

information has been obtained from different studies of this regulatory system, many points remain unclear and require further study (Pragman and Schlievert, 2004).

1.1.5.5 Effects of metabolic alterations on virulence factor expression

Bacteria can select the preferred source of carbon from the surrounding environment in order to facilitate its rapid growth. This selection is associated with shifting the metabolic processes in accordance with nutrient availability (Görke and Stülke, 2008). For example, most bacteria prefer glucose as a carbon source for quick growth, and they can metabolize glucose either by glycolysis and the citric acid cycle (tricarboxylic acid TCA, Krebs cycle) (Görke and Stülke, 2008). For the quick growth of *S. aureus*, the glycolysis processes are used to produce ATP and other molecules such as pyruvate and NADH. After that, pyruvate is converted to acetyl-coenzyme A then to acetylphosphate and acetate. The acetylphosphate is used to produce more ATP, whereas the acetate is accumulated in the media during the growth.

After the exponential growth phase and the complete consumption of the glucose, the bacteria encounter nutrient limitation conditions and begin to consume the accumulated acetate as a carbon source. This requires a fully functional TCA cycle. The TCA cycle produces ATP with more NADH and $FADH_2$ that are used in the electron transport chain in the aerobic condition, to produce more quantities of ATP (Görke and Stülke, 2008, Somerville *et al.*, 2002). Genes that encode enzymes that are involved in the metabolism shifting and activity are controlled by regulatory proteins such as CcpA and CodY. These can sense the nutritional status of the cell (Sonenshein, 2007).

(A) Catabolite control protein A (CcpA)

CcpA is a global regulator of carbon-metabolism pathways in many Gram-positive bacteria. It has been shown to contribute to the regulation of more than 100 genes involved in carbon

acquisition, transport and metabolism in *B. subtilis* (Sonenshein, 2007). CcpA can be either a positive or negative transcriptional regulator. For instance, this regulatory protein can activate genes that are involved in the synthesis of pyruvate and acetylA, and repress genes that are involved in the TCA cycle such as *citB* and *citZ* (Sonenshein, 2007). CcpA binds to a specific site in the targeted gene promoter entitled the catabolite responsive element (*cre-site*), and its activity is promoted via HPr (histidine containing protein) which is activated by ATP and fructose‑1,6-bisphosphate (FBP) (Görke and Stülke, 2008). The abundant availability of ATP and the FBP, as well as the HPr, is important for the CcpA with regard to its targeted genes (Sonenshein, 2007).

In addition to the CcpA role in metabolism regulation, this protein has been found to play a role in virulence factor regulation. For example, in *S.aureus,* the CcpA binding site (*cre*) has been identified in the TSST-1 gene (*tst*), and CcpA has been found to be a transcription inhibitor for *tst*. In addition, inactivation of the CcpA increases the transcription of *tst*, which suggests a direct effect of the CcpA on *tst* (Görke and Stülke, 2008, Seidl *et al.*, 2008a). Moreover, biofilm formation in *S.aureus* is promoted via CcpA as it increases *icaA* expression and PIA production (Seidl *et al.*, 2008b). Also, the CcpA protein seems to promote antibiotic resistance as its inactivation remarkably reduces the resistance levels for methicillin and teicoplanin in two different resistant strains of *S. aureus* and the mechanism of this effect is not fully understood (Seidl *et al.*, 2006). Additionally, CcpA positively regulate the *ilv* operon which is involved in branched chain amino acid biosynthesis that plays animportant role to activate another regulatory protein called CodY.

(B) CodY

CodY is a metabolic responsive protein that regulates a number of genes which are involved

in nitrogen and carbon metabolism. The first report of this regulatory protein was in *B.subtilis* as a nutrient limitation and stress condition responsive protein (Sonenshein, 2005).

This regulatory protein activates genes encoding enzymes that are involved in glucose metabolism to produce energy. For example, in *B. subtilis* during glucose abundance, CodY positively regulates genes that are coding for acetate kinase *(ackA)* enzyme that contributes to the conversion of pyruvate to acetate in order to produce ATP via glycolysis during the exponential growth phase (Shivers *et al.*, 2006). Additionally, CodY in cooperation with CcpA represses expression of genes that involved in TCA cycle in the glucose rich environment (Shivers *et al.*, 2006). Also, CodY represses amino acid synthesis and transportation, post-exponential virulence factors and nitrogen component metabolism during the exponential phase (Sonenshein, 2005).

CodY binds to its targeted DNA at the A/T rich region. This regulator possesses the ability to sense nutrient availability in the environment via two effector molecules, guanosine triphosphate (GTP) and branched-chain amino acids (BCAA), in particular isoleucine or valine (Ratnayake-Lecamwasam *et al.*, 2001). Intracellular pool concentration of these two ligands affects both independently and additively, the affinity of CodY to bind its targeted genes and regulate its transcription. For instance, when bacterial cells encounter nutrition limitations, these two effectors are not available in abundance, and consequently the affinity of CodY to bind DNA is reduced and de-repression of CodY-repressed genes occurs (Ratnayake-Lecamwasam *et al.*, 2001, Sonenshein, 2007).

CodY homologues have been reported in many other Gram positive bacteria such as *Streptococcus pneumoniae*, *Clostridium difficile* and *S. aureus*. In *S. aureus*, more than 200 genes that are involved in different functions, including amino acid biosynthesis and

transportation, nitrogen compound metabolism and virulence factors have been found to be down-regulated via CodY, either directly or indirectly (Majerczyk *et al.*, 2010).

Recently, Majerczyk and colleagues (2008), conducted a comparative study of clinical isolates of *S. aureus* and a *codY* mutant, and reported that CodY has an obvious influence on the phenotypic and genotypic characteristics of *S. aureus*. For example, a *S. aureus codY* mutant showed a significant increase in the hemolysin and protease gene transcription in the exponential and stationary phases. Moreover, they claimed that RNAII and RNAIII transcript molecules from the *agr* locus were de-repressed during the exponential phase. This shows an obvious alteration in virulence factors regulation (Majerczyk *et al.*, 2008).

The up-regulation of haemolysin and protease genes can play a role in providing peptides or amino acids to the bacteria during the nutrient limitation (Anderson *et al.*, 2006). CodY appears to play a crucial role in linking metabolism with virulence factors in *S. aureus,* in particular during the stationary phase and in nutrient limitation or stress conditions.

In general, the regulatory effect of CcpA and CodY proteins is initiated in the presence of their metabolic component activators (HPr, FBP and ATP for the CcpA, and GTP, isoleucine for CodY protein). In addition, both CcpA and CodY are DNA-binding proteins that can directly or indirectly affect some of the virulence factors genes transcription, whether in a positive or a negative manner, by sensing their metabolic activators which forms a link between the metabolic status of the cell and regulation of its virulence factors.

Furthermore, these proteins enhance the carbohydrate metabolism which facilitates rapid growth through the exponential phase. This might affect the expression of some virulence factors genes that are controlled in a growth-dependent manner. Figure 5 illustrates some of the regulatory effects of CcpA and CodY.

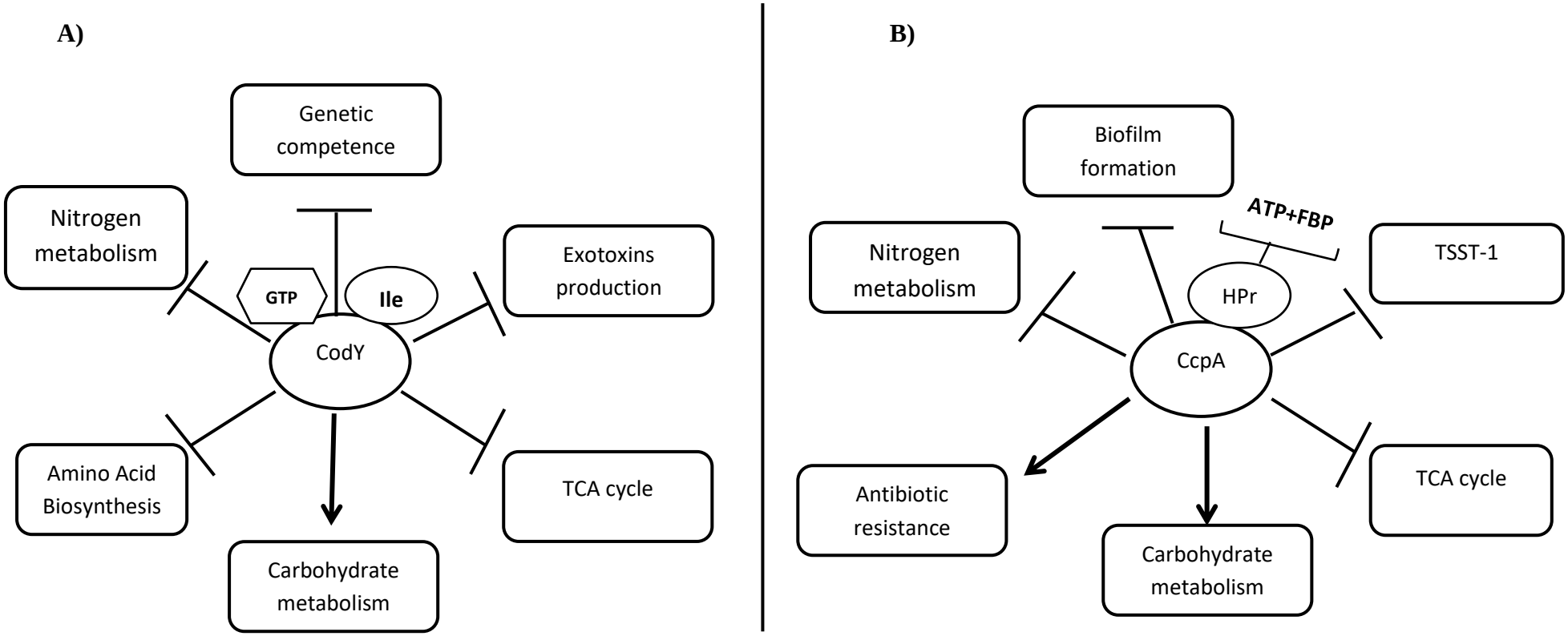


Figure 5 Effect of the two metabolic responsive proteins CcpA and CodY on different molecular functions

A) CodY regulation is promoted by GTP and isoleucine (Ile) during exponential growth, **B)** the CcpA regulatory effect is enhanced via histidine containing protein (HPr) that is activated by fructose−1,6-bisphosphate (FBP) and ATP during exponential growth in carbohydrate abundance. Arrows represent promotional effect; bars represent inhibitory effect.

1.2 Mupirocin

Mupirocin (pseudomonic acid) is an antibacterial agent produced by the Gram-negative bacterium *Pseudomonas fluorescens*. This antimicrobial agent displays a broad spectrum of inhibitory activity against Gram-negative bacteria such as *E.coli* and Gram-positive bacteria, including the staphylococci and streptococci. However, it is much less active against the majority of Gram-negative bacilli and anaerobes (Sutherland *et al.*, 1985, Gurney and Thomas, 2011).

The first reported research of mupirocin was in 1971then investigations discovered a mixture of pseudomonic acids (A-D) that was collectively named mupirocin. This antibacterial agent consists of highly unusual polyketide metabolites and the highest proportion (95%) consisting of pseudomonic acid A(Chain and Mellows, 1974). The structure of mupirocin is composed of monic acid containing a pyran ring, attached to 9-hydroxy-nonanoic acid via an ester linkage (Figure 6) (Chain and Mellows, 1974, Chain and Mellows, 1977).

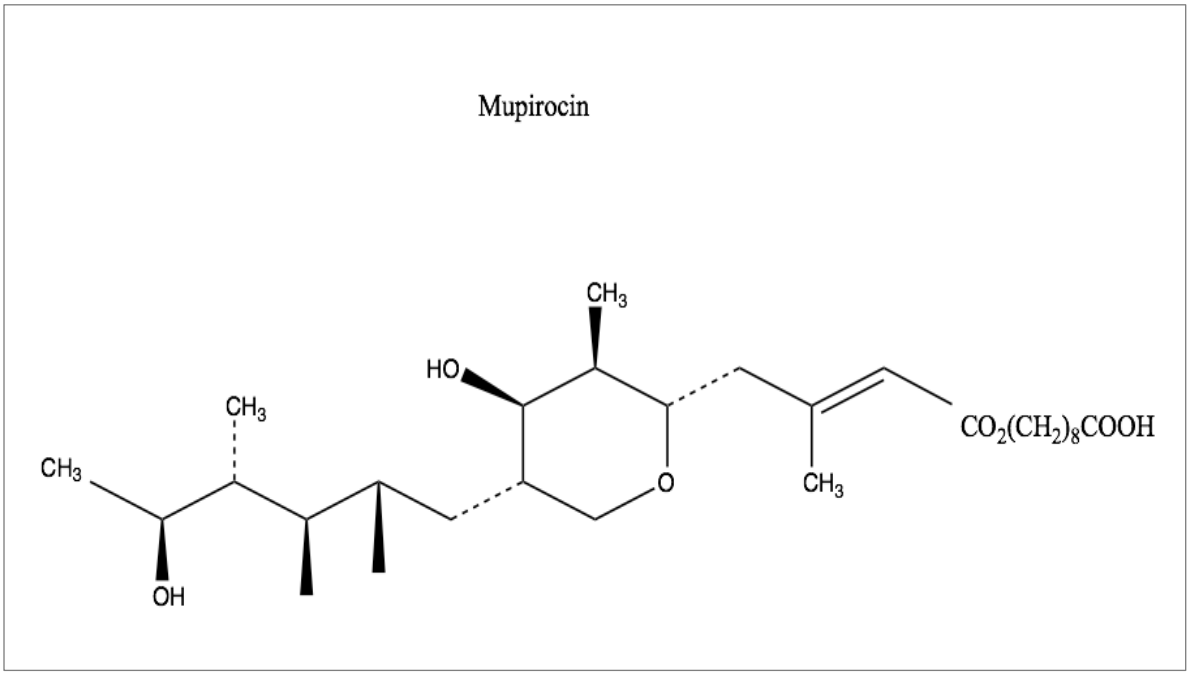


Figure 6 mupirocin chemical structure (drawn using ChemoDraw)

1.2.1. Mechanism of action of mupirocin

Mupirocin uniquely affects bacterial protein synthesis by inhibiting aminoacyl-tRNA synthetase enzyme which plays a crucial role in pairing the $tRNA^{ile}$ to its cognate amino acid isoleucine (Hughes and Mellows, 1978b, Hughes and Mellows, 1980). Normally, the aminoacyl-tRNA synthetase enzyme binds its amino acid and ATP at a specific site to form an aminoacyl-adenylate and release inorganic pyrophosphate (PPi). Then the adenylate-aminoacyl-tRNA synthetase complex binds its cognate tRNA molecule to transfer/charge the amino acid onto its tRNA to be ready for the translation processes (Figure 7) (Woese *et al.*, 2000).

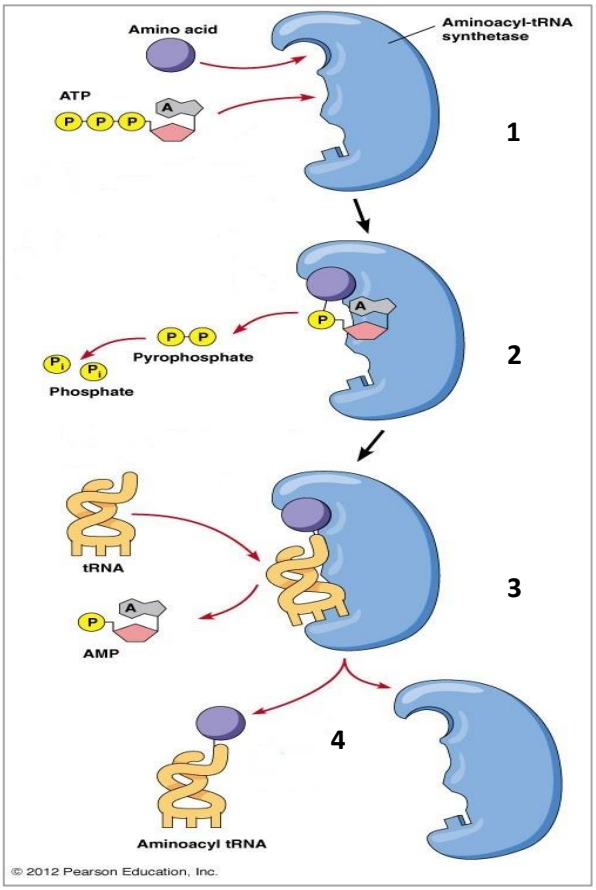


Figure 7 Lodging t-RNA with its cognate amino acid process.

1) ATP and amino acid occupy the active site of the catalyzing enzyme, 2) Amino acid is activated by AMP and pyrophosphate are released, 3) AMP is displaced with compatible cognate tRNA, 4) Aminoacylated tRNA is released from the aminoacyl-tRNA synthetase enzyme.(Anon. 2012).

Mupirocin binds to isoleucyl transfer-RNA synthetase in a specific and reversible way and inhibits the formation of the adenylate- aminoacyl-tRNA synthetase complex as a competitive inhibitor but not the amino acid transfer to the tRNA step (Hughes and Mellows, 1978a). The monic acid terminus resembles the side-chain structure of isoleucine (Figure 8) which enables it to compete with isoleucine for the binding site in the enzyme. Competitive inhibition was shown because increasing the isoleucine pool after mupirocin treatment at low concentrations reversed the inhibition in *E.coli* (Hughes and Mellows, 1978a).

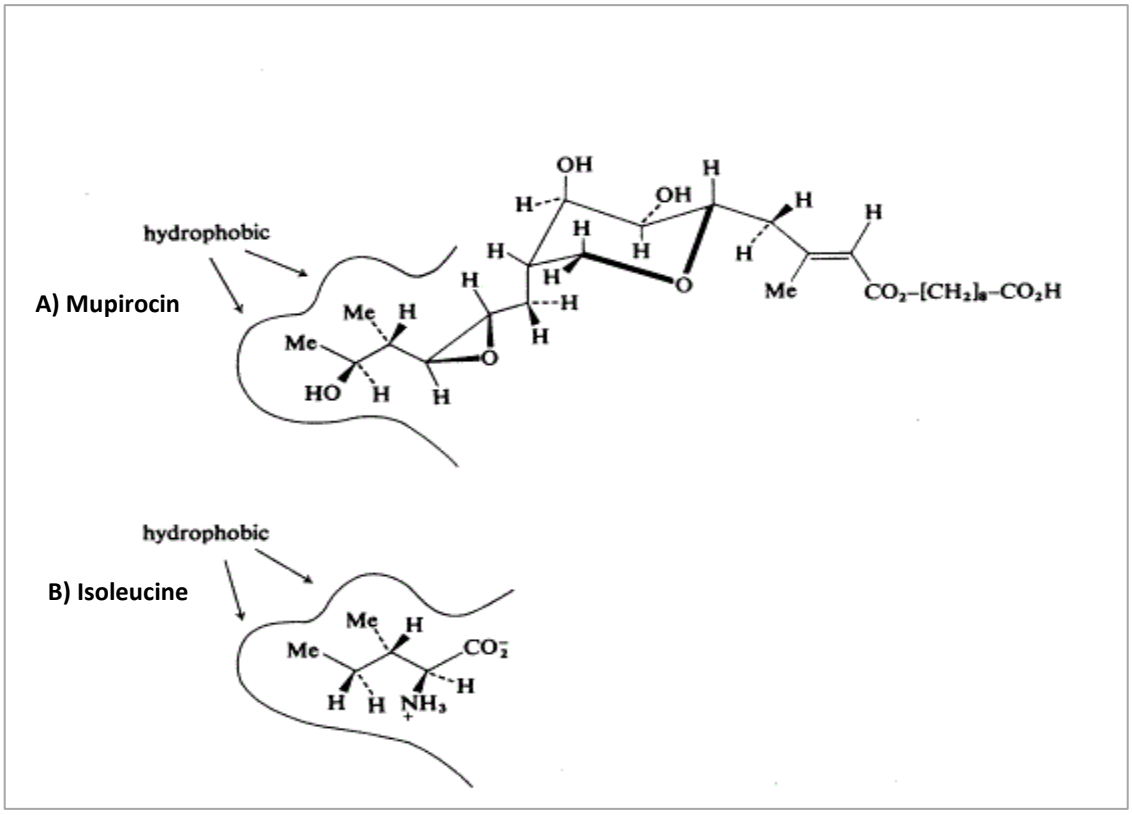


Figure 8 Similarity of the hydrophobic-binding domains in the monic acid terminus of mupirocin(A) and the carbon skeleton of L-isoleucine(B) (Hughes and Mellows, 1978a)

1.2.2 Medical applications and resistance

Mupirocin has been used as a treatment for staphylococual infections since 1985. Topical treatment is the only possible way of use since mupirocin shows a high affinity for serum

proteins, thus it not used systematically and it is applied against skin infections. In addition, preventing nasal carriage of *S. aureus* including MRSA strains was used to tackle infection transmission in hospitals and nursing homes (Sutherland *et al.*, 1985, Hetem and Bonten, 2013). A combination of mupirocin nasal decolonization and chlorhexidine body washing showed 37% reduction of MRSA carriage among nursing home residents (McDanel *et al.*, 2013).

Mupirocin is useful in decolonization regimes in the inpatient setting for limited periods of time. For instance, short term use of intranasal mupirocin was used as a preoperative treatment for elective surgical patients to prevent *S. aureus* post-surgical infections. This has been associated with a 58% reduction in post-surgical *S. aureus* infections and incidence of mupirocin resistance occurred only rarely with this regimen in the Netherlands (Bode *et al.*, 2010). In addition, nasal decolonization of MRSA regime using mupirocin showed effective results to eradicate nasal carriage of MRSA in intensive care unit (ICU) patients and inclusion of mipirocin in an oral paste and intranasally reduced MRSA lung infections (Coates *et al.*, 2009, Nardi *et al.*, 2001). Moreover, mupirocin prophylaxis showed effective results for dialysis patients as the antimicrobial had reduced the risk of developing *S. aureus* infections by 60% among those patients (Evelina *et al.*,2007).

Furthermore, mupirocin can be used to eradicate community- associated MRSA infections in selected communities. For example, mupirocin showed effective results to prevent community-associated MRSA infections among military trainees after single 5 days course of nasal mupirocin, where *S. aureus* skin infection is highly endemic, (Ellis *et al.*,2007).

However, mupirocin resistance appeared two years after it was first introduced (Rahman *et al.*, 1987). The resistance is characterized in two distinctive levels, low level resistance (between 8-256 µg/ml), which occurs as a result of spontaneous mutation in the antibiotic target (*IleS*), and high level resistance (>500 µg/ml), which is displayed by the production of a

plasmid-encoded gene, *mupA*, is responsible for the production of a novel isoleucyl-tRNA synthetase that is insensitive to mupirocin. In other words, when the organism possesses this novel (*IleS*), the mupirocin mechanism of action is deactivated and a high level of mupirocin resistance occur (Gilbart *et al.*, 1993).

Prolonged usage of mupirocin or routine application to control *S. aureus* endemics for inpatients can lead to the emergence of resistance, particularly with unrestricted policies that allow widespread mupirocin usage for prolonged periods (Patel *et al.*, 2009). Restricted mupirocin use to only those patients who had no skin lesion or previous staphylococcal infection is been applied in some countries and showed mupirocin resistance decline (Patel *et al.*, 2009). In the UK 888 MRSA isolates, from nursing homes in England, were screened for antimicrobial susceptibility and mupirocin resistant isolates presents 5% of them. (McDanel *et al.*, 2013, Horner *et al.*, 2013). Recent screening for antimicrobial susceptibility in Libya showed that mupirocin resistance occurred in 15% of MRSA isolates(86) used in the study where restricted policy for mupirocin prescription is not applied (Mohamed *et al.*, 2010). More details of mupirocin resistance and its prescription policy are discussed in chapter 3.

1.2.3 The effects of mupirocin on *S. aureus* virulence factors

Several antibacterial agents such as gentamicin, clindamycin and others have displayed inhibitory activity on protein synthesis which leads to inhibition of toxin synthesis without affecting cell growth (Schlievert and Kelly, 1984). A previous study by Edwards-Jones and Foster (1994) reported that toxic shock syndrome toxin (TSST-1) synthesis was inhibited under the effect of a sub-inhibitory concentration of Bactroban, a cream for treatment of burns and other infections in which the active ingredient was mupirocin (Edwards-Jones and Foster, 1994). In addition, mupirocin exhibits an inhibitory activity on other virulence factors in *S.aureus* such as enterotoxin and haemolysin production and the effects of mupirocin on *S. aureus* varied from one strain to another (Edwards-Jones thesis, 1997).

The mechanism of action of mupirocin mimics amino acid limitation thus, it has been applied to induce a bacterial stringent response *in vitro* in Gram negative bacteria such as *E. coli* and Gram positive bacteria including *B. subtilis* and *S. aureus,* also its influence on nucleosides intracellular pool has been observed (Cassels *et al.*, 1995, Crosse *et al.*, 2000b). These effects are discussed in details below.

1.3 Introduction to the stringent response

The stringent response is a physiological state that bacteria exhibit to survive when they encounter extreme conditions, such as nutrient limitation. In this circumstance, bacteria can economise their nutrient consumption by reducing metabolic activities to a minimal rate until the surrounding environment is improved. This stress adaption is achieved by alterations in bacterial gene regulation, in which the bacteria up-regulate specific genes and simultaneously down-regulate others (Chatterji and Kumar Ojha, 2001, Takahashi *et al.*, 2004).

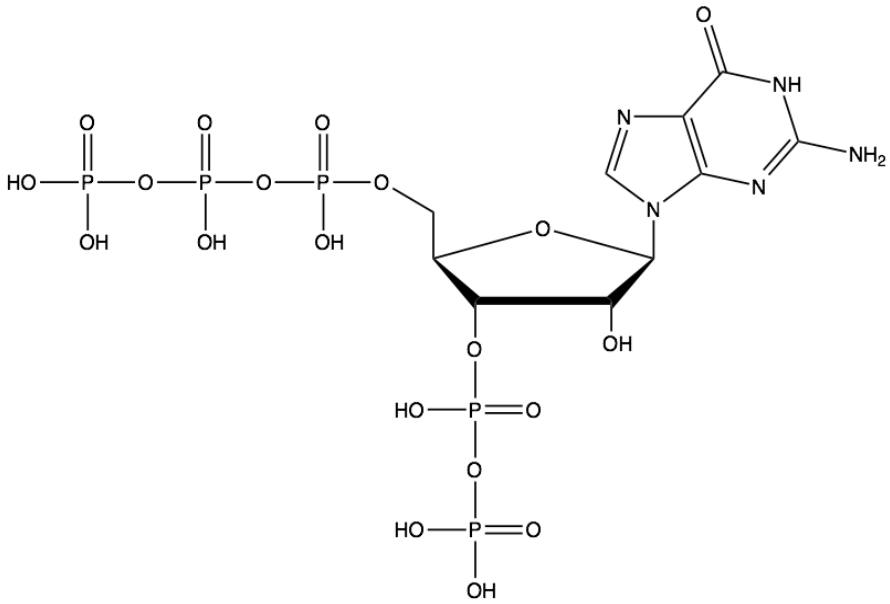
In 1960's Marchel Cashel observed two spots on thin-layer chromatography plates called ”magic spots” 1 and 2 subsequently identified as the two nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Figure 9) which accumulated rapidly in *E.coli* after nutrient limitation by amino acid starvation, that triggered a stringent response state (Cashel and Gallant, 1969). Subsequent studies have shown that (p)ppGpp is accumulated not only in *E. coli*, but in other species of bacteria and also fungi and plants. However, until the present time, there has been no evidence for the existence of (p)ppGpp in mammals (Braeken *et al.*, 2006, Takahashi *et al.*, 2004).

After much of research, it has become evident that the hallmark of the stringent response is (p)ppGpp, which acts as an alarmone responsible for regulating global intracellular changes

including transcription, translation, replication, virulence induction in pathogenic bacteria, differentiation and latency through various mechanisms (Wu and Xie, 2009).

A) Guanosine pentaphophate

pppGpp



B) Guanosine tetraphosphate

ppGpp

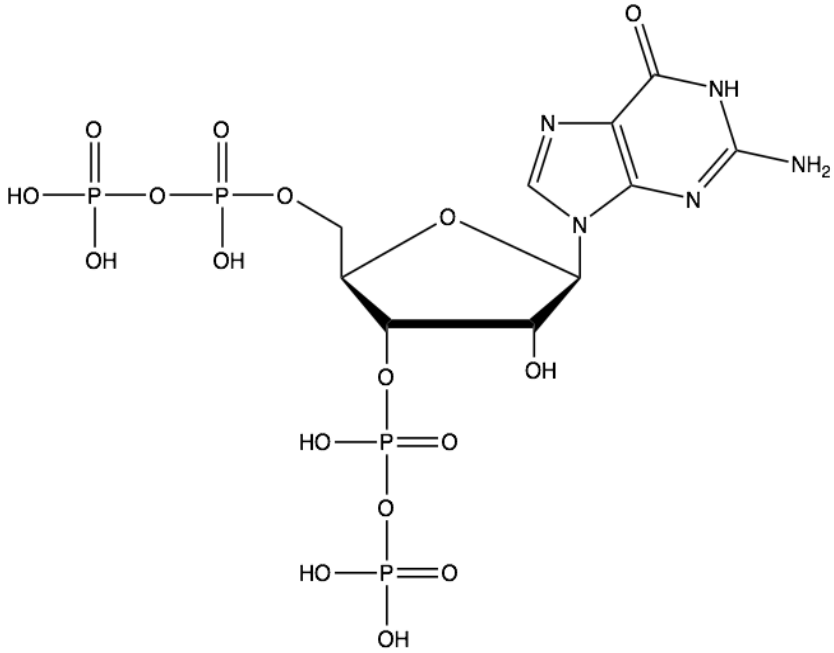


Figure 9 chemical structure for pppGpp (A) and ppGpp (B) (drawn by ChemoDraw)

1.3.1 (p)ppGpp synthesis

Haseltine and Block (1973) showed that (p)ppGpp synthesis in *E.coli* is triggered when an uncharged tRNA attaches to the A-site of the 50S ribosome. This, blocks protein synthesis on the ribosome, which consequently, triggers an idling reaction whereby RelA (the first known (p)ppGpp synthase) catalyses the enzymatic phosphorylation of GDP and GTP to ppGpp and pppGpp respectively, by using ATP as a phosphate donor (Haseltine and Block, 1973).

Further studies have revealed that *E.coli* shows two different pathways to produce (p)ppGpp, RelA and SpoT pathways. The relaxed gene or r*elA* that codes for the RelA enzyme which is a (p)ppGpp synthase is activated in response to amino acid starvation through the mechanism described above. SpoT, in contrast to the RelA enzyme, is a bifunctional enzyme that has the ability to affect (p)ppGpp in two ways : synthetic activity and hydrolytic activity by hydrolysing, pppGpp and ppGpp, to GTP/GDP + PPi (Figure 10) (Chatterji and Kumar Ojha, 2001, Wu and Xie, 2009).

Moreover, the SpoT enzyme seems to produce ppGpp in most stress and nutrient limitation conditions other than from amino acid deprivation, such as phosphorous, iron and carbon starvation (Murray and Bremer, 1996). However, less is known about the mechanism behind SpoT-dependent production but inactivation of *spoT* and *relA* genes in *E.coli* by mutation made the cell entirely unable to produce (p)ppGpp and, consequently, unable to display the stringent response (Magnusson *et al.*, 2003).

1.3.2 (p)ppGpp role in the stringent response

Many studies have reached the consensus that (p)ppGpp displays many of its effects on gene regulation in the stationary phase, which enables it to play a crucial role in bacterial survival

during growth arrest by controlling the expression of a number of genes (Wu and Xie, 2009, Nyström, 2004).

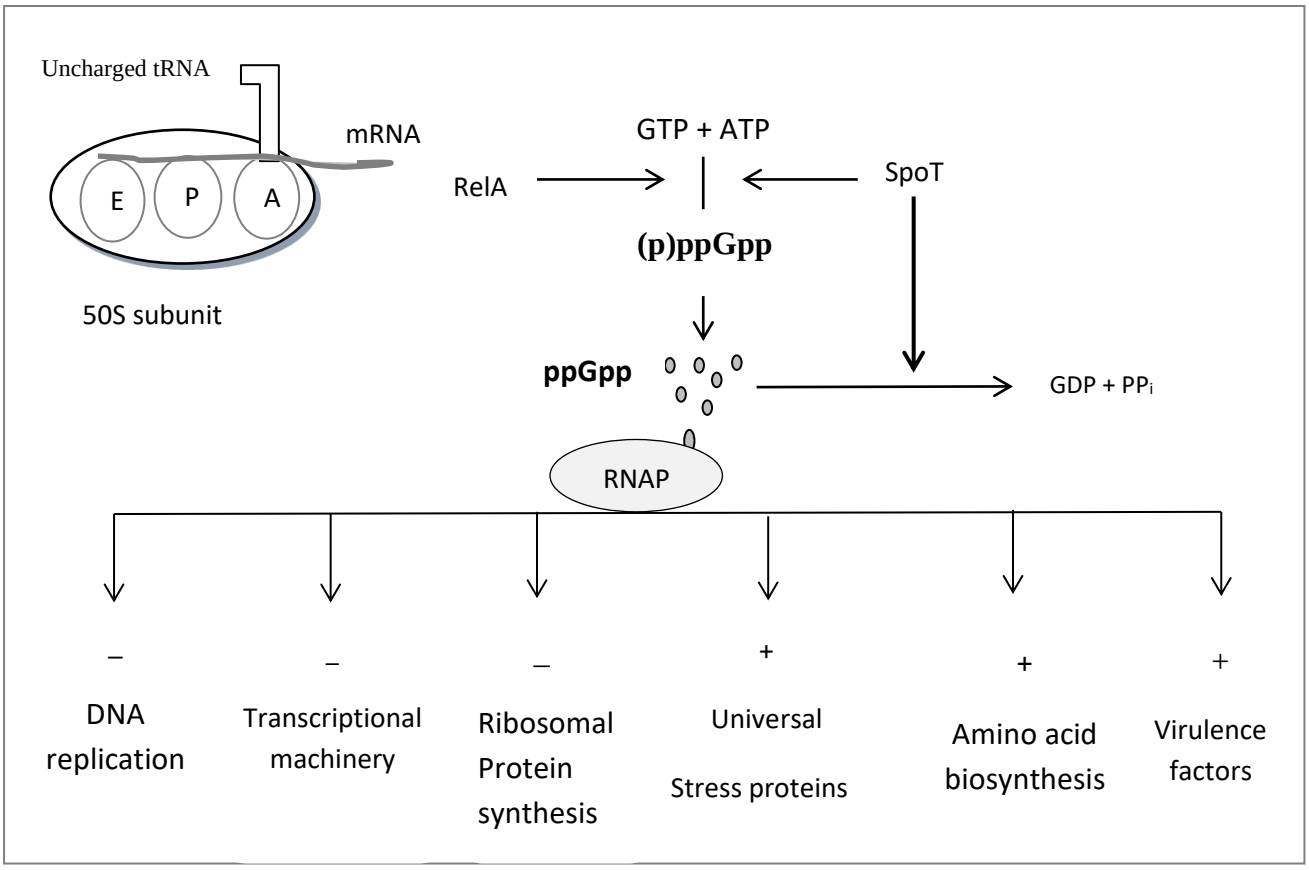


Figure 10 (p)ppGpp synthesis, hydrolysis and its influences on regulation of gene expression in *E.coli*

Binding of the uncharged tRNA to A-site of 50S subunit activates relA enzyme to catalyse (p)ppGpp synthesis. SpoT enzyme also synthesises and hydrolyses (p)ppGpp. ppGpp attach to RNAP to regulate different genes functions transcription. (Auther drawing).

This alteration of gene expression has been observed by comparing bacterial cells that are capable of producing (p)ppGpp (wild type cell) and mutants that lacked the ability to produce (p)ppGpp (p)ppGpp^0. Both cell types were exposed to carbon starvation in the same growing conditions. As these cells entered the stationary phase, their proteomic profiles indicated that (p)ppGpp^0 cells exhibited proteomic profiles similar to those observed during growth, however, in contrast, the other cells showed a stationary phase proteomic profile. Moreover,

the alteration of gene expression for these cells was investigated by a DNA microarray technique and the results supported the proteomic data(Magnusson *et al.*, 2003) (Chang *et al.*, 2002). These observations suggested an obvious correlation between (p)ppGpp production and gene expression alterations that result in the stationary phase proteomic profile which might enable bacteria to adapt to stress conditions in the surrounding environment and survive. Conversely, the proteomic profile that has been shown by the(p) $ppGpp^0$ cells (growth state profile) explains their failure to survive, since the cells display no response (no gene expression alteration) to the stress condition, carbon deprivation, which leads to cell death (Chang *et al.*, 2002, Magnusson *et al.*, 2003). These results have provided sufficient explanation for the (p)ppGpp influence on gene expression, when bacterial cells encounter stressful conditions, and have encouraged researchers to further observe and understand the roles and influences of (p)ppGpp.

1.3.3 Effects of (p)ppGpp on cell functions

The accumulation of (p)ppGpp inhibits the synthesis of ribosomal and transfer RNA, and thus profoundly affects translation as well as other cellular processes. In addition to its repressive activity, (p)ppGpp exhibits a positive effect on genes that are involved in the stress response such as, stress resistance and starvation survival genes(Magnusson *et al.*, 2005) as illustrated in figure 10. In *E.coli* (p)ppGpp effects on different cellular processes such as transcription, translation and DNA replication have been studied in detail and are described below.

1.3.3.1 Effects of (p)ppGpp on transcription

The effect of (p)ppGpp in the transcriptional process is described as an interaction between different elements, (p)ppGpp, RNA polymerase (RNAP), promoters of the ribosomal RNA and amino acid biosynthesis genes, sigma factors and other cofactors. Most transcriptional regulatory factors bind to the DNA's promoter to recruit or exclude RNAP whereas,

(p)ppGpp seems to bind directly to the RNAP in order to regulate the promoter and ppGpp tends to binds to the β, β' and σ^{70} subunits on the RNAP (Magnusson *et al.*, 2005, Chatterji and Kumar Ojha, 2001). However, Artsimovitch and colleagues (2004) claimed that ppGpp binds to the near active site of the RNAP where it can interact with both β, β' subunits (Artsimovitch *et al.*, 2004)

In addition, (p)ppGpp shows a strong effect on transcription initiation for amino acid biosynthesis promoters *in vivo*, but this effect was undetectable *in vitro* until the discovery of the cofactor DksA which is considered as a crucial element for (p)ppGpp in *E.coli* to initiate transcription for amino acid synthesis and to inhibit rRNA expression either *in vivo* or *in vitro* (Paul *et al.*, 2005).

For example, in the amino acid histidine gene transcription, (p)ppGpp and DksA bind to the RNAP and redirect it to attach to the discriminator sequence, transcriptional site of the histidine operon (an AT-richregion), which increases the formation rate of the open complexes to trigger the transcriptional initiation of the histidine operon, stimulating transcription (Paul *et al.*, 2005). Conversely, in the case of rRNA deactivation, (p)ppGpp and DksA reduce the stability of the open complexes which inhibits the transcription initiation and deactivate the *rrn* operon transcription by binding to a GC rich region (Figure 11B;)(Barker *et al.*, 2001).

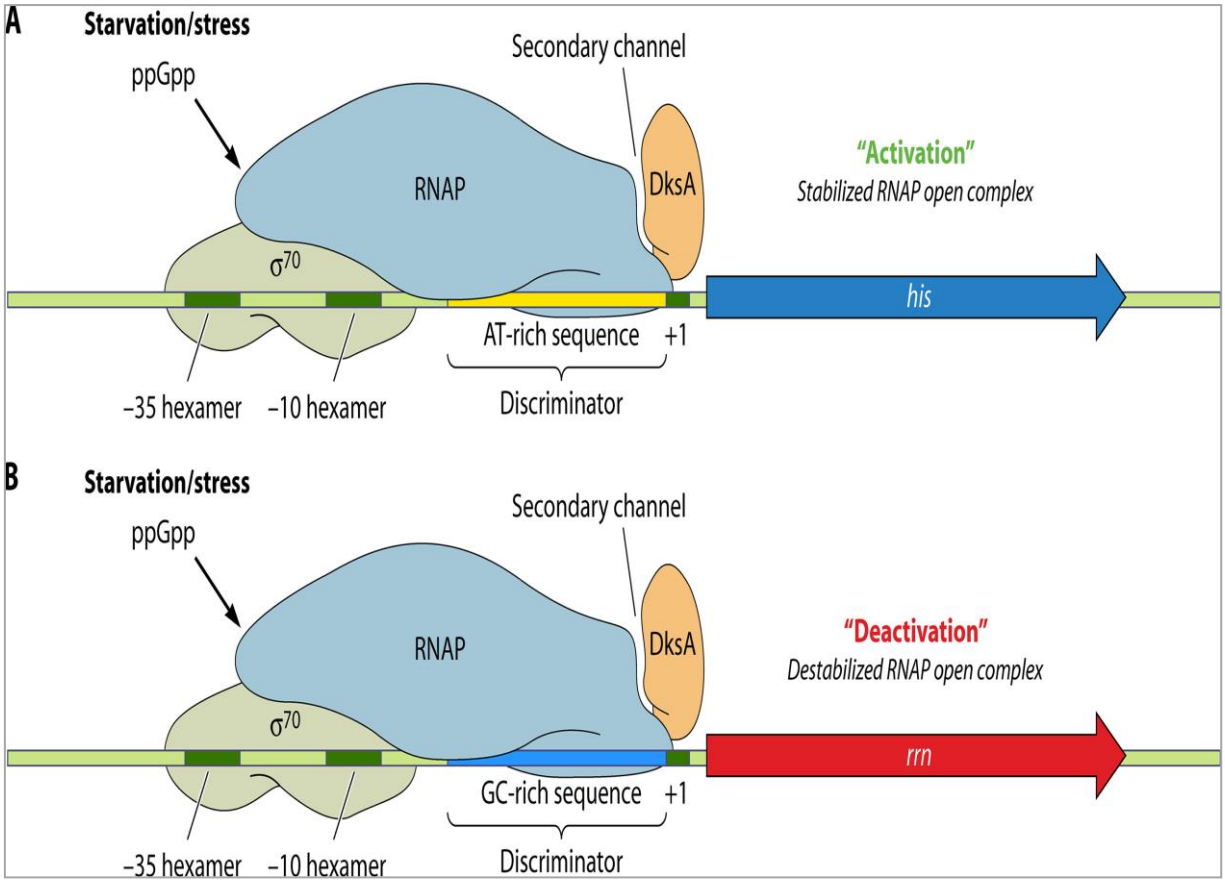


Figure11 The mechanism of ppGpp regulation of the expression of his and rrn promoters

A; ppGpp and DksA bind to the RNAP and redirect it to attach to the discriminator sequence site of the histidine operon (AT-rich), which leads promoter transcription activation. B; ppGpp and DksA reduce the stability of the open complexes, which inhibit the transcription initiation and deactivates the *rrn* operon (Dalebroux *et al.*, 2010)

Another mechanism by which (p)ppGpp can inhibit the transcription is via freeing RNAP from σ^{70}-dependant genes (most growth genes) that allows competition amongst other sigma factors and increases the possibility of RNAP attaching to other sigma factors, such as sigma S, 32 and 54 (Srivatsan and Wang, 2008). For example, specialized sigma factors bind the promoters of genes that are appropriate for the environmental conditions and consequently initiate the transcription for those sigma factor dependent genes. In particular (p)ppGpp can activate sigma S factor, which plays a crucial role in transcription initiation under stress conditions and at the

stationary phase in *E.coli* . Moreover, sigma S-dependent promoters require (p)ppGpp for their activation. In other words, ppGpp is involved in the production and activation of sigma S factor, which indicates its indirect effect on transcription (Gentry *et al.*, 1993, Kvint *et al.*, 2000).

1.3.3.2 Effects of (p)ppGpp on translation

The repression activity that (p)ppGpp shows on the synthesis of the stable RNA (tRNA and rRNA) is a crucial early effect that leads to inhibition of the initiation of the translation process. Another way that (p)ppGpp can affect the translation process is by reducing and inhibiting the activity of the translation elongation factors EF-Tu and EF-G, which results in major disruption of the translation process (Figure 12) (Srivatsan and Wang, 2008). In addition, a recent paper by Milon and his colleagues revealed that (p)ppGpp interacts with IF2 (initiation factor responsible for the translation initiation) and consequently, translation is inhibited as (p)ppGpp binds the same site of IF2 that is targeted by GTP in normal translation processes. In other words, (p)ppGpp competes with GTP to attach to the IF2 factor **(**Figure 12) (Milon *et al.*, 2006).

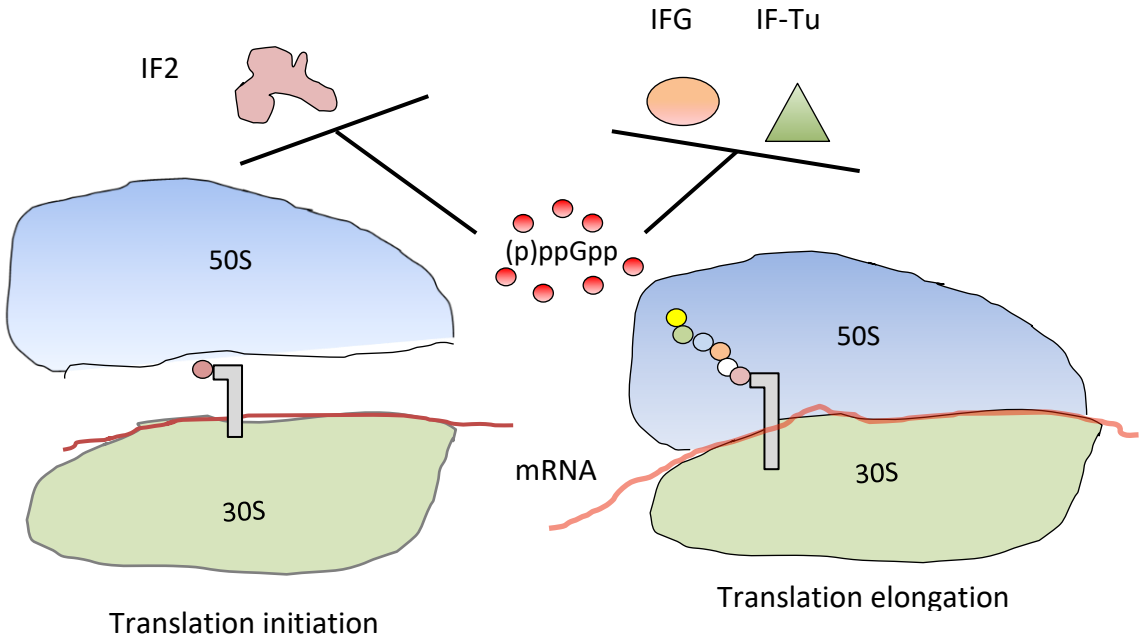


Figure 12 Effects of (p)ppGpp on translation

(p)ppGpp inactivates translation by interrupting the IF2 function and also *via* inhibition of elongation factors(IFG, IF-Tu), bars represent inhibitory effect. (Auther drawing)

1.3.3.3 Effects of (p)ppGpp on DNA replication

Glaser *et al* (1995) have claimed that, (p)ppGpp plays a role in affecting growth of *E.coli* by inhibiting the initiation of DNA replication which is a crucial point in DNA replication (see section 1.1.4.1). They also reported that the concentration of (p)ppGpp was inversely correlated with the initiation of new DNA replication(Figure13) (Schreiber *et al.*, 1995b). However, another way in which(p)ppGpp affects DNA replication is by inhibition of the replication elongation irrespective of the replication fork position on the chromosome (Figure13) (Levine *et al.*, 1991).

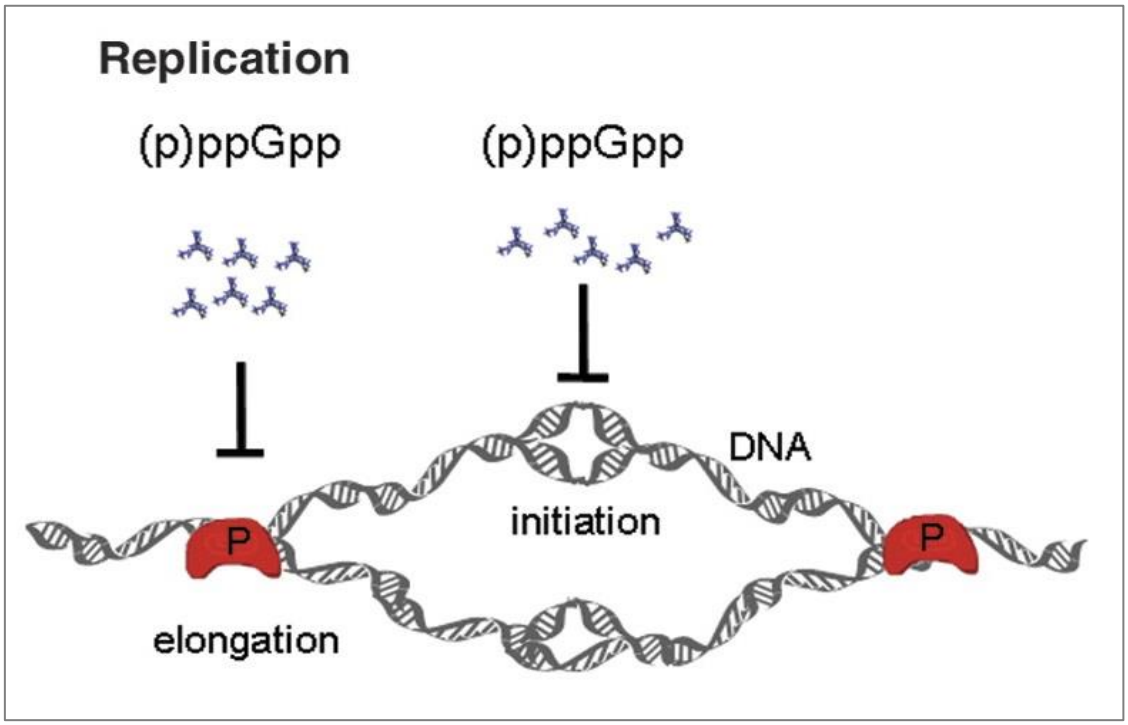


Figure13 Effects of (p)ppGpp on DNA replication

(p)ppGpp impedes DNA replication by inhibiting replication initiation and by stalling replication elongation. Modified from (Srivatsan and Wang, 2008).

A summary of the proposed effects of (p)ppGpp on the cells molecular activity is shown in Figure 14.

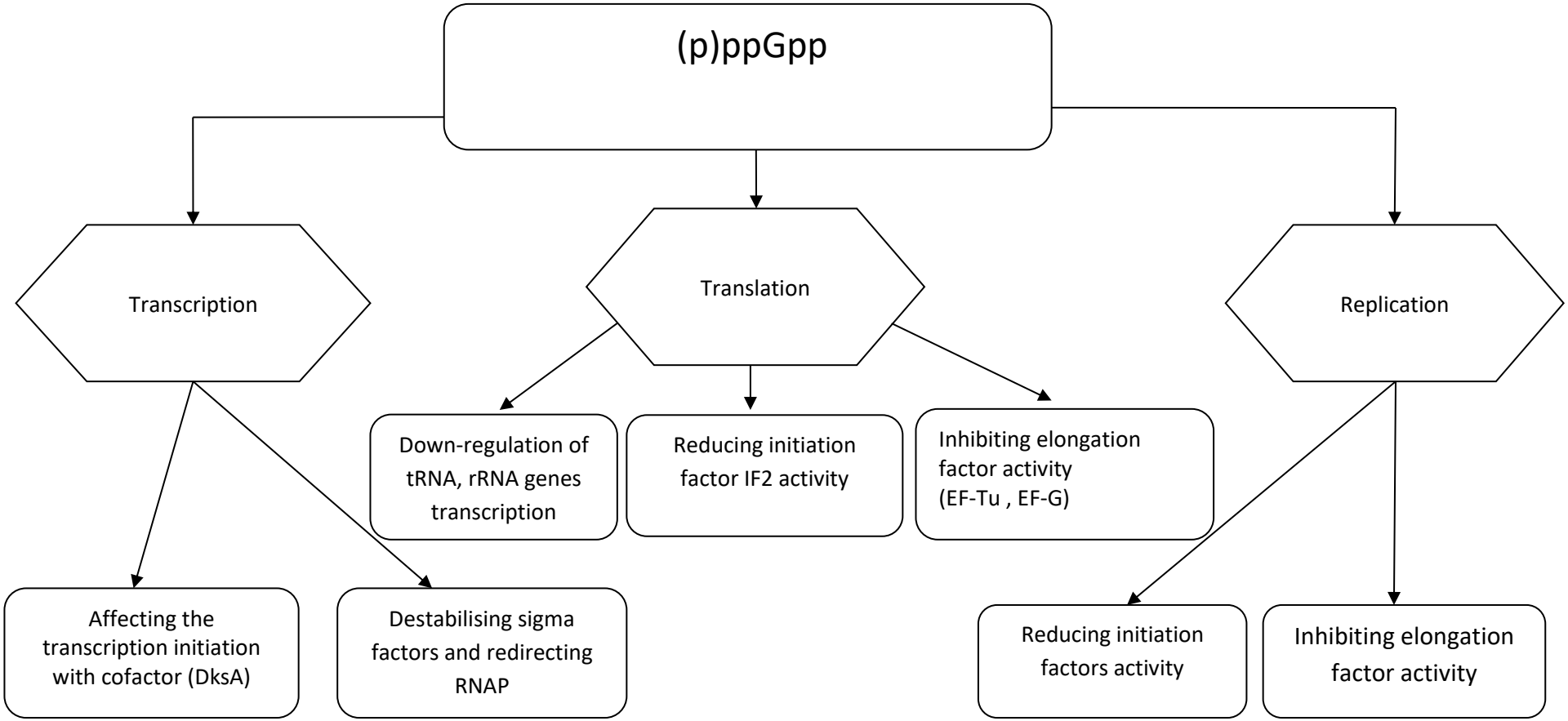


Figure 14 Summary of various proposed effects of (p)ppGpp effects on *E.coli* as a prokaryote model

1.4 (p)ppGpp in Gram-positive bacteria

A wide range of Gram-positive bacteria display the ability to produce (p)ppGpp under stress conditions including *B.subtilis, Listeria monocytogenes,* streptococci and staphylococci. (Lemos *et al.*, 2008, Nanamiya *et al.*, 2008, Taylor *et al.*, 2002, Cassels *et al.*, 1995). The mechanism and effect of (p)ppGpp produced in Gram-positive bacteria was thought to be similar to that of Gram-negative bacteria, but there is growing evidence that there are fundamental differences between these two groups of bacteria in their (p)ppGpp synthesis, influences and functions (Wolz *et al.*, 2010).

1.4.1 (p)ppGpp synthesis in Gram-positive bacteria

RelA and SpoT are the enzymes responsible for synthesis of (p)ppGpp in Gram-negative bacteria. A homologous enzyme named RSH (RelA/SpoT Homologue) is responsible for (p)ppGpp synthesis in Gram-positive bacteria although the (p)ppGpp alarmone is derived from GTP/GDP by phosphorylation by ATP as in *E. coli* (Wolz *et al.*, 2010, Potrykus and Cashel, 2008). RSH displays bifunctional activity as it synthesises and hydrolyses (p)ppGpp and similar to RelA and SpoT, RSH is composed of a C-terminal regulatory domain (CTD) as well as a N-terminal enzymatic domain (NTD) (Hogg *et al.*, 2004). The CTD is assumed to be involved in the reciprocal regulation activity states (p)ppGpp hydrolyse OFF/synthesis ON and (p)ppGpp hydrolyse ON/synthesis OFF (Hogg *et al.*, 2004, Mechold *et al.*, 2002). Interestingly, RSH has demonstrated no (p)ppGpp synthesis activity when expressed in *E. coli* and exposed to amino acid starvation, which suggests that its activity is limited to its native genetic background (Mechold *et al.*, 2002).

1.4.2 Mechanisms of action of (p)ppGpp in Gram-positive bacteria

The mechanisms of action that (p)ppGpp shows in Gram positive bacteria differs from Gram negative bacteria in several ways. For example, (p)ppGpp and DksA show direct

effects on amino acid synthesis when these two elements bind to RNA polymerase in order to either up-regulate or down-regulate promoters in *E. coli* (section1.3.4.1) By contrast, the DksA cofactor is not present in Gram-positive bacteria (Wolz *et al.*, 2010, Krásný and Gourse, 2004).

Another example of the variations is that (p)ppGpp affects DNA replication at a different stage in Gram-positive bacteria. For instance, in *E. coli,* (p)ppGpp affects the DNA replication at an earlier stage as it blocks DNA replication initiation at the oriC site which consequently prevents the DNA replication. In contrast, (p)ppGpp in *B. subtilis* exhibits inhibitory activity against primase, which leads to interruption of the elongation stage, subsequently, DNA replication is stalled (Levine *et al.*, 1991).

Furthermore, the decreased concentrations of GTP in the intracellular pool in *B. subtilis* and *S.aureus* seem to play an important role in the stringent response effects. For instance, the decreased level of GTP pool can repress genes that use this nucleotide as a transcriptional initiator. In *B. subtilis* rRNA gene promoter *(rrn)* activity is correlated with the GTP intracellular concentration as it tends to be up-regulated in the abundance of the nucleotide and repressed in the low concentrations of GTP (Krásný and Gourse, 2004, Tojo *et al.*, 2010).

In addition, GTP plays an important role in the translation initiation and translocation (section 1.1.4.5). A further important consequence that might occur in low concentrations of GTP is the reactivation of CodY-repressed genes. GTP enhances affinity of CodY to its target genes and decrease in GTP intracellular concentration would affect the repressive activity of this global regulatory protein and lead to huge transcriptional alterations, in particular for genes that are involved in nitrogen and amino acid metabolism as well as those encoding virulence factors (Sonenshein, 2005). During the stringent response the

GTP pool is rapidly decreased as a result of its consumption through (p)ppGpp synthesis (Geiger *et al.*, 2010a). In addition Kriel and colleagues (2012) claimed that (p)ppGpp is a posttranscriptional inhibitor for the GTP synthesis pathway in *B.subtilis* as it inhibits the activity of three enzymes, guanylate kinase (Gmk), hypoxanthine phosphoribosyltransferase (HprT) and IMP dehydrogenase (GuaB), that are involved in the GTP synthesis pathway (Figure15). It's worth noting that, IMP accumulates during the stringent response as a result of (p)ppGpp inhibitory effect, subsequently the IMP , as it is a precursor for ATP synthesis, may be recruited in the ATP synthesis pathway leading to increased ATP pools during the stringent response (Kriel *et al.*, 2012).

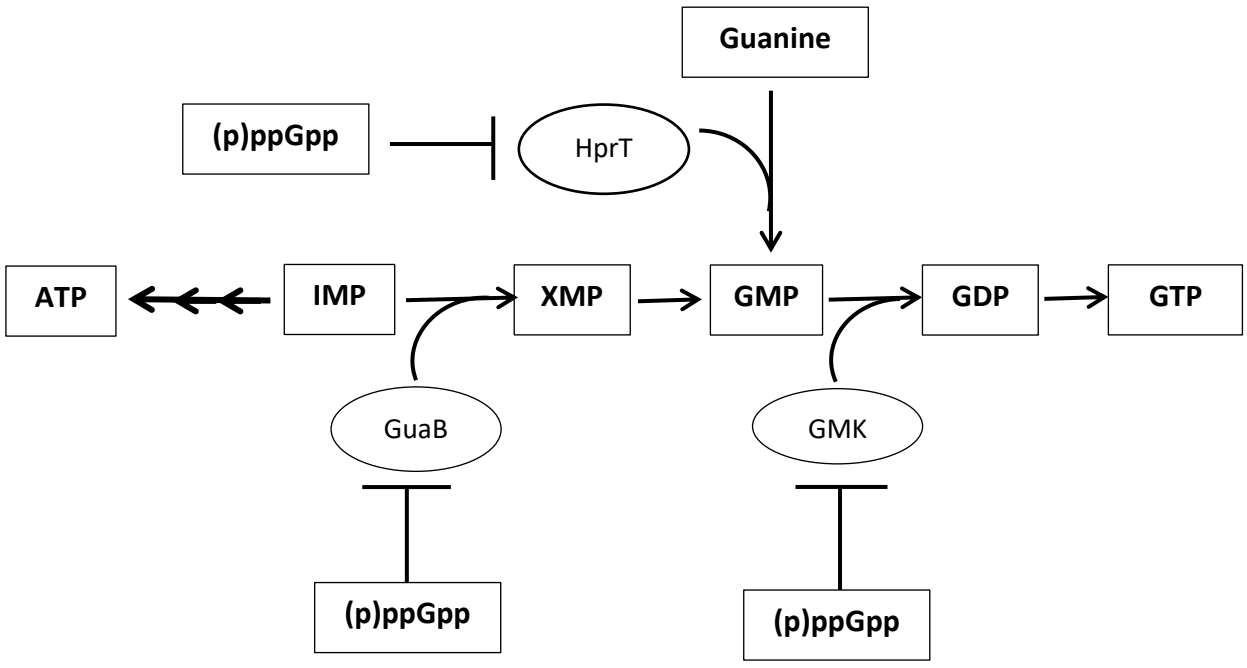


Figure15 Effect of (p)ppGpp on the GTP synthesis pathway

During the stringent response (p)ppGpp directly inhibits *GuaB* (IMP-Dehydrogenase), *Gmk* (guanylate kinase) and *HprT* (hypoxanthine phosphoribosyltransferase) enzymes that are involved in GTP *de novo* biosynthesis which results in declined GTP and increased ATP pools. Arrows represent positive effect, bars represent inhibition. (Auther drawing)

A summary of (p)ppGpp mechanisms of action during the stringent response in Gram-positive bacteria is shown in Figure 16.

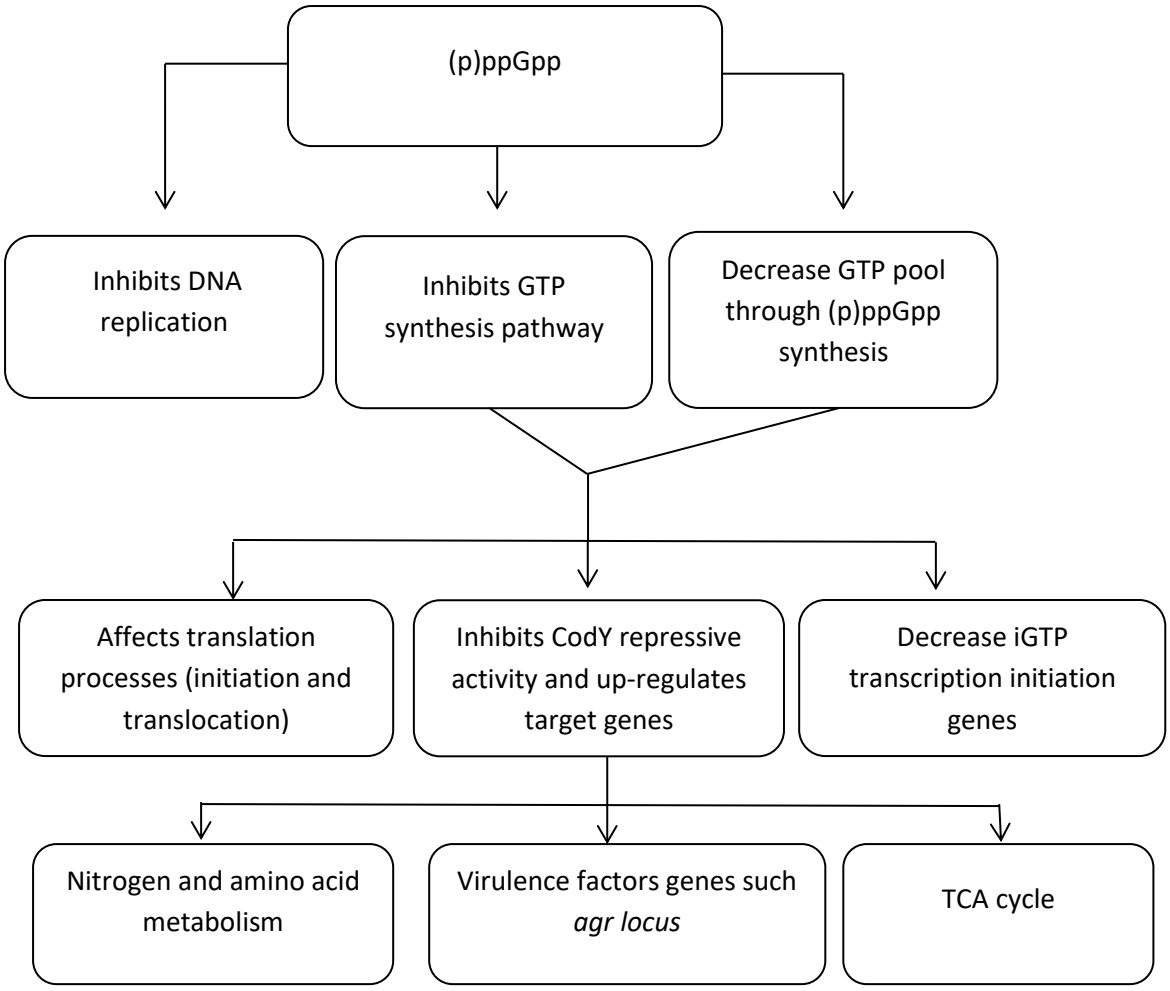


Figure 16 Summary of (p)ppGpp mechanism effects in Gram positive bacteria

DNA replication, GTP synthesis and pool are directly affected by (p)ppGpp. Decreased levels of GTP affects transcription initiation for some genes and would also trigger large alterations in metabolism and virulence factors expression through deactivating CodY repressive effect.

1.4.3 (p)ppGpp and pathogenicity in Gram-positive bacteria

After much research on Gram-positive (p)ppGpp, a consensus has been arrived at that there is an obvious link between possessing the *rel* gene, which is responsible for (p)ppGpp

production, and the pathogenicity of these bacteria. For example, inactivation of *relA* gene in *Streptococcus pyogenes* results in expression of several virulence factors such as streptolysin S, streptolysin O, streptodornase, and streptokinase which are involved in the *Streptococcus pyogenes* pathogenicity (Steiner and Malke, 2001). In addition, in *B.subtilis* a *Rel* mutant lacked detectable (p)ppGpp and failed to form spores and survive under stress conditions (Wendrich and Marahiel, 1997).

In contrast with other bacteria, an early studies on (p)ppGpp in *S. aureus* revealed that the *rel* gene in *S. aureus* is essential for the organisms viability as the *rel* mutant was not able to remain viable (Gentry *et al.*, 2000). Recent work by Wolz and his research group showed that, rsh_{syn} mutant cell stayed viable and they attributed the essentiality of the *rel* gene in cell viability to the hydrolase activity domain of RSH enzyme which seems responsible to prevent toxic level of (p)ppGpp that can be produced from other enzymes such as RelQ and RelP (Geiger *et al.*, 2010a). The stringent responses influence on pathogenicity has been explained by a series of intracellular actions. As mentioned previously, (p)ppGpp synthesis is accompanied by a significant decrease in the GTP intracellular pool. GTP has a crucial role on CodY function, CodY is a regulatory protein that is involved in carbon and nitrogen metabolism and is responsible for repression of virulence genes in some Gram-positive bacteria,(see section 1.1.5.5.B).The decrease in GTP concentration inactivates the repressive function of CodY and subsequently expression of virulence genes is increased which might increase the virulence properties of the bacteria (Handke *et al.*, 2008, Majerczyk *et al.*, 2010).

In recent years, the obvious correlation between (p)ppGpp production and pathogenicity in Gram-positive bacteria has opened doors to study this phenomenon closely and especially in *Staphylococcus aureus*.

1.4.4 Stringent response in *S. aureus*

Like many Gram-positive bacteria, *S. aureus* exhibits a stringent response after exposure to stressful conditions such as amino acid starvation and has the ability to produce (p)ppGpp. This was reported by Cassels and colleagues(1995), who observed (p)ppGpp accumulation in 28 different strains from six species of staphylococci including *S. aureus* (15 strains), *S. saprophyticus* (2 strains), *S. warneri* (1strain), *S. epidermidis* (7 strains), *S. hominis* (1 strain) and *S. haemolyticus* (2 strains) after inducing the stringent response *in vitro* by adding mupirocin (Cassels *et al.*, 1995)

Subsequent studies by Crosse and his team (2000) showed that the accumulation of (p)ppGpp in *S. aureus* could be induced by different types of nutrient deprivation such as, total amino acids, glucose, carbon and isoleucine (*via* mupirocin treatment). In addition, they showed that the Rel/SpoT enzyme exhibited dual functions (synthesis and hydrolysis of (p)ppGpp) which was earliest observation about the mechanism of effect for this enzyme in *S.aureus* (Crosse *et al.*, 2000b).

1.4.5 (p)ppGpp synthesis in *S. aureus* via mupirocin treatment

The mechanism of (p)ppGpp accumulation in *S.aureus* after mupirocin treatment occurs when this antibacterial agent inhibits the bacterial isoleucyl tRNA synthetase, which leads to uncharged tRNA binding to the A-site of the 50 S ribosome unit (Figure 17). Uncharged tRNA in the ribosome is sensed by the C-terminal domain in the RSH then the N-terminal enzymatic domain is shifted towards synthesis activity and begin to catalyse (p)ppGpp synthesis from GTP/GDP using ATP (Geiger *et al.*, 2010a). In contrast to *E.coli* which produces ppGpp dominantly, *S. aureus* showed ability to produce both ppGpp and pppGpp after mupirocin treatment. For example, pppGpp and ppGpp have been detected after mupirocin treatment (3 x MIC) and pppGpp appeared to be produced in higher

concentrations than ppGpp (Reiß *et al.*, 2011). However, other researchers detect ppGpp as well as pGpp and they claimed no pppGpp was present after stringent response induction by mupirocin (Crosse *et al.*, 2000b). Hydrolysis of pppGpp to form ppGpp and technical variations in detection methods for these nucleotides leads to these contrary results. This point is discussed in more detail in Chapter 4.

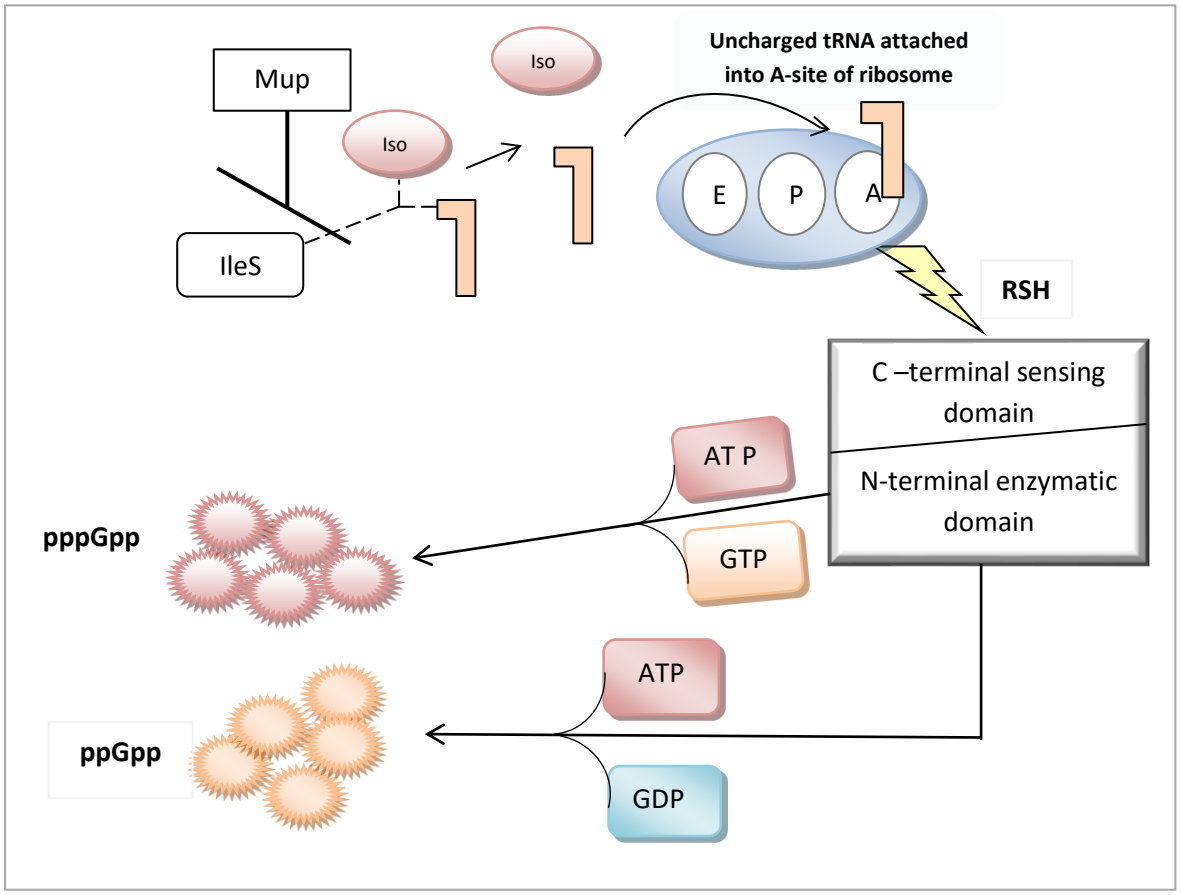


Figure 17 Stringent response induction by mupirocin in *S. aureus*

Mupirocin inhibits isoleucyl-tRNA synthase (*IleS*) activity which leads to accumulation of uncharged tRNA and free amino acid, isolecine (*Ile*). The C-terminal regulatory domain (CTD) in RSH (RelA/SpoT homologue), senses the uncharged tRNA accumulation then N-terminal enzymatic domain shifts toward synthesis formation function in order to synthesise (p)ppGpp by deriving it from GTP and GDP using ATP as energy source and phosphate donor. Dotted line indicates inhibited activity of IleS enzyme. (Auther drawing)

1.4.6 Global alteration during stringent response

Recent work by Reib and colleagues (2011) have used transcriptomic and proteomic techniques to observe the effect of mupirocin (at 3 x MIC) on *S. aureus* COL strain. They reported that most of the repressed genes during the stringent response were those involved in the regulation, structure and function of the cell transcription, translation and replication machineries (Reiß *et al.*, 2011). In addition, remarkable repression had occurred on genes that encoded products that were essential in rapid growth as well as for genes that were involved in RNA and DNA synthesis pathways. Also, they observed a significant reduction (50%) in protein synthesis rate relative to control cells after 10 min of mupirocin addition (Reiß *et al.*, 2011).

Anderson and colleagues (2006) observed the stress responses in *S. aureus* that were induced in different ways such as, heat shock, cold shock and nutrient limitation and they reported significant increases in mRNA stability (89 to 100%) of stress-induced transcripts after stringent response induction compared to control cells and they suggested that might be related to the inhibition of protein synthesis during the stringent response (Anderson *et al.*, 2006). In addition, groups of stress response genes are induced during the stringent response including the, universal stress proteins family (Usp) that is involved in different functions such as oxidative-stress resistance, stationary-phase survival and adhesion (Kvint *et al.*, 2003).

Production of toxin-antitoxin systems is another of the stringent response features that occurrs in *E.coli, B subtilis* and *S. aureus* (Fu *et al.*, 2007, Gerdes *et al.*, 2005). Most toxin-antitoxin loci contain two genes that encode a stable toxin and an unstable antitoxin that seizes the toxins by direct protein–protein interaction and they are degraded by cellular proteases (Gerdes *et al.*, 2005, Hayes and Van Melderen, 2011). Chromosomally encoded toxin antitoxin systems contribute to stringent response by inhibiting important cellular

processes such as translation. For example, in *S. aureus* $MazEF_{Sa}$ is a toxin-antitoxin sequence-specific endoribonuclease which cleaves mRNA and inhibits translation during the stringent response (Fu *et al.*, 2007). Recent work has suggested an important role of the toxin antitoxin system in the *S. aureus* fitness following mupirocin exposure (Reiß *et al.*, 2012a).

Global transcriptional alterations occurred through the stringent response. Spx is a global transcriptional regulator that can activate or repress transcription of wide range of genes involved in growth and stress responses in *S. aureus* (Pamp *et al.*, 2006). However, Most of the up-regulated genes during the stringent response in *S.aureus* were found to encode hypothetical proteins of yet undetermined functions that might be related to cell adaption during the stress response (Anderson *et al.*, 2006, Reiß *et al.*, 2011).

1.4.7 Effects of the stringent response on amino acid pools and mupirocin susceptibility

During the stringent response induced by mupirocin, Reib and colleagues claimed that isoleucyl tRNA synthetase IleS gene (*ileS*) and genes that were involved in branched-chain amino acid (BCAA) biosynthesis such as *ilv-leu* were transcribed at high levels and their expression was associated with slight increase in the intracellular concentrations of these amino acids in comparison to control cells which might reflect their important role during stringent response in *S. aureus* (Reiß *et al.*, 2011). This finding is in agreement with other observations that amino acid biosynthesis and transport genes transcription increased after stringent response induction by mupirocin (60µg/ml^{-1}) (Anderson *et al.*, 2006). In addition, it was suggested that up-regulation of genes encoding proteases may increase digestion of extracellular proteins within the bacteria milieu in order to recover from the amino acid deficit caused by the stringent response (Anderson *et al.*, 2006).

In another study, two different strains of *S. aureus* (Newman and HG001) rsh_{syn} mutant exhibited lower MIC (up to 4 fold) toward mupirocin than the wild type strain which led to speculation that (p)ppGpp can up-regulate genes involved in isoleucine biosynthesis in wild type strains which might elevate isoleucine level to antagonise the activity of mupirocin, thereby reducing mupirocin influence in the wild type cells (Geiger *et al.*, 2010a).

1.4.8 Regulation of virulence factor expression by the stringent response

S. aureus virulence factor genes are mostly regulated in a growth-dependent manner. For example cell surface components that facilitate host tissue attachment are expressed within the exponential growth phase whereas exoproteins and toxins are preferentially expressed in the post-exponential growth phase (Cheung *et al.*, 2004).

During the stringent response a number of genes that encode adhesin proteins such as intercellular adhesion protein A-B (*ica A-B),* fibrinogen-binding protein (*fbp*) and fibronectin-binding proteins A and B (*fnbA, B*) were up-regulated (Anderson *et al.*, 2006, Reiß *et al.*, 2011). In addition, genes that encoded toxins such as TSST-1 (*tst*), staphylococcus enterotoxin B (*seb*), and proteases (*sspA, sspB* and *sspC*) genes were also transcribed during the stringent response. Further, genes that encoded two components regulatory systems which contribute to virulence genes regulation were up-regulated during the stringent response. These regulatory systems include the quorum sensing *agr locus* and its effector regulator RNAIII, Sar family and Sae systems (Anderson *et al.*, 2006, Reiß *et al.*, 2011, Geiger *et al.*, 2010a).

The stringent response in *S. aureus* also exhibits clear effects on the pathogenicity of the organism through decreasing the level of GTP which influences on the regulatory protein CodY repressive activity on number of virulence factors genes (see section 1.1.5.5.B)

(Majerczyk *et al.*, 2008). Recent work by Wolz and colleagues (2010) used a *rsh* $_{syn}$ mutant strain of *S. aureus* and they found that it was attenuated in an animal model of infection. Then they introduced a *codY* mutation into the rsh_{syn} mutant strain and they observed an increased level of virulence in this strain and they attributed this to the loss of CodY protein repressive activity on virulence factor gene expression (Geiger *et al.*, 2010a). In addition, *codY* gene has been reported to be repressed at both transcription and translation levels during the stringent response induced *via* mupirocin (3 x MIC) (Reiß *et al.*, 2011)

Furthermore, Wolz and his research group (2012) have observed the effect of the stringent response on virulence factors in *vivo* and they claimed that induction of the stringent response in *S. aureus* was also important for the cell survival after phagocytosis (Geiger *et al.*, 2012). They found that, uptake of wild type *S. aureus* in neutrophils could trigger the stringent response and cytotoxic phenol-soluble modulins (PSMs), peptides that promote neutrophil lysis after phagocytosis, were found to be expressed and produced which enabled *S. aureus* to survive from phagocytosis in contrast to *psm* mutant, *rsh* mutant, *rsh* and *codY* double mutant strains (Geiger *et al.*, 2012).

Another mechanism that has been shown to increase pathogenicity of *S. aureus* during the stringent response is *via* stabilization of mRNA for those genes encoding important virulence regulators such as RNAIII, SarA and SaeRS (Geiger *et al.*, 2010a). The obstructed ribosome with uncharged tRNA was assumed to increase the lifetime of the bacterial mRNA as the ribosome can protect mRNA from the RNase degradation activity (Anderson *et al.*, 2006). However, it is not clear if the increased stabilization of mRNA during mupirocin treatment is paralleled by increased protein synthesis in *S. aureus.*

On the other hand, during the stringent response the organism's pathogenicity can be reduced in different aspects. For instance, repression of genes encoded for products that contribute in the translation machinery such as rRNA, obstruction of ribosomes by uncharged tRNA and lack of essential nutrient components such as amino acids would affect the virulence product synthesis for the bacteria such as exo-proteins and enzymes. In *S. aureus,* a reduction (50%) in protein synthesis during stringent response triggered *via* mupirocin had been reported (Reiß *et al.,* 2011). In addition, the associated growth inhibition through the stringent response may affect the pathogenicity of invasive infections that *S. aureus* might exhibit.

1.4.9 transcritomic technologies to observe the global alteration of gene expression during the stringent response

The majority of previous studies that have observed the effect of the stringent response on gene expression in *S. aureus* have used microarray techniques (Anderson *et al.,* 2006, Geiger *et al.,* 2010, Reiß *et al.,* 2011). Microarray (hybridization-based technology) was the most advanced tool that could be used for transcriptomic study in the last few years. However, the advent of next generation sequencing (high throughput sequencing technology) in particular RNA-seq has changed the way in which transcriptomes are observed (Nagalakshmi *et al.,* 2010, Wang *et al.,*2009).

RNA-seq, also called "Whole Transcriptome Shotgun Sequencing", is a technology that applies sequencing to a library of cDNA, obtained from RNA, utilizing the capabilities of next-generation sequencing (high-throughput DNA sequencing technologies). This is followed by mapping the sequencing reads to a reference genome to provide precise quantification of global gene transcription (Wang *et al.,* 2009).

There is some kind of similarity between microarray and RNA-seq methods. For example, both of them can be conducted using the same work flow starting from experimental

design; data acquisition, and finally analysis and interpretation. However, key differences between these technologies make the RNA-seq advantagous over microarray. For instance, using microarray technology, transcript detection is limited as specific oligonucleotides probes are hybridized with their corresponding genes; while in the RNA-seq known transcript as well as unknown can be detected which makes RNA-seq technology also ideal for discovery-based experiments (Wang *et al.*,2009, Arnoud and Vliet., 2010).

In addition, microarrays can measure the relative level of RNA only whereas; absolute rather than relative values can be quantified by RNA-seq with a large dynamic range of expression levels. Also, *de novo* transcriptome assembly and differential expression analysis can be performed for organisms lacking a reference genome by RNA-seq technology (Arnoud and Vliet., 2010). Another advantage of RNA-seq over microarray is that mismatches affect the hybridization efficiency which lead to high signal background in the microarray technology while RNA-seq delivers low background signal as cDNA sequences can be mapped unambiguously to unique regions of the genome which enable easy elimination of the noise during analysis (Wang *et al.*,2009).
In this study, the RNA-seq technology was performed to observe the global alteration on gene expression after exposure to a sublethal concentration of mupirocin which has not been used in previous observations.

1.4.10 Summary

An increasing number of observations on the stringent response in *S. aureus* have only been reported recently despite of the clinical importance of this pathogen. Most of these studies used multiples of MIC to induce the stringent response in *S. aureus* using mupirocin which lead to cell death after few hours (Cassels *et al.*, 1995, Crosse *et al.*, 2000b, Anderson *et al.*, 2006). During the stringent response, global phonotypic and

genotypic alterations occur such as inhibited growth and global gene expression alterations (Reiß *et al.*, 2011) (Geiger *et al.*, 2010a).However, similar alterations have been reported in different strains of *S. aureus* in several studies after exposure to sub-inhibitory concentration of antibiotics. For instance, early production of TSST-1 after sub-inhibitory concentration of silver sulphadizine and prolonged lag phase with reduced TSST-1 production after mupirocin treatment was reported early by Edwards-Jones and Foster (Edwards-Jones and Foster, 1994). Subsequent work observed increasing toxins production of *S. aureus* (TSST-1, PVL alpha haemolysin) after exposure to sub-inhibitory concentration of non-protein targeting agents in particular nafcillin (Stevens *et al.*, 2007). Conversely reduced toxin production but not transcription following treatment with sub-inhibitory concentration of protein synthesis inhibitor such as clindamycin and linezolid was reported (Stevens *et al.*, 2007). Suppression of toxin production in pathogenic organisms is important particularly during infections caused via toxins such as necrotizing infections (Zimbelman *et al.*, 1999, Stevens *et al.*, 1987). Reduced toxins production and growth inhibition that occurred after sub-inhibitory concentration of proteins synthesis inhibitor agents in particular mupirocin can be related to the stringent response as these feature were also exhibited after stringent response induction *via* high inhibitory concentrations (Reiß *et al.*, 2011).

Here, the effect of sub-inhibitory concentration of mupirocin on *S. aureus* global transcription has been studied including the influence of this concentration on the bacterial virulence factors. Linking the stringent response that *S. aureus* shows and its regulation of virulence factors might improve our understanding for the organism pathogenicity and can help to draw up new treatment approaches that can consider the response of the organism during different conditions and its effects on pathogenicity.

1.5 Aims and objectives

This project aimed to carry out a comprehensive investigation of the effect of Mupirocin on *S. aureus* when used at sub-inhibitory concentration. A particular focus of the study was the stringent response and the expression of virulence factors.

Specific Aims:

1. To investigate the susceptibility of recent and previous clinical isolates (147) of *S. aureus* to Mupirocin in order to assess the usefulness of this antibacterial in clinical use.
2. To verify the hypothesis that sub-inhibitory concentrations of mupirocin are capable of triggering the stringent response in *S. aureus using strain 8325-4* as a model organism.
3. To quantify the influence of the sub-inhibitory concentration of mupirocin on *tst* gene transcription and TSST-1 synthesis and link that with the stringent response effect on virulence in a TSST-1 producing clinical isolate of *S. aureus* (B49).
4. To observe the effects of the stringent response (if it was indeed induced) on the global gene transcription in general, with particular focus on genes that encode virulence factors.

Objectives;

The project objective was to determine the influence of a sub-inhibitory concentration of mupirocin on metabolic activity, gene expression and toxin synthesis in relation to induction of the stringent response and that would include;

1. Determination of minimum inhibitory concentration, MIC, of mupirocin for *S. aureus* 8325-4, B49 and 147 clinical isolates via micro-dilution methods to determine their susceptibility to mupirocin.
2. Detection of the effect of sub-inhibitory concentration of mupirocin on the guanosine nucleotides pool and stringent response nucleotide (p)ppGpp production *via* High Performance Liquid Chromatography (HPLC).

3. Investigation of the effect of sub-inhibitory concentrations of mupirocin on expression of particular virulence genes in *S. aureus* including TSST-1 and RNAIII by real time reverse transcription (RT)-PCR and the influence on toxin synthesis via Reverse Passive Latex Assay (RPLA).

4. Observation of the global transcriptional alterations that sub-inhibitory concentration of mupirocin influence at different time points during the stringent response, tolerance and recovery periods using RNA- sequencing technology.

Chapter 2

Material and methods

2.1 Sample collection and sources

Staphylococcus aureus 8325-4 is a useful model strain that been used in previous works (Cassels *et al.,* 1995, Crosse *et al.,* 2000) to observe the stringent response induction. *S. aureus* (B49) is a TSST-1 producer clinical isolate from burn unite in Booth Hall hospital isolated in 1992 (Edwards-Jones 1997) and used to study the effect of sublethal concentration of mupirocin on TSST-1 production. These *S. aureus* strains (8325-4 and B49) are mupirocin sensitive and they been provided by Professor Howard Foster for use in this study. In addition, 147clinical isolates of MSSA and MRSA were used in this study to determine their mupirocin susceptibility regardless to their caused infections. These clinical isolates were divided to three groups according to their isolation year and source. Group A included 44 clinical isolates (2011) of *S. aureus* from the Health Protection Agency in the UK. Group B includes 43 samples of *S. aureus* isolated from Bury General Hospital in 1999 in the UK. The third group C included 60 samples isolated in Libya (2009-2010), kindly donated by Saeed Waerg and Professor Howard Foster.

2.2 *Staphylococcus aureus* identity verification

All the samples were grown over night at 37°C on nutrient agar then their identification was confirmed by Gram stain and Staphaurex*(Remel) latex kit according to the manufacturer's instructions. All isolates were then preserved on beads in tryptone glycerol buffer and stored at -80°C until they were needed.

2.3. Determination of samples MICs for mupirocin (chapter3)

A microtiter plate assay was conducted to determine the MIC (Minimum Inhibitory Concentration) of mupirocin (supplied by Glaxo Smith Kline) for each isolate. Single and double strength Iso-sensitest broth medium (Oxoid) was prepared according to the manufacturer's instructions. One hundred microliters of the double strength Iso-sensitest

broth was added to the first well in each row in the microtiter plate. The remaining wells were loaded with 100 µl single strength Iso-sensitest broth. Then 100µl of mupirocin (1 mg ml^{-1}) was added to the first well of each row then mixed. After that, the tip was replaced with a new one, and 100 µl was transferred to the next well and so on in order to set up the serial dilution of mupirocin as follows: 1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0161, 0.008, 0.004, 0.002, 0.001, 0.0005, 0.00025, 0.00012 mg ml^{-1}. The final 100 µl taken from the last dilution was discarded. From 14 -16 h incubated culture 1ml was diluted to OD= 2 (6.1 x 10^8 cfu/ ml^{-1}) then each well in the plate was inoculated with 10 µl of the diluted stock to reach OD ≈ 0.5 at 600nm (1.3x 10^7 cfu ml^{-1}). Each well in the plate presented different dilutions of the mupirocin against the same bacterial sample through the column. Two columns were considered as controls. One was a broth containing the inoculum and the other was not inoculated broth in order to check for contamination (negative control).

The plate was incubated for 24 h at 37°C. After that digital reading was taken by using a microtiter plate reader (MultiskanFC, Thermo scientific) at 570 nm was obtained. The MIC was determined as the last well showing no growth. These procedures were applied in three independent triplicates whenever the test had been performed.

2.4. Disc diffusion assay

From overnight culture grown on Nutrient Agar, a few colonies were taken using a sterile loop and inoculated into 1ml of sterile distilled water to reach OD = 0.15 – 0.2 at 600nm (10^8 cfu/ml) which is approximate to 0.5 McFarland standard. Then using a sterilized cotton swab, the bacterial cells were spread onto a Nutrient Agar (NA) plate. After that different antibiotic discs, listed in table 1 (provided by OXOID LTD UK), were dispensed on the surface of the plate and incubated for 24 h at 37◦C, zones diameter surrounding each antibiotic discs were measured , and the state of susceptibility was determined

according to National Committee for Clinical Laboratory Standards NCCLS. Three replicated were performed for each clinical isolates.

Table (1) antibiotics list used in the disc diffusion assay.

Antibiotics discs and concentrations		
Vancomycin 30µg	Cefoxitin 30 µg	Gentamycin 10µg
Fusidic acid 10µg	Erythromycin 15µg	Streptomycin 10µg
Chloramphenicol 30µg	Clindamycin 2µg	Ciprofloxacin 5µg

2.5. Induction of stringent response by mupirocin and observation of its effects (Chapter 4)

The purpose of this experiment was to verify whether the sub-inhibitory concentration of mupirocin is capable of triggering the stringent response in *S.aureus* 8325-4. The experiment was divided into three stages; 1) triggering stringent response by adding sublethal concentration of mupirocin to cultures, 2) observation of the effect of mupirocin on cell growth OD_{600nm}, 3) observation of the effect of mupirocin on ppGpp production and related nucleosides, GTP and ATP, intracellular pools. The detection was performed for 1, 2, 3 and 4 h samples after mupirocin addition then at 12 and 24 h time points.

2.5.1. Stringent response induction Three-hundred millilitres of Iso-sensitest broth was inoculated with one bead of *S.aureus* 8325-4 in a 500 ml flask, and incubated in a rotary incubator for 18 hours at 37°C and 200 r.p.m. Then, 40 ml of the broth culture adjusted to OD_{600nm} = 0.5 ($1.3x\ 10^7$cfu ml^{-1}) by spectrophotometer (CAMSPEC M330) was prepared then mupirocin was added to a final concentration 0.25µg/ml (0.5 MIC) to the bacterial broth. Flasks were incubated in the rotary incubator for the intervals needed and negative control broth cultures were placed in parallel with treated cultures with mupirocin. In addition, OD_{600nm} reading was taken for each time pointes for control and test cultures whenever the test had been performed.

2.5.2. Mupirocin effect on growth influence of the sub-inhibitory concentration of mupirocin on cell growth was observed at the desired time points. One hundred microliter of the cell culture was added to 900 µl of the same non inoculated medium into 1 ml cuvette and placed into spectrophotometer. Three different reading of the OD_{600nm} were taken and mean of them was multiplied by the dilution factor. Similar processes were followed to measure OD_{600nm} for the control cell culture.

2.5.3. ppGpp and other nucleoside extraction and detection Cells were harvested at the determined time points and nucleotides extraction was performed following the protocol of (Greenway and England, 1999) with slight alterations. Bacterial broth (40ml) was filtered through filter paper (Millipore 0.45µm pore size). Then 5 ml of 2M ice cold formic acid was added to the culture and incubated on ice for 1 h. Cell debris was removed by centrifugation for 10 min at 6000 x g, and the supernatant was filtered through a nitrocellulose membrane (Whatman 0.45µm pore size). The filtrates were then frozen using liquid nitrogen, dried by lyophilisation (Coolsafe TM, SCANVAC) for 48 h and then kept at 4°C.

2.5.4. Nucleotide fractionation the lyophilised extract was dissolved in 3 ml of highly purified de-ionised distilled water. One millilitre was then injected into High Performance Liquid Chromatography (HPLC) (Polaris,VARIAN) using a Partisil 10 SAX column, anion exchanger (Whatman) and eluted with a with gradient of 7mM K_2HPO_4, pH 4.0 to 0.5 M KH_2PO_4/0.5 M Na_2SO_4, pH 5.4 at a flow rate of 1 ml min^{-1}. The detection wavelength (UV) was 254nm. Nucleotide concentrations were expressed relatively to the cell dry weight.

2.5.5 Standards nucleotides calibration curves

Standard nucleotides, AMP, ADP, ATP, GMP, GDP, GTP (Sigma-Aldrich,UK) and ppGpp (TrilinK,USA) were used as controls in the HPLC technique to determine their

retention time and calculated the nucleotide concentration in the intracellular pool of the cells. Different concentrations of each standard was prepared in the range of 1.1-0.010 Nano mole (concentration for each standard are illustrated in chapter 4). Then standards were injected in the HPLC instrument, using the same conditions mentioned above, and retention times determined for each standard. Different concentrations of each standard were also fractionated in order to allow quantitative determination.

2.5.6 Measuring Cell Dry Weight

Forty ml of the broth culture adjusted to OD= 0.5 was centrifuged for 5 min at 10,000g. a washing step with 99% ethanol was conducted then the cell suspension was transferred into glass vials and dried at 60°C until a constant weight was achieved. The weight difference between dried vials with and without cells was used for CDW determination.

2.6 Observing the effect of sub-inhibitory concentration of mupirocin on *tst* gene transcription and TSST-1 production (Chapter 5 experiment)

In this experiment a sub-inhibitory concentration of the mupirocin was added to exponentially growing cell of *S.aureus* B49 (TSST-1 producer) and mupirocin effect on these cells was observed in three aspects; 1- effect on cell growth, 2- influence on the TSST-1 production and 3- Effect on *tst* gene transcription. All these observations were conducted at 5 different time points 0, 1, 8, 16 and 24 h for the treated cell with sub-inhibitory concentration of mupirocin in parallel with control cell culture in three independent triplicates.

2.6.1 Effect of mupirocin treatment on cell growth

Similar processes described above in section (2.5.2) were applied for exposed cells and untreated cultures.

2.6.2 Influence of mupirocin on TSST-1 production

Production of TSST-1 was investigated in test cells and compared with control cells to observe mupirocin effect on the toxin production. Briefly, *S. aureus* B49 was grown in 5

ml of Brain Heart Infusion Broth overnight in a rotary incubator at 37°C. Cell were washed with PBS then inoculated into 20 ml BHI and OD adjusted to 0.5 at 600nm and mupirocin was added to a final concentration 0.12µg/ml-1(0.5 MIC) in the test culture and this step was omitted in control culture. Cultures were incubated for the desired time points then TSST-1 was detected using a reverse passive latex assay RPLA (OXOID, TD0940, LTD UK), according to the manufacturer's instructions. In this assay latex particles are sensitised with purified antiserum from rabbits immunised with purified TSST-1 and the agglutination occurs in the presence of TSST-1. Three millilitres of culture were centrifuged for 15 minutes at 10,000 r.p.m and the supernatant was transferred to new tube and used as a sample. Using V-bottomed microtiter plate (Sterilin, Fisher Scientific) each sample occupied two rows. 25µl of Phosphate buffered saline (PBS) was dispensed in each well of the first two rows except the first well in each row. Then 25 µl of the sample was added into the first and second well of each row. Doubling dilution was made by transferring 25µl from the first well to the second and so on up to well number 7. After that 25µl sensitized latex added to each well in the first row and 25µl of the control latex was added to each well in the second row. Then the plate was rotated in the micromixer for 1 min and left at room temperature overnight. After that the agglutination reaction was observed and recorded. The titre was taken as the last dilution that showed agglutination. The assay sensitivity was 2ng /µl-1. Positive and negative controls provided with the kit were setup whenever the test was performed. Each reading was taken after 18-24h as recommended and the experiment was carried out in independent triplicates.

2.6.3 Total RNA extraction and Real Time PCR

Cell aliquots from cultures exposed to sunlethal concentration of mupirocin for (0, 1, 8, 16 and 24h) were harvested (3ml) and stored in -80°C until use. RNA extraction was performed following the instructions of RNAprotect® Bacteria Reagent and RNeasy®

Mini Kit (Qiagen). DNase I (Qiagen) was included in the RNA extraction procedures as instructed to avoid DNA contamination. Purity and concentration of RNA were verified using a Nanodrop spectrophotometer 2000 (Thermo Scientific). Then cDNA were obtained using QuantiTect®Reverse transcription two steps (Qiagen). After that primers and probes for *tst*, *RNAIII* and 16S reference gene (Vaudaux *et al.*, 2002, Fosheim *et al.*, 2011) listed in table 3, were checked for efficiency replication with *S. aureus* B49 and used in the real time PCR in this work. PCR reagents, primers and probes concentration as well as the real time PCR cycling program is illustrated in table 2 and 3 respectively. The expression ratio for each gene was relatively calculated using *16S* as a reference gene by $2^{-\Delta\Delta CT}$ for *RNAIII* and Pfaffl for *tst* gene appropriately to their replication efficiency (Livak and Schmittgen, 2001 and Pfaffi, 2001). Ct values were collected for a minimum of three independent experiments.

Table 2 real time PCR reagents mix.Component	**Volume**	**Final concentration**
2x Quanti Tech PCR master mix	12.5µl	1 x
Primer forward	1µl	0.4µM
Primer reverse	1µl	0.4µM
Probe	5µl	0.2 µM
Template cDNA	2µl	4 ng
DNase RNase free water	3.5µl	
Total reaction volume	25µl	

Table 3 program cycle for the real time PCR.

Step	**Time**	**Temperature**
PCR initial activation step	15 min	95°C
2-step cycling		
Denaturation	15s	95°C
Annealing/ extension	60s	60°C
Number of cycles	35-45	

Table 4 Primers and probes used in the real time PCR .

Gene	Primer forward	Primer reverse	Probe
16S	**551F-** GGCAAGCGTTATCCGGAATT	**651R-** GTTTCCAATGACCCTCCACG	**573T-** CCTACGCGCGCTTTACGCCCA[a]
tst	**STSTQF** CCCTTTGTTGCTTGCGACA	**STSTQR** GCTTTTGCAGTTTTGATTATT	**STSTQT** TCGCTACAGATTTTCACCCCT GTT CCCTTATCAT[a]
RNAIII	**367F-** TTCACTGTGTCGATAATCCA	**436R-** TGATTTCAATGGCACAAGAT	**388T-** TTTACTAAGTCACCGATTGTTGAAATGA[a]

a 5’FAM and 3’TAMRA labelled.

2.7 Observation the effect of sub-inhibitory concentration of mupirocin on the global transcription for *S.aureus* 8325-4 via Next generation sequencing technique (RNA-seq) (chapter 6)

RNA-sequencing was carried out by the Centre for Genomic Rresearch (CGR) at the University of Liverpool to observe the effect on the global transcription of *S.aureus* 8325-4 after treatment with sub-inhibitory concentration of mupirocin treatment. By RNA sequencing technology wide view of the transcription alterations that mupirocin might cause it obtained. The technique and the analysis of the results were performed at Centre for Genomic Rresearch (CGR). The whole genome sequence of *S.aureus* 8325-4 was not available and for that reason *S.aureus* 8325 whole genome sequence was used as reference in this test as *S.aureus* 8325-4 is derivative from *S.aureus* 8325, two differences between these strains first, sigB is defective in 8325-4 second, 8325-4 is cured from prophages ϕ11, ϕ12 and ϕ13(O'Neill 2010). However, the compatibility of this with this transcriptomic analysis was checked with different expertise in the department, University of Leeds (Dr,O'Neill) and the CGR staff. Then the experimental design was approved.

2.7.1 Mupirocin treatment and RNA extraction

Sub-inhibitory concentration of mupirocin was added to exponentially growing cells (OD 0.5) in Iso-sensitest broth and cells were harvested at three different time points 1, 12 and 24h in parallel with control cells and their OD were recorded. After that, normalization for the cell was applied by adjusting all the cultures to OD = 0.5 at 600nm. RNA was extracted as described above. This was carried out for treated and control cells in independent triplicates of each time point, (table 5), RNAs were sent to the CGR for RNA-seq.

2.7.2 RNA sequencing

Verification of RNA integrity was conducted via Bioanalyzer 2100(Agilent technology) at the CGR then non-coding RNA was depleted from the samples via RiboZero Magnetic kit (Bacteria) (Catalogue No. MRZB12424) from Epicentre using 2μg of starting material. RNA–Seq libraries

were prepared from 20-25 ng of the enriched material using the Epicentre ScriptSeq v2 RNA-Seq Library Preparation Kit (Catalogue No. SSV21106). Following 13 cycles of amplification, libraries were purified using AMPure XP beads. Each library was quantified using Qubit and the size distribution assessed using the Agilent 2100 Bioanalyser. These final libraries were pooled in equimolar amounts using the Qubit and Bioanalyzer data. The quantity and quality of each pool was assessed by Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa (KK4854) on a Roche Light Cycler LC480II according to manufacturer's instructions. The resultant pools were sequenced on 3 lanes of the HiSeq 2000 using 2x100 bp paired-end sequencing with v3 chemistry. The mapping tool was Mapper Bowtie 2.1.0 using paired-end mapping mode. Analysis software was R version 3.0.1 and edgeR package version 3.0.4. Differentially expressed genes were identified based on FDR (False Discovery Rate) values and fold change, the cut-off is at FDR ≤ 0.05 PLUS 2-fold change. After obtaining the data from the CGR genes were grouped based on their function using the DAVID server (http://david.abcc.ncifcrf.gov/home.jsp). Figure 18 summaries the processes for this experiment.

Table 5, RNAs samples details

Sample No	Condition	Time point	Replication	Group
1	C	1h	**A**	N_1h
2	T	1h	**A**	P_1h
3	C	12h	**A**	N_12h
4	T	12h	**A**	P_12h
5	C	24h	**A**	N_24h
6	T	24h	**A**	P_24h
7	C	1h	**B**	N_1h
8	T	1h	**B**	P_1h
9	C	12h	**B**	N_12h
10	T	12h	**B**	P_12h
11	C	24h	**B**	N_24h
12	T	24h	**B**	P_24h
13	C	1h	**C**	N_1h
14	T	1h	**C**	P_1h
15	C	12h	**C**	N_12h
16	T	12h	**C**	P_12h
17	C	24h	**C**	N_24h
18	T	24h	**C**	P_24h

Control cells either C/N, treated cells either T/P

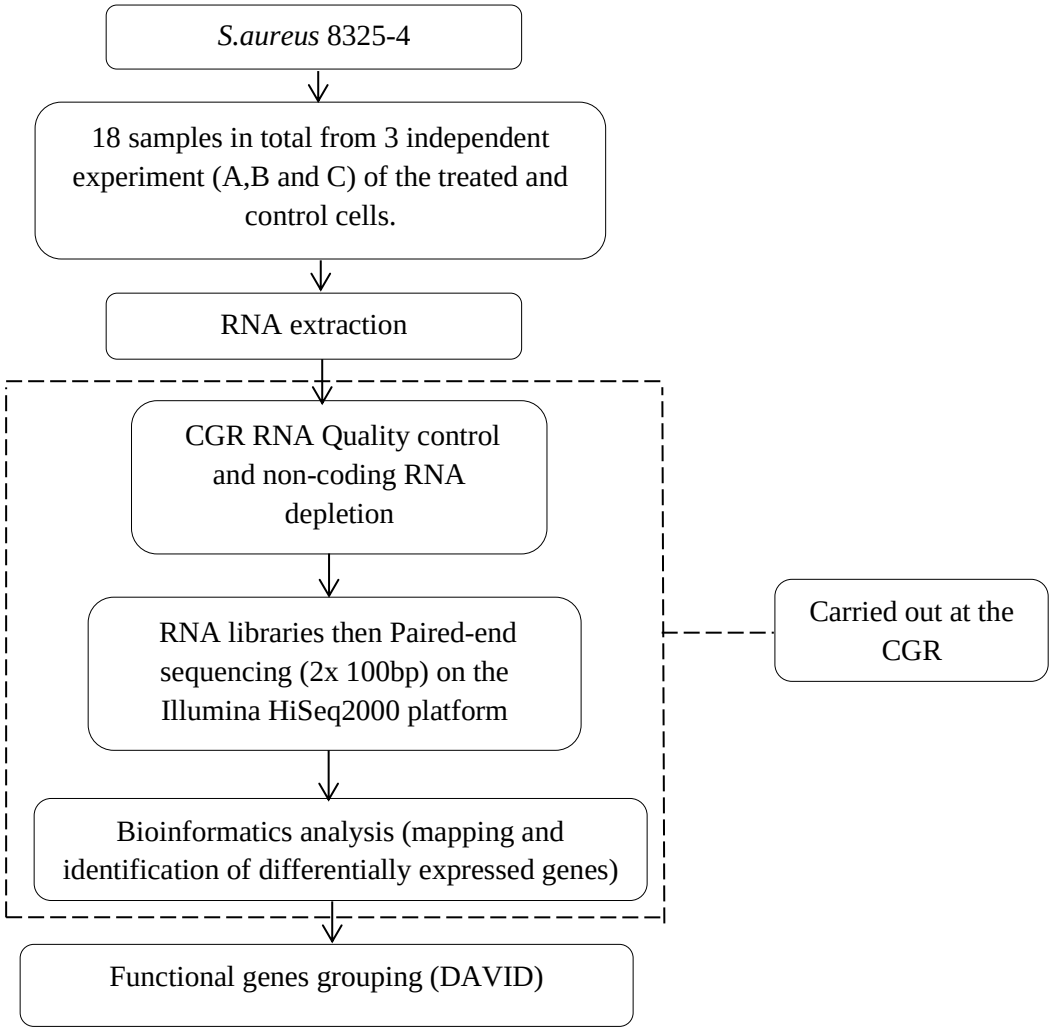


Figure 18 flow work for observing the transcriptional alteration in *S.aureus* 8325-4 after sub-inhibitory concentration of mupirocin treatment via RNA sequencing technique. Dotted box indicates the part of RNA-seq processes that carried out at the CGR.

2.8 Statistical analysis

Where possible experiments where performed in triplicate and the results were analyzed statistically using the student t-test on Excel program particularly in chapters 3-5. For chapter 6 (RNA-seq) false discovery rate (FDR) was used to obtain p-value multiple testing and was carried out by the CGR group.

Chapter 3

Mupirocin susceptibility

3.1 Introduction

Mupirocin is widely used to treat superficial wound infections, for decolonization of nasal *S. aureus* carriage and controlling outbreaks of methicillin-resistant *S. aureus* (MRSA) (Hetem and Bonten, 2013). Mupirocin impairs protein synthesis by inhibiting the bacterial isoleucyl tRNA synthetase leading to a block in isoleucyl tRNA formation (chapter 1section 1.2.1).

The frequency of using mupirocin in hospitals has increased as it been use for eliminating nasal carriage of MRSA before elective operations see (1.1.2). Therefore, resistance to mupirocin may be expected to rise in recent clinical isolates due to its increased usage.

In this work, three groups of *S. aureus* isolates including MSSAs and MRSAs were subjected to MIC measurements via micro-dilution methods to determine their susceptibility to mupirocin. Forty three of these *S. aureus* strains were isolated in the UK in the early nineteen-nineties and 44 obtained in 2011. Another 60 samples isolated in Libya in 2009-2010 were also investigated for their susceptibility to mupirocin.

3.2 Results

One hundred and forty seven clinical isolates were screened for their mupirocin susceptibility by determining their MICs as the cutoff is 4µg ml^{-1}(≤ 4µg ml^{-1} sensitive and > 4µg ml^{-1} resistant). These isolates were categorised in three groups; group A 44 recent UK isolates, group B 43 early UK isolates and group C contains 60 recent isolates from Libya. Mupirocin MICs for these groups were determined and are shown in table 3, 4, 5 and Figures 19 and 20. Figure 19 shows a summary of the results. Mupirocin resistant strains were only present in the isolates from Libya, all the older and recent UK isolates were sensitive. Examining the results more closely results revealed that *S. aureus* in group A (recent UK isolates) were all susceptible to mupirocin as their MICs were ≤ 4 µg ml^{-1}. However, 3 isolates of this group exhibited MICs 2 fold less than the resistance threshold (Table 6 and Figure 20). Group B (UK early isolates) were all susceptible to mupirocin and half of them had MICs that were 3 fold less than the resistance threshold (Table 7 and Figure 20). In contrast, mupirocin resistance was detected in group C (Libya isolates) and 30 % (n=18) of the clinical isolates showed MIC > 4µg ml^{-1} Table 6 and Figure 20). In addition, another 5 isolates had MICs at the resistance threshold.

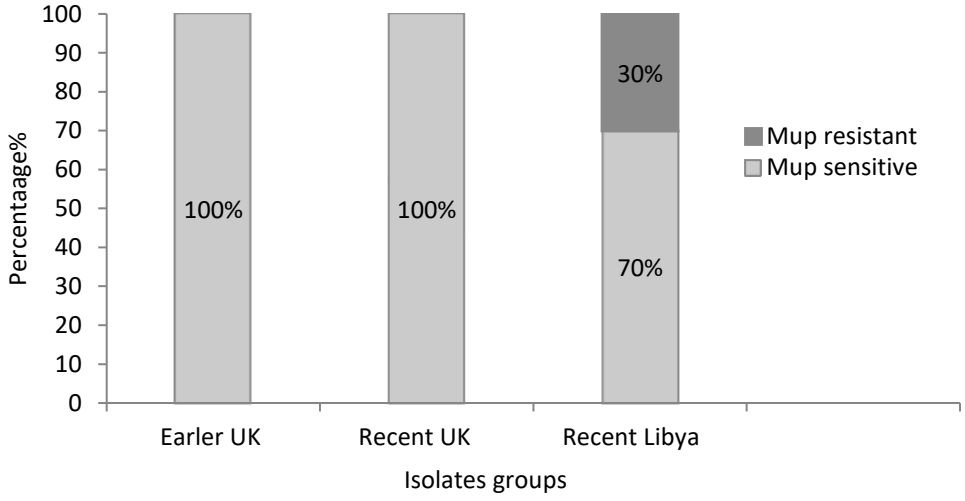


Figure 19 Mupirocin susceptibility for the three different groups of *Staphylococcus aureus* clinical isolates.

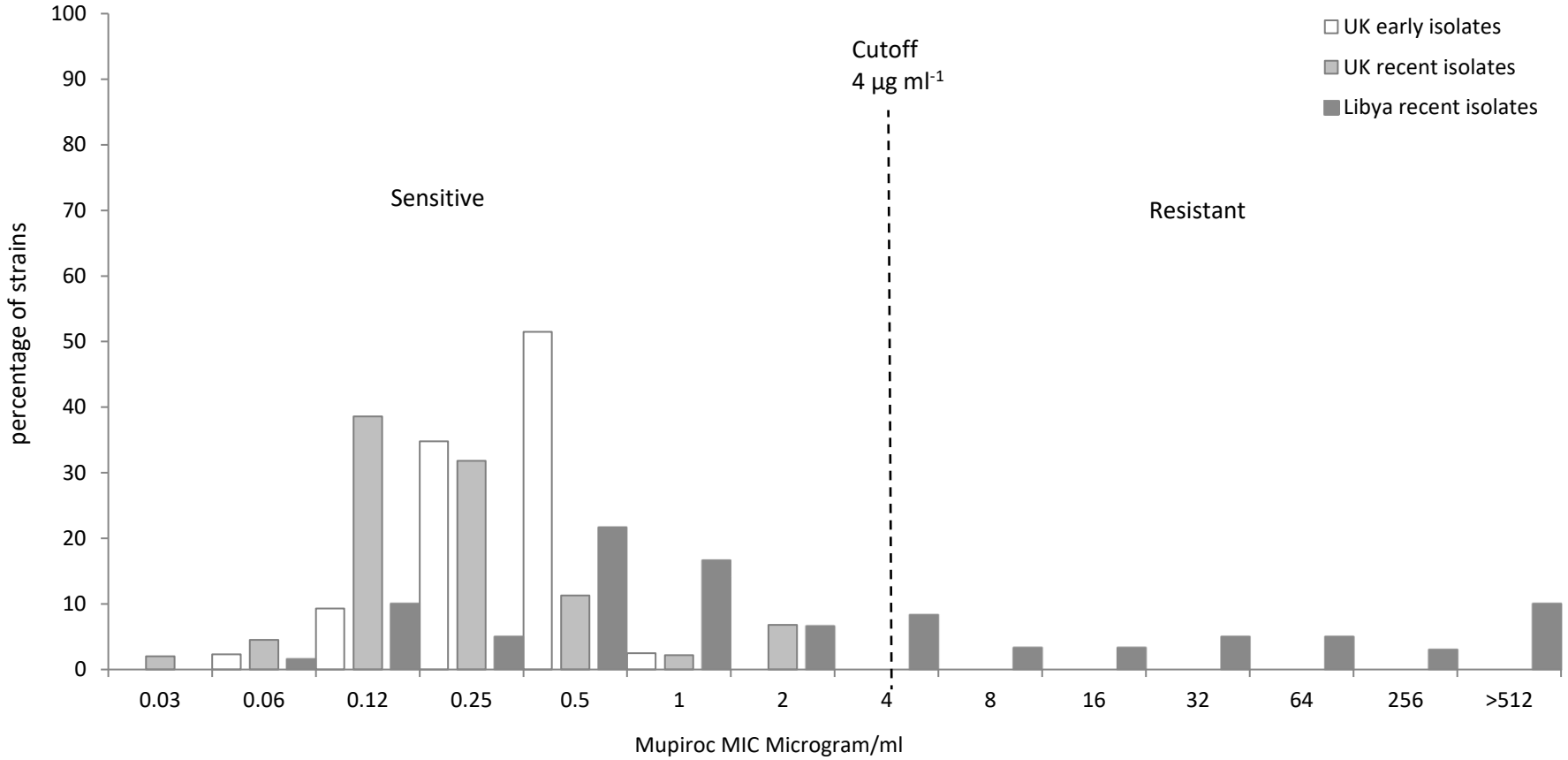


Figure 20 percentages of MICs for *Staphylococcus aureus* isolates in the different groups. The value cutoff values for resistant or sensitive($4\ \mu g\ ml^{-1}$) is according to BSAC.

Table 6 Mupirocin MICs of recent UK isolates of *Staphylococcus aureus* .

Sample No	MIC µg /ml	Sample No	MIC µg /ml	Sample No	MIC µg /ml
1	0.25	**16**	0.25	**31**	0.12
2	0.25	**17**	0.25	**32**	0.25
3	0.5	**18**	0.25	**33**	0.12
4	0.031	**19**	0.25	**34**	0.25
5	0.5	**20**	0.25	**35**	0.12
6	0.5	**21**	0.12	**36**	0.12
7	2	**22**	0.5	**37**	0.12
8	2	**23**	0.12	**38**	0.25
9	0.5	**24**	0.12	**39**	0.12
10	2	**25**	0.12	**40**	0.12
11	0.5	**26**	1	**41**	0.12
12	0.25	**27**	0.25	**42**	0.12
13	0.12	**28**	0.25	**43**	0.06
14	0.12	**29**	0.12	**44**	0.06
15	0.25	**30**	0.12		

Table 7 Mupirocin MICs of early UK isolates of *Staphylococcus aureus.*

Sample No	MIC µg /ml	Sample No	MIC µg /ml	Sample No	MIC µg /ml
1	0.12	**16**	0.5	**31**	0.5
2	0.25	**17**	0.5	**32**	0.5
3	0.5	**18**	0.5	**33**	0.25
4	0.12	**19**	0.5	**34**	0.5
5	0.06	**20**	0.5	**35**	0.5
6	0.12	**21**	0.5	**36**	0.25
7	0.12	**22**	1	**37**	0.25
8	0.5	**23**	0.5	**38**	0.5
9	0.25	**24**	0.25	**39**	0.5
10	0.5	**25**	0.25	**40**	0.5
11	0.5	**26**	0.25	**41**	0.25
12	0.5	**27**	0.25	**42**	0.5
13	0.5	**28**	0.25	**43**	0.25
14	0.25	**29**	0.25		
15	0.25	**30**	0.5		

Table 8 Mupirocin MICs for recent Libyan isolates of *Staphylococcus aureus*.

Sample No	MIC µg /ml	Sample No	MIC µg /ml	Sample No	MIC µg /ml
1	0.12	**21**	0.5	**41**	0.12
2	0.06	**22**	0.25	**42**	2
3	0.12	**23**	1	**43**	16 LLR
4	> 256 HLR	**24**	0.5	**44**	16 LLR
5	0.25	**25**	1	**45**	4
6	31 LLR	**26**	64 LLR	**46**	64 LLR
7	0.5	**27**	0.5	**47**	8 LLR
8	4	**28**	4	**48**	0.5
9	4	**29**	> 256 HLR	**49**	0.5
10	1	**30**	1	**50**	0.12
11	> 256 HLR	**31**	64 LLR	**51**	> 256 HLR
12	0.5	**32**	1	**52**	0.5
13	0.5	**33**	> 256 HLR	**53**	0.12
14	0.5	**34**	2	**54**	0.5
15	1	**35**	250 LLR	**55**	0.5
16	0.5	**36**	2	**56**	1
17	0.25	**37**	4	**57**	1
18	1	**38**	250 LLR	**58**	8 LLR
19	1	**39**	31 LLR	**59**	2
20	31 LLR	**40**	> 256 HLR	**60**	0.12

HLR, high level of resistance . LLR, low level of resistance

Of the 18 resistant isolates in group C, 12 had a low level of resistance as their MICs were between 8-256 µg ml^{-1} and the remaining 6 showed high level of resistance as their MICs were >256 µg ml-^{1}

Furthermore, antibiotic resistance profiles for the resistant strains were obtained. Their susceptibility to vancomycin, chloramphenicol, gentamicin, fusidic acid, erythromycin, streptomycin, cefoxitin, clindamycin and ciprofloxacin was investigated and the results are shown in Table 9. The results revealed that, among mupirocin resistant isolates, 16, were MRSA as they showed resistance to Cefoxitin and only 2 were MSSA with low level of mupirocin resistance. In addition, 6 MRSAs showed high level of mupirocin resistance and they exhibited MDR (multi drugs resistance) results as they were resistance to several antibiotics such as clindamycin, erythromycin and fusidic acid (Table 9).

Table 9 Antibiotics profile for mupirocin resistant isolates from group C (Libya isolates).

Sample No	Mupirocin MIC	Vancomycin 30µg	Cefoxitin 30 µg	Gentamycin 10µg	Fusidic acid 10µg	Erythromycin 15µg	Streptomycin 10µg	Chloramphenicol 30µg	Clindamycin 2µg	Ciprofloxacin 5µg
50	LLR	R	R	S	R	R	S	S	R	R
64	LLR	R	R	S	R	S	S	S	R	S
70	LLR	R	R	R	R	S	S	S	R	S
75	LLR	S	S	S	R	S	S	S	S	S
79	LLR	S	S	S	R	R	R	S	S	S
82	LLR	S	R	S	R	R	R	R	R	S
83	LLR	R	R	R	R	R	R	R	S	R
87	LLR	R	R	S	R	R	S	R	R	S
88	LLR	R	R	R	R	R	R	R	R	S
90	LLR	R	R	S	R	R	R	R	R	R
91	LLR	R	R	R	R	R	R	R	S	R
102	LLR	R	R	R	R	S	S	S	R	S
48	HLR	R	R	S	R	R	R	R	R	S
55	HLR	R	R	S	R	R	R	R	R	S
73	HLR	S	R	R	R	R	S	S	R	S
77	HLR	S	R	R	R	R	R	R	R	R
84	HLR	R	R	S	R	R	R	R	R	R
95	HLR	R	R	R	R	R	R	R	R	S

R, resistant. S, sensitive. HLR, high level of resistance . LLR, low level of resistance.

3.3 Discussion

In this part of the study the susceptibility to mupirocin was determined for three different groups of isolates, early and recent isolates from the UK as well as isolates of *S.aureus* from hospitals in Libya collected in 2010-11.

In the UK mupirocin can be prescribed to treat soft tissue lesion infections or to reduce *S. aureus* carriage including mupirocin-susceptible MRSA strains. Systemic use of mupirocin is not recommended by UK Guidelines as it is not effective. Using systemically active agents beside mupirocin is suggested as it can improve clearance rates (Gould *et al.,* 2009). In addition, mupirocin prophylaxis for elective surgical patients and in outbreak situations, is only performed under strict infection control team advice. The UK guidelines restrict the use of mupirocin to 2%, applied to the inner surface of each nostril three times daily for five days only. The use of 4% chlorhexidine gluconate aqueous solution is recommended in addition to mupirocin (Coia *et al.,* 2006, Gould *et al.,* 2009). There is no evidence that these restrictions are in place in Libia.

In this work early isolates from the UK (n=43) were all sensitive to mupirocin as well as the recent UK isolates (n=44) which could be a result of the successful guidelines for mupirocin prescription and application despite of its increased usage in hospital for nasal decolonization and in controlling nosocomial infections.

However, that was not the case for the Libyan isolates as only 70% (n=42) of them showed sensitivity to mupirocin whereas 30 % (n=18) were mupirocin resistant (2 are MSSA and 16 are MRSA). Twelve of the 18 isolates exhibited low level of resistance and 6 showed high level of resistance. In addition, 26 of 60 isolates (43.3%) were MRSA 10 of them were mupirocin sensitive and all the isolates with HLR to mupirocin were MRSA and multidrug

resistant strains which is in line with previous studies (Cadilla *et al.*, 2011, Han *et al.*, 2007) when significant rate of high level of mupirocin resistance occurred among MDR strains.

Lack of restricted policy for mupirocin use in Libya might be a reason for the emergence of mupirocin resistance especially the over-the-counter availability of antibiotics in Libya and use by the general public that all can increase resistance to mupirocin and other antibiotics resistance in the country. Setting up restricted policy for mupirocin usage can reduce its resistance as had been reported in different countries for example, in New Zealand 1999 after 8 years of mupirocin usage 28 % of *S.aureus* were resistant to mupirocin and that was attributed to the usage of mupirocin in the community without prescriptions (Upton *et al.*, 2003). In addition, in 1993 high levels of mupirocin resistance in Australia increased to 15% among medical isolates of MRSA. After that, guidance on limiting mupirocin use in the community was issued by the health department in the country which decreased the resistance rate to 0.3% in 4 years (Torvaldsen *et al.*, 1999). In addition, common use of mupirocin in hospitals to treat skin and soft tissue infections and to eradicate *Staphylococcal c*arriage in health care worker and patients can increase the mupirocin resistance (Upton *et al.*, 2003, Simor *et al.*, 2007, McNeil *et al.*, 2011) . Applying restriction on antibiotics prescriptions in hospitals and markets might help to reduce mupirocin resistance as the Australian trial above.

Mupirocin susceptibility can be classified in three categories; Firstly, sensitive when the MIC is ≤ 4 μg ml^{-1} which was found for 87.7% (n=129) of the isolates used tested. Secondly, low level resistance when the MIC is between 8-256 μg ml^{-1}, spontaneous mutation in the antibiotic target (*IleS*) can be responsible for this resistance level (Antonio *et al.*, 2002). In addition, Ramsey and colleagues claimed that, *mupA* which encodes an additional modified isoleucyl-tRNA synthetase, can results in low level of resistance when it located in the chromosome of the low resistance strains (Ramsey *et al.*, 1996). Interestingly, subsequent observation by Driscoll and others reported susceptible strain of *S. aureus* (MB1348) despite

of its positive results for *mupA* in PCR results. This finding was attributed to single base-pair deletion in the *mupA* gene that resulting in truncated mupA protein leading to mupirocin susceptible phenotype result (Driscoll *et al.*, 2007). The third category is high level resistance when the MIC is >500 µg ml^{-1} due to a plasmid-mediated gene *mupA*, that is encoding alternate *IleS2* which leads to mupirocin high resistance (Hodgson *et al.*, 1994). In addition, recent work revealed that, a novel locus, *mupB*, was responsible for high level resistance to mupirocin in *S. aureus* and it has been assumed that the *mupB* is located on non-conjugative plasmid (Seah *et al.*, 2012). Summary of mupirocin susceptibility models are shown in figure 21.

In conclusion, no *S. aureus* of UK early and recent isolates in this work showed resistance to mupirocin which indicates that mupirocin could be a good choice to eliminate *S. aureus* nasal carriage, treat wounds and soft tissue infections. The mupirocin resistance that occurred among 18 Libya isolates could be investigated via different molecular techniques such as PCR to detect the presence of either *mupA* or *mupB* genes. Furthermore, sequencing of these genes as well as verifying their location whether on conjugative, non-conjugative plasmid or on chromosome of positive PCR isolates alongside pulsed-field gel electrophoresis (PFGE) typing can give clearer picture of mupirocin resistance mechanism for these isolates.

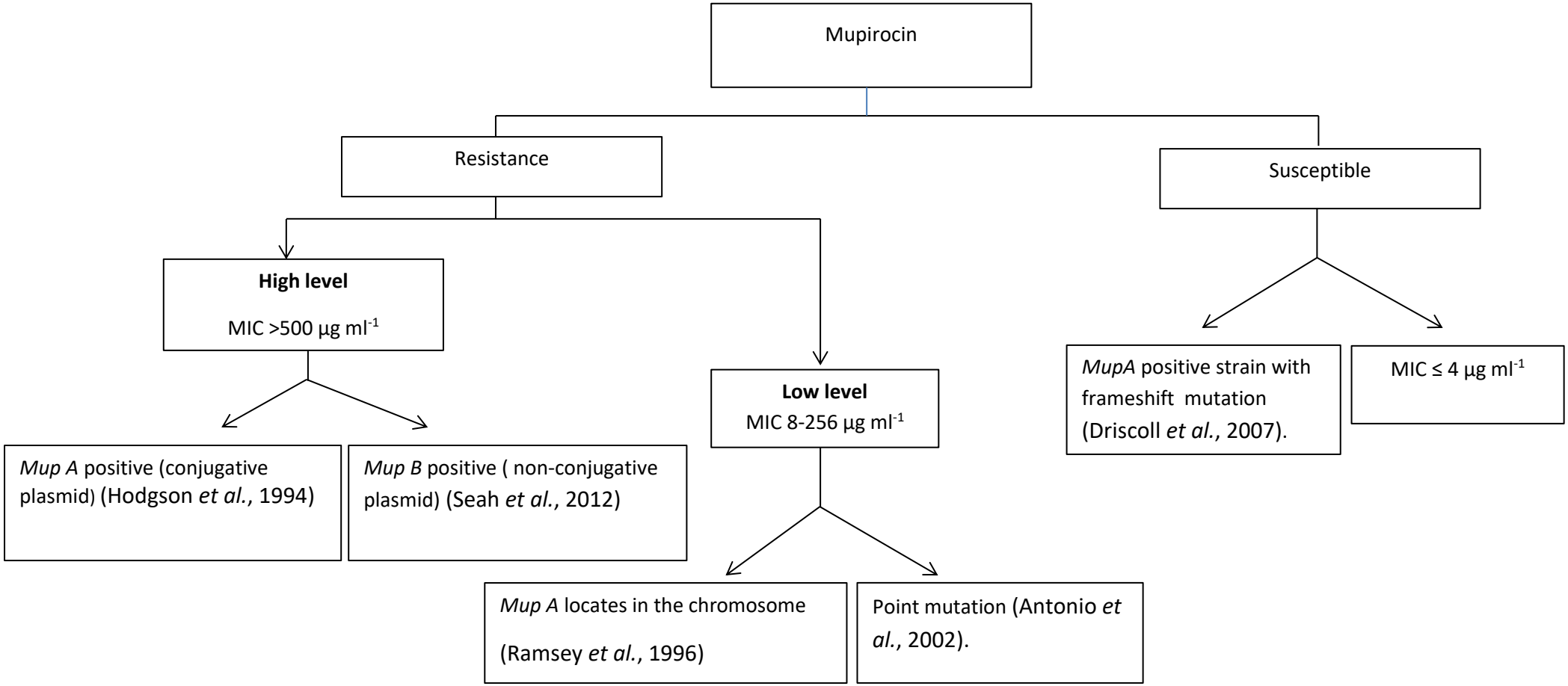


Figure 21 reported mechanisms of mupirocin resistance in *Staphylococcus aureus*.

Chapter 4

Stringent response induction

4.1 Introduction

Stress responses such as the stringent response are physiological states that bacteria exhibit and which aid survival when they encounter extreme conditions such as nutrient limitation and heat or cold shock. ppGpp is a hallmark of the stringent response, which is a small nucleoside/alarmone responsible for regulating global intracellular changes including transcription, translation, replication and virulence induction in pathogenic bacteria (Wolz *et al.*, 2010) (See Chapter 1 section 1.3).

Like many bacteria, *S. aureus* exhibits a stringent response after exposure to stressful conditions and has the ability to produce the global regulator (p)ppGpp (Anderson *et al.*, 2006, Cassels *et al.*, 1995). Mupirocin has been used in several studies at bactericidal concentration that mimics amino acid limitation conditions to trigger the stringent response (Crosse *et al.*, 2000b, Cassels *et al.*, 1995). Relatively little is known about the stringent response in *S. aureus*, in particular the effects of sub-inhibitory concentrations of mupirocin have not been studied either at short or long term of exposure.

In this work, *S. aureus* strain 8325-4 treated with sub-inhibitory concentrations of mupirocin was observed for short (up to 4 h) and long periods (up to 72 h). Significant differences in growth rate occurred up to 24 h in tested cultures relative to controls. In addition, an intracellular concentration of ppGpp after exposure (determined by HPLC) reached a maximum after 1 h then decreased after 4 h but was not detectable at 12 h and later comaring to control cultures. Detection of ppGpp after sub-lethal concentration of mupirocin treatment as well as significant growth inhibition in the first few hours of observation suggest the capability of this concentration to trigger the stringent response in *S. aureus* 8325-4 which has not been previously reported.

4.2 Results

4.2.1 Effects of Sub-inhibitory concentration of mupirocin effect on cell growth

The effects of the sub-inhibitory concentration of mupirocin (0.5 MIC = 0.25µg/ml^{-1}) on *S. aureus* 8325-4 growth was observed throughout 1, 2, 3 and 4 h in parallel with control cultures (Figure 22). Comparison between the optical density of control and test cultures revealed that there was a highly significant effect of the sublethal concentration of mupirocin on growth for these time points. For instance, at 1 h the control growth OD reached 1.2 while the treated cells OD was 0.5 (P value < 0.01). In addition, significant growth inhibition had occurred for 2 h samples when the OD of treated cell reached 1.6 and the test cells were at 0.57 OD (P value < 0.01). After 3 h of sub-inhibitory concentration of mupirocin treatment the cell OD showed slight increase 0.62 whereas the control culture OD was 2.2 making the P value < 0.01. Furthermore, significant inhibition for the treated cell was shown at 4 h where the control cells OD was 2.56 and the OD of test cells was 0.78 (P value < 0.01) Figure 22.

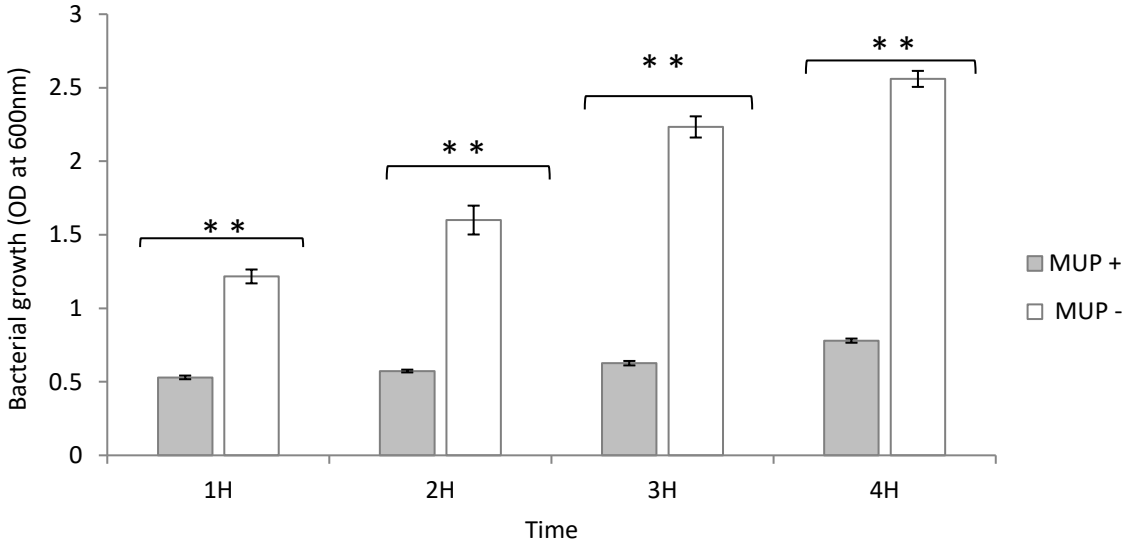


Figure 22 Effect of the sublethal concentration of mupirocin on *Staphylococcus aureus* 8325-4 growth. OD600 . ▭ mupirocin treated, OD600 ▭ controls; Values are given as mean from three independent replicates and the error bars present standard error. Statistical significant differences (T-test: ** p-value ≤ 0.01).

4.2.2 Standards calibration for ppGpp and other nuclesides

Nucleosides and ppGpp standards were prepared in different concentrations to plot a calibration curve for each component (in triplicate and their means are expressed in table 1-4 and figures 2-5. These calibration curves as well as response factors for each standard were used to calculate the unknown concentration of the components in each bacterial sample run. Response factor for components was calculated using the formula

$$\text{Response factor} = \frac{concentration}{peak\ area}$$

Following tables and graphs illustrate the experiment results for each standard.

1- ATP

Table 10 ATP values and peak area in HPLC.

	ATP				
Values pmol	195	393	591	789	1183
Average of UV absorbance	8.9	17.95	27	35.8	53.8

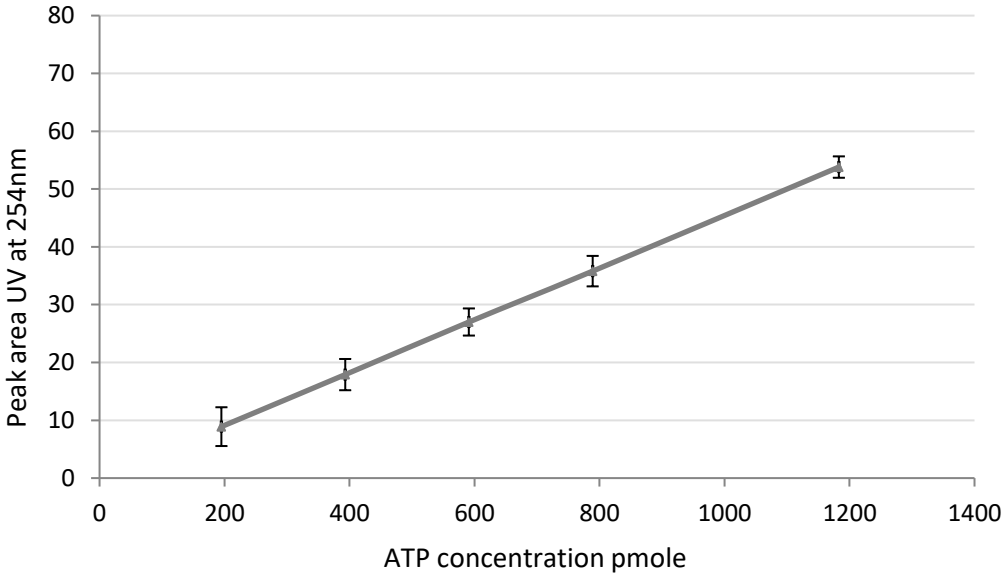


Figure 23 ATP calibration curve.
Values are given as mean from triplicates and error bars present standard deviation.

2-GDP

Table 11 GDP values and peak area in HPLC.

	GDP				
Values pmole	225.6	451.2	667	902.5	1128
Average of UV absorbance	15	30.3	45.4	60.6	57.7

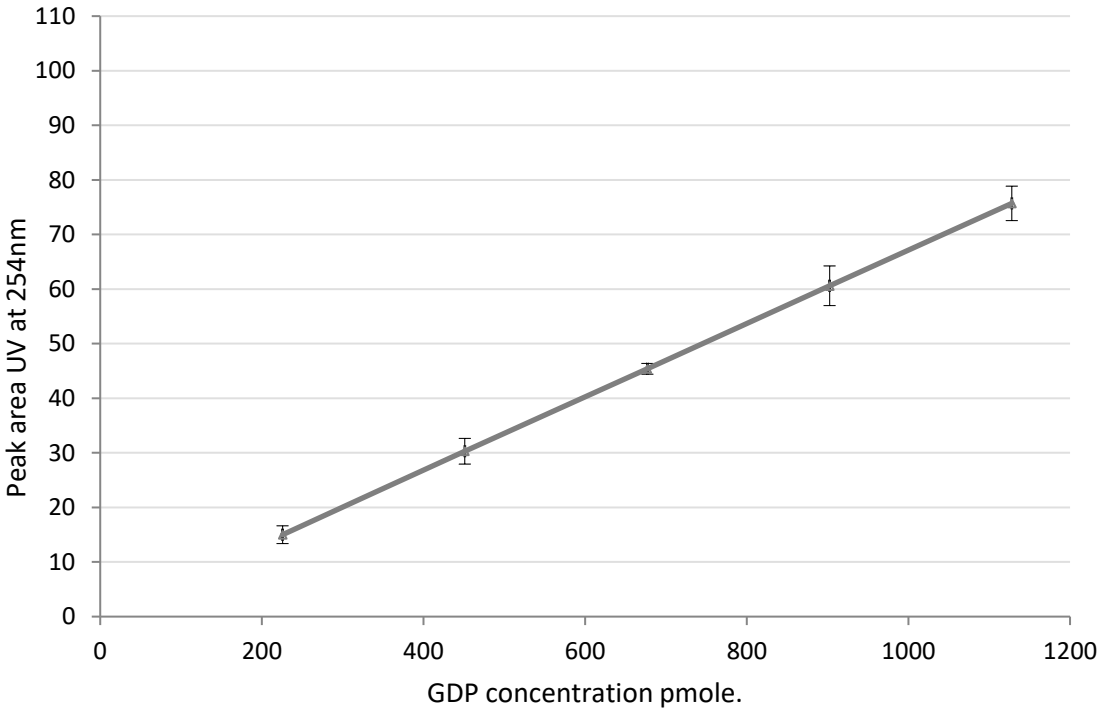


Figure 24 GDP calibration curve.
Values are given as mean from triplicates and error bars present standard deviation.

3- GTP

Table 12 GTP values and peak area in HPLC.

	GTP				
Values pmo	100	250	500	750	1000
Average of UV absorbance	2.4	6.1	12.2	18.37	24.3

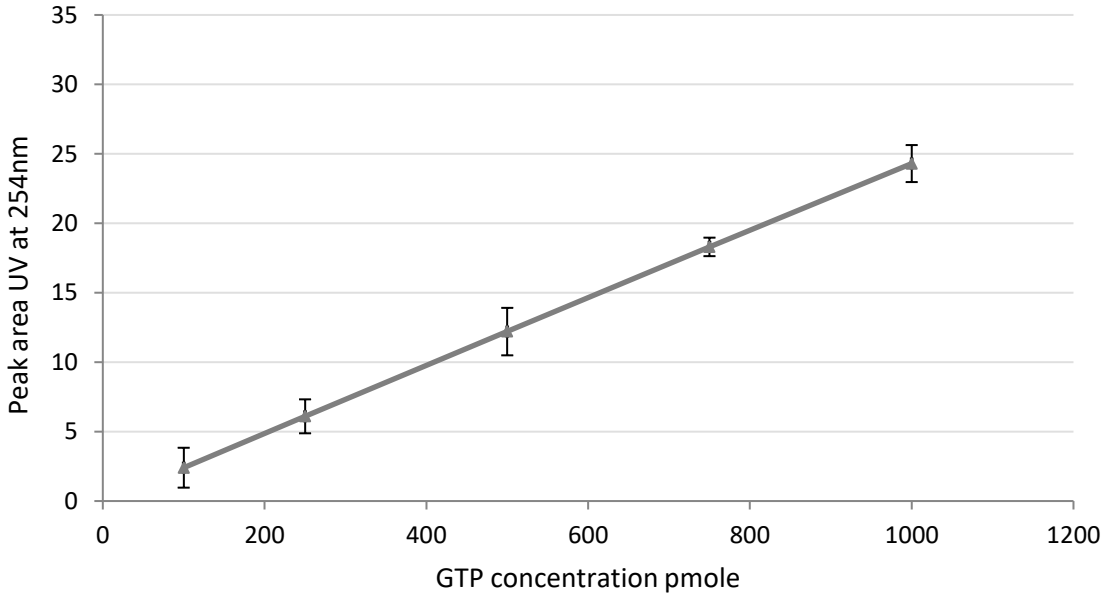


Figure 25 GTP calibration curve.
Values are given as mean from triplicates and error bars present standard deviation.

ppGpp table 13 ppGpp values and peak area in HPLC.

	ppGpp				
Values pmo	100	250	500	750	1000
Average of UV absorbance	2.4	6	12	18	24

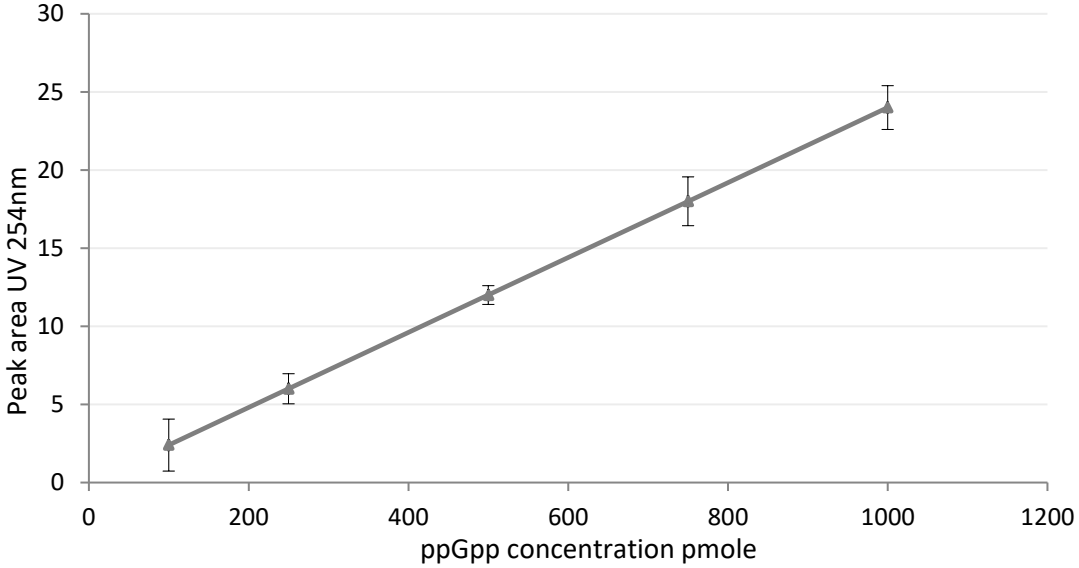


Figure 26 ppGpp calibration curve.
Values are given as mean from triplicates and error bars present standard deviation.

4.2.3 ppGpp detection

Following the significant inhibition of cell growth that sub-inhibitory concentration of mupirocin had shown, ppGpp was detected *via* HPLC SAX10 column throughout 1, 2, 3 and 4 h samples to verify that this concentration of mupirocin was capable of triggering the stringent response in *S. aureus* 8325-4 (prescriptive figures 27 and 28).

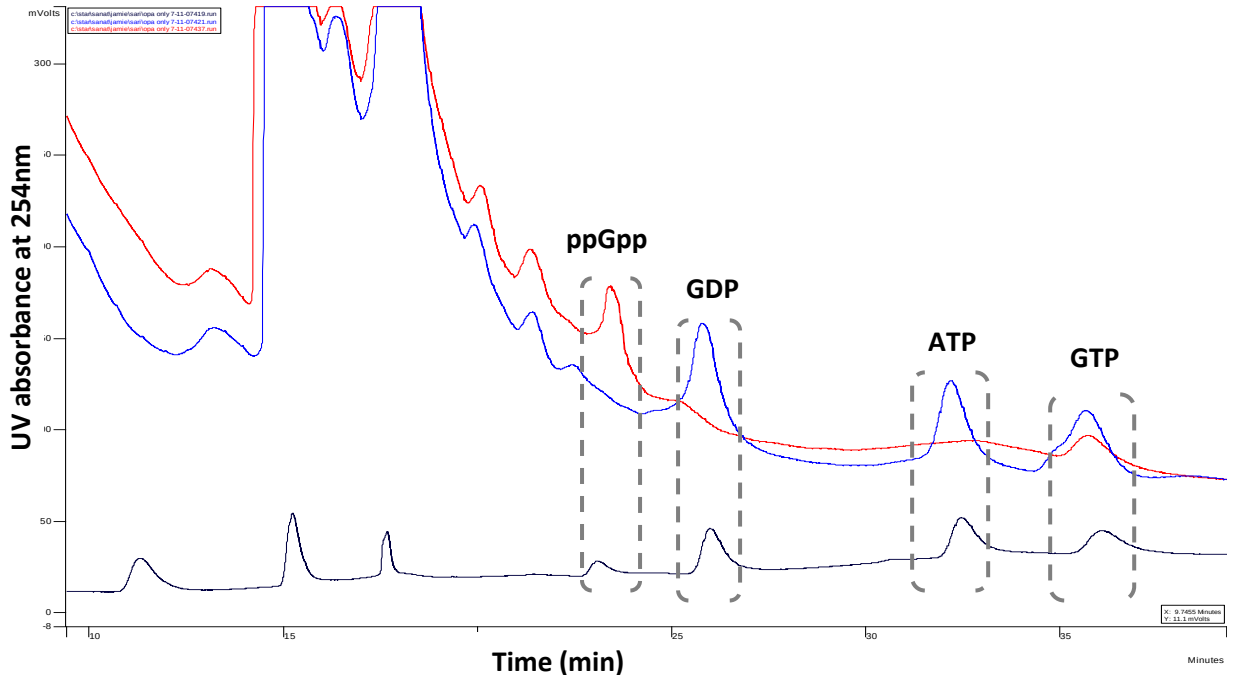


Figure 27 Detection of metabolic nucleosides for *Staphylococcus aureus* 8325-4.
Control (blue) and treated cells (red) with 0.5xMIC of mupirocin at 1 h. black chromatogram present standards.

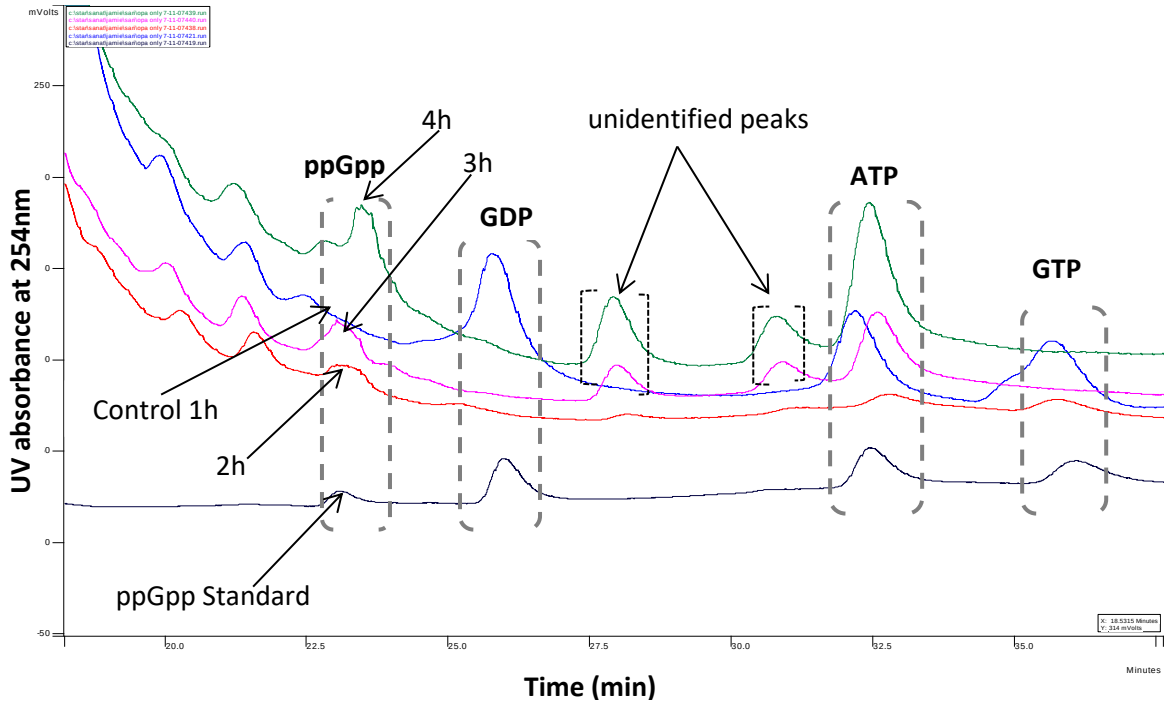


Figure 28 Detection of ppGpp and metabolic nucleosides for *Staphylococcus aureus* 8325-4 treated with 0.5xMIC of mupirocin at 2, 3 and 4 h.
Chromatograms; Standards in black, control(1 h) in blue, test for 2 h in red, test for 3 h in pink and test cells after 4 h in green.

In addition, ppGpp concentration varies slightly through these time points after the sub-lethal concentration of mupirocin treatment. For example, ppGpp concentration at 1 h was 3.99 nmole/mg CDW which was the highest concentration through these samples. After 2 h of mupirocin exposure ppGpp concentration was 3.63 nmole/mg CDW and a slight decrease occurred to 3.46 nmole/mg CDW after 3 h. In addition, ppGpp concentration decreased in the 4 h sample to 2.4 nmole/mg CDW (Figure 29).

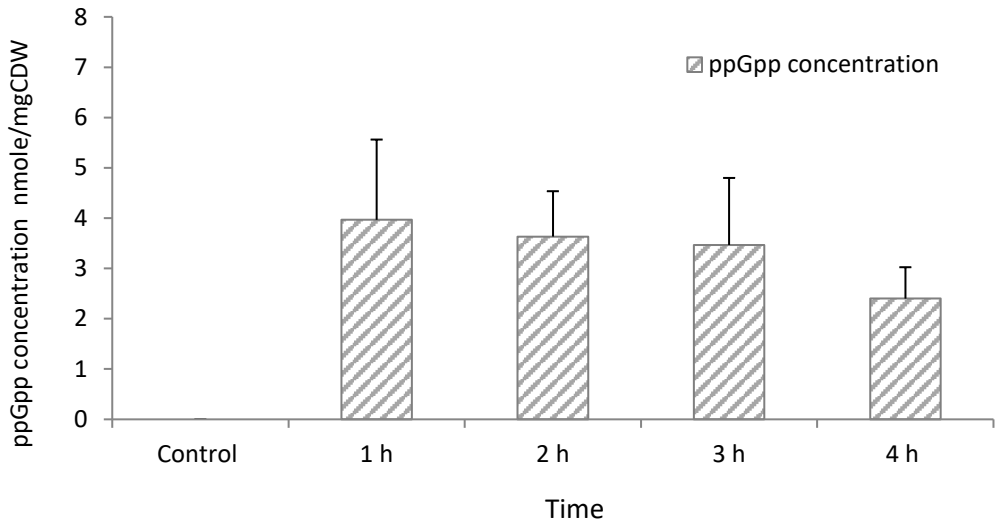


Figure 29 Effect of sub-inhibitory concentration of mupirocin on ppGpp production in for *Staphylococcus aureus* 8325-4.
Values are given as mean from three independent replicates and the error bars present standard deviation.

A negative correlation occurred between the growth and ppGpp concentration in the treated cells results. For example, the OD of the treated cells at 1 h was 0.5 and the ppGpp concentration was 3.99 nmole/mgCDW. In 2 h sample results slight increase in the OD occurred and slight decrease in ppGpp was observed. In addition, the OD for 4 h cells increased to 0.78 whereas the ppGpp concentration dropped to 2.4 nmole/mgCDW (Figures 30 and 31).

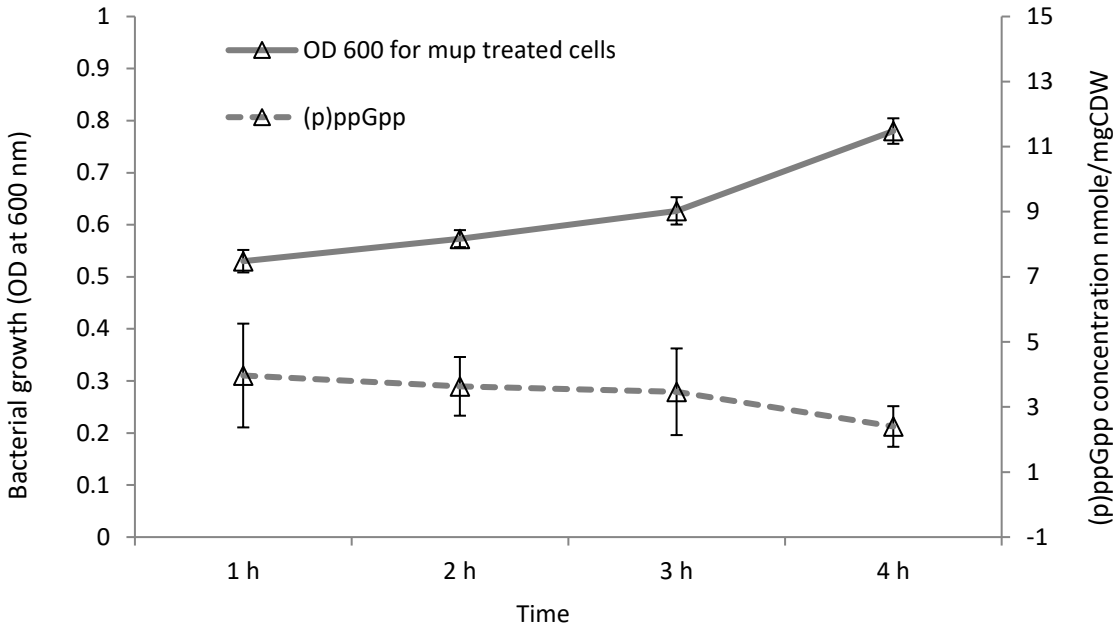


Figure 30 reciprocal results of growth and ppGpp production in *S.aureus* 8325-4 after sub-inhibitory concentration of mupirocin treatment. Values are given as mean from three independent replicates and the error bars present standard deviation. Dotted line represents (p)ppGpp concentration, normal line represents mupirocin treated cell growth.

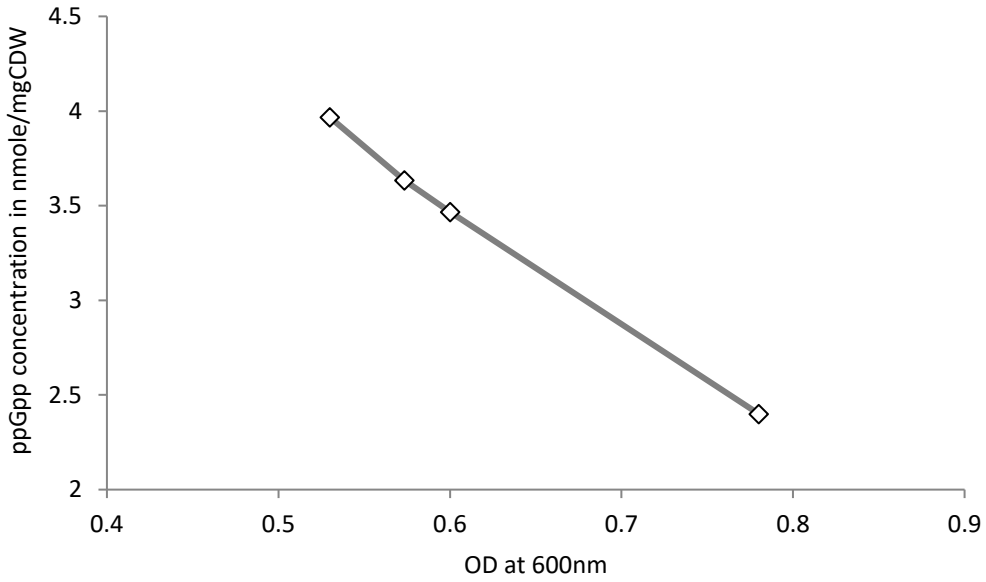


Figure 31 negative correlation between growth and ppGpp production in *S. aureus* 8325-4 after sub-inhibitory concentration of mupirocin treatment.

4.2.4 Effect of ppGpp production on pools of other nucleotides

(p)ppGpp synthesis relies on the enzymatic phosphorylation of the GDP and GTP to produce ppGpp and pppGpp respectively using ATP as a phosphate donor. Thus the concentrations of these nucleotides were observed in the culture treated with sublethal concentrations of mupirocin. For instance, a significant decrease in the GDP intracellular pool had occurred in 1 h treated cell (0.83 nmole/mgCDW) in contrast to control cells where GDP concentration was 4.3 nmole/mgCDW (P value < 0.01, t-test) . In addition, GDP concentrations remained at a low level through 2, 3 and 4 h after mupirocin treatment, 0.62, 0.56 and 0.38 nmole/mgCDW respectively (Figure 33). Moreover, GTP concentration was 6.8 nmole/mg-CDW in control cells at 1 h but a significant decrease had occurred when it dropped to 2.4 nmole/mgCDW in the treated cells (P value < 0.05). Notably, [GTP] was greatly decreased and it fell to undetectable levels at 3 and 4 h in test cells. Furthermore, a highly significant decrease in ATP concentration appeared in the treated cells, 0.5 nmole/mgCDW, relatively to control cells 4.6 nmole/mgCDW, at 1 h (P value < 0.01) then increased in 4 h sample to 3.4 nmole/mgCDW.

It important to note that the significant decrease in these nucleotides concentrations through 1, 2, 3 and 4 h of mupirocin treatment was associated with the increased concentration of ppGpp (Figure 32).

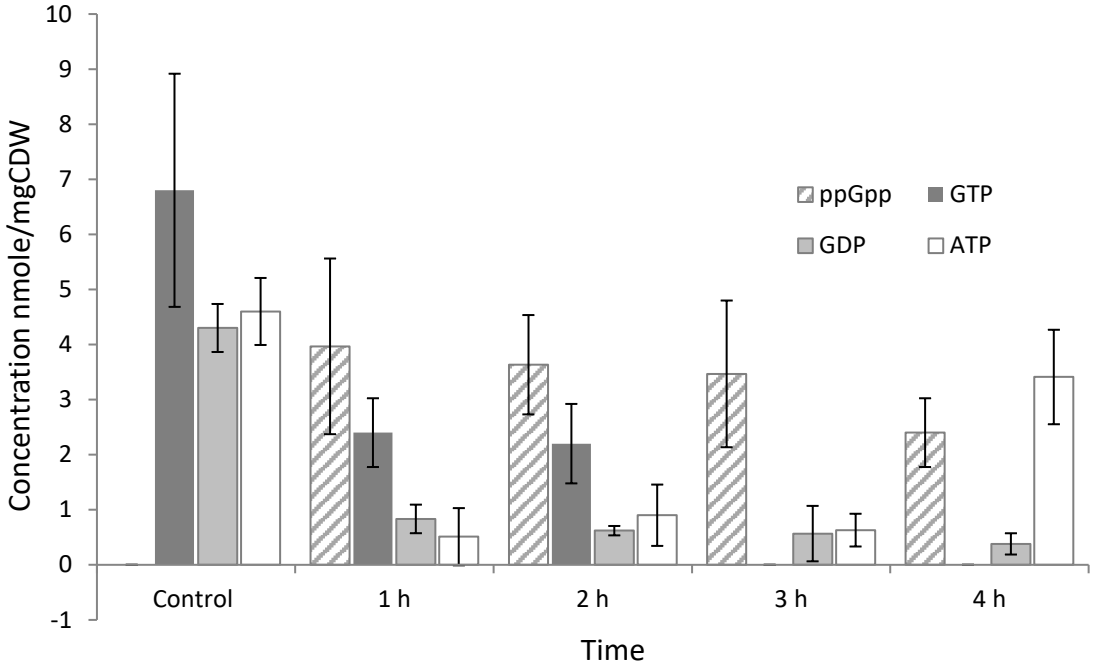


Figure 32 alterations on nucleosides and ppGpp intracellular pool concentration in *S. aureus* 8325-4 after sublethal concentration of mupirocin treatment.
Values are given as means from three independent replicates and the error bars present standard deviation. GTP , ppGpp , GDP , ATP ,

4.2.5 Extended observing of mupirocin effect on growth and ppGpp production

Observing the effect on growth and ppGpp production of the sublethal concentration of mupirocin was extended to 72 h for the test and control cells to obtain a complete picture of the mupirocin effect. ppGpp was not detected through 12, 24, 48 and 72 h for control and test cells whereas an effect on growth had occurred on test cells OD. For instance, At 12 h high significant effect of mupirocin on growth appeared as the control cells OD reached 6.7 and the test cells OD was 2.6 (P value < 0.01). Similarly, significant influence of mupirocin on growth was seen at 24 h samples when the OD of the test cells reached to 5.9 while the control cell OD was 10.4 making the P value < 0.01. Both cultures reached stationary phase at 48 and 72 h and no significant difference on their OD occurred between test and control cells through these time points (Figure 33).

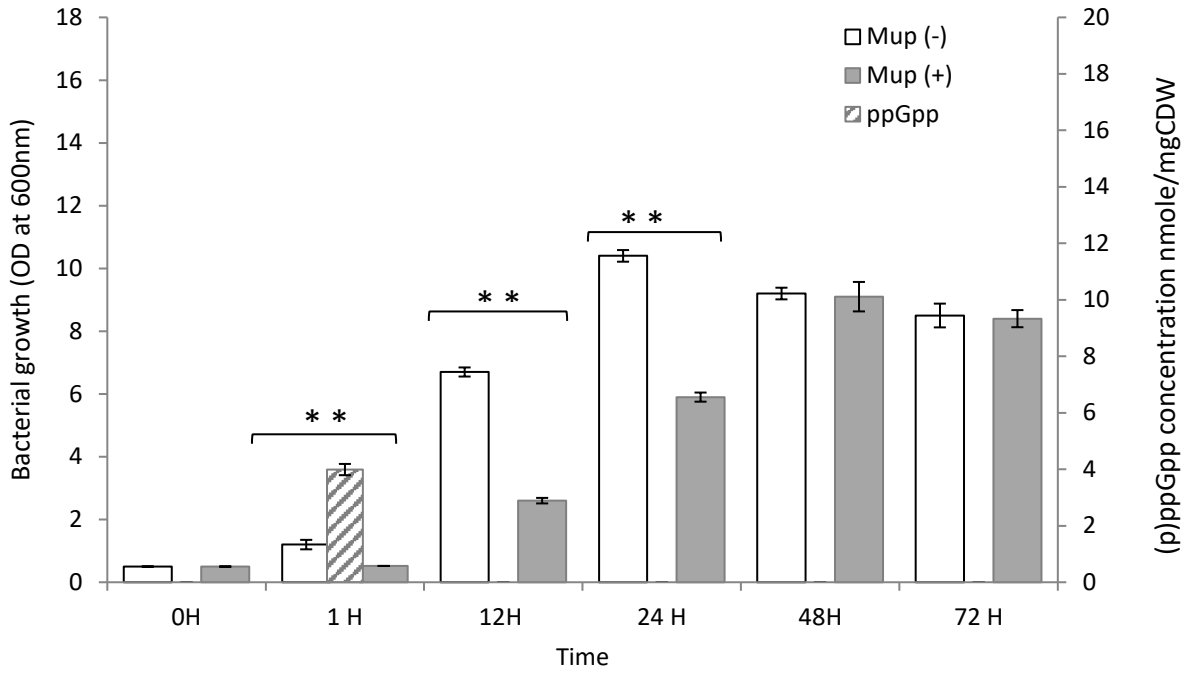


Figure 33 effect of the sublethal concentration of mupirocin on *Staphylococcus aureus* 8325-4 growth up to 72 h.

OD_{600} mupirocin treated , OD600 controls , ppGpp concentration . Values are given as mean from three independent replicates and the error bars present standard error statistical significant differences (T-test: ** p-value ≤ 0.01).

4.3 Discussion

In previous studies *S. aureus* showed the ability to produce (p)ppGpp during the stringent response condition triggered by bactericidal concentrations of mupirocin (Cassels *et al.*, 1995, Geiger *et al.*, 2010a, Reiß *et al.*, 2011). In this work, a sub-inhibitory concentration of mupirocin also showed the ability to trigger the stringent response in *S. aureus* strain 8325-4 as ppGpp was detected by HPLC after mupirocin exposure. Using sub-inhibitory concentration of mupirocin to trigger the stringent response in *S. aureus* has not been conducted in previous work.

A rapid synthesis of ppGpp has been reported by previous studies when *S. aureus* was exposed to bactericidal concentrations of mupirocin (Reiß *et al.*, 2011, Geiger *et al.*, 2010a, Crosse *et al.*, 2000b) which is in agreement with the current work results as ppGpp was detected after 1 h of sub-inhibitory concentration of mupirocin. Also, ppGpp was detected up to 4 h. The production of ppGpp was associated with significant growth inhibition for the treated cells throughout these time points. Previous studies found ppGpp to be responsible for remarkable down-regulation of genes that are involved in replication, transcription and translation during the stringent response in *S. aureus, B. subtilis* and other bacteria (Geiger and Wolz, 2014, Wang *et al.*, 2007). Furthermore, a recent investigation (Geiger *et al.*, 2012) showed that ppGpp directly represses genes coding for ribosomal proteins, translation initiation, elongation factors and DNA replication in *S. aureus* during the stringent response. Non-transcription of these genes under (p)ppGpp regulation during the stringent response might explain the significant growth inhibition of the test cells in this work. Although only a slight decrease of ppGpp had occurred between 3 and 4 h the OD of the treated cell showed a slight increase which may indicate a negative correlation between the stringent response and cell growth. The negative effect of ppGpp on replication (Wang *et al.*, 2007, Geiger *et al.*, 2012) might explain the reciprocal change in (p)ppGpp concentration and the bacterial growth rate. Indeed, extended observation on the sublethal concentration of mupirocin effect on

growth revealed that ppGpp was not detectable at 12, 24, 48 and 72 h in the treated cells while the cell growth had resumed (Figure 33). However, at these time points significant difference in cell growth had occurred in test cells relative to control up to 24 h results. In an earlier study by Edwards-Jones (thesis1997) and (Edwards-Jones and Foster, 1994b) a prolonged lag phase for *S. aureus* was seen after sublethal concentration of mupirocin treatment which is in agreement with the results obtained here as the OD of the treated culture were significantly inhibited at 1, 2, 3 and 4 h. The significant difference in growth rate between treated and control cells that was seen up to 24 h might be a consequence of prolonged lag that occurred in the first few hours (1, 2, 3 and 4 h) in the test cells which can be attributed to the negative effects of ppGpp on growth and replication.

In addition, the resumed growth in the treated cell after few hours of sublethal concentration of mupirocin treatment reported here is in agreement with (Edwards-Jones and Foster, 1994b). Further investigation and proposed model for *S. aureus* tolerance and recovery from stringent response is discussed in detail in Chapter 6.

During the stringent response in *S. aureus*, large decreases in GDP and GTP intracellular pools have been reported in previous observations (Reiß *et al.*, 2011, Geiger *et al.*, 2010a). In this work, a drop in the GDP and GTP intracellular pool appeared throughout 1, 2, 3 and 4 h after mupirocin treatment. For example, in the treated cells GTP decreased greatly in contrast to 1 h untreated samples. Decreased level of GTP continued up to 2 h then GTP could not be detected at 3 and 4 h. This sharp drop in GTP pool might be a result of synthesizing pppGpp. Cassles and colleagues had claimed that pppGpp was produced in higher concentration (4.2 fold) than ppGpp in *S. aureus* 8325-4 after mupirocin treatment and they showed increasing pppGpp spot densities in TLC in parallel with increasing mupirocin concentration (Cassels *et al.*, 1995). Conversely, Cross and his research group have used the same strain and they detected ppGpp and pGpp but not pppGpp after mupirocin treatment which agrees with

current work results as pppGpp could not be detected (Crosse *et al.*, 2000b). One reason for this contrary result might be the different techniques used to detect (p)ppGpp in these studies. For instance, in the present study and (Crosse *et al.*, 2000b) work a HPLC SAX 10 column was used and the pppGpp was not detected whereas (Cassels *et al.*, 1995) used TLC and was able to detect both pppGpp and ppGpp. However, recent work on the stringent response in *S. aureus* used Ion Paired Liquid Chromatography accompanied by Mass Spectrometry (IP-LC-MS) to detect (p) ppGpp and both pppGppp and ppGpp were detectable after mupirocin treatment (Reiß *et al.*, 2011, Geiger *et al.*, 2010a).

In addition, a significant decline in the ATP intracellular pool occurred through 1, 2 and 3 h while ppGpp concentration increased. The ATP decrease might be as a result of its use as a phosphate donor in (p) ppGpp synthesis. However, an increase in ATP intracellular pool had occurred by 4 h. In *B.subtilis,* GTP and ATP intracellular pool levels change reciprocally during amino acid deprivation (Lopez *et al.*, 1981) which in agreement with their results here at 4 h where the GTP was not detectable and [ATP] increased (Figure 32). The alteration of these nucleosides is mediated by (p)ppGpp inhibitory effect on three enzymes that are involved in GTP synthesis pathway which results in inosine monophosphate (IMP) dehydrogenase accumulation that recruited in ATP synthesis processes which leads to its increased level (Kriel *et al.*, 2012) (see Chapter 1 section 1.4.2).

In conclusion, the detection of ppGpp in the intracellular pool and alteration of the nucleosides concentrations ATP, GDP and GTP as well as the significant growth inhibition of treated cell with sublethal concentration of mupirocin in this work imitates the effect of the stringent response triggered via bactericidal concentration of mupirocin on the same and different strains of *S. aureus* in previous studies (Reiß *et al.*, 2011, Geiger *et al.*, 2010a, Cassels *et al.*, 1995, Crosse *et al.*, 2000a). This confirms the capability of sublethal concentrations of mupirocin to evoke the stringent response in *S. aureus* 8325-4.

Chapter 5

Effect of sub-inhibitory concentration of mupirocin on the TSST-1expression and production

5.1 Introduction

The wide range of virulence factors that *S. aureus* possesses and expresses during infection together with the emergence of antibiotic resistance have increased the incidence of morbidity and mortality caused by the organism worldwide. *S. aureus* pathogenicity is attributed to the wide array of virulence factors that can be secreted extracellularly during the infection. Expression of most of the genes that encode virulence factors are tightly regulated to the growth stage of the cells.

In this work, *S. aureus* B49, which is a clinical isolate from a burns patient that produces toxic shock syndrome toxin TSST-1 (Edwards-Jones 1997), was used to observe the effect of sub-lethal concentration of mupirocin on the TSST-1 production. The effect of this concentration on cell growth, transcription of TSST-1 and RNAIII the global regulator for *S. aureus* virulence factors was determined throughout 1, 8, 16 and 24 h using relative quantitative RT-PCR. TSST-1 production was measured using RPLA (Reverse Passive Latex Assay) to obtain a complete picture for the effects of mupirocin on toxin synthesis.

Significant growth inhibition occurred in the first few hours of observation in mupirocin treated cells which was associated with remarkable early transcription for RNAIII and *tst* genes that are normally expressed post exponentially. Despite the up-regulation of *tst* and RNAIII, TSST-1 synthesis was inhibited as it could not been detected during the early times of observation. Similar to chapter 4 results, significant growth inhibition for *S. aureus* (clinical isolates) had occurred after sub-inhibitory concentration of mupirocin treatment which suggests also induction of stringent response at least at 1 h of exposure. Early transcription of *tst* and *RNAIII* as well as the inhibited translation for TSST-1 might be attributed to stringent response effect. Explanation of the link between stringent response and transcription of these genes is discussed here. In addition, proposed mechanisms that might elucidate the inhibition of TSST-1 synthesis are suggested.

5.2 Results

5.2.1 Effect of sub-lethal concentration of mupirocin on cell growth

The sublethal concentration of mupirocin was determined for *S. aureus* (B49) to be 0.12µg/ml^{-1} (0.5 MIC). The effect on cell growth was observed throughout 24 h in parallel with control growth and the results are shown in Figure 34. A comparison between the optical density of control and test cultures revealed that there was a highly significant effect of the sublethal concentration of mupirocin on growth up to 8 h of incubation as the growth OD reached 7.1 for the control cell and 1.3 in the treated cell culture (P value < 0.01). Then treated cells with mupirocin started to show growth through 16 and 24 h, reaching OD 6.6 and 8.1 respectively. However, the sublethal concentration of mupirocin still showed a significant effect on the growth in comparison to control culture (OD 8.1, 9;.P = 0.05; Figure 34).

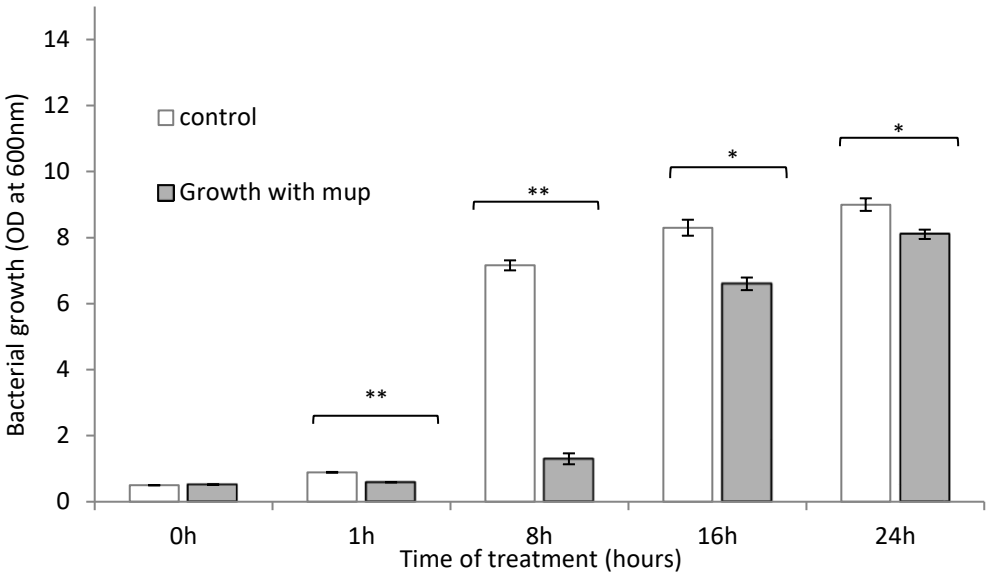


Fig 34 Effect of the sublethal concentration of mupirocin on *Staphylococcus aureus* B49 (clinical isolate) growth, mupirocin treated ▩, control□ .

Values are given as mean from three independent replicates and the error bars present standard error. Statistical significant differences (T-test: ** p-value ≤ 0.01).

In addition, the observation was extended to 36 h to obtain a clearer picture. Interestingly, cells treated with a sublethal concentration of mupirocin showed continuous increasing growth to

reach OD= 8.5) after 28 h (Figure 35). After that the growth dropped slightly and remained constant, OD= 8, for 32 and 36 h of incubation. In contrast, the growth of control culture showed slight decrease and stayed constant OD = 8, up to 36 h (Figure 35).

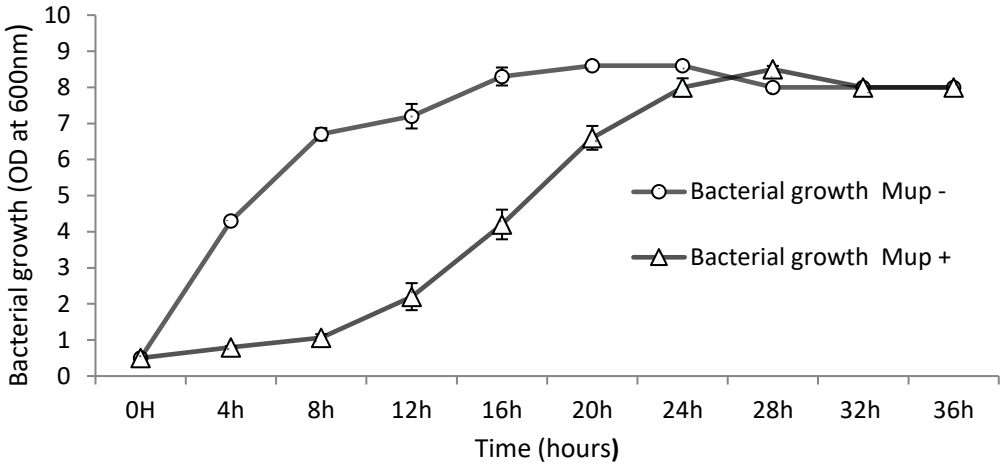


Figure 35 effect of sublethal concentration of mupirocin on bacterial growth throughout 36 h.

Values are given as mean from three independent replicates and the error bars present standards deviation from three independent triplicates. ○ Growth in control condition, Δ Growth with mupirocin.

5.2.2 Toxin production

To observe the influence of sublethal concentrations of mupirocin on TSST-1 synthesis, two sets of exponentially growing cells were prepared and sublethal concentration of mupirocin was added to one of them and the other was used as control broth. Supernatants from these cultures were harvested at different time points (0, 1, 8, 16, and 24 h) from both cultures.

Production of TSST-1 was detectable in the control broth after the first hour of incubation with low titre 3.3 (the sensitivity of the test latex suspension is 2 ng/ml).as cells exponentially grown OD= 0.9, then toxin production increased more than 6 folds as titres were >256 for 8, 16 and 24 h (Figure 36).

However, that was not the case of the cell treated with sublethal concentrations of mupirocin as they showed an obvious delay in toxin production as shown in figure 37. TSST-1 was not

detectable in the supernatant of the 1 and 8 h broths, OD 0.5 and 1.3 respectively. Although the OD of the 1 h of control broth was 31% less than the OD of 8 h of treated cells, TSST-1 was detectable in the supernatant of 1 h control broth.

Furthermore, after 16 h of incubation for the treated cells (OD=6.6) the TSST-1 started to be detectable (titre 16; Figure 37), which was at least 4 fold less than the control for the same time point (Figure 36). However, a large increase in the toxin produced occurred in 24 h supernatant (titre >256), Figure 37).

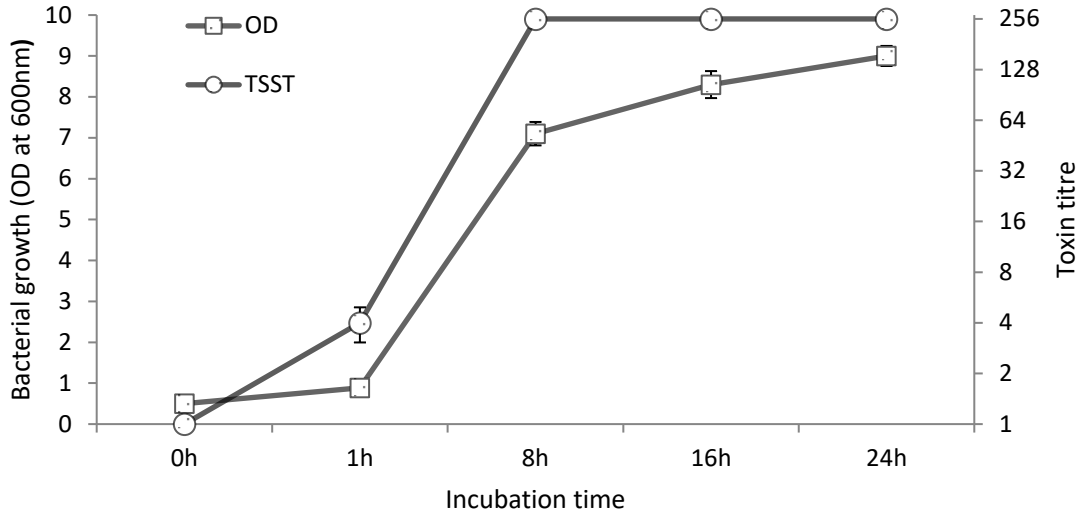


Figure 36 growth and toxin production in control condition.

Values are given as mean from three independent replicates and the error bars present standards deviation from three independent triplicates. TSST-1 production is expressed as a titre. The sensitivity of the test latex suspension is 2 ng/ml.
⊡ Optical density of bacterial growth, ⊙ TSST-1 production titre.

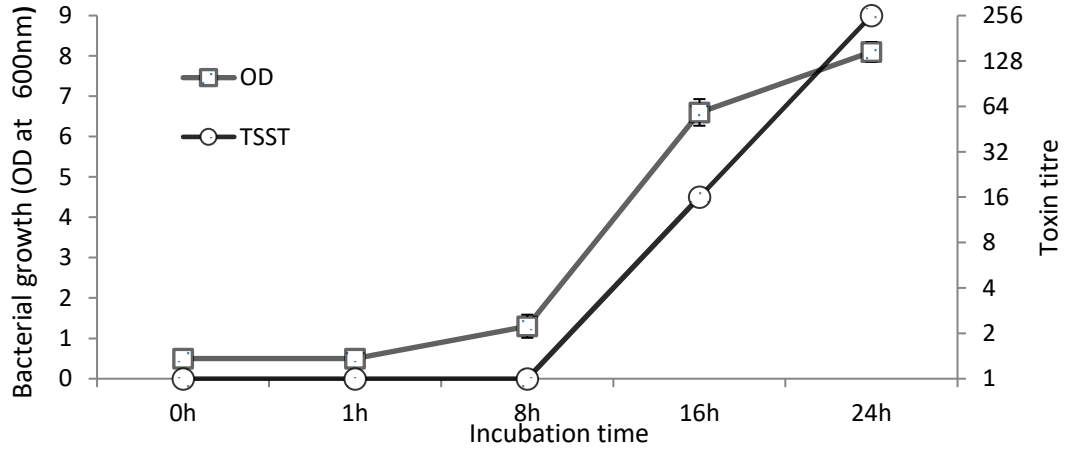


Figure 37 Effects of sublethal concentration of mupirocin on bacterial growth and TSST-1 production.

Values are given as mean from three independent replicates and the error bars present standards deviation from three independent triplicates. TSST-1 production is expressed as a titre. The sensitivity of the test latex suspension is 2 ng/ml.

⊡ Optical density of bacterial growth, ⊙ TSST-1 production titre.

5.2.3 Transcription observation

Quantitative real time PCR was performed in order to detect expression of the genes for *tst* and *RNAIII* relative to the control cells in order to detect the effect of sublethal concentrations of mupirocin on the transcription of *tst*, which are the genes that encode TSST-1 and the global regulator of *agr* system in *S. aureus* RNAIII that plays essential role in *tst* regulation, respectively. Total RNA was harvested from control and treated cells after 1, 8, 16 and 24 h. The expression ratio for *tst* and *RNAIII* transcription was quantified relative to untreated cells and results are illustrated in Figure 38. Cycle threshold values for *tst* and *RNAIII* genes were normalized by *16S* ct value and the expression ratio of them in the treated culture with sub-blethal muopirocin was calculated relative to untreated cultures. Depending on the amplification efficiency (E) to RNAIII the ratio was calculated using the $2^{-\Delta\Delta Ct}$ equation where $\Delta\Delta Ct = \Delta Ct(\text{treated}) - \Delta Ct(\text{untreated})$. $\Delta Ct(\text{treated}) = Ct(\text{target gene},) - Ct(\text{reference gene})$ and $\Delta Ct(\text{untreated}) = Ct(\text{target gene}) - Ct(\text{reference gene})$ (Livak and Schmittgen, 2001). Depending on the amplification efficiency (E) for *tst* gene the expression ratio was obtained using the Pffafi method (Pfaffi, 2001) where

$$\text{Ratio} = \frac{(\text{E target})^{(\Delta Ct \text{ target (untreated - treated)}}}{(\text{E reference})^{(\Delta Ct \text{ reference (untreated - treated)}}}$$

At the first time point of harvesting the expression ratio for both genes, *tst* and *RNAIII*, was 0.93 compared to control cells which indicates no significant change in expression level (< 2 fold). Although not reaching the significant level of expression fold change, this expression was associated with inhibited bacterial growth (Figure 34), which indicates significant alteration on their regulation as they have been reported to be controlled in growth dependent manner (Arvidson and Tegmark, 2001). However, after 8 h of treatment with mupirocin *tst* and *RNAIII* showed a slight increase in expression relative to the control.Ratios decreased in comparison to the 1 h ratio (Figure 38), particularly for the *tst* gene. Notably, cells treated with sub-lethal

concentrations of mupirocin showed a gradual increase in density throughout 1 and 8 h as their OD increased from 0.5 to 1.3 respectively (Figure 34).

RNA harvested from cells at 16 h showed a significant increase (p value <0.05) for *tst* and *RNAIII* transcriptions as their mRNA increases were 1.36 and 1.48 fold respectively. In addition, *tst* expression for 24 h had increased markedly, more than 5 fold (p value <0.01) and *RNAIII* increased slightly to 1.6 fold. The increased expression of these genes during 16 and 24 h were associated with exponential growth of treated cells (OD 1.3 to 8.1).

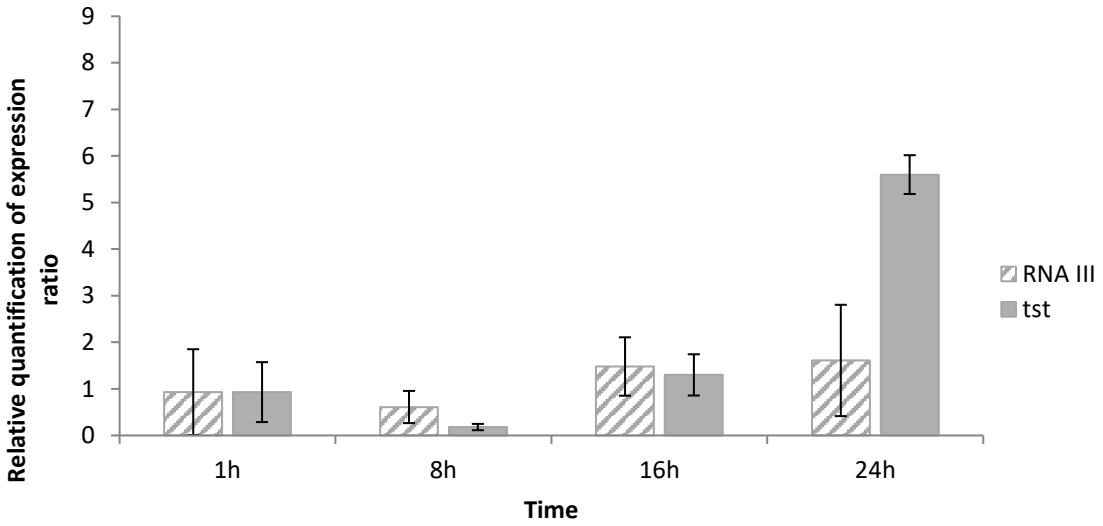


Figure 38 Expression ratios for *tst* and *RNAIII* transcription after sublethal concentration of mupirocin. *tst* gene expression ratio , RNAIII gene expression ratio ,Values represent mean gene expression ratio calculated relative control condition after normalization by using 16S gene. Error bars represent standard deviation from independent triplicates.

5.2.4 Protease inhibition

Real time PCR results showed increased ratio of *tst* expression after 1 and 8 h of sublethal concentration treatment whereas TSST-1 was not detectable during these time points. These results raised an obvious question that needed to be answered which is, "if the *tst* gene was transcribed why it is not detected in the broth? One possible scenario (discussed in detail below) to answer this question was that the toxin might be synthesised and secreted outside of the cells but it was degraded by protease. To verify that, protease inhibitor (1mM) was added to treated with mupirocin. Results from both cultures (treated cells with mupirocin and treated cells with

mupirocin + protease inhibitor) showed identical RPLA results at same time points which suggests that there was no degradative activity of protease on TSST-1 during these time points (1 and 8 h) Table 14.

Table14 Optical density and TSST-1 titre for mupirocin treated *S. aureus* with and without protease addition (1mM).Values are from independent triplicates.

Incubation time	OD 600nm		TSST titre	
	Mup	Mup + protease inhibitor	Mup	Mup + protease
0 h	0.5	0.5	Negative	Negative
1 h	0.5	0.5	Negative	Negative
8 h	1.3	1.5	Negative	Negative
16 h	6.6	6.9	16	16
24 h	8.1	7.9	256	256

5.3 Discussion

In this part of the study the effects of sublethal concentrations of mupirocin on a clinical isolate of *S. aureus* showed significant growth inhibition the first few hours of mupirocin exposure from (1 to 8 h). In addition, up-regulation of *tst* and *RNAIII* genes occurred as they were detected by relative quantitative RT-PCR during this inhibited growth. Although, up-regulation of *tst* and its activator *RNAIII* has appeared, TSST-1 synthesis seems to be inhibited as it not been detected during these time points (Figure 37).

The inhibited growth and delayed toxin production here is in agreement with previous observation on different clinical isolate T4 when Edwards Jones reported prolonged lag phase of growth and delayed TSST-1 production after sublethal concentration of mupirocin exposure (Edwards Jones 1997). Furthermore, Edwards Jones and Foster had spotted earlier and increasing TSST-1 production in this *S. aureus* clinical isolate after exposure to sublethal concentration of silver sulphadiazine (Edwards-Jones and Foster, 1994b)which is in contrast to the effects of mupirocin.

As mentioned earlier in Chapter 4, the laboratory strain of *S. aureus* 8325-4 showed inhibited growth after sublethal concentration of mupirocin treatment and ppGpp was detected during growth inhibition up to 4 h from adding sublethal concentration of mupirocin to *S. aureus* 8325-4 broth culture. In addition, in previous work 15 different strains of *S. aureus* were capable of producing (p)ppGpp after mupirocin treatment (Cassels *et al.*, 1995). Recently, Wolz and colleagues have proven that inhibitory concentration of mupirocin (3 × MIC) is capable of triggering the stringent response in *S. aureus* strain COL and microarray results showed down-regulation of nucleotide synthesis, translation and energy metabolism genes occurred after only 10 min of expose to inhibitory concentration of mupirocin (Reiß *et al.*, 2011). Furthermore, in *B. subtilis* (p)ppGpp directly inhibited primase, an essential component for replication, that consequently inhibited the replication machinery (Wang *et al.*, 2007). Recent work has shown (p)ppGpp inhibits the DNA replication elongation as well as replication initiation in *E.coli* and *B.subtilis* (DeNapoli *et al.*, 2013) leading to inhibited growth and one can assume the same mechanism may occur in *S. aureus* which may accont for the significant growth inhibition in in the early stage (1- <8 h) observed in this study. Relative quantification for RT-PCR revealed that *tst* and *RNAIII* genes were up-regulated shortly after mupirocin addition particularly after 1 h. Early up-regulation of *agr* locus product and its positively affected target genes such as *tst* have been reported in different studies after exposure to sub inhibitory concentrations of protein synthesis targeting antibiotics such as clindamycin and linezolid (Stevens *et al.*, 2007, Ohlsen *et al.*, 1998). These virulence genes (*tst* and *RNAIII*) are tightly regulated in growth dependent manner and they are transcribed in post exponential phase of growth in normal condition under *agr* locus (Novick *et al.*, 1995b, Novick *et al.*, 1993). Despite the growth inhibition throughout 1 to 8 h, early transcription of *tst* and *RNAIII* has occurred indicating a remarkable alteration on virulence genes regulation. This finding raises a question which is how the regulation of these genes had been altered and why that happened?

An answer for the question might come from the growing evidence of CodY protein effects on *S. aureus* virulence factors and its crucial role in linking between metabolism and virulence factor regulation (Pohl *et al.*, 2009, Majerczyk *et al.*, 2010). CodY protein represses a wide range of virulence factors in *S. aureus* during exponential growth. The abundance of GTP in the intracellular pool plays a central role in increasing the affinity of CodY to bind its DNA target genes during exponential growth (Majerczyk *et al.*, 2008, Majerczyk *et al.*, 2010). Recently, early transcription of the *agr* locus and some exoproteins virulence genes in different strains of *S. aureus* has been reported after introducing *codY* mutations (Rivera *et al.*, 2012). This early transcription of *agr* in *codY* mutant strains is in agreement with qRT-PCR results in this work. Therefore, the results suggest that the stringent response affects CodY repressive activity indirectly as triggering the stringent response would commence (p)ppGpp synthesis which consumes GTP from the intracellular pool rapidly after mupirocin addition. This is reported in chapter 4 when the GTP intracellular concentration decreased markedly after 1 h of sublethal concentration of mupirocin and then was not detectable after 3 and 4 h. In addition, Wolze and colleagues have reported significant decrease in GTP pool after only 10 min of exposure to inhibitory concentration of mupirocin. (Reiß *et al.*, 2012b, Geiger *et al.*, 2010b). Hence, GTP consumption during (p)ppGpp synthesis and the inhibition of the GTP synthesis pathway that (p)ppGpp exhibit would obviously decreased the GTP pool and lead to de-repression of CodY target genes such as the *agr* locus and activation of *RNAIII* transcript begin to occur in the early stage of growth which may up-regulate other virulence factors that are under *agr* regulation such as TSST-1 and other toxins. In other words CodY seems to shape and contribute to virulence gene regulation in *S. aureus* after mupirocin treatment through losing its repressive effect on some of them such as *agr* (Geiger *et al.*, 2010b, Reiß *et al.*, 2012b). This proposed mechanism and CodY role is illustrated in (Figure 39).

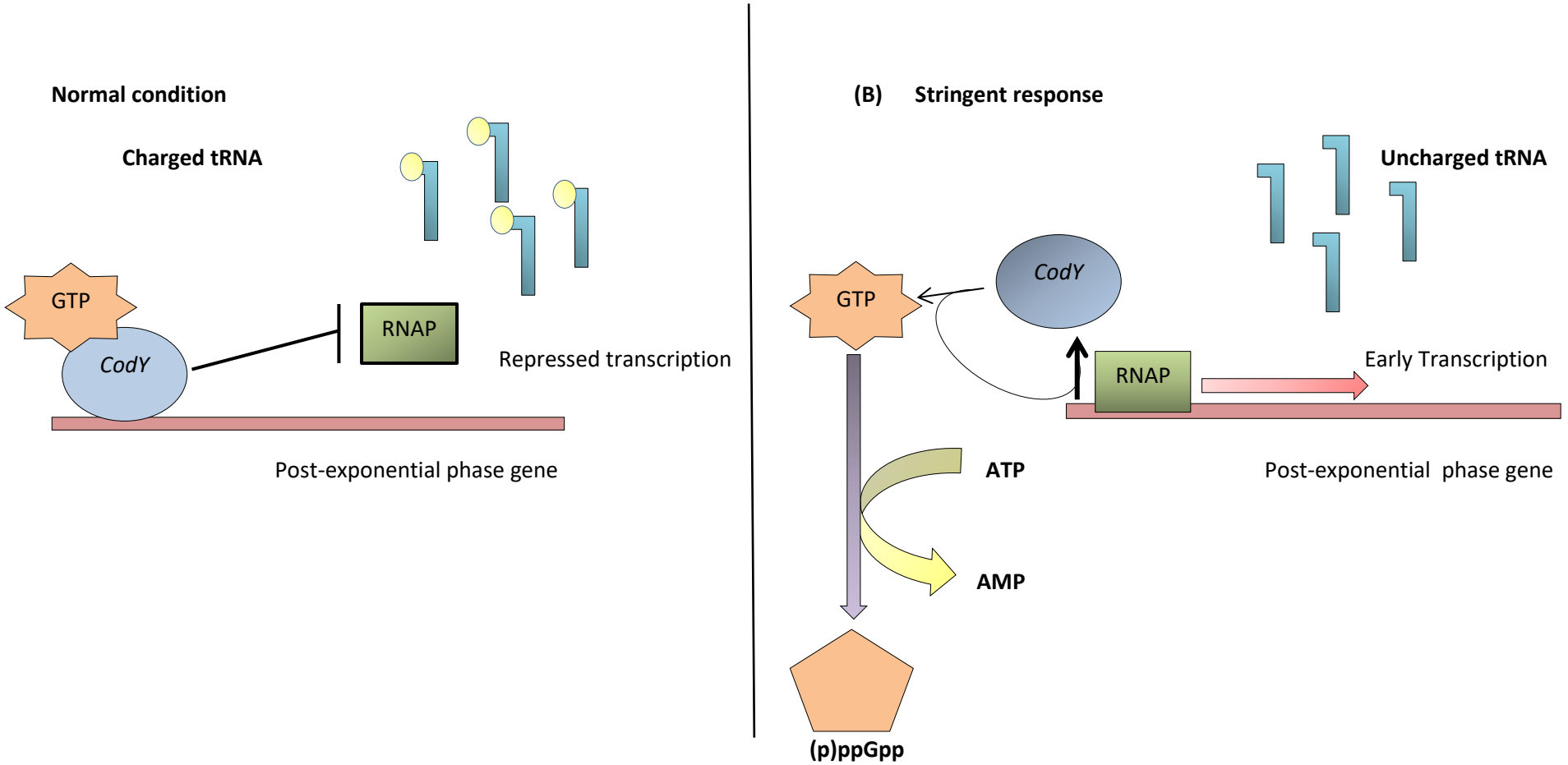


Figure 39 effect of CodY on genes regulation in exponential growth at untreated condition and at inhibited growth at stringent response state.

(A) In nutrient rich conditions GTP increases CodY affinity to its target DNA resulting in repressed transcription. **(B)** In amino acid starvation, caused by mupirocin, stringent response is triggered by uncharged tRNA and intracellular GTP is utilized in (p)ppGpp synthesis which reduces CodY affinity to its target DNA particular post-exponential phase genes resulting in early transcription

Interestingly, TSST-1 production was not detected during the early time of exposure to mupirocin (1-8 h) despite its mRNA transcription up-regulated at that time. This can be attributed to the mupirocin mechanism of action that would lead to attachment of de-aminocylated tRNA to the A-site of the 50S ribosome unit which consequently obstructs the translation processes(Parenti *et al.*, 1987). Shortage of charged isoleucyl-tRNA in the intracellular pool as results of mupirocin effect can be a cause for inhibition of translation of the *tst* mRNA particularly when isoleucine one of the main amino acids that comprise TSST-1 (Sunohara *et al.*, 2004). In addition, ribosomes paused by uncharged tRNA have been reported to induce mRNA cleavage near the paused ribosome site in bacteria which would lead to inhibition of translation of mRNA even though its transcript had been up-regulated (Sunohara *et al.*, 2004).

Furthermore, stalling ribosomes during amino acid starvation can induce the toxin and antitoxin system that decays mRNAs(Christensen *et al.*, 2003). Recently, three toxin-antitoxin systems in *S. aureus* that are involved in endoribonuclease activity and translation initiation inhibition have been shown to be up-regulated during mupirocin treatment which may result in preventing protein synthesis including that of TSST-1 (Reiß *et al.*, 2012b, Fu *et al.*, 2007, Yoshizumi *et al.*, 2009)

Another possible reason for the inhibited translation might be the decreased level of GTP which plays an important role in translation as energy source for, binding tRNA to the A-site of the ribosome unite as well as its role in translocation of the ribosome toward the 5' of the mRNA(Gualerzi and Pon, 1990). Moreover, insufficient ribosomes might lead to inhibited translation as recent observation reported down regulation for ribosomal genes under the effect of stringent response triggered by inhibitory concentration of mupirocin (Reiß *et al.*, 2012b). The following diagram summarises these proposed mechanisms for the absence of TSST-1 synthesis in this work.

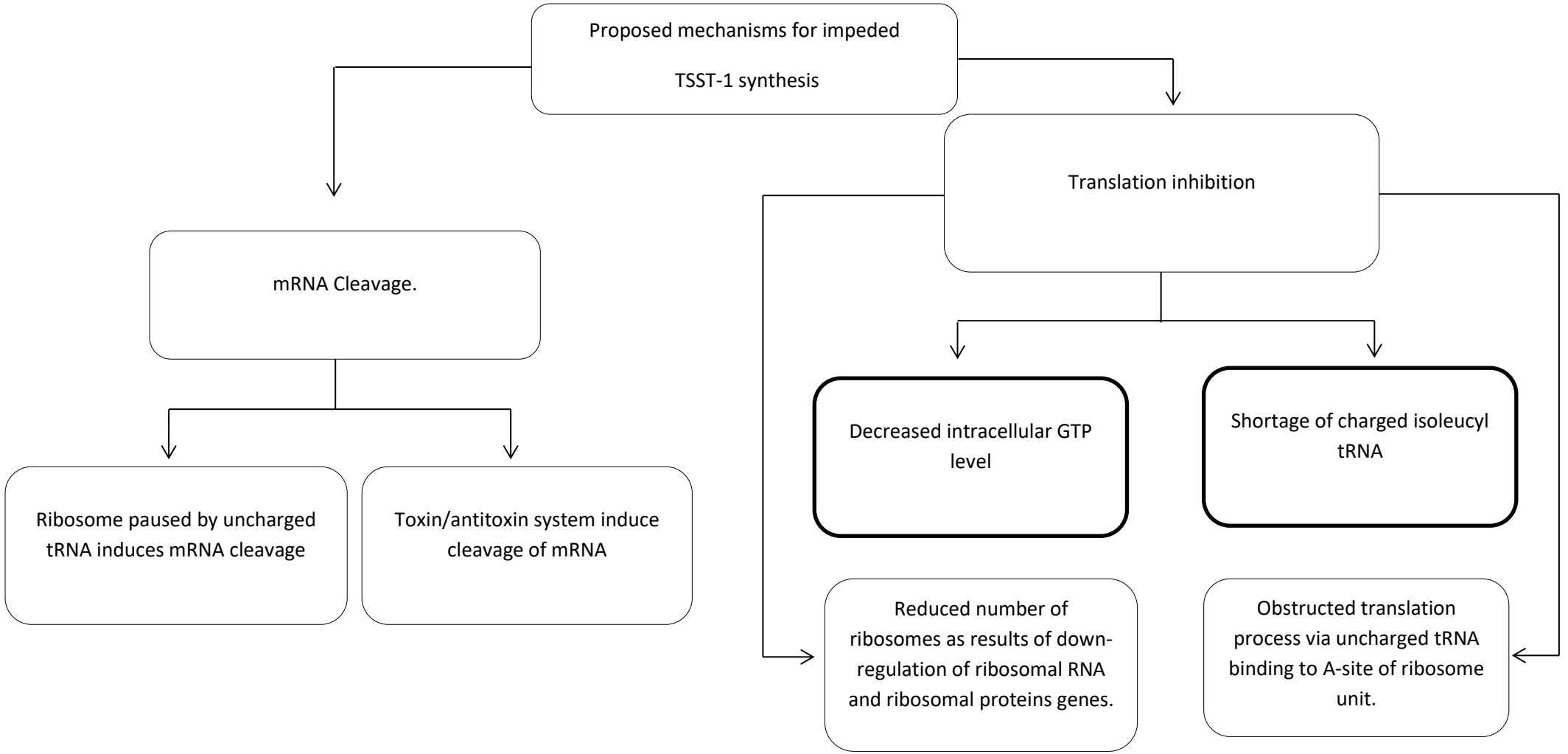


Figure 40 proposed mechanisms that can result in impeded TSST-1 synthesis after treatment with sublethal concentrations of mupirocin

Extracellular TSST-1 degradation could be another reason for undetectable TSST-1 production as different studies have reported the up regulation of the *HtrA* gene during stress response in *S. aureus* which encodes serine protease and has the capability to digest TSST-1 (Bore *et al.*, 2007, Lawellin *et al.*, 1989). The degradation of TSST-1 by serine protease inhibitor is unlikely as there were no significant difference in the TSST-1 production between broth supplemented with protease inhibitor and controls after mupirocin addition. A further but unlikely possibility is that TSST-1 was synthesised but not excreted. This was not investigated here but there are no reports of inhibition of protein secretion by (p)ppGpp.

Regarding to 16 and 24 h results, sublethal concentration of mupirocin seemed to prolong the lag phase which consequently delayed the exponential and the stationary phases for treated cell in comparison to control cells. This variation in growth phases between the two cultures would simply explain the contrasts of their *tst* and *RNAIII* transcripts and TSST-1 production as regulation of these genes tightly dependent on growth phase.

The effects of inhibitory concentrations of different antibacterial agents have been observed in several studies. Antibiotic mode of action and regulation of virulence factors play important role in response to the treatment. Steven and colleagues have noticed increase transcription of exoprotein genes associated with inhibited translation when exposed to proteins synthesis inhibitors such as clindamycin and linezolid(Stevens *et al.*, 2007). Conversely, TSST-1 and exoproteins were produced at increasing levels in the early stage of growth after exposure to inhibitory concentration of non-protein targeting agent such as silver sulphadiazine, nafcillin and other β-lactam antibiotics(Edwards-Jones and Foster, 1994a, Edwards-Jones and Foster, 2002, Subrt *et al.*, 2011, Ohlsen *et al.*, 1998). Therefore, bactericidal and bacteriostatic effects for the antibacterial agent are not the only

strategy that should be considered for treatment. Effects of the antibiotic on virulence factor regulation and their production should be taken into consideration in particular during treating infection caused by exoprotein producing bacteria.

In summary, despite the increased transcription of *tst* and RNAIII in early stage of growth by sublethal concentrations of mupirocin, TSST-1 production was inhibited. Mupirocin, similarly to clindamycin, appears to be able to inhibit TSST-1 production at sublethal concentrations which might make it suitable to treat wound infections caused by *S. aureus* toxin producing strains even if sublethal concentrations occur at some sites in the wound.

Chapter 6

Observing the global transcription alterations after sub-inhibitory concentration of mupirocin treatment

6.1 Introduction

Mupirocin has been used in different works to trigger the stringent response via inhibition of isoleucine aminoacylation leading to unchanged tRNA accumulation which trigger the stringent response in *S. aureus* and other bacteria. Observations of global alterations in gene transcription after mupirocin treatment have been conducted in previous studies via DNA microarray and most of these observations were at bactericidal concentrations of mupirocin for less than 10 h, as treated cells entered the death phase after a few hours of exposure (Reiß *et al.*, 2011, Geiger *et al.*, 2010a).

In the present work, *S. aureus* 8325-4, a lab strain derived from *S.aureus* 8325 and it is sigB defective and cured from prophages ϕ11, ϕ12 and ϕ13 that present in 8325 (O'Neill 2010), was exposed to sub-lethal concentrations of mupirocin, and transcriptional profiles were obtained via high throughput RNA-sequencing at three different time points, 1, 12 and 24 h, for treated and control cells, to investigate the global effect of sub-lethal concentrations of mupirocin on gene expression. This observation also focused on differentially expressed genes in treated cultures and especially on the expression of virulence factor genes throughout these time points. In addition, genes whose expression overlapped for the different time points were identified to track the regulation alterations caused by mupirocin by comparison with the control cells and they were classified into groups according to their involvement in the different biological functions.

S. aureus recovery from the stringent response has not been reported in previous studies which are related to the mupirocin concentration that leaded to cell death. In this work, the recovery from the stringent response after exposure to sub-lethal concentrations of mupirocin is discussed in phenotypic and genotypic aspects and a scenario of recovery is suggested.

6.2 Results

6.2.1 Effect of mupirocin and ppGpp on the growth of *S. aureus* 8325-4

The effect of a sublethal (0.5 MIC) concentration of mupirocin (0.12 μg/ml^{-1}) on growth of *S. aureus* 8325-4 was observed throughout the three time points 1, 12 and 24 h in parallel with growth of control cultures and the results are shown in Figure 41. Comparison between the optical density of the control and treated cultures showed that there was a significant effect of the sublethal concentration of mupirocin on growth up to 24 h. The main effect was to prolong the lag phase for the treated cells. In the first time point the control cell OD reached 1.2 whereas the treated cells remained constant at 0.5, a significant difference (P= 0.02). Then mupirocin had a highly significant effect (P = 0.0006) on 12 h cultures as the control cells OD reached 6.5 whereas the treated cells OD was only 2.6. In addition, after 24 h control cells OD reached 10.4 and the test cells rose to 5.8, significantly lower than the controls (P = 0.002). In treated cells the optical density for 24 h culture increased to OD 5.8 which is similar to the OD of 12 h control cells (Figure 41). Furthermore, (p)ppGpp nucleotide was detected after 1 h of sublethal concentration of mupirocin exposure but was not detectable after 12 or 24 h (Figure 41).

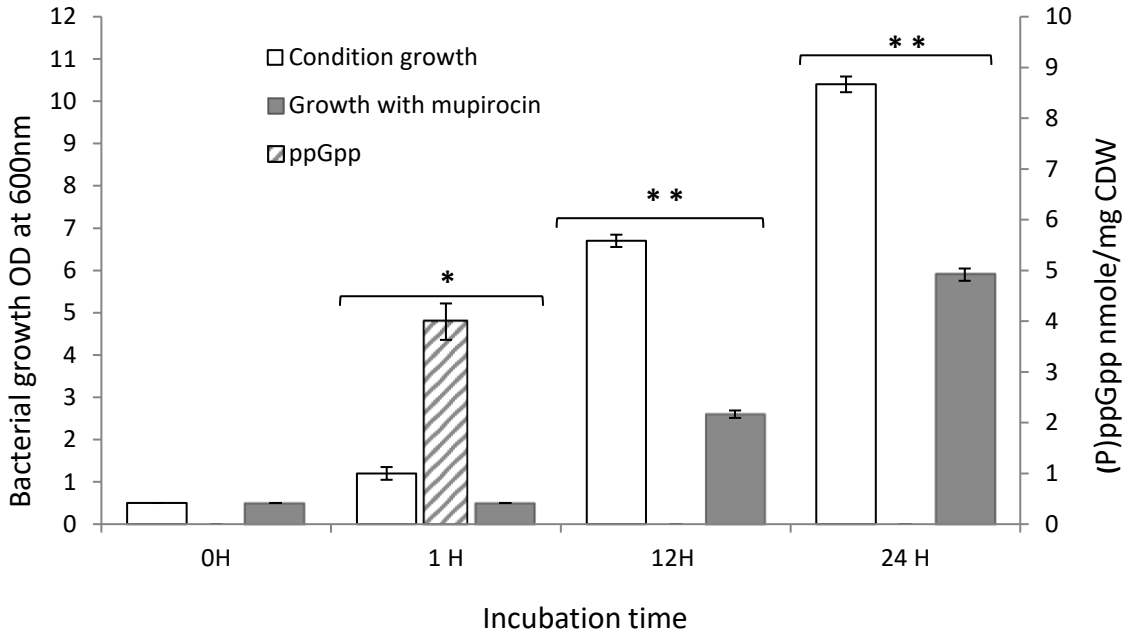


Figure 41 effects of the sublethal concentration of mupirocin on the growth and ppGpp production by *Staphylococcus aureus* 8325-4

, OD_{600} mupirocin treated , OD600 controls; , ppGpp concentration . Values are given as mean from three independent replicates and the error bars present standard error Statistical significant differences (T-test:*, p-value <0.05, ** p-value <0.01).

RNA was extracted from cells treated with sublethal concentration of mupirocin and controls at the time points shown in Figure 42 and was analysed by high throughput sequencing technique (RNA sequencing) to observe the global alterations in transcription for *S. aureus* 8325-4. Differentially expressed genes were defined by using a 2 fold change as the threshold for the criterion of a significant change and the False Discovery Rate (FDR) had to be ≤0.05.

6.2.2 Comparison between gene expression in test and controls at different times

Expression data was obtained for 2860 genes and of these 869 (30.3%) showed a significant change in their expression (Figure 2) and the remaining genes showed either no change or no significant alteration on their transcription after 1 h of sublethal concentration

of mupirocin treatment. Approximately half of the differentially expressed genes 55.12% (n=479) were up-regulated and 44.8% (n=390) genes were down-regulated.

After 12 h of mupirocin exposure, the differentially expressed genes were 33.8% (n=967) approx. 3.5% more than after 1 h (Figure 42). Again more of the differentially expressed genes were up-regulated 52.9% (n= 512) whereas 47.1% (n=455) were down-regulated.

The differentially expressed genes 39.3% (n=1125) after 24 h had increased 9% and 5.5% higher than at the 1 and 12 h time points respectively (Figure 42). Up-regulated genes were 56.6% (n=637) and the down-regulated genes were 43.3% (n=488) of the differentially expressed genes.

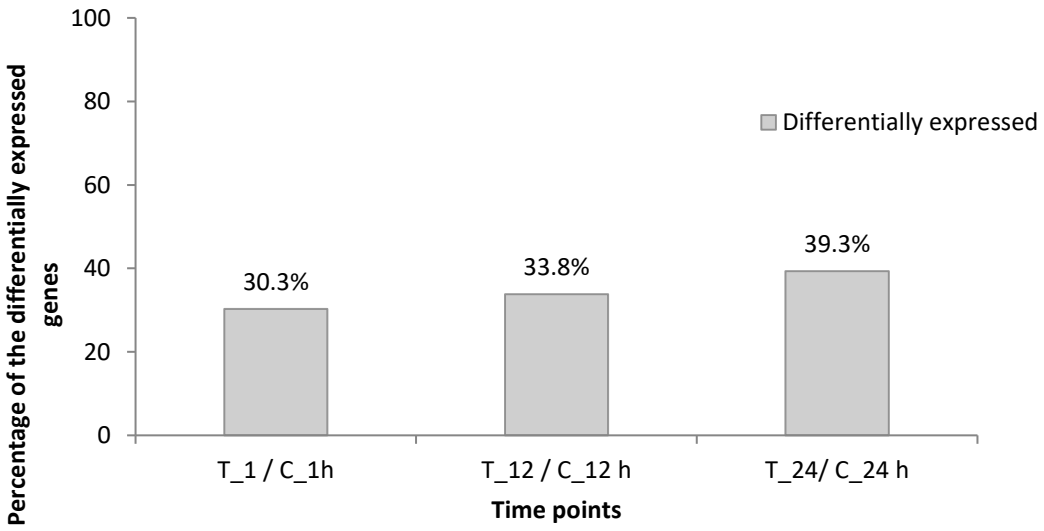


Figure 42 Total differentially expressed genes percentage based in contrast between mupirocin treated and control cells of *S. aureus* 8325-4 for each time point.

Differentially expressed gene data is shown in (Figure 43) and genes that were differentially expressed only at one time were determined. For example, at the 1 h time point data showed that 146 genes were positively induced and 206 were negatively controlled. In addition, 279 gens were up-regulated and 311 genes were down-regulated at 12 h. Moreover, 508 genes

were shown to be activated and 443 were inhibited in 24 h data (Figure 43). However, genes that show overlap in gene expression between the different time points are reported. For instance, gene expression overlap occurred between 1 and 12 h time points for 229 up-regulated genes and 142 down-regulated genes. Gene expression overlap between 1 h and 24 h occurred in transcription of 125 up-regulated genes and 43 down-regulated genes, respectively. Among positively regulated gene, gene expression overlap between 12 h and 24 h were 25 genes and negatively regulated genes between them were 3.

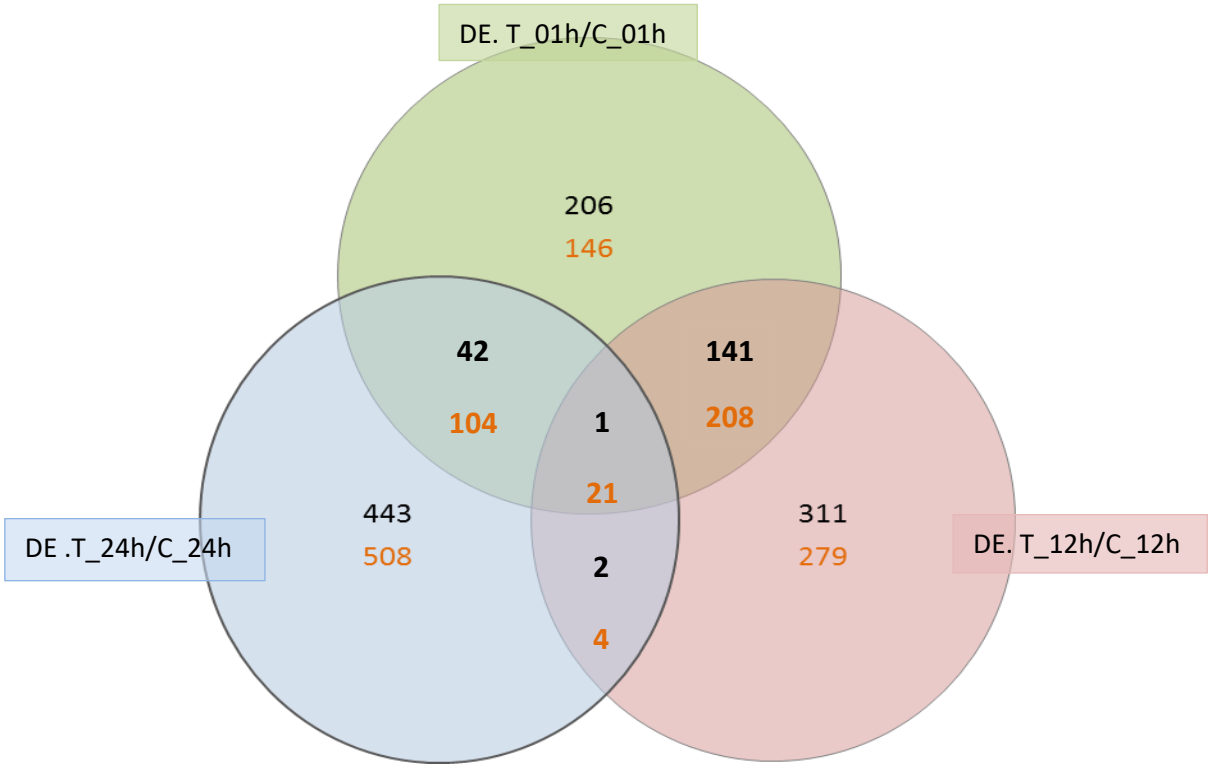


Figure 43 differentially expressed genes of *S.aureus* 8325-4 treated with sub-lethal concentration of mupirocin for 1, 12 and 24 h compared with control (non- treated) samples for each time point.

Key: Up-regulated orange, down-regulated in black. All genes were subjected to a twofold differential expression cut-off at FDR ≤0.05, DE =differentially expressed, T= mupirocin treated and C= controls.

In addition, based on fold changes for genes, transcription profiles were obtained and comparisons of these profiles were made between all samples irrespective of their time point and growth condition in order to observe correlation between their transcription profiles. In the following heatmap each square illustrates the level of correlation between the corresponding samples (Figure 44). Level of correlation expressed in colours as red colour means high correlation and dark blue for low correlation. For example, arrow (A) on the heatmap indicates high level of similarity (as the square appears in dark orange and red) of the transcriptional profile for control cell at 12 h and the transcriptional profile for treated cells with mupirocin at 24 h. Furthermore, significant similarity of transcriptional profiles has occurred among treated cells with mupirocin for samples at 1 and 12 h arrow (B) which indicate similar expression regulation for their genes (Figure 44).

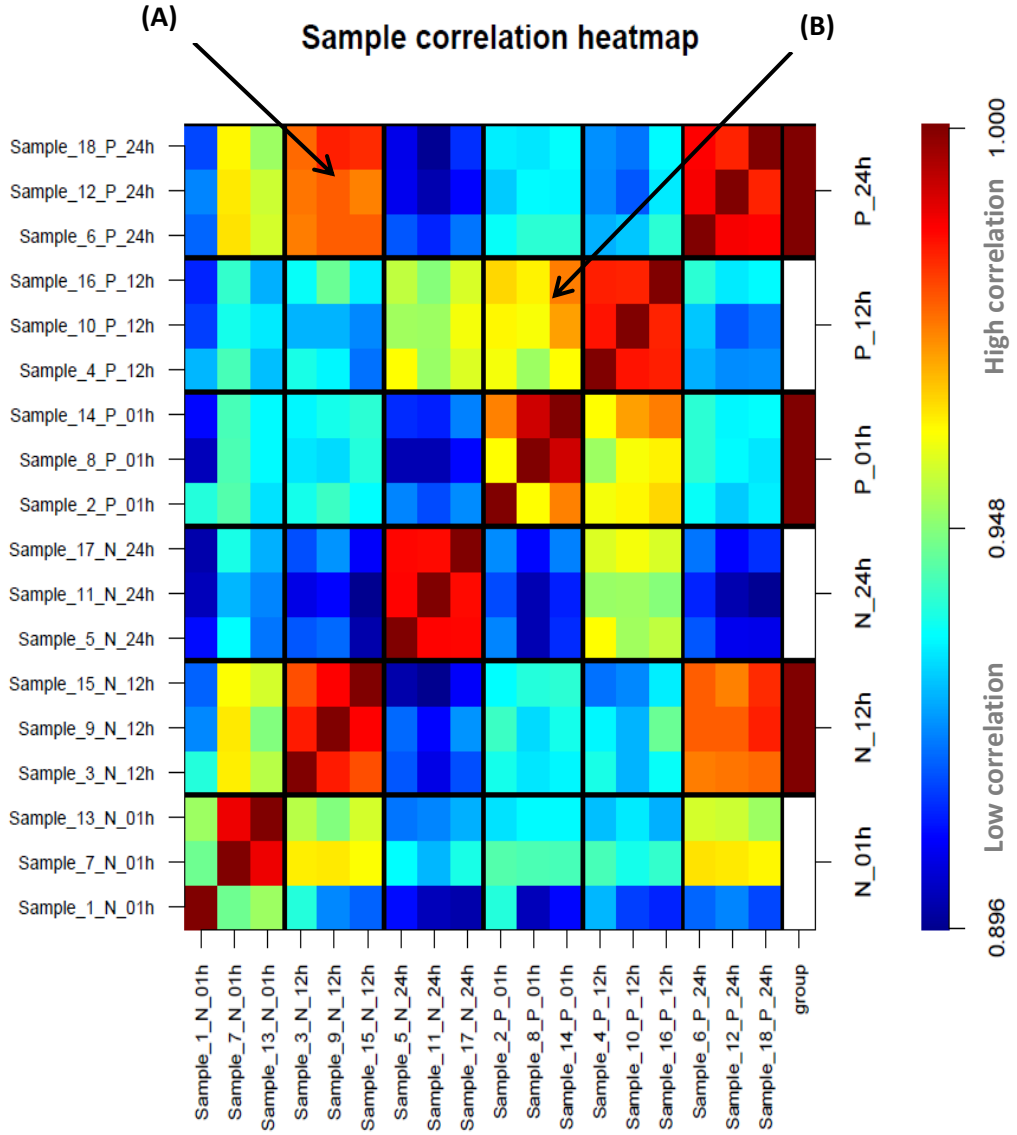


Figure 44 sample correlation heatmap based on fold change in gene expression of *S.aureus* 8325-4 treated with sub-lethal concentration of mupirocin (P) for 1, 12 and 24 h compared with control samples (N) at the same time points.

All genes were subjected to a twofold differential expression cut-off at FDR ≤0.05. The diagonal (from bottom left to top right) shows self- correlation. Level of correlation from red (high) to dark blue (low). Samples are from independent triplicates.

6.2.3 Functions of unique and overlapping genes expression

Differentially expressed genes that showed unique regulation among the time points were classified for gene function using gene functional classification toll server (DAVID

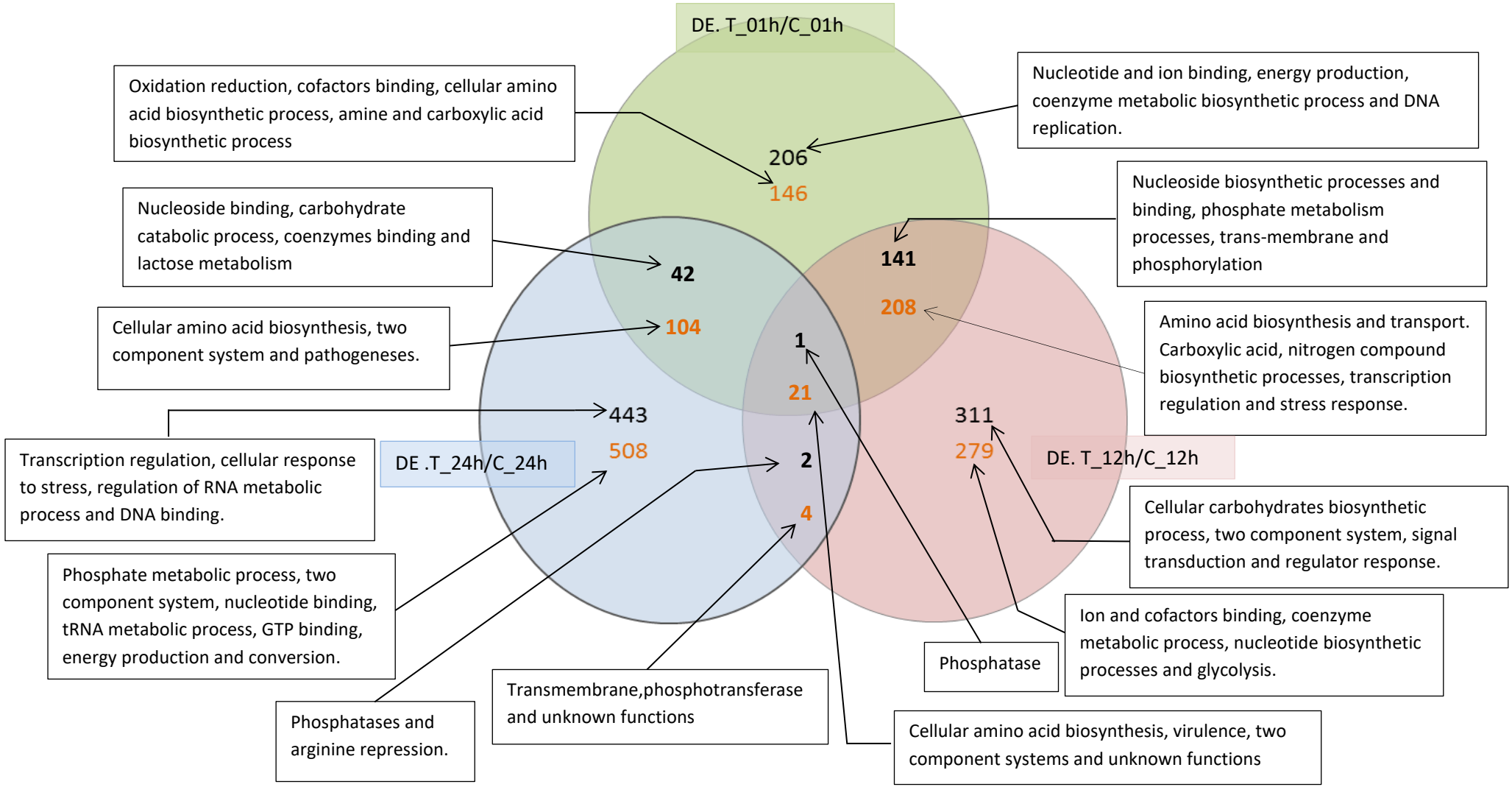
Bioinformatics Resource). For example, at 1 h genes that were involved in different functions including cofactors binding, cellular amino acid biosynthetic process and carboxylic acid biosynthetic processes were positively induced and negative regulation had occurred for genes that contribute in energy production, coenzyme metabolic biosynthetic process and DNA replication Figure 46. In addition, at 12 h genes that were involved in ion and cofactors binding, coenzyme metabolic process, nucleotide biosynthetic processes and glycolysis were up-regulated whereas, those involved in cellular carbohydrates biosynthetic process, two component system, signal transduction and regulator response were down-regulated. Genes that contribute in phosphate metabolic process, two component systems, nucleotide binding, GTP binding, energy production and conversion were up-regulated at 24 h and those involved in transcription regulation, cellular response to stress, regulation of RNA metabolic process and DNA binding were down-regulated Figure 45.

Genes which showed overlap in expression as shown in Figure 43 were studied in more detail by observing their up- or down-regulation and investigating their functions to obtain a clearer picture to more fully understand the effects of the stringent response (Figure 45), an annotated version of Figure 43. For instance, Comparing 1 and 12 h transcriptome results from treated and control cells showed overlapping occurred for up-regulated genes that contribute in branched chain amino acid (BCAAs) biosynthesis processes, carboxylic acid and nitrogen compound biosynthetic processes, transcription regulation and stress response (Table 15). On the other hand, there was overlapping of negatively controlled genes for those involved in variety of functions such as nucleoside and nucleotide biosynthetic processes and binding, phosphate metabolism processes, trans-membrane and phosphorylation (Figure 45).

In addition, up-regulated overlapped genes for 1 h and 24 h time points were involved in different functions including cellular amino acid biosynthesis, two component system, and pathogenesis. However, overlapping of down-regulated genes occurred for genes that are engaged in cellular functions including carbohydrate metabolic processes, nucleotide and coenzyme binding and lactose metabolism (Figure 45).

Genes overlapping between 12 and 24 h occurred (Figure 45), among few positively induced genes that were involved in functions such as transmembrane, amine biosynthetic processes, cellular amino acid biosynthesis, virulence and two component systems.

Furthermore, overlapping of up-regulated genes throughout the three time point had occurred (n=21 genes) 10 of which coded unknown function products whereas the remaining included genes that involved in cellular amino acid biosynthesis, virulence and two component systems (Table 16).



Figur 45 differentially expressed genes of *S. aureus* 8325-4 strains treated with sub-lethal MIC of mupirocin for 1, 12 and 24 h compared with control (non- treated) samples for each time points(up-regulated in orange, down-regulated in black.).

All genes were subjected to a twofold differential expression cut-off at FDA ≤0.05. DE =differentially expressed, T= test sample and C= control condition.

Table 15 Transcription alteration of genes involved in important functions following exposure to sub-lethal concentrations of mupirocin.

Locus tag	log_2FC.P_01h/N_01h	log_2FC.P_12h/N_12h	log_2FC.P_24h/N_24h	Description
Cellular amino acid biosynthesis and transport.				
SAOUHSC_02288	5.558383707	0.16090767	2.348077015	isopropylmalate isomerase small subunit LueD
SAOUHSC_01396	4.634513538	1.615679676	1.761330946	dihydrodipicolinate synthase
SAOUHSC_01395	4.272831445	1.718137546	1.38266185	aspartate semialdehyde dehydrogenase
SAOUHSC_01322	4.116077558	1.321428234	-0.819926157	homoserine kinase
SAOUHSC_01397	3.996076025	1.512043862	1.70815607	dihydrodipicolinate reductase
SAOUHSC_01321	3.773442547	1.466197942	-0.452475385	threonine synthase
SAOUHSC_01159	3.28512134	2.109141364	2.378937576	isoleucyl-tRNA synthetase
SAOUHSC_01319	2.746277235	2.08075022	-0.685131176	aspartate kinase
SAOUHSC_02741	1.981726437	1.644037233	0.500963974	amino acid ABC transporter permease
SAOUHSC_02743	1.714682862	1.403793953	-0.337621794	amino acid ABC transporter permease
SAOUHSC_02742	1.629843628	1.209543086	0.08294902	amino acid transporter
SAOUHSC_00536	1.061068082	1.880059929	-0.649915225	branched-chain amino acid aminotransferase
carboxylic acid biosynthesis process				
SAOUHSC_01398	4.387817621	1.069213935	1.164675724	tetrahydropyridine-2-carboxylate N-succinyltransferase
SAOUHSC_01395	4.272831445	1.718137546	1.38266185	aspartate semialdehyde dehydrogenase
SAOUHSC_01319	2.746277235	2.08075022	-0.685131176	aspartate kinase
SAOUHSC_02244	1.096630469	1.760287155	0.38189061	succinyl-diaminopimelate desuccinylase
SAOUHSC_02716	1.057146	2.245993715	-4.083664854	dethiobiotin synthase
SAOUHSC_01635	1.007339187	1.16215325	0.585340173	shikimate kinase

(FC) fold change a – means down-regulation, (p) Treated cells with mupirocin, N Control cells.

Table 15, continue.

Locus tag	log_2FC.P_01h/N_01h	log_2FC.P_12h/N_12h	log_2FC.P_24h/N_24h	Description
Stress response				
SAOUHSC_00715	4.524388932	1.501906714	2.20621774	response regulator
SAOUHSC_02757	4.058647623	3.140527115	-3.376450823	Toxin/antitoxin system protein
SAOUHSC_01819	3.779090714	1.454848755	-0.607804235	Universal stress protein
SAOUHSC_00992	3.155331132	3.254186318	-0.997848819	MarR family transcriptional regulator
SAOUHSC_02692	3.058061862	2.49048462	-2.651989113	Toxin/antitoxin system protein
SAOUHSC_00934	2.248105826	0.891111754	1.627478342	transcriptional regulator Spx
SAOUHSC_01685	2.087166309	5.287314939	-5.928228587	heat-inducible transcription repressor HrcA
SAOUHSC_01281	1.878196259	-0.500067106	0.509305484	host factor 1 protein
SAOUHSC_02664	1.424576532	0.894209915	0.824786568	transcriptional regulator
SAOUHSC_00505	1.339454735	5.23664769	-6.636074044	endopeptidase
SAOUHSC_00935	1.316914929	3.309088494	-3.241761051	adaptor protein
Nitrogen component biosynthetic processes				
SAOUHSC_01395	4.272831445	1.718137546	1.38266185	aspartate semialdehyde dehydrogenase
SAOUHSC_01397	3.996076025	1.512043862	1.70815607	dihydrodipicolinate reductase
SAOUHSC_01321	3.773442547	1.466197942	-0.452475385	threonine synthase
SAOUHSC_02244	1.096630469	1.760287155	0.38189061	succinyl-diaminopimelate desuccinylase
SAOUHSC_02716	1.057146	2.245993715	-4.083664854	dethiobiotin synthase

(FC) fold change, (p) Treated cells with mupirocin, N Control cells.

Table 16 overlapping up-regulated genes throughout 1, 12 and 24 h.

Locus tag	Log_2FC.P_01h/N_01h	Log_2FC.P_12h/N_12h	Log_2FC.P_24h/N_24h	Description
SAOUHSC_00401	6.553871845	2.630380209	4.439239373	hypothetical protein
SAOUHSC_00717	6.162806261	3.028173651	2.153450097	hypothetical protein
SAOUHSC_02160	5.698850429	1.698809391	2.224018723	hypothetical protein
SAOUHSC_02161	5.42152956	1.219541744	2.462834269	MHC class II analog protein
SAOUHSC_01114	5.261024517	1.593980386	3.040741108	fibrinogen-binding protein
SAOUHSC_00716	5.183185291	1.666778008	2.45608222	hypothetical protein
SAOUHSC_01396	4.634513538	1.615679676	1.761330946	dihydrodipicolinate synthase
SAOUHSC_00715	4.524388932	1.501906714	2.20621774	response regulator
SAOUHSC_01398	4.387817621	1.069213935	1.164675724	tetrahydropyridine-2-carboxylate N-succinyltransferase
SAOUHSC_00714	4.253441498	1.127500378	2.158843762	sensor histidine kinase SaeS
SAOUHSC_01397	3.996076025	1.512043862	1.70815607	dihydrodipicolinate reductase
SAOUHSC_02773	3.643517026	1.442373114	2.285008828	transporter
SAOUHSC_00367	3.473836595	1.61971871	2.77915483	hypothetical protein
SAOUHSC_01159	3.28512134	2.109141364	2.378937576	isoleucyl-tRNA synthetase
SAOUHSC_02112	2.921384751	2.274531859	1.475211248	hypothetical protein
SAOUHSC_00961	2.753277716	1.898520437	2.364803055	hypothetical protein
SAOUHSC_02566	2.524505653	1.902989861	1.207635927	hypothetical protein
SAOUHSC_02334	2.33900609	1.223618318	2.131598941	bacteriophage L54a single-stranded DNA binding protein
SAOUHSC_02731	2.248023772	1.371139687	1.0624403	hypothetical protein
SAOUHSC_02012	1.67574536	1.258524884	1.433805033	glycosyltransferase
SAOUHSC_01707	1.64945833	1.152647828	1.630139022	hypothetical protein

(FC) fold change, (p) Treated cells with mupirocin, N Control cells

6.2.4 Regulation of virulence factor genes

In this study, transcription of 60 virulence factor genes were examined at 1, 12 and 24 h after exposure to sub-inhibitory concentration of mupirocin *via* the RNA-seq analysis to observe their regulation relatively to normal conditions. Again, differentially expressed genes were defined by using a 2 fold change as the threshold for the criterion of a significant change and a False Discovery Rate (FDR) and P value had to be ≤ 0.05

The effect of sub-inhibitory concentration of mupirocin showed clear alteration to virulence factor gene regulation in *S. aureus*. For instance, some genes that are normally expressed during post-exponential /stationary phase in normal conditions were transcribed at 1 h of mupirocin exposure including *agr* locus and genes coding for lipase, capsular polysaccharide biosynthesis protein Cap5B, staphylocoagulase and alpha-hemolysin toxin. Conversely, genes that are normally tightly controlled in a growth dependent manner such as those coding for clumping factor, catalase and fibronectin-binding protein, up-regulated during exponential phase in normal condition, appear to be up-regulated while the cell growth was inhibited during stringent response. However, genes were divided into four groups according to their regulation manner through the time points as follows;

Group A genes that were up-regulated after 1 h then down-regulated after 12 h and up-regulated again after 24 h (Table 17). These included genes such as the *agr* locus, lipase, serine protease *SpIA* and alpha-hemolysin. These genes are normally expressed in the late exponential phase or early of stationary phase. Interestingly, the up-regulation of these genes is associated with the stringent condition where the cell growth has been inhibited.

Group B included genes that were up-regulated during 1 and 12 h then repressed at 24 h (Table 18). These genes encoded products that are involved in the stress response such as, heat shock protein *GrpE* and protein export protein *PrsA* and encoded proteins that are

involved mainly in cell binding such as fibronectin-binding protein, intercellular adhesion protein C, extracellular matrix and plasma binding protein. TRAP gene which encoding for RNAIII activating protein that plays a crucial role in *S. aureus* toxin production was also regulated in this manner.

Group C genes were up-regulated throughout all time points (Table 19). These genes encoded for binding proteins such as penicillin-binding protein 2 *(SAOUHSC_01467)* and fibrinogen-binding protein-like protein *(SAOUHSC_01110)* or involved in response regulation and host immune cells evasion such as *SAOUHSC 00715* and *SAOUHSC 01115* respectively. Further, some of genes are expressed at 1 h much more than 12 and 24 such as genes that involved in immune evasion, formyl peptide receptor-like1 inhibitory protein, MHC class II analog protein, response regulator immunoglobulinG-binding protein Sbi.

Furthermore, transcription results showed another virulence gene grouping (D) that was positively induced at 1 h without reaching the cut off threshold (2 fold) such as phenol-soluble modulin 1-2 and serine protease genes SplB, C, D, E and F. Regulation for most of these genes was similar to group A. However, some genes in this group were up-regulated at 1 and 12 h such as clumping factor gene, enterotoxin family protein and then they were down-regulated at 24 h similar to group B genes regulation (Table 20).

Table 17 Group A virulence factors genes regulation

Locus tag	1h			12h			24h			Description
	FC	p-value	FDR	FC	p-value	FDR	FC	p-value	FDR	
SAOUHSC_00300	16.9	6.28E-14	3.99E-12	-4.43	1.74E-05	0.0001	93.7	1.01E-26	1.16E-24	lipase
SAOUHSC_00115	8.3	7.90E-12	3.12E-10	-4.85	1.20E-07	1.42E-06	9.8	2.40E-13	6.03E-12	capsular polysaccharide biosynthesis protein Cap5B
SAOUHSC_00192	7.5	3.76E-05	0.0002	-1.35	0.49	0.65	2.4	0.05	0.11	staphylocoagulase
SAOUHSC_00114	6.4	2.70E-09	5.95E-08	-2.6	0.0004	0.002	5.9	3.90E-09	4.43E-08	capsular polysaccharide biosynthesis protein
SAOUHSC_01121	5.8	2.16E-06	2.36E-05	-1.93	0.063	0.13	70.5	3.36E-22	2.18E-20	alpha-hemolysin
SAOUHSC_02709	5.3	1.29E-05	0.0001	-1	0.97	1	6.3	3.60E+06	2.18E-05	leukocidin s subunit
SAOUHSC_01281	3.6	1.61E-06	1.84E-05	-1.4	0.188	0.319	1.5	0.181	0.27	host factor 1 protein
SAOUHSC_02262	2.9	0.0008	0.004	-18.4	3.39E-16	2.11E-14	50.9	9.31E-25	8.33E-23	hypothetical protein/ AgrD
SAOUHSC_02265	2.86	0.0001	0.001	-7.67	5.20E-12	1.62E-10	18.3	1.31E-20	7.86E-19	accessory gene regulator protein A
SAOUHSC_01942	2.7	0.0001	0.0008	-2.1	0.004	0.015	1.6	0.05	0.1	serine protease SplA
SAOUHSC_02264	2.6	0.0006	0.003	-10.9	3.53E-14	1.60E-12	29.8	2.97E-25	2.93E-23	accessory gene regulator protein C
SAOUHSC_02261	2.3	0.005	0.018	-12.1	2.95E-14	1.36E-12	42.2	1.07E-25	1.18E-23	accessory gene regulator protein B
SAOUHSC_00130	2.3	0.01	0.034	-1.1	0.77	0.86	1.3	0.37	0.48	heme-degrading monooxygenase IsdI
SAOUHSC_02119	2.2	0.0015	0.0068	-1	0.88	0.94	3.5	5.31E-07	3.93E-06	high affinity proline permease
SAOUHSC_02314	2.1	0.004	0.01	-5.27	1.44E-09	2.62E-08	10.2	4.54E-16	1.66E-14	sensor protein KdpD
SAOUHSC_01953	2.1	0.004	0.0166	-1.55	0.079	0.16	1.5	0.09	0.16	gallidermin superfamily epiA protein

(FC) fold change. (FDR) False Discovery Rate. (–) Down regulated.

Table 18 Group B virulence factors gene regulation

Locus tag	1h			12h			24h			Description
	FC	p-value	FDR	FC	p-value	FDR	FC	p-value	FDR	
SAOUHSC_02803	45.2	6.45E-19	1.08E-16	6.9	3.63E-07	3.77E-06	-1.2	0.55	0.65	fibronectin-binding protein
SAOUHSC_00703	9	6.67E-08	1.08E-06	3	0.004	0.013	-1	0.69	0.78	quinolone resistance protein NorA
SAOUHSC_00992	8.8	1.56E-16	1.79E-14	9.5	2.30E-17	1.73E-15	-2	0.005	0.01	MarR family transcriptional regulator
SAOUHSC_01127	6.6	1.76E-11	6.38E-10	2.7	0.0001	0.0007	-1.52	0.11	0.18	superantigen-like protein
SAOUHSC_01124	6.1	2.00E-10	5.20E-09	4.3	2.55E-07	2.74E-06	-1.1	0.72	0.8	superantigen-like protein
SAOUHSC_01125	6.1	4.77E-11	1.47E-09	2.9	4.66E-05	0.0002	-1.55	0.084	0.15	superantigen-like protein
SAOUHSC_00620	5.8	4.91E-06	4.84E-05	4.5	8.27E-05	0.0004	-6	1.95E-06	1.25E-05	accessory regulator A(SarA)
SAOUHSC_03004	4.9	6.08E-05	0.0004	1.8	0.1	0.202	-1.2	0.5	0.61	intercellular adhesion protein B
SAOUHSC_01964	3.7	0.01	0.05	2.6	0.0001	0.0006	-2.2	0.002	0.007	RNAIII-activating protein TRAP
SAOUHSC_01684	3.2	0.0006	0.003	36.7	5.79 E19	5.92E-17	-55.7	4.96E-22	3.15E-20	heat shock protein GrpE/ thermosensor
SAOUHSC_00816	3	0.0005	0.003	1.15	0.653	0.781	-1.1	0.67	0.76	extracellular matrix and plasma binding protein
SAOUHSC_01327	2.8	0.004	0.01	5.3	8.52E-06	6.22E-05	-4	3.39E-05	0.0001	catalase
SAOUHSC_03005	2.7	0.0005	0.002	2.1	0.01	0.03	-1.6	0.09	0.16	intercellular adhesion protein C
SAOUHSC_00505	2.5	0.006	0.021	36.7	1.04E-19	1.36E-17	-97	4.24E-27	5.27E-25	endopeptidase
SAOUHSC_01972	2.4	0.001	0.006	6.6	2.41E-10	5.22E-09	-1.7	0.83	0.88	protein export protein PrsA
SAOUHSC_02696	2.1	0.001	0.005	2.3	0.0003	0.001	-2.2	0.0006	0.002	methicillin resistance determinant protein FmhA

(FC) fold change. (FDR) False Discovery Rate. (–) Down regulated.

Table 19 Group C virulence factors genes regulation

Locus tag	1h			12h			24h			Description
	FC	p-value	FDR	FC	p-value	FDR	FC	p-value	FDR	
SAOUHSC_01115	55.7	2.64E-15	2.36E-13	2.6	0.02	0.05	8.5	1.78E-06	1.16E-05	immune evasion
SAOUHSC_01112	51.2	5.68E-32	1.09E-28	2.2	0.003	0.012	1.4	0.16	0.25	formyl peptide receptor-like 1 inhibitory protein
SAOUHSC_02161	42.8	1.72E-25	6.18E-23	2.3	0.005	0.017	5.5	5.82E-08	5.17E-07	MHC class II analog protein
SAOUHSC_00715	22.9	3.78E-18	5.70E-16	2.82	0.001	0.004	4.6	2.73E-06	1.69E-05	response regulator
SAOUHSC_02706	19.2	4.79E-08	7.98E-07	1.8	0.20	0.33	5.2	0.0009	0.003	immunoglobulin G-binding protein Sbi
SAOUHSC_00714	19	4.35E-15	3.55E-13	2.2	0.018	0.049	4.4	1.29E-05	6.85E-05	sensor histidine kinase SaeS
SAOUHSC_01110	13.4	5.0E-06	4.91E-05	3	0.03	0.078	5.2	0.003	0.01	fibrinogen-binding protein-like protein
SAOUHSC_02802	7.3	2.61E07	3.63E-06	1.7	0.13	0.25	1.3	0.47	0.58	fibronectin binding protein B
SAOUHSC_02566	5.65	2.03E-06	2.23E-06	3.7	0.0002	0.003	2.3	0.017	0.041	SarR
SAOUHSC_02710	5.1	1.98E-05	0.0001	1.1	0.69	0.81	4.6	6.41E-05	0.0002	leukocidin f subunit
SAOUHSC_02333	4.8	0.0001	0.0008	1.6	0.20	0.33	2.6	0.01	0.03	transglycosylase SceD
SAOUHSC_02171	3.2	0.28	0.44	-1.5	0.70	0.82	-12	0.0004	0.001	staphylokinase
SAOUHSC_02708	2.8	0.001	0.005	1	0.88	0.95	-1.1	0.60	0.70	gamma-hemolysin h-gamma-II subunit
SAOUHSC_01467	2.2	0.01	0.04	1	0.46	0.61	3.1	7.05E-05	0.0003	penicillin-binding protein 2

(FC) fold change. (FDR) False Discovery Rate. (–) Down regulated.

Table 20 Group D virulence factors genes regulation

Locus tag	1 h			12 h			24 h			Description
	FC	p-value	FDR	FC	p-value	FDR	FC	p-value	FDR	
SAOUHSC_01952	1.994	0.003	0.012	-2.1	0.001	0.005	1.05	0.87	0.81	lantibiotic epidermin biosynthesis protein EpiB
SAOUHSC_01561	1.931	0.493	0.651	2	0.489	0.632	8	0.15	0.089	PVL orf 50-like protein
SAOUHSC_00394	1.87	0.005	0.020	2.46	0.0005	0.002	-2.2	0.0004	0.001	superantigen-like protein
SAOUHSC_00812	1.87	0.022	0.062	3	7.97e-05	0.0004	-7.1	4.57e-10	2.57e-11	clumping factor
SAOUHSC_01135	1.5	0.156	0.286	-194	2.04e-42	2.92e-39	7.43	4.89e-38	5.13e-41	phenol-soluble modulin1
SAOUHSC_01705	1.50	0.098	0.198	1.3	0.274	0.420	-1.98	0.0147	0.005	enterotoxin family protein
SAOUHSC_01949	1.47	0.136	0.257	-1	0.993	1	1.18	0.61	0.506	intracellular serine protease
SAOUHSC_01939	1.42	0.205	0.348	-1.7	0.062	0.132	1.8	0.07	0.036	serine protease SplC
SAOUHSC_01938	1.24	0.449	0.610	-1.6	0.084	0.170	1.68	0.13	0.073	serine protease SplD
SAOUHSC_01954	1.23	0.504	0.661	1.14	0.677	0.799	-1.36	0.43	0.32	leukotoxin LukD
SAOUHSC_01136	1.23	0.438	0.599	-168.8	1.09e-50	2.88e-47	75.1	2.02e-37	2.82e-40	phenol-soluble modulin2
SAOUHSC_01936	1.07	0.766	0.868	-1.52	0.12	0.22	1.53	0.19	0.11	serine protease SplE
SAOUHSC_01941	1.06	0.842	0.916	-1.63	0.145	0.260	1.32	0.50	0.39	serine protease SplB
SAOUHSC_01935	1.06	0.842	0.916	-1.3	0.416	0.574	1.32	0.46	0.35	serine protease SplF

(FC) fold change. (FDR) False Discovery Rate. (–) Down regulated.

6.2.5 Effect of sublethal concentration of mupirocin on the gene expression throughout time points in the treated cultures

Gene expression throughout the time points was recorded and comparisons made relative to each other in order to observe the effect of the sublethal concentration of mupirocin on gene expression.

Firstly, comparison data set of differentially expressed genes was for 12 h transcriptome results relative to 1 h. Among 2784 genes, 526 (18.3%) showed a significant difference in expression which indicates high similarity between these two time points which also can be seen in the heatmap arrow (B; Figure 44). Up-regulated genes were 265 (50.3%) and 261 (49.6%) were down-regulated.

Comparison between 24 h and 1 h transcriptome data showed an increased number of the differentially expressed genes 897 (31.3%) where 374 genes (41.7%) were positively regulated and 523 genes (58.3%) were negatively regulated. The third measurement was between the transcript data for 24 h relative to 12 h and the differentially expressed genes were 1060 which was 37% of the total transcriptome data. It is worth noting that a high similarity between 12 h and 1 h is reflected clearly in the percentage of the differentially expressed genes among 24/1h and 24/12h data sets (Figure 46).

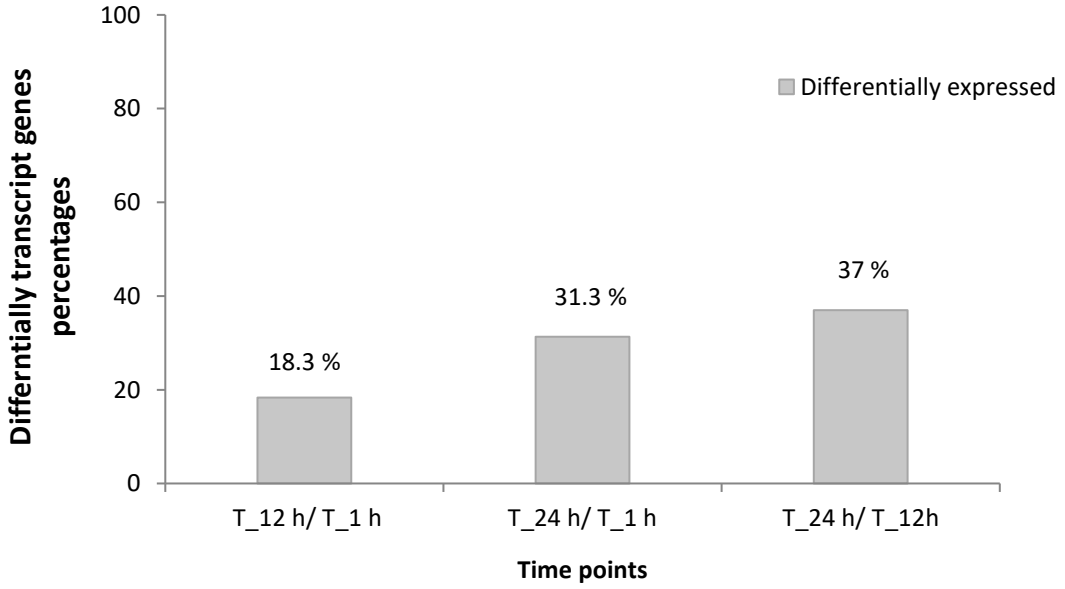


Figure 46 total differentially expressed genes in mupirocin treated cells (T) of *S. aureus* 8325-4 at different times after exposure to mupirocin in the treated cultures

6.2.6 Unique and overlapped genes expression

Overlapping and unique differentially expressed genes were determined and illustrated in Figure 47. Comparison between 12 / 1 h and 24 / 1 h data showed 43 overlapped expressions of up-regulated genes and 83 genes were down-regulated. In addition, overlapping of differentially expressed genes between 24/1 h and 24/12 h had occurred, 309 genes were down-regulated and 259 up-regulated. Moreover, 35 genes expression showed overlap in all three sets of data (Figure 47). Among them, 28 genes were up-regulated and 7 were down-regulated.

However, unique differentially expressed genes were spotted in the three data sets. For example, 12/1 h results showed 215 up-regulated and 150 down-regulated genes. Also, 206 and 65 genes where negatively and positively regulated respectively, in 24/ 1h comparison set. Highest number of unique gens occurred in 24h/12h data when 249 were positively induced and 208 were negatively controlled (Figure 47).

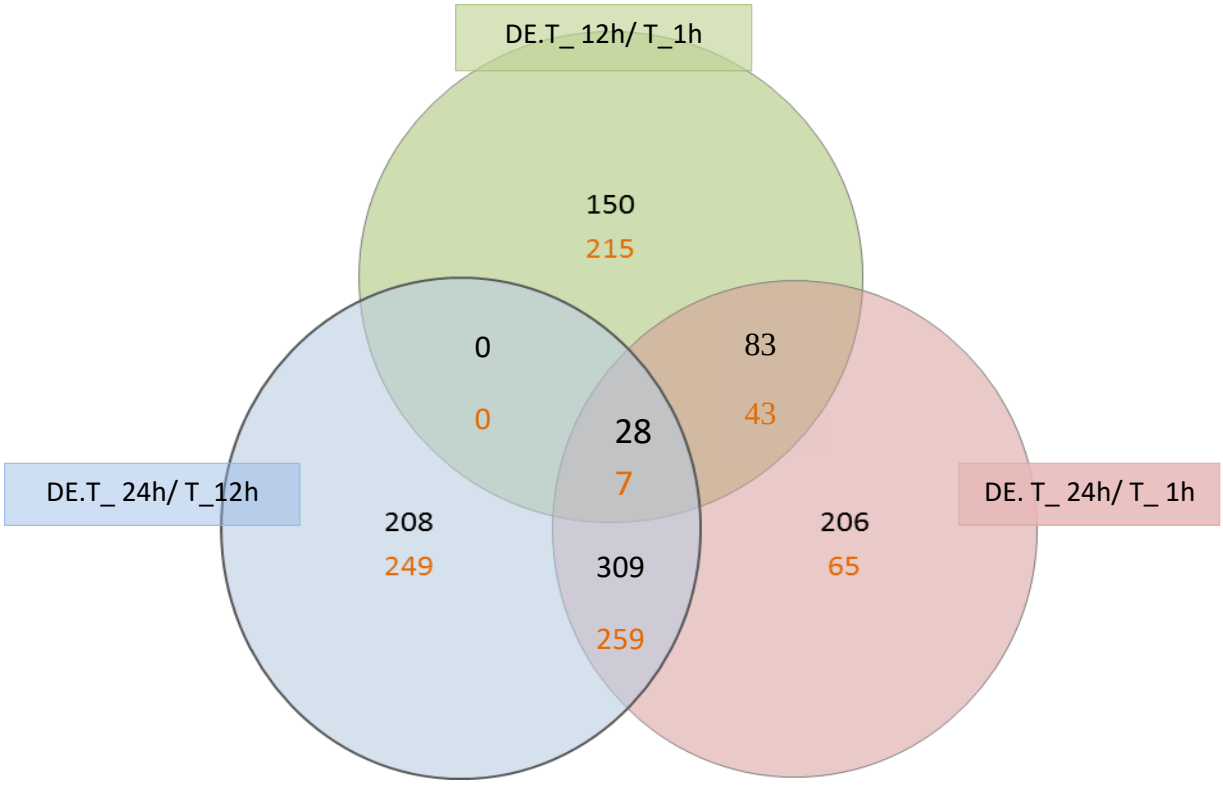


Figure 47 differentially expressed genes of *S. aureus* 8325-4 strains treated with sub-lethal concentration of mupirocin for 1, 12 and 24 h.

Key: Up-regulated red , down-regulated in black. All genes were subjected to a twofold differential expression cut-off at FDR ≤0.05 DE =differentially expressed, T= test sample.

6.2.7 Functions of unique and overlapping genes

Genes that showed overlapping and unique expression profiles were studied in more detail by investigating their functions to obtain a clearer picture of their expression throughout the three time points. For instance, overlap of differentially expressed genes between 12 / 1 h and 24 / 1 h data revealed that 43 overlapping up-regulated genes were involved in different functions and molecular processes such as nucleotide and nucleoside binding, ATPase activity and DNA metabolic processes. Conversely, 83 negatively regulated genes were involved in carboxylic acid processes, cellular amino acid biosynthesis and regulation of transcription overlapped.

Furthermore, overlapping of 259 positively regulated genes had occurred between 24/1 h and 24/12 h. Some of these genes were involved in nucleotide, nucleoside and nucleobase biosynthetic processes, transmembrane, phosphorylation, energy production and ATP synthesis and transport. On the other hand, 309 genes that were involved in DNA metabolism and binding, carbohydrate transport and galactose metabolism were down-regulated.

Moreover, overlap of 28 down-regulated genes in all sets of data was observed Table 21. Several of these genes involved in amino acid biosynthesis, nitrogen compound processes and ABC transporter activities (Figure 48). In contrast, only 7 genes were positively controlled through these data groups some of them were involved in amine biosynthetic process and cellular amino acid derivative metabolic process (Figure 48) and three of them coding for hypothetical proteins (Table 21). It is important to note that genes that encoded for unknown function proteins represented the highest proportion of these genes with overlapping expression profiles among all data sets above.

However, among differentially expressed gene groups of them showed no overlapping in the three comparisons sets. For example, 12/1 h results showed 215 genes were positively transcribed and some were involved in ion and metal binding, stress response and cellular carbohydrate metabolism also 150 negatively induced genes were reported in this set that involved in different function include nitrogen compound biosynthetic process, nucleotide biosynthetic process, phosphate metabolism and two component systems (Figure 48). Results of 24/1 displayed 65 positively activated genes contribute in DNA metabolic process and binding, signals, cell membrane activity and nucleotides binding. In addition, 206 genes were negatively expressed in this set and some of them were involved in transcription regulation , DNA binding, carboxylic acid processes, cellular amino acid biosynthesis, Nitrogen compound biosynthetic process. Moreover, 249 genes that involved in energy production, hydrogen ion transport and phosphorylation and other functions were up-regulated in 24/12

cooperation set whereas, 208 genes contribute in different function such as amino acid transport and metabolism, ion binding, glycolysis, cofactor and coenzyme catabolic process were down-regulated.

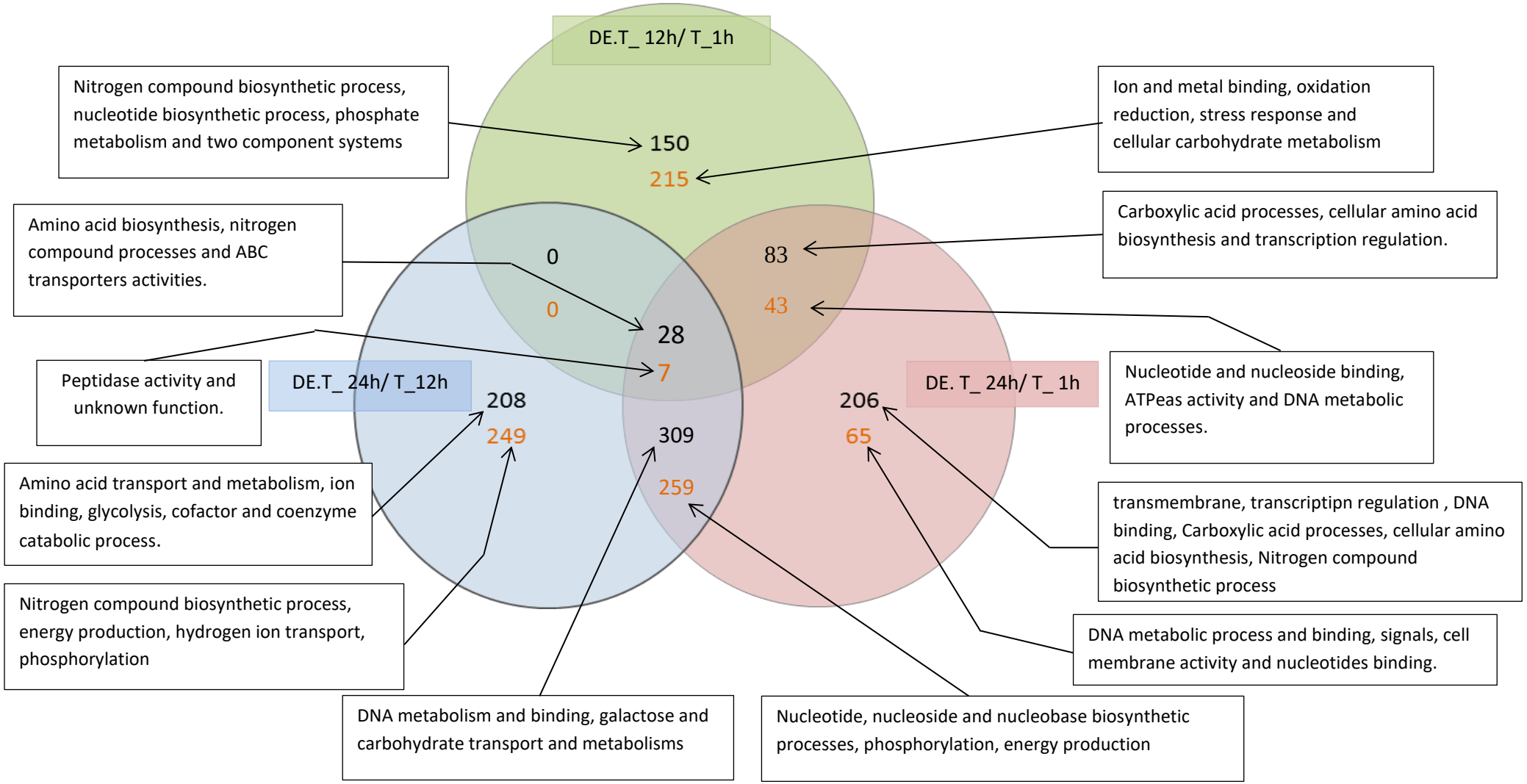


Fig 48 differentially expressed genes of *S.aureus* 8325-4 strains treated with sub-lethal concentration of mupirocin for 1, 12 and 24 h in contracts for each other(up-regulated in orange, down-regulated in black).

All genes were subjected to a twofold differential expression cut-off at FDA ≤0.05, DE =differentially expressed, T= test sample.

Table 21 Down-regulated genes with overlapping expression between1, 12 and 24 h in cells treated with mupirocin

Locus tag	Log_2FC.P_12h/P_01h	Log_2FC.P_24h/P_01h	Log_2FC.P_24h/P_12h	Description
SAOUHSC_02821	-3.977941175	-5.877848673	-1.899907498	membrane spanning protein
SAOUHSC_02820	-3.428483047	-5.136049803	-1.707566756	hypothetical protein
SAOUHSC_00069	-2.931446441	-5.552842891	-2.62139645	protein A
SAOUHSC_00070	-2.148894144	-3.621500987	-1.472606844	accessory regulator-like protein
SAOUHSC_00010	-2.131739265	-3.46063222	-1.328892955	hypothetical protein
SAOUHSC_00012	-2.079906748	-3.204339165	-1.124432416	hypothetical protein
SAOUHSC_00844	-2.021422707	-4.560196467	-2.53877376	hypothetical protein
SAOUHSC_00354	-1.985741158	-3.655028707	-1.669287549	hypothetical protein
SAOUHSC_02756	-1.979295444	-6.060329121	-4.081033677	hypothetical protein
SAOUHSC_00842	-1.850067143	-4.484710613	-2.63464347	ABC transporter ATP-binding protein
SAOUHSC_02757	-1.828043916	-5.96305768	-4.135013764	hypothetical protein
SAOUHSC_01159	-1.732399582	-2.894024985	-1.161625403	isoleucyl-tRNA synthetase
SAOUHSC_00167	-1.695239595	-4.825830101	-3.130590506	peptide ABC transporter ATP-binding protein

(FC) fold change.(p) treated cell with mupirocin.

Table 21, (continued) Down-regulated genes with overlapping expression between 1, 12 and 24 h in cells treated with mupirocin.

Locus tag	Log_2FC.P_12h/P_01h	Log_2FC.P_24h/P_01h	Log_2FC.P_24h/P_12h	Description
SAOUHSC_00843	-1.681073287	-4.456045096	-2.774971809	hypothetical protein
SAOUHSC_00424	-1.617393935	-5.542062461	-3.924668525	ABC transporter permease
SAOUHSC_01396	-1.514932166	-3.826305602	-2.311373436	dihydrodipicolinate synthase
SAOUHSC_00717	-1.502964184	-3.243040647	-1.740076463	hypothetical protein
SAOUHSC_01398	-1.412403513	-3.33084284	-1.918439327	tetrahydropyridine-2-carboxylate N-succinyltransferase
SAOUHSC_01322	-1.362524116	-3.632662051	-2.270137935	homoserine kinase
SAOUHSC_02803	-1.356948563	-3.650587966	-2.293639403	fibronectin-binding protein
SAOUHSC_02704	-1.341508438	-4.123448248	-2.781939811	hypothetical protein
SAOUHSC_01835	-1.282238036	-2.805534286	-1.52329625	hypothetical protein
SAOUHSC_00825	-1.274624343	-2.363821735	-1.089197392	hypothetical protein
SAOUHSC_02922	-1.242271622	-2.973801478	-1.731529856	L-lactate dehydrogenase
SAOUHSC_02755	-1.232184445	-3.631839405	-2.39965496	hypothetical protein
SAOUHSC_02692	-1.157100186	-3.632861121	-2.475760935	hypothetical protein
SAOUHSC_00989	-1.126055473	-3.121480569	-1.995425096	hypothetical protein
SAOUHSC_01964	-1.044134361	-2.717998459	-1.673864099	hypothetical protein

(FC) fold change.(p) treated cell with mupirocin.

Table 22 Up-regulated genes with overlapping expression between 1, 12 and 24 h in cells treated with mupirocin.

Locus tag	Log_2FC.P_12h/P_01h	Log_2FC.P_24h/P_01h	Log_2FC.P_24h/P_12h	Description
SAOUHSC_02933	1.723713716	4.35289399	2.629180274	betaine aldehyde dehydrogenase
SAOUHSC_02932	1.381943964	4.824866303	3.442922339	choline dehydrogenase
SAOUHSC_01255	1.279436349	2.800045076	1.520608727	hypothetical protein
SAOUHSC_01257	1.256345315	2.775002766	1.51865745	hypothetical protein
SAOUHSC_00120	1.242429351	3.89892197	2.656492619	UDP-N-acetylglucosamine 2-epimerase
SAOUHSC_01256	1.158286253	3.014956836	1.856670583	hypothetical protein
SAOUHSC_01611	1.088060446	2.186102915	1.098042469	2-oxoisovalerate dehydrogenase%2C E2 component%2C dihydrolipoamide acetyltransferase

(FC) fold change.(p) treated cell with mupirocin.

6.3 Discussion

6.3.1 Differentially expressed genes relative to control

6.3.1.1 Differentially expressed genes at 1 h

At the first time point (1 h), differentially expressed genes were 869 of the total of 2860 genes. More than half of these differentially expressed genes, 55.12%, were positively regulated and 44.8% were negatively regulated. From the up-regulated genes, some of them were involved in amino acid biosynthesis process and genes encoding products that have the ability to degrade host tissue or digest extracellular proteins were observed with 2 fold changes Table 12. Induction of these genes during the stress response may be attributed to the deactivation of CodY repressive activity as a result of decreased level of the CodY ligand, GTP, during (p)ppGpp synthesis processes (Figure 50) (Geiger *et al.*, 2012, Pohl *et al.*, 2009).

Up-regulation of amino acid biosynthesis and protease genes is in line with previous work that used mupirocin to trigger the stringent response (Reiß *et al.*, 2011, Geiger *et al.*, 2010). Positive induction of these genes might indicate how treated cells cope with amino acid limitation, mimicked by mupirocin treatment, by increasing free cellular amino acids levels through increasing their biosynthetic processes as well as providing them from the surrounding environment.

Moreover, groups of stress adaption and regulation genes were up-regulated, such as *spxA* (4.5 fold), which plays a role in transcription inhibition and *SAOUHSC 01819* (13 fold) that encodes one of the universal stress proteins family which enhances cell survival rate when it exposed to prolonged stress conditions. This has been reported by Drumm to help to establish chronic persistent infection in *M. tuberculosis* (Drumm *et al.*, 2009).Toxin antitoxin system genes, *SAOUHSC 02692*(8 fold) and *SAOUHSC 02757* (16 fold) were also up-regulated, which is also a classical feature of stress response (Reiß *et al.*, 2011). Furthermore, Hfq gene, which

encodes host factor 1 protein, that is involved in stress response and virulence factors in *S.aureus* was increased 3.6 fold. Most of these genes have been shown to be up-regulated during different stress response conditions, such as oxidative stress, nutrient deprivation, acidic and alkaline shocks, and their activation mechanism is not yet known (Anderson *et al.*, 2006, Reiß *et al.*, 2011). However, recent work has revealed that, during amino acid starvation, the universal stress protein gene *SAOUHSC 01819* seems to be up-regulated independently from CodY in *S. aureus* (Geiger *et al.*, 2012). Increasing transcription of these genes can obviously indicate the huge alteration in genes regulation that is needed to adjust the cell physiology and its molecular functions in order to allow the cell to survive in this condition.

Nevertheless, the highest proportions of the positively expressed genes encoded hypothetical proteins (63.8%), which reflect the lack of knowledge of the stress response process. Rieb and his colleagues have suggested that hypothetical proteins might play a role in adapting cell physiology to stress response (Reiß *et al.*, 2011). Another possibility is that they might contribute in regulating gene transcription, translation and other biological processes that need to be tuned with the surrounding environmental conditions.

On the other hand, there were 390 significantly differently down-regulated genes at the first time point. Among these genes 63.5% were known to be involved in essential biological activity, such as replication, transcription, translation machineries, RNA and DNA pathway precursors, metabolic pathways and transport systems, which is in agreement with previous authors (Geiger *et al.*, 2012, Geiger *et al.*, 2010a, Reiß *et al.*, 2011) who have reported down-regulation of replication, transcription, translation machinery genes after mupirocin treatment. In *B.subtilis* most of rRNA gene transcription starts with guanosine nucleotides, which can subsequently be influenced via GTP intracellular levels (Krásný and Gourse, 2004). Furthermore, the transcriptional start of two rRNA operons in *S.aureus* have been mapped and have confirmed GTP's role in initiating their primary promoters, which can explain the GTP

effect on their expression (Geiger *et al.*, 2012, Krásný *et al.*, 2008). Down-regulation of these genes is highly conserved in Gram positive bacteria during the stringent response which can justify the stalled cell growth at this time point,1 h , for the treated cells. Furthermore, functions of 36.4% of the genes differentially down-regulated are unknown yet. One can speculate that these genes may either contribute in the molecular functions mentioned above, or may be involved in repressive activity on stress response genes during normal conditions.

It is worth mentioning that Anderson and his colleagues claimed that inhibitory concentrations of mupirocin did not increase *relA* transcript titre appreciably (Anderson *et al.*, 2006). In this work transcription of the gene that is responsible for ppGpp synthesis, RSH was increased 1.2 relatively to control. Although this was not a significant increase according to the criteria used here, the hallmark of stringent response ppGpp was detected *via* direct detection using HPLC after 1 h of sublethal concentration of mupirocin treatment this low level of expression might be related to the level of the stringent response that caused by the sublethal mupirocin. Furthermore, up-regulation of genes that are involved in amino acid biosynthesis, transcription regulation, stress response, virulence factors and the down-regulation of genes that contribute to transcription, translation and replication are in agreement with previous observations using bactericidal concentrations of mupirocin (Anderson *et al.*, 2006, Geiger *et al.*, 2012, Reiß *et al.*, 2011), which strongly supports the fact that the sublethal concentration of mupirocin used here was capable of triggering the stringent response during at least 1 h of treatment which has not been reported in previous studies and supports the HPLC results in chapter 4.

6.3.1.2 Differentially expressed genes at 12 h

Transcriptome analysis results for the second time point (12 h) showed some similarity to the 1 h results where 47.8% (229 genes) of the significantly differentially up-regulated genes during 1 h induction remained positively expressed at 12 h. Several up-regulated genes during this

time point were involved in amino acid biosynthesis and transport processes, universal stress proteins, toxin/antitoxin system, regulation genes and some virulence factors genes that reflect classical stringent response features. Positive regulation of these genes may indicate an extended effect of the stringent response on cell physiology that is particularly noticed on the slowly growing cells at 12 h. This finding is in line with Reib and colleagues (Reiß *et al.*, 2011), who speculated that secondary stress responses occurred when different genes that were involved in the stringent response in *S. aureus* such as IleS and adaption protein gene were induced at different times of stringent response state. Notably, transcription of the RSH gene (*SAOUHSC 1742*), encoding the enzyme (RelA/SpoT) that is responsible for both synthetase/hydrolase activity for (p)ppGpp, was up-regulated 1.5 fold at 12 h which is slightly higher than 1 h transcript (1.2 fold), but (p)ppGpp was not detectable by the SAX 10 column at this time point. This can lead to the assumption that the Rel/SpoT enzyme might be switched to hydrolase activity ON/synthetase activity OFF at this time point, which may explain the deficiency of its detection at 12 h.

In addition, a number of genes that were up-regulated only at 12 h were involved in different functions such as ion and cofactors binding, coenzyme metabolic process, nucleotide biosynthetic processes and glycolysis. Slow increasing OD at 12 h can be attributed to the induction of these genes. However, 66% (n=151 genes) of up-regulated genes during this time point encoded hypothetical proteins with unknown functions. One can speculate that some of these proteins may play a role in maintaining the cell physiology during the stringent response.

455 genes were down-regulated genes at this time point. Clusters of them genes involved in cell membrane and trans-membrane activities, two components transduction system, and phosphorylation which are consistent with the stringent response state.

6.3.1.3 Differentially expressed genes at 24 h

The effect of the sub-lethal concentration of mupirocin seemed to disappear after 24 h of exposure. Differentially expressed genes were 39.3% of the whole transcript: 637 genes were up-regulated and 488 down-regulated. Most of the positively expressed genes were involved in essential biological activity, such as replication, transcription, translation machineries, RNA and DNA pathway precursors, as the growth of the bacteria seems to be in the exponential phase. In addition, the heatmap for the transcription showed considerable similarity between transcriptome profile for the 12 h control and 24 h test cells which indicates that a treated cell with sublethal concentration of mupirocin after 24 h had recovered from the stringent response and resumed growth, This may explain previous observations by Edwards-Jones (thesis 1997) and Edwards-Jones and Foster (1994) that sub-inhibitory concentrations of mupirocin had no effect on growth after 24 h (Edwards-Jones and Foster, 1994b). Furthermore, stress response genes that had been up-regulated at 1 and 12 h, such as universal stress proteins, heat-inducible transcription repressor *HrcA*, toxin antitoxin system and adaptor protein genes, were down-regulated which may confirm cell recovery from the stress response. Restoring GTP levels might be one of the possibilities for cell recovery after stringent response particularly because genes such as *HprT* and *GmK* that contribute to GTP synthesis (Geiger and Wolz, 2014, Kriel *et al.*, 2012) were up-regulated (1.2 fold) and (2.4 fold) respectively.

6.3.2 Gene expression overlapping

Transcriptional observation on the three time points revealed gene overlapping among them for instance, at 1 and 12 h overlapping of up-regulated genes occurred for those that contribute to branched chain amino acid (BCAAs) biosynthesis processes, carboxylic acid and nitrogen compound biosynthetic processes, transcription regulation and cofactors binding activities. On the other hand, there was overlapping of negatively controlled genes that involved in variety of

functions such as nucleoside and nucleotide biosynthetic processes and binding, phosphate metabolism processes, trans-membrane and phosphorylation. The regulation manner of these genes is reported in previous works as being characteristic of alterations for the stringent response (Anderson *et al.*, 2006, Reiß *et al.*, 2011). This overlapping might be attributed to the positive induction for genes that contribute in the stringent response in particular, those involved in transcription regulation such as adaptor protein and heat-inducible transcription repressor *HrcA.* In addition, some genes showed constant level of transcription through these time points such as *MarR* family transcriptional regulator and genes involved in amino acid synthesis which can enhance the number of overlapping genes expression between these time points.

Secondary stress response was claimed by Rieb and colleagues after mupirocin treatment when they noted *IleS* transcription increased more rapidly after 10 min than after 60 min whereas some genes that contribute in stress protection or adaptation where transcription after 60 min was higher than after 10 min (Reiß *et al.*, 2011). In this work, adaptor protein and heat-inducible transcription repressor *HrcA* regulation increased at 12 h more than 1 h which is in agreement with their finding and the secondary stress response might increase the number of genes with overlapping transcription patterns between these time points.

In addition, among 1 and 24 h time point up-regulated genes, overlapping occurred for post-exponential phase genes such as cellular amino acid biosynthesis, two component system and virulence factors. The effects of decreased intracellular levels of the [GTP] during the stationary phase of growth for 24 h control cells, and rapid decrease of this nucleotide after 1 h of mupirocin treatment, a result of (p)ppGpp synthesis (Geiger *et al.*, 2010a, Reiß *et al.*, 2011), may be a reason for this overlapping, particularly when most of these genes are under CodY regulation which has been shown to be influenced by intracellular abundance of [GTP] (Geiger *et al.*, 2012). Moreover, negatively regulated overlapping genes expressions were reported for

exponential phase genes such as those engaged in carbohydrate metabolic processes, nucleotide and coenzyme binding and lactose metabolism. CodY loses its positive influence on carbon metabolism and pathway during the stress response and in the stationary phase, where the intracellular pool of GTP is decreased, which might affect carbohydrate metabolic processes (Sonenshein, 2005, Pohl *et al.*, 2009). Furthermore, recent work has shown that (p)ppGpp negatively controls genes that involved in physiological processes such as coenzyme biosynthesis, nucleotide binding and DNA replication in *S. aureus* and *B.subtilis* (Geiger *et al.*, 2012, Wang *et al.*, 2007) which may explain their down-regulation during the 1 h time point results.

6.3.4 Regulation of virulence factors genes

During the stress response, GTP concentration in the intracellular pool is reduced considerably through the ppGpp synthesis process as shown in the current work (chapter 4) and in other previous studies (Geiger *et al.*, 2010a, Reiß *et al.*, 2011). Decreased levels of GTP caused by mupirocin treatment (see chapter 4) would decrease CodY affinity to bind its target DNA, resulting in de-repression for amino acid biosynthesis and transport genes as well as many virulence factor genes in *S. aureus* (Majerczyk *et al*., 2010). Quorum sensing, particularly the *agr* locus and other virulence genes, are clearly influenced during the stringent response as a result of CodY deactivation, which might explain the correlation between nutrient limitation and virulence factors activation during the stress response (Majerczyk *et al.*, 2010) (Pohl *et al.*, 2009).

In this work, 60 genes that coded for proteins involved in diverse *S. aureus* pathogenesis such as stationary phase virulence factors including *agrBCDA*, *SarA*, enterotoxins and capsular polysaccharide biosynthesis protein Cap5B genes were up-regulated which is in line with previous studies (Geiger *et al.*, 2010a, Reiß *et al.*, 2011). Most of these genes are regulated

dependently on CodY protein via *agr* locus product RNAIII (Majerczyk *et al.*, 2010) and this finding is in line with the up-regulation of RNAIII genes in the clinical isolates of *S. aureus* (B49) in Chapter 5. However, all these genes were negatively transcribed after 12 h while cell OD begun to rise gradually. Intracellular levels of GTP may be increased at this point and thus CodY may have resumed its repressive activity on these genes. After that, at 24 h they were up-regulated again and some of them, such as *agrBCDA* and alpha-hemolysin, displayed large changes. Negative regulation in slow growing cells (12 h) and up-regulation at mid exponential growth (24 h) for these genes simulates their regulation in a normal condition that is tightly controlled by the growth phase. This finding along with the heatmap results where there was a similar transcriptome profile between 12 h control cells and 24 h test cells, both indicate cell recovery from the stringent response effect.

Exponential phase regulated virulence genes such as fibronectin-binding protein, fibrinogen-binding protein and catalase, were up-regulated at 1 h which also reported in previous work results (Reiß *et al.*, 2011), and the author suggested that during amino acid starvation, *S. aureus* binds to host tissue by these binding proteins then begins to produce some tissue destructive virulence factors such as alpha hemolysin and proteases to hydrolyse proteins in order to obtain the needed amino acids. Conversely, Wolz and colleagues 2009 have speculated that down-regulation of cell wall proteins due to *agr* locus repressive activity during the stringent response would give the cells the ability to escape from limited nutrition environments (Pohl *et al.*, 2009). It worth noting that, despite the repressive activity of RNAIII on some exponentially expressed virulence genes such as fibronectin-binding protein A (Novick, 2003). The transcription of this gene increased 45 fold at 1 h. *sar* gene was up-regulated at this time point, Wolze and colleagues (Wolz *et al.*, 2000) have shown that, SarA plays important role in *FnbpA* activation which might explain this result. Another explanation might be that RNAIII

repressive activity on these genes has been either weakened or deactivated during the stringent response.

Positive induction for these genes had occurred at 12 h as the cell OD increased. A previous study (Pohl *et al.*, 2009) showed that, catalase and intracellular adhesion proteins (*Ica*ADBC) are regulated by CodY dependently which may explain their up-regulation at this time point, they were then all negatively controlled when the growth rose dramatically at 24 h. This regulation manner at 12 h and 24 h can be attributed to their tight growth phase-dependent regulation pattern.

A third group of virulence genes were positively controlled throughout all the time points. Some of them were involved in binding such as fibronectin binding protein B *(SAOUHSC_02802*), in virulence such as staphylokinase (*SAOUHSC_02171*) and in response such as (*SAOUHSC_00715*). The changes in expression were the highest at 1 h and lowest at 12 h, and at 24 h they were intermediate. It is difficult to interpret this result but it is possible that genes of this group are under several regulators, so that each one of them affects expression at different growth phases/conditions. Another possibility might be that up-regulation of these genes is related to maintaining gene regulation and cell adaption to different environment conditions, besides their involvement above. However, it is crucial to remember that gene transcription does not imply translation to its protein as discussed in chapter 5 results earlier and opposite regulation between transcription and translation has been reported in a previous study (Reiß *et al.*, 2011).

Group D virulence factors genes were up-regulated at 1 h but the fold change of their transcription did not reach the threshold of the cut-off (2 fold) and were therefore not significant at this time point. Throughout the time points some of them showed a transcription manner similar to group A genes such as serine protease SplB,C,D F and phenol-soluble

modulins 1 and 2. Others displayed a transcription manner similar to group B such as clumping factor and enterotoxin family protein. However, the transcription level of some of these genes had reached a significant level of fold change at other time points such as clumping factor which increased 3 fold change at 12 h and phenol-soluble modulin1 at 24 h (7.4 fold). A recent study (Geiger *et al.*, 2012) showed that phenol-soluble modulins 1and 2 was regulated independently from CodY which may explain its different transcription level at 1 h in comparison to other stationary phase virulence genes in group A that are regulated by CodY . Furthermore, reasons for the insignificant level of transcription at 1 h for other genes are not clear but it is possible that these genes are induced via cofactors or regulators in excess nutrient conditions and, as a result of stringent response, these cofactors or regulators are inhibited which may reduce the transcription level of group D genes at 1 h.

In conclusion, the results suggest that the regulation of virulence genes in *S. aureus* during stringent response is related to CodY which, due to reduction in [GTP], loses its affinity to bind its target genes, such as the *agr* locus, leading to activated transcription of this locus with consequent up-regulation a variety of virulence genes via its effector RNAIII. On the other hand, restoring intracellular GTP/BCAAs to normal levels after recovery from the stringent response would reactivate CodY repressive activity on its targeted virulence genes, resulting in reduced transcription as reported above for stationary phase virulence genes at 12 h. A proposed model for this regulation is shown in Figure 49.

6.3.5 Differentially expressed genes for test cells

The effects of sublethal concentrations of mupirocin on cell growth and gene regulation throughout different time points was investigated in this part of the work.

ppGpp, was detected 1 h after mupirocin exposure which in line with previous data and HPLC analysis. A comparison between treated and untreated cell transcriptome results revealed that

remarkable repression of genes that were involved in various cellular functions, and processes such as transcription, translation, replication, energy production and transport had occurred, which may explain the stalled cell growth for treated cells after 1 h. Conversely, genes that were involved in stress response adaption, amino acid biosynthesis, and transports and virulence genes, were up-regulated.

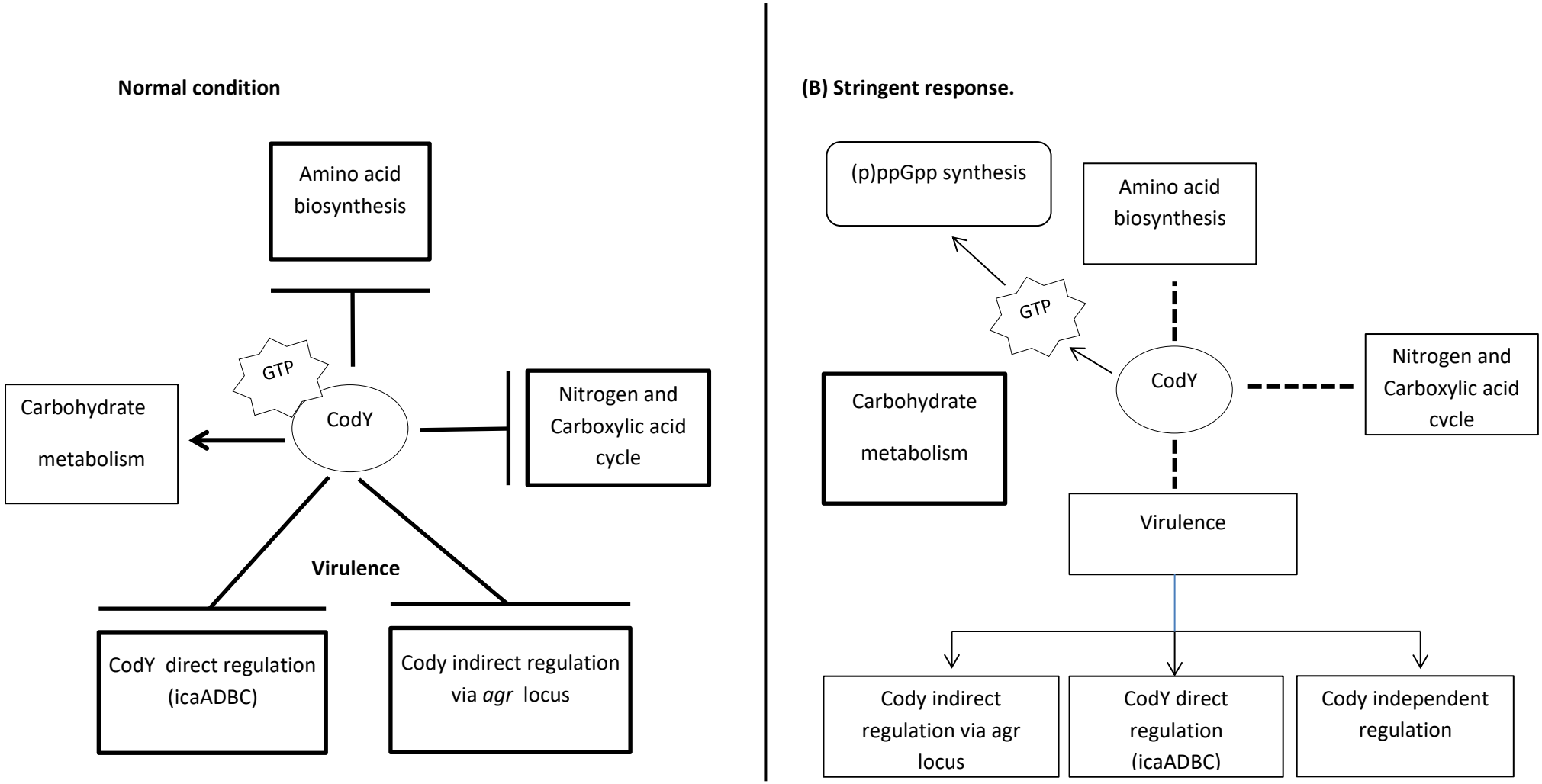


Figure 49 proposed model for effects of CodY on *S. aureus* gene expression. (A) In excess nutrient GTP induces CodY repressive activity on targeted genes (bold squares). **(B)** In stringent response condition, CodY loses its ligand, GTP which would deactivate its repressive activity on its target genes resulting in up-regulation of these genes. bold Arrowheads indicate positive induction and T-junctions indicate negative regulation. Dotted lines indicate CodY deactivated repressive activity .

The contrast between treated cells results shows that the 12 h transcriptome had similarities to 1 h, as the percentage of differentially expressed genes between the two was 18% of the complete transcriptome profile. In addition, visualising the transcriptome data on the heatmap, based on fold changes in gene expression displayed the similarity between these time points. It is worth noting that, transcription of *codY* gene (*SAOUHSC_01228*) was down-regulated at 1 and 12 h but increased at 24 h (2 fold). Down regulation of *codY* gene might reduce its repressive activity on the targeted genes during 1 and 12 h. Conversely, its positive transcription might play a role in restoring its repressive activity on its targeted genes. For example, several clusters of genes that were involved in amino acids including, BCAAs biosynthesis and metabolism processes, carboxylic acid system, and nitrogen component metabolism, were induced positively at 1 and 12 h. Most of these genes are under CodY regulation (Geiger *et al.*, 2012, Pohl *et al.*, 2009, Majerczyk *et al.*, 2010), up-regulation of these genes at these time points would increase the similarity between their transcriptome profiles.

Nevertheless, some transcriptional regulators genes that were up-regulated during 1 h, such as *SAOUHSC_02664*, *SAOUHSC_00934* and host factor protein gene *SAOUHSC_01281* (regulates transcription of some virulence genes), were significantly down-regulated at 12 h. Furthermore, several clusters of genes that were involved in molecular functions, such as carbohydrate transport and galactose metabolism, energy, DNA, nucleotide metabolic and binding processes were considerably up-regulated at 12 h only, which may contribute to eliciting the increasing growth at this time point. Cells at 12 h are clearly entering recovery processes from the stringent response, which might explain these results. More details about the stringent response recovery are discussed below.

Differentially expressed genes rate increased up to 31.3% and 37% in a contrast between 24/1h and 24/12h respectively. Genes that were activated at 1 and 12 h, as mentioned above, were

negatively controlled with overlapping, in particular, for these were significantly increase during stringent response and that can be attributed to cell growth at 24 h. Conversely, genes that were involved in replication, trans-membrane activity, nucleotide, nucleoside and nucleobase biosynthetic processes were positively activated. In addition, the increasing in OD at 24 h and up-regulation of these genes can indicate that cell recovery from the stringent response had occurred.

6.3.6 Stringent response tolerance and recovery

Most of the previous studies on triggering the stringent response in *S. aureus* via mupirocin were not able to observe cell tolerance and recovery from stringent response as they used bactericidal concentration of mupirocin that lead to cell death (Crosse *et al.*, 2000a, Anderson *et al.*, 2006, Reiß *et al.*, 2011), However, resuming growth after mupirocin treatment was reported by Edwards-Jones (thesis 1997) and Edwards-Jones and Foster (1994) when they claimed that sub-inhibitory concentrations of mupirocin had no effect on growth after 24 h (Edwards-Jones and Foster, 1994b) which agrees with the results of the current work.

Here, treated cell growth increased slowly after 12 h of sub-lethal concentration of mupirocin, from OD_{600} 0.5 after 1 h to 2.6 after 12 h. This growth was associated with decreased level of RSH transcript, and ppGpp was not detected at 12 h, which suggests that (p)ppGpp hydrolysis had occurred. The reason for down-regulated transcription of RSH is not clear yet but one can assume that stopping ppGpp synthesis can be achieved via reducing its inducer, deacylated tRNA in the A-site of ribosome that is caused by mupirocin treatment. As described in chapter 1 (section 1.2.1) mupirocin works as a reversible competitive enzyme inhibitor that inhibits the synthesis of the isoleucyl-tRNA synthase *IleS*, which plays a crucial role in catalysing the transfer of isoleucine onto its cognate tRNA. Recovery from such competitive enzyme inhibitor

effect can be achieved *via* increasing either the concentration of the targeted enzyme, *IleS*, or its substrate, isoleucine (Hughes and Mellows, 1978b).

In recent study *IleS* coding gene was reported as one of the highest up-regulated genes after mupirocin treatment (Reiß *et al.*, 2011). In this work, transcriptome data revealed that the coding gene for *IleS* (*SAOUHSC_01159*) was up-regulated at 1, 12 and 24 h time points (9.7, 4.3 and 5.2 fold) respectively, which may suggest that a cell treated with mupirocin is trying to overcome the inhibitory effect of mupirocin by producing higher concentrations of *IleS* enzyme than its inhibitor. Moreover, the remarkable up-regulation for genes that are involved in BCAAs (isoleucine, valine and leucine) cellular biosynthesis and metabolism processes, as well as genes that coding for proteases and amino acid transport, support the assumption stated earlier that *S. aureus* tries to compensate for the lack of amino acids quickly via cellular biosynthesis as well as through importing amino acids from the surrounding environment or host tissue. Indeed, early increase of BCAAs was detected after 30 min of mupirocin treatment (Reiß *et al.*, 2011), which is in agreement with the gene transcription results above.

In addition, genes that are involved in tRNA metabolism and processes, such as *SAOUHSC_01726* and *SAOUHSC_01599*, were up-regulated at 12 h, increasing 2.9 and 7.4 fold respectively, which show that the cells are producing new tRNA instead of impeded tRNA in the A-site of ribosomal proteins. Also, more than 30 genes that encoded for ribosomal proteins were significantly up-regulated after 24 h which can be related to compensating blocked ribosomes with un-charged tRNA in order to resume the machinery function. However, Shyp and colleagues 2012 have suggested that increasing amino acid concentration and aminocylated tRNA would resume translation (Shyp *et al.*, 2012). Indeed, activation of genes that are involved in transcription, translation, replication, energy production and transport were seen in the transcriptome data for 12 h, 24 h or both, and can be reflected on the cell growth at these time points. Early study on *E.coli* stringent response claimed that cessation

of (p)ppGpp synthesis and accumulation would occur as long as ribosomes are actively engaged in protein synthesis which might interpret the undetectable (p)ppGpp at these time points (Cashel and Gallant, 1969). Activation of these genes mentioned above, can be attributed to either losing ppGpp repressive activity on them after its hydrolysis (Geiger *et al.*, 2012, Reiß *et al.*, 2011) or might be related to increasing GTP intracellular level via up-regulation of its synthetic genes. For example, genes that contribute to GTP synthesis were up-regulated at 12 and 24 h including *HprT* (1.2 fold) , *GmK* (2.4 fold) and (1.2 fold), (2.4 fold) respectively.

All these alterations in different genes functions mentioned above suggest a scenario that could explain how the cells tolerate the stringent conditions as well as reprograming gene regulation to recover and resume its growth and replication. A model for these processes is shown in Figure 50.

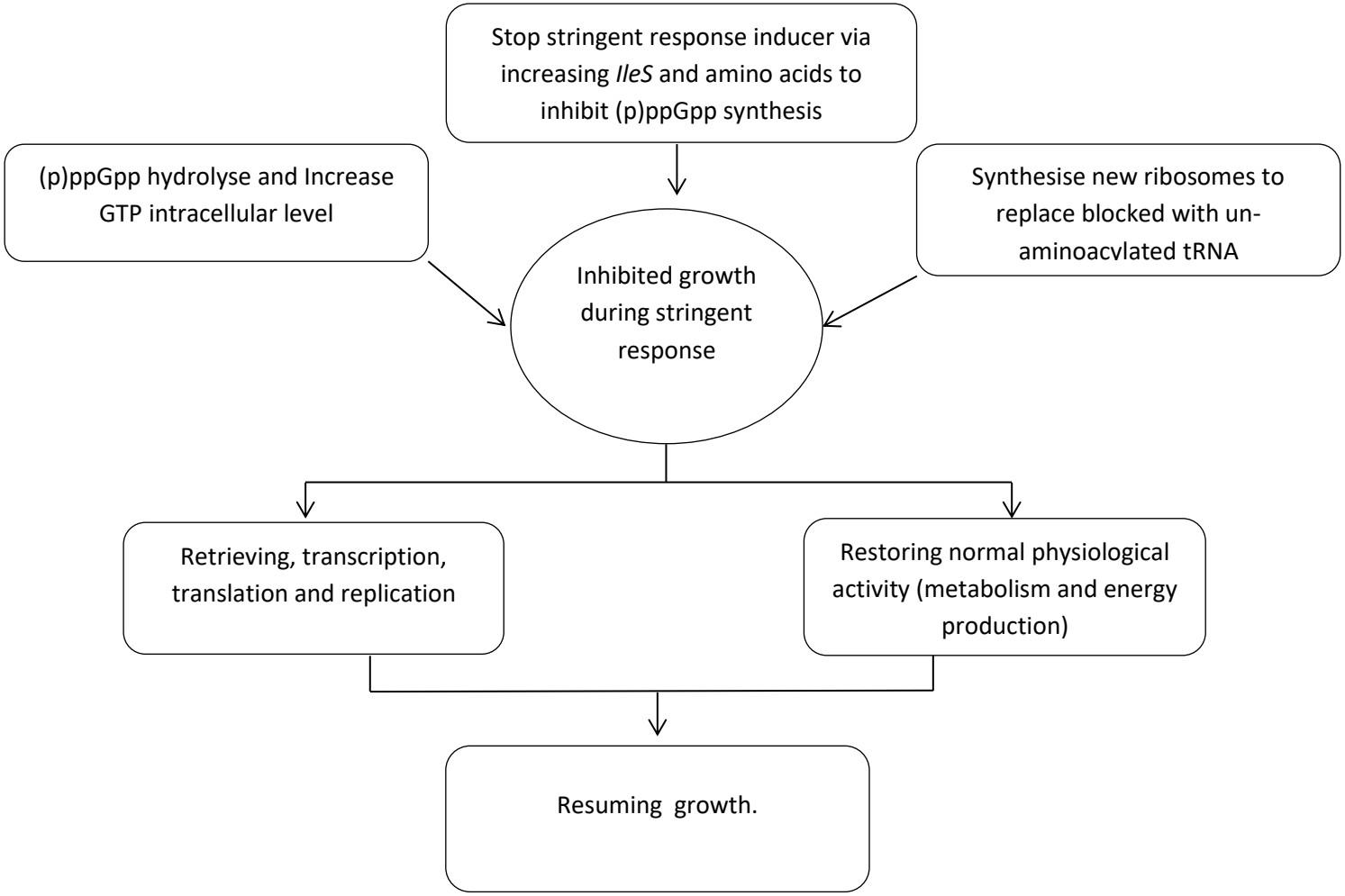


Figure 50 proposed mechanisms that can result in tolerance and recovery processes from the stringent response after treatment with sublethal concentrations of mupirocin.

Chapter 7

General discussion and conclusion

7 General discussion and conclusions

The results in this study show that the increased clinical use of mupirocin in the UK has not lead to a gross increase in mupirocin resistance in MRSA isolates although the sample size was relatively small. However there was evidence of mupirocin resistance in MRSA isolates from Libya. The difference may be that use of mupirocin to eliminate carriage before elective surgery is carefully controlled and the patients are checked to confirm clearance and are subjected to further treatment if unsuccessful (Coates *et al.*, 2009). Such careful use helps to prevent resistance and resistant strains may be eliminated by other treatments. In contrast in Libya, antibiotics are available without prescription and may be used indiscriminately by the general public to treat a variety of skin conditions. A similar increase in resistance was seen in New Zealand when mupirocin was made available over the counter (Upton *et al.*, 2003). Reduction in availability can led to a reduction in resistance as seen in Australia (Torvaldsen *et al.*, 1999). These results suggest that careful stewardship e.g. control of availability and careful use of mupirocin is needed to prevent increased prevalence of resistance.

Relatively few previous studies have been carried out on the stringent response in *S. aureus.* The majority of these studies triggered the stringent response either by depleting branched chain amino acids from growth media or *via* adding inhibitory concentrations of mupirocin (Cassels *et al.*, 1995, Crosse *et al.*, 2000).

The present study has confirmed that sub-inhibitory concentrations of mupirocin were also able to trigger the stringent response. For the first time, the ability of sub-inhibitory concentrations to inhibit TSST-1 synthesis and global effects on gene expression were studied by RT-PCR and RNA-seq analysis respectively. Even at sub-inhibitory concentrations there were significant phenotypic and genotypic alterations during the first few hours of treatment including growth inhibition, ppGpp synthesis and global

transcriptional alterations. Similar alterations had been reported when the stringent response was triggered by bactericidal concentrations of mupirocin in previous studies (Anderson *et al.*, 2006, Geiger *et al.*, 2010a, Reiß *et al.*, 2011). Furthermore, observation of the effects of this concentration was continued for 12 and 24 h after mupirocin addition and cells showed gradual increasing growth which indicates that the cells were able to recover from the stringent response, something not possible with cells treated with inhibitory concentrations as this leads to cell death.

In addition, a TSST-1 producing clinical isolate of *S. aureus* (B49) was been treated with the sub-inhibitory concentration of mupirocin to investigate the effects of this concentration on TSST-1 production. Substantial changes relatively to the control had appeared including growth inhibition, early transcription of the toxin gene (and RNAIII) but toxin synthesis was delayed which was also the result of the stringent response.

7.1 Summary of the key finding in stringent response effects

The sub-inhibitory concentration of mupirocin in this work was capable of inducing the stringent response and several features of stringent response were seen after the treatment.

7.1.1 Effect on cell growth

Significant growth inhibition was seen through the first 4 h of mupirocin exposure compared to control cells. That this was due to the stringent response was confirmed by detection of ppGpp which probably causes this effect by affecting DNA replication as has been shown in *B. subtilis* and *E.coli* (Schreiber *et al.*, 1995a, Levine *et al.*, 1991). However, the effective mechanism of ppGpp on DNA replication in *S. aureus* is unknown at the present, but transcriptome profile for the treated cell at 1 h showed down-regulation for genes that are involved in nucleoside biosynthetic processes and DNA replication including genes coding for replication initiation

proteins and DNA gyrase subunit A-B. These findings are in line with the decreased cell density seen at 1 h in treated cells compared to controls.

7.1.2 Effect on gene transcription

Transcriptional activities are affected after mupirocin treatment and differentially expressed genes reached 30.3% of the total transcriptome. GTP pool concentrations play a crucial role in transcription alteration and an obvious decrease in GTP intracellular pool was seen up to 4 h which was probably associated with ppGpp synthesis. GTP is consumed during ppGpp synthesis and ppGpp also inhibit enzymes that are involved in GTP synthesis pathway at the posttranscriptional level which can explain the decrease in the intracellular pool of GTP (Kriel *et al.,* 2012).

A high GTP intracellular pool is important for many genes that need this nucleotide to initiate their transcription such as rRNA proteins genes (Krásný and Gourse, 2004, Tojo *et al.*, 2010). Further effect of the GTP pool in transcription can be seen on the virulence genes regulation as the metabolic responsive protein CodY loses its repressive activity on virulence genes which leads to transcriptional activation for global virulence genes regulatory systems and proteins such as *agr* system and SarA (Majerczyk *et al.*, 2010). The transcriptome profile during this time in this work showed up-regulation of more than 50 genes that involved in *S. aureus* virulence factors.

7.1.3 Effect on gene translation

Protein synthesis is affected during then stringent response in *S. aureus* which can be related to several events. For example, mupirocin plays a role in inhibiting translation as accumulated uncharged tRNA binds to A-site of the ribosome and obstructs the translation machinery (Hughes and Mellows, 1978a). In addition, an abundant GTP pool is important for the translation processes as GTP is utilized during initiation and translocation steps of

translation (Luchin *et al.*, 1999, Laalami *et al.*, 1996). Transcriptional data showed down-regulation of genes that encode translation initiation factor 2 (IF2). Furthermore, de-activation of genes that encode for ribosomal proteins as results of declined GTP pool would significantly affect the translation processes.

In general, after sub-inhibitory concentration of mupirocin addition, significant growth inhibition and global transcriptional alterations as well as negative influence on translation key factors such as GTP pool and IF2, in *S. aureus* 8325-4 was seen.

These changes on the cell growth, transcription and the effect on translation key factors that occurred after exposure to sub-inhibitory concentration of mupirocin are typical features of the stringent response and they are adequate evidences to claim that, this concentration is capable to trigger the stringent response in *S. aureus* 8325-4.

7.2 Recovery from stringent response

Triggering the stringent response in *S. aureus* in previous studies was performed by using bactericidal concentration of mupirocin which resulted in cell death few hours of treatment and squandered the opportunity to verify the cell ability to recover from stringent response with exception of Edward-Junes and Foster work (Edwards-Jones and Foster, 1994b). However, in the current work treated cells with sub-inhibitory concentration of mupirocin showed slow and gradual growth after 12 h of exposure then the growth reached expositional phase 24 h which indicated cell recovery from stringent response.

Transcriptome profile at these time points showed several indications which might allow production of a scenario for recovery. For example, significant activation for clusters of genes that are involved in different functions including amino acid biosynthesis and transport, protease activity and (IleS) gene all may antagonise the effects of mupirocin. Products of

these genes might tackle the stringent response induction by decreasing the accumulation of uncharged tRNA which is the trigger for ppGpp synthesis.

Another group of genes activated such as genes that code for enzymes that facilitate the GTP biosynthesis pathway and which can increase the GTP intracellular pool and subsequently increase translation machinery activity. Furthermore, increasing the GTP pool would restore the global regulatory protein CodY activity either in positive manner such as for genes that involved in carbohydrate metabolism to facilitate growth (Shivers *et al.*, 2006), or in negative manner such as repressing the virulence factor transcription and other stationary phase genes (Pohl *et al.*, 2009).

Nevertheless, it is essential to verify the synthesis of these genes product in order to support this scenario. However, as mentioned above cells density at these time points increased slightly at 12 h and exponential at 24 which is in line, in general, with their transcriptome profiles.

7.3 Sub-inhibitory concentration influence on TSST-1 production

The clinical isolate and TSST-1 producer of *S. aureus* was also exposed to a sub-inhibitory concentration of mupirocin to verify the effect of this concentration on TSST-1 production. A prolonged lag phase occurred between 1 and 8 h then the cells OD increased dramatically through 16 and 24 h in comparison to control cells. Transcriptomic results by q-PCR showed increased transcription ratio had occurred for *tst* and its regulator RNAIII during the inhibited growth (1, 8 h). These genes are regulated in a growth-dependent manner and they are usually transcribed in the post-exponential phase of growth also Toxin synthesis was inhibited as no toxin was detected during growth inhibition despite the upregulation of transcription.

7.4 Future work

A proteomics study would provide a more complete picture of the effect of the sub-inhibitory concentration of mupirocin in *S. aureus*. This is particularly for those virulence genes that to determine whether increased transcription leads to increased production of e.g. toxins. Two-dimensional gel electrophoresis (2D-E) and Mass spectrometry (MS) can be used to detected protein synthesis. Also, confirmation for RNA-seq results for some important genes in the stringent response such as *relA* and *IleS* as well as *codY* and *sar* genes that involved in virulence factors regulation in *S. aureus* during stringent response via RT-PCR would be useful.

7.5 General conclusion

Sub-inhibitory concentrations of mupirocin were able to trigger the stringent response in *S. aureus* 8325-4. Although the stringent response and consequent reduction in the GTP pool induce virulence factors transcription, significant negative effects on the transcription of translation machinery elements was observed and toxin synthesis was reduced. In addition, observation of the effects of this concentration of mupirocin on TSST-1 production showed delayed synthesis of TSST-1 despite its early transcription. Delaying toxin synthesis might cause an immediate reduction of pathogenicity of the bacterium in particular for diseases caused by toxin production. Also, quick growth inhibition caused by the sub-inhibitory concentration of mupirocin can contribute effectively in treatment combination with other antibacterial agent that would target other points such as cell wall synthesis. Thus in clinical use even if some cells are not exposed to inhibitory concentrations, the inhibition of growth will contribute to the overall clearance of the organism.

References

Alarcon, B., Vicedo, B. & Aznar, R. 2006. PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. *Journal of Applied Microbiology,* 100**,** 352-364.

Anderson, K. L., Roberts, C., Disz, T., Vonstein, V., Hwang, K., Overbeek, R., Olson, P. D., Projan, S. J. & Dunman, P. M. 2006. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *Journal of Bacteriology,* 188**,** 6739-6756.

Andrey, D. O., Renzoni, A., Monod, A., Lew, D. P., Cheung, A. L. & Kelley, W. L. 2010. Control of the *Staphylococcus aureus* toxic shock tst promoter by the global regulator SarA. *Journal of Bacteriology,* 192**,** 6077-6085.

Anfinsen, C. B. 1968. Characterization of *staphylococcal* nuclease and the status of studies on its chemical synthesis. *Pure and Applied Chemistry,* 17**,** 461-488.

Antonio, M., Mcferran, N. & Pallen, M. J. 2002. Mutations affecting the Rossman fold of isoleucyl-tRNA synthetase are correlated with low-level mupirocin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 46**,** 438-442.

Antoun, A., Pavlov, M. Y., Andersson, K., Tenson, T. & Ehrenberg, M. 2003. The roles of initiation factor 2 and guanosine triphosphate in initiation of protein synthesis. *The EMBO Journal,* 22**,** 5593-5601.

Archer, G. L. 1998. *Staphylococcus aureus*: A Well-Armed Pathogen. *Clinical Infectious Diseases,* 26**,** 1179-1181.

Argudín, M. Á., Mendoza, M. C. & Rodicio, M. R. 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins,* 2**,** 1751-1773.

Arnoud, H. M and van Vliet. 2010. Next generation sequencing of microbial transcriptomes: challenges and opportunities. *FEMS Microbiology Letters*, 302, 1-7.

Artsimovitch, I., Patlan, V., Sekine, S.-I., Vassylyeva, M. N., Hosaka, T., Ochi, K., Yokoyama, S. & Vassylyev, D. G. 2004. Structural Basis for Transcription Regulation by Alarmone ppGpp. *Cell,* 117**,** 299-310.

Arvidson, S. & Tegmark, K. 2001. Regulation of virulence determinants in *Staphylococcus aureus*. *International Journal of Medical Microbiology,* 291**,** 159-170.

Baker, T. A. & Bell, S. P. 1998. Polymerases and the replisome: machines within machines. *Cell,* 92**,** 295-305.

Barker, M. M., Gaal, T., Josaitis, C. A. & Gourse, R. L. 2001. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. *Journal of Molecular Biology,* 305**,** 673-688.

Bernheimer, A. & Schwartz, L. L. 1963. Isolation and composition of *staphylococcal* alpha toxin. *Journal of General Microbiology,* 30**,** 455-468.

Bhakdi, S., Muhly, M. & Füssle, R. 1984. Correlation between toxin binding and hemolytic activity in membrane damage by *staphylococcal* alpha-toxin. *Infection and Immunity,* 46**,** 318-323.

Bhatia, A. & Zahoor, S. 2007. *Staphylococcus aureus* enterotoxins: A review. *J Clin Diag Res,* 3**,** 188-97.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bächi, B. & Projan, S. 2004. Microarray-based analysis of the S*taphylococcus aureus* σB regulon. *Journal of Bacteriology,* 186**,** 4085-4099.

Blomster-Hautamaa, D., Kreiswirth, B., Kornblum, J., Novick, R. & Schlievert, P. 1986. The nucleotide and partial amino acid sequence of toxic shock syndrome toxin-1. *Journal of Biological Chemistry,* 261**,** 15783-15786.

Bode, L. G., Kluytmans, J. A., Wertheim, H. F., Bogaers, D., Vandenbroucke-Grauls, C. M., Roosendaal, R., Troelstra, A., Box, A. T., Voss, A. & Van Der Tweel, I. 2010. Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. *New England Journal of Medicine,* 362**,** 9-17.

Bore, E., Langsrud, S., Langsrud, Ø., Rode, T. M. & Holck, A. 2007. Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis. *Microbiology,* 153**,** 2289-2303.

Borukhov, S., Lee, J. & Laptenko, O. 2005. Bacterial transcription elongation factors: new insights into molecular mechanism of action. *Molecular Microbiology,* 55**,** 1315-1324.

Bowler, P., Duerden, B. & Armstrong, D. 2001. Wound microbiology and associated approaches to wound management. *Clinical Microbiology Reviews,* 14**,** 244-269.

Braeken, K., Moris, M., Daniels, R., Vanderleyden, J. & Michiels, J. 2006. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends in Microbiology,* 14**,** 45-54.

Brantl, S. & Wagner, E. G. H. 2000. Antisense RNA-mediated transcriptional attenuation: an in vitro study of plasmid pT181. *Molecular Microbiology,* 35**,** 1469-1482.

Bronner, S., Monteil, H. & Prévost, G. 2004. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiology Reviews,* 28**,** 183-200.

Browning, D. F. & Busby, S. J. 2004. The regulation of bacterial transcription initiation. *Nature Reviews Microbiology,* 2**,** 57-65.

Bukowski, M., Wladyka, B. & Dubin, G. 2010. Exfoliative toxins of *Staphylococcus aureus*. *Toxins,* 2**,** 1148-1165.

Bussiere, D. E. & Bastia, D. 1999. Termination of DNA replication of bacterial and plasmid chromosomes. *Molecular Microbiology,* 31**,** 1611-1618.

Cadilla, A., David, M. Z., Daum, R. S. & Boyle-Vavra, S. 2011. Association of high-level mupirocin resistance and multidrug-resistant methicillin-resistant *Staphylococcus aureus* at an academic center in the midwestern United States. *Journal of Clinical Microbiology,* 49**,** 95-100.

Cashel, M. & Gallant, J. 1969. Two Compounds implicated in the Function of the RC Gene of *Escherichia coli*. *Nature,* 221**,** 838-841.

Cassels, R., Oliva, B. & Knowles, D. 1995. Occurrence of the regulatory nucleotides ppGpp and pppGpp following induction of the stringent response in *staphylococci*. *Journal of Bacteriology,* 177**,** 5161-5165.

Chain, E. B. & Mellows, G. 1974. Structure of pseudomonic acid, an antibiotic from *Pseudomonas fluorescens*. *J. Chem. Soc., Chem. Commun.***,** 847-848.

Chain, E. B. & Mellows, G. 1977. Pseudomonic acid. Part 1. The structure of pseudomonic acid A, a novel antibiotic produced by *Pseudomonas fluorescens*. *J. Chem. Soc., Perkin Trans. 1***,** 294-309.

Chang, D.-E., Smalley, D. J. & Conway, T. 2002. Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. *Molecular Microbiology,* 45**,** 289-306.

Chatterji, D. & Kumar Ojha, A. 2001. Revisiting the stringent response, ppGpp and starvation signaling. *Current Opinion in Microbiology,* 4**,** 160-165.

Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H. & Xiong, Y.-Q. 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunology & Medical Microbiology,* 40**,** 1-9.

Cheung, A. L., Nishina, K. A., Trotonda, M. P. & Tamber, S. 2008. The SarA protein family of *Staphylococcus aureus*. *The International Journal of Biochemistry & Cell Biology,* 40**,** 355-361.

Cheung, A.L., Schmidt, K., Bateman, B. and Manna, A.C. 2001. SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. Infection and Immunity, 69, 2448–2455.

Cheung, A. L. & Ying, P. 1994. Regulation of alpha-and beta-hemolysins by the *sar* locus of *Staphylococcus aureus*. *Journal of Bacteriology,* 176**,** 580-585.

Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T., Vandenesch, F. & Romby, P. 2010. *Staphylococcus aureus* RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. *PLoS Pathogens,* 6**,** 3 e1000809.

Christensen, S. K., Pedersen, K., Hansen, F. G. & Gerdes, K. 2003. Toxin–antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *Journal of Molecular Biology,* 332**,** 809-819.

Coates, T., Bax, R. & Coates, A. 2009. Nasal decolonization of *Staphylococcus aureus* with mupirocin: strengths, weaknesses and future prospects. *Journal of Antimicrobial Chemotherapy***,** 64, 9-15.

Coia, J., Duckworth, G., Edwards, D., Farrington, M., Fry, C., Humphreys, H., Mallaghan, C., Tucker, D. 2006. Guidelines for the control and prevention of meticillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. Journal of Hospital Infection, 63, 1-44

Collier, M. 2004. Recognition and management of wound infections. *World Wide Wounds***,** 1-10.

Cosgrove, K., Coutts, G., Jonsson, M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J. & Foster, S. J. 2007. Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 1025-1035.

Crosse, A. M., Greenway, D. & England, R. 2000a. Accumulation of ppGpp and ppGp in *Staphylococcus aureus* 8325-4 following nutrient starvation. *Letters in Applied Microbiology,* 31**,** 332-337.

Cunningham, L., Catlin, B. W. & De Garilhe, M. P. 1956. A Deoxyribonuclease of *Micrococcus pyogenes*1. *Journal of the American Chemical Society,* 78**,** 4642-4645.

Dalebroux, Z. D., Svensson, S. L., Gaynor, E. C., Swanson, M.S. 2010. ppGpp conjures bacterial virulence. *Microbiology and Molecular Biology Reviews*, 74, 171–199.

David Greenwood, Richard Slack and John Peutherer (2002). Medical Microbiology. 16th ed. London, England : Churchill Livingstone. 690.

Denapoli, J., Tehranchi, A. K. & Wang, J. D. 2013. Dose-dependent reduction of replication elongation rate by (p) ppGpp in *Escherichia coli* and *Bacillus subtilis*. *Molecular Microbiology,* 88**,** 93-104.

Diekema, D. J., Pfaller, M. A., Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N. & Beach, M. 2001. Survey of infections due to *Staphylococcus* dpecies: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the western pacific region for the SENTRY antimicrobial surveillance program, 1997–1999. *Clinical Infectious Diseases,* 32**,** S114-S132.

Dinos, G., Kalpaxis, D. L., Wilson, D. N. & Nierhaus, K. H. 2005. Deacylated tRNA is released from the E site upon A site occupation but before GTP is hydrolyzed by EF-Tu. *Nucleic Acids Research,* 33**,** 5291-5296.

Doery, H. M., Magnusson, B., Cheyne, I. & Sulasekharam, J. 1963. A phospholipase in staphylococcal toxin which hydrolyses sphingomyelin. *Nature*, 198**,** 1091-1092.

Driscoll, D. G., Young, C. L. & Ochsner, U. A. 2007. Transient loss of high-level mupirocin resistance in *Staphylococcus aureus* due to *MupA* polymorphism. *Antimicrobial Agents and Chemotherapy,* 51**,** 2247-2248.

Drumm, J. E., Mi, K., Bilder, P., Sun, M., Lim, J., Bielefeldt-Ohmann, H., Basaraba, R., So, M., Zhu, G. & Tufariello, J. M. 2009. *Mycobacterium tuberculosis* universal stress protein Rv2623 regulates bacillary growth by ATP-Binding: requirement for establishing chronic persistent infection. *PLoS Pathogens,* 5**,** 5 e1000460.

Durand, G., Bes, M., Meugnier, H., Enright, M. C., Forey, F., Liassine, N., Wenger, A., Kikuchi, K., Lina, G., Vandenesch, F. & Etienne, J. 2006. Detection of new Methicillin-Resistant *Staphylococcus aureus* clones containing the Toxic Shock Syndrome Toxin 1 gene responsible for hospital- and community-acquired infections in France. *Journal of Clinical Microbiology,* 44**,** 847-853.

Dziejman, M. & Mekalanos, J. J. 1995. Two-component signal transduction and its role in the expression of bacterial virulence factors. *Two-component signal transduction. ASM Press, Washington, DC***,** 305-317.

Edwards-Jones, V. & Foster, H. A. 1994. The effect of topical antimicrobial agents on the production of toxic shock syndrome toxin-1. *Journal of Medical Microbiology,* 41**,** 408-13.

Edwards-Jones, V. Toxic shock syndrome toxin in relation to burned patients. PhD thesis. 1997.

Edwards-Jones, V. & Foster, H. A. 2002. Effects of silver sulphadiazine on the production of exoproteins by *Staphylococcus aureus*. *Journal of Medical Microbiology,* 51**,** 50-55.

Elek, S. D. 1959. *Staphylococcus pyogenes* and its relation to Disease. *Ulster Medical Journal,* 28, 75-76

Ellis, M. W., Griffith, M. E., Dooley, D. P., McLean, J. C., Jorgensen, J. H., Patterson, J. E., *et al*. 2007. Targeted intranasal mupirocin to prevent colonization and infection by community-associated methicillin-resistant *Staphylococcus aureus* strains in soldiers: a cluster randomized controlled trial. *Antimicrobial Agent Chemotherapy,* 5, :3591-3598

Engels, W., Kamps, M. & Van Boven, C. 1978. Influence of cultivation conditions on the production of staphylocoagulase by *Staphylococcus aureus* 104. *Journal of General Microbiology,* 109**,** 237-243.

Eron, L. & Block, R. 1971. Mechanism of Initiation of Repression of In Vitro Transcription of the Lac Operon of *Escherichia coli*. *Proceedings of the National Academy of Sciences,* 68**,** 1828-1832.

Evelina, T., Yehuda, C., Anthony, A., Gabriela, F., Marilyn, F., and Erika, A. 2003. Mupirocin Prophylaxis to Prevent *Staphylococcus aureus* Infection in Patients Undergoing Dialysis: A Meta-analysis. *Clinical Infectious Diseases*, 37, 1629–1638.

Fosheim, G., Nicholson, A., Albrecht, V. & Limbago, B. 2011. Multiplex real-time PCR assay for detection of methicillin-resistant *Staphylococcus aureus* and associated toxin genes. *Journal of Clinical Microbiology,* 49**,** 3071-3073.

Foster, T. J. 2005. Immune evasion by *staphylococci*. *Nature Reviews Microbiology,* 3**,** 948-958.

Foster, T. J. & Höök, M. 1998. Surface protein adhesins of *Staphylococcus aureus*. *Trends in Microbiology,* 6**,** 484-488.

Foster, T. J. & Mcdevitt, D. 1994. Surface-associated proteins of *Staphylococcus aureus*: Their possible roles in virulence. *FEMS Microbiology Letters,* 118**,** 199-205.

Fraser, J. D. & Proft, T. 2008. The bacterial superantigen and superantigen-like proteins. *Immunological Reviews,* 225**,** 226-243.

Fu, Z., Donegan, N. P., Memmi, G. & Cheung, A. L. 2007. Characterization of MazFSa, an endoribonuclease from *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 8871-8879.

Fuda, C., Suvorov, M., Vakulenko , S.B., & Mobashery, S. 2004. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *Journal of Biological Chemistry,* 279, 40802–40806.

Geiger, T., Francois, P., Liebeke, M., Fraunholz, M., Goerke, C., Krismer, B., Schrenzel, J., Lalk, M. & Wolz, C. 2012. The stringent response of *Staphylococcus aureus* and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. *PLoS Pathogens,* 8**,** e1003016.

Geiger, T., Goerke, C., Fritz, M., Schäfer, T., Ohlsen, K., Liebeke, M., Lalk, M. & Wolz, C. 2010a. Role of the (p) ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. *Infection and Immunity,* 78**,** 1873-1883.

Geiger, T., Goerke, C., Fritz, M., Schäfer, T., Ohlsen, K., Liebeke, M., Lalk, M. & Wolz, C. 2010. Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. *Infection and Immunity,* 78**,** 1873-1883.

Geiger, T. & Wolz, C. 2014. Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. *International Journal of Medical Microbiology,* 304**,** 150-155.

Gentry, D., Li, T., Rosenberg, M. & Mcdevitt, D. 2000. The rel gene is essential for in vitro growth of *Staphylococcus aureus*. *Journal of Bacteriology,* 182**,** 4995-4997.

Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B. & Cashel, M. 1993. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. *Journal of Bacteriology,* 175**,** 7982-7989.

Gerdes, K., Christensen, S. K. & Løbner-Olesen, A. 2005. Prokaryotic toxin–antitoxin stress response loci. *Nature Reviews Microbiology,* 3**,** 371-382.

Giacometti, A., Cirioni, O., Schimizzi, A., Del Prete, M., Barchiesi, F., D'errico, M., Petrelli, E. & Scalise, G. 2000. Epidemiology and microbiology of surgical wound infections. *Journal of Clinical Microbiology,* 38**,** 918-922.

Gilbart, J., Perry, C. R. & Slocombe, B. 1993. High-level mupirocin resistance in *Staphylococcus aureus*: evidence for two distinct isoleucyl-tRNA synthetases. *Antimicrobial Agents and Chemotherapy,* 37**,** 32-38.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J.-C., Lina, G., Bes, M., Vandenesch, F., Piémont, Y., Brousse, N. & Floret, D. 2002. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *The Lancet,* 359**,** 753-759.

Görke, B. & Stülke, J. 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nature Reviews Microbiology,* 6**,** 613-624.

Gould, F., Brindle, R., Chadwick, P., Fraise, A., Hill, S., Nathwani, D. et al. 2009. Guidelines (2008) for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the United Kingdom. *Journal of Antimicrobial Chemotherapy*, 63, 849–861.

Götz, F. 2002. *Staphylococcus* and biofilms. *Molecular Microbiology,* 43**,** 1367-1378.

Greenway, D. & England, R. 1999. ppGpp accumulation in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* subjected to nutrient limitation and biocide exposure. *Letters in Applied Microbiology,* 29**,** 298-302.

Gross, C., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J. & Young, B. 1998. The functional and regulatory roles of sigma factors in transcription. Cold Spring Harbor symposia on quantitative biology. *Cold Spring Harbor Laboratory Press*, 141-156.

Gualerzi, C. O. & Pon, C. L. 1990. Initiation of mRNA translation in prokaryotes. *Biochemistry,* 29**,** 5881-5889.

Gurney, R. & Thomas, C. M. 2011. Mupirocin: biosynthesis, special features and applications of an antibiotic from a Gram-negative bacterium. *Applied Microbiology and Biotechnology,* 90**,** 11-21.

Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. 1996. Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infection and Immunity,* 64**,** 3512-3517.

Han, L. L., Mcdougal, L. K., Gorwitz, R. J., Mayer, K. H., Patel, J. B., Sennott, J. M. & Fontana, J. L. 2007. High frequencies of clindamycin and tetracycline resistance in methicillin-resistant *Staphylococcus aureus* pulsed-field type USA300 isolates collected at a Boston ambulatory health center. *Journal of Clinical Microbiology,* 45**,** 1350-1352.

Handke, L. D., Shivers, R. P. & Sonenshein, A. L. 2008. Interaction of *Bacillus subtilis* CodY with GTP. *Journal of Bacteriology,* 190**,** 798-806.

Haseltine, W. A. & Block, R. 1973. Synthesis of guanosine tetra-and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proceedings of the National Academy of Sciences,* 70**,** 1564-1568.

Hawiger, J., Timmons, S., Strong, D., Cottrell, B., Riley, M. & Doolittle, R. 1982. Identification of a region of human fibrinogen interacting with *staphylococcal* clumping factor. *Biochemistry,* 21**,** 1407-1413.

Hayes, F. & Van Melderen, L. 2011. Toxins-antitoxins: diversity, evolution and function. *Critical Reviews in Biochemistry and Molecular Biology,* 46**,** 386-408.

Heinzelmann, M., Scott, M. & Lam, T. 2002. Factors predisposing to bacterial invasion and infection. *The American Journal of Surgery,* 183**,** 179-190.

Hetem, D. J. & Bonten, M. J. M. 2013. Clinical relevance of mupirocin resistance in *Staphylococcus aureus*. *Journal of Hospital Infection,* 85**,** 249-256.

Higgins, J., Loughman, A., Van Kessel, K. P., Van Strijp, J. A. & Foster, T. J. 2006. Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiology Letters,* 258**,** 290-296.

Hodgson, J., Curnock, S., Dyke, K., Morris, R., Sylvester, D. & Gross, M. 1994. Molecular characterization of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870. *Antimicrobial Agents and Chemotherapy,* 38**,** 1205-1208.

Hogg, T., Mechold, U., Malke, H., Cashel, M. & Hilgenfeld, R. 2004. Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. *Cell,* 117**,** 57-68.

Horner, C., Parnell, P., Hall, D., Kearns, A., Heritage, J. & Wilcox, M. 2013. Meticillin-resistant *Staphylococcus aureus* in elderly residents of care homes: colonization rates and molecular epidemiology. *Journal of Hospital Infection,* 83**,** 212-218.

Hu, C., Xiong, N., Zhang, Y., Rayner, S. & Chen, S. 2012. Functional characterization of lipase in the pathogenesis of *Staphylococcus aureus*. *Biochemical and Biophysical Research Communications,* 419**,** 617-620.

Hughes, J. & Mellows, G. 1978a. Inhibition of isoleucyl-transfer ribonucleic acid synthetase in italic *Echerichia coli* by pseudomonic acid. *Biochem. J,* 176**,** 305-318.

Hughes, J. & Mellows, G. 1978b. On the mode of action of pseudomonic acid: inhibition of protein synthesis in *Staphylococcus aureus*. *The Journal of Antibiotics,* 31**,** 330-335.

Hughes, J. & Mellows, G. 1980. Interaction of pseudomonic acid A with *Escherichia coli B* isoleucyl-tRNA synthetase. *Biochem. J,* 191**,** 209-219.

Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B. & Ehresmann, C. 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *The EMBO Journal,* 24**,** 824-835.

Huseby, M., Shi, K., Brown, C. K., Digre, J., Mengistu, F., Seo, K. S., Bohach, G. A., Schlievert, P. M., Ohlendorf, D. H. & Earhart, C. A. 2007. Structure and biological activities of beta toxin from *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 8719-8726.

Imlay, J. A. 2003. Pathways of oxidative damage. *Annual Reviews in Microbiology,* 57**,** 395-418.

Janda, J., Abbott, S. & Brenden, R. 1997. Overview of the etiology of wound infections with particular emphasis on community-acquired illnesses. *European Journal of Clinical Microbiology and Infectious Diseases,* 16**,** 189-201.

Ji, G., Beavis, R. C. & Novick, R. P. 1995. Cell density control of *staphylococcal* virulence mediated by an octapeptide pheromone. *Proceedings of the National Academy of Sciences,* 92**,** 12055-12059.

Kamio, Y., Rahman, A., Nariya, H., Ozawa, T. & Izaki, K. 1993. The two *staphylococcal* bi-component toxins, leukocidin and gamma-hemolysin, share one component in common. *FEBS Letters,* 321**,** 15-18.

Kaneko, J. & Kamio, Y. 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Bioscience, Biotechnology, and Biochemistry,* 68**,** 981-1003.

Kantor, H. S., Temples, B. & Shaw, W. V. 1972. *Staphylococcal* delta hemolysin: purification and characterization. *Archives of Biochemistry and Biophysics,* 151**,** 142-156.

Kapral, F. & Miller, M. M. 1971. Product of *Staphylococcus aureus* responsible for the scalded-skin syndrome. *Infection and Immunity,* 4**,** 541-545.

Kerrigan, S. W., Kaw, G., Hogan, M., Penadés, J., Litt, D., Fitzgerald, D. J., Foster, T. J. & Cox, D. 2002. Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine–aspartate repeat protein SdrE and protein A. *Molecular Microbiology,* 44**,** 1033-1044.
Kozak, M. 1999. Initiation of translation in prokaryotes and eukaryotes. *Gene,* 234**,** 187-208.

Krásný, L. & Gourse, R. L. 2004. An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *The EMBO Journal,* 23**,** 4473-4483.

Krásný, L., Tišerová, H., Jonák, J., Rejman, D. & Šanderová, H. 2008. The identity of the transcription+ 1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. *Molecular Microbiology,* 69**,** 42-54.

Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., Rendon, S., Chen, R., Tu, B. P. & Wang, J. D. 2012. Direct regulation of GTP homeostasis by (p) ppGpp: a critical component of viability and stress resistance. *Molecular Cell,* 48**,** 231-241.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., Golda, A., Maciag-Gudowska, A., Brix, K. & Shaw, L. 2008. A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One,* 3**,** e1409.

Kuehnert, M. J., Kruszon-Moran, D., Hill, H. A., Mcquillan, G., Mcallister, S. K., Fosheim, G., Mcdougal, L. K., Chaitram, J., Jensen, B. & Fridkin, S. K. 2006. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001–2002. *Journal of Infectious Diseases,* 193**,** 172-179.

Kvint, K., Farewell, A. & Nyström, T. 2000. RpoS-dependent Promoters Require Guanosine Tetraphosphate for Induction Even in the Presence of High Levels of σs. *Journal of Biological Chemistry,* 275**,** 14795-14798.

Kvint, K., Nachin, L., Diez, A. & Nyström, T. 2003. The bacterial universal stress protein: function and regulation. *Current Opinion in Microbiology,* 6**,** 140-145.

Laalami, S., Grentzmann, G., Bremaud, L. & Cenatiempo, Y. 1996. Messenger RNA translation in prokaryotes: GTPase centers associated with translational factors. *Biochimie,* 78**,** 577-589.

Lawellin, D. W., Franco-Buff, A. & Todd, J. K. 1989. Alteration of proteins reacting with antibody to toxic shock syndrome toxin 1 by endogenous proteases of *Staphylococcus aureus*. *Review of Infectious Diseases,* 11**,** S117-S124.

Lebeau, C., Vandenesch, F., Greenland, T., Novick, R. P. & Etienne, J. 1994. Coagulase expression in *Staphylococcus aureus* is positively and negatively modulated by an agr-dependent mechanism. *Journal of Bacteriology,* 176**,** 5534-5536.

Lebowitz, J. & Mcmacken, R. 1986. The *Escherichia coli* dnaB replication protein is a DNA helicase. *Journal of Biological Chemistry,* 261**,** 4738-4748.

Lemos, J. A., Nascimento, M. M., Lin, V. K., Abranches, J. & Burne, R. A. 2008. Global regulation by (p) ppGpp and CodY in *Streptococcus mutans*. *Journal of Bacteriology,* 190**,** 5291-5299.

Levine, A., Vannier, F., Dehbi, M., Henckes, G. & Séror, S. J. 1991. The stringent response blocks DNA replication outside the region in *Bacillus subtilis* and at the origin in *Escherichia coli*. *Journal of Molecular Biology,* 219**,** 605-613.

Lina, G., Gillet, Y., Vandenesch, F., Jones, M. E., Floret, D. & Etienne, J. 1997. Toxin involvement in staphylococcal scalded skin syndrome. *Clinical Infectious Diseases,* 25**,** 1369-1373.

Lina, G., Piémont, Y., Godail-Gamot, F., Bes, M., Peter, M.-O., Gauduchon, V., Vandenesch, F. & Etienne, J. 1999. Involvement of Panton-Valentine leukocidin—producing *Staphylococcus aureus i*n primary skin infections and pneumonia. *Clinical Infectious Diseases,* 29**,** 1128-1132.

Lindmark, R., Thorén-Tolling, K. & Sjöquist, J. 1983. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *Journal of Immunological Methods,* 62**,** 1-13.

Lindsay, J. A., Ruzin, A., Ross, H. F., Kurepina, N. & Novick, R. P. 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology,* 29**,** 527-543.

Livak, K., and Schmittgen, T. 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods,* 25,402-408.

Lopez, J. M., Dromerick, A. & Freese, E. 1981. Response of guanosine 5′-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. *Journal of Bacteriology,* 146**,** 605-613.

Low, D. K. & Freer, J. H. 1977. The purificaton of β-lysin (spingomyelinase C) from *staphylococcus aureus*. *FEMS Microbiology Letters,* 2**,** 139-143.

Lowy, F. D. 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. *Journal of Clinical Investigation,* 111**,** 1265-1273.

Luchin, S., Putzer, H., Hershey, J. W., Cenatiempo, Y., Grunberg-Manago, M. & Laalami, S. 1999. In vitro study of two dominant inhibitory GTPase mutants of *Escherichia coli* translation initiation factor IF2 direct evidemce that GTP hydrolysis is necessary for factor recycling. *Journal of Biological Chemistry,* 274**,** 6074-6079.

Magnusson, L. U., Farewell, A. & Nyström, T. 2005. ppGpp: a global regulator in *Escherichia coli*. *Trends in Microbiology,* 13**,** 236-242.

Magnusson, L. U., Nyström, T. & Farewell, A. 2003. Underproduction of ς70 Mimics a Stringent Response A PROTEOME APPROACH. *Journal of Biological Chemistry,* 278**,** 968-973.

Majerczyk, C. D., Dunman, P. M., Luong, T. T., Lee, C. Y., Sadykov, M. R., Somerville, G. A., Bodi, K. & Sonenshein, A. L. 2010. Direct targets of CodY in *Staphylococcus aureus*. *Journal of Bacteriology,* 192**,** 2861-2877.

Majerczyk, C. D., Sadykov, M. R., Luong, T. T., Lee, C., Somerville, G. A. & Sonenshein, A. L. 2008. *Staphylococcus aureus* CodY negatively regulates virulence gene expression. *Journal of Bacteriology,* 190**,** 2257-2265.

Malys, N. & Mccarthy, J. E. 2011. Translation initiation: variations in the mechanism can be anticipated. *Cellular and Molecular Life Sciences,* 68**,** 991-1003.

Marinas, K. 1992. Prokaryotic DNA replication. *Annual Review of Biochemistry,* 61**,** 673-719.

Marrack, P. & Kappler, J. 1990. The staphylococcal enterotoxins and their relatives. *Science,* 248**,** 705-711.

Matsunaga, T., Kamata, S.-I., Kakiichi, N. & Uchida, K. 1993. Characteristics of *Staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. *The Journal of Veterinary Medical Science/the Japanese Society of Veterinary Science,* 55**,** 297-300.

Mccormick, J. K., Yarwood, J. M. & Schlievert, P. M. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annual Reviews in Microbiology,* 55**,** 77-104.

Mcdanel, J. S., Murphy, C. R., Diekema, D. J., Quan, V., Kim, D. S., Peterson, E. M., Evans, K. D., Tan, G. L., Hayden, M. K. & Huang, S. S. 2013. Chlorhexidine and mupirocin susceptibilities of methicillin-resistant *Staphylococcus aureus* from colonized nursing home residents. *Antimicrobial Agents and Chemotherapy,* 57**,** 552-558.

Mcdevitt, D., Francois, P., Vaudaux, P. & Foster, T. 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Molecular Microbiology,* 11**,** 237-248.

Mcdevitt, D., Vaudaux, P. & Foster, T. 1992. Genetic evidence that bound coagulase of *Staphylococcus aureus* is not clumping factor. *Infection and Immunity,* 60**,** 1514-1523.

Mcneil, J. C., Hulten, K. G., Kaplan, S. L. & Mason, E. O. 2011. Mupirocin resistance in *Staphylococcus aureus* causing recurrent skin and soft tissue infections in children. *Antimicrobial Agents and Chemotherapy,* 55**,** 2431-2433.

Mechold, U., Murphy, H., Brown, L. & Cashel, M. 2002. Intramolecular Regulation of the Opposing (p)ppGpp Catalytic Activities of RelSeq, the Rel/Spo Enzyme from *Streptococcus equisimilis*. *Journal of Bacteriology,* 184**,** 2878-2888.

Melles, D. C., Van Leeuwen, W. B., Boelens, H., Peeters, J. K., Verbrugh, H. A. & Van Belkum, A. 2006. Panton-Valentine leukocidin genes in *Staphylococcus aureus*. *Emerg Infect Dis,* 12**,** 1174-5.

Messer, W. 2002. The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiology Reviews,* 26**,** 355-374.

Meyer, R. R. & Laine, P. S. 1990. The single-stranded DNA-binding protein of *Escherichia coli*. *Microbiological Reviews,* 54**,** 342.

Miller, M. B. & Bassler, B. L. 2001. Quorum sensing in bacteria. *Annual Reviews in Microbiology,* 55**,** 165-199.

Milon, P., Tischenko, E., Tomšic, J., Caserta, E., Folkers, G., La Teana, A., Rodnina, M. V., Pon, C. L., Boelens, R. & Gualerzi, C. O. 2006. The nucleotide-binding site of bacterial translation initiation factor 2 (IF2) as a metabolic sensor. *Proceedings of the National Academy of Sciences,* 103**,** 13962-13967.

Mohamed, O., Abdulbaset, A., Mohamed, A., Asma, E., Samira, A., Ezzeddin, A., et al . 2010. Misidentification of methicillinresistant *Staphylococcus aureus* (MRSA) in hospitals in Tripoli,Libya. *Libyan Journal of Medicine,* 5, 1-4.

Moreillon, P., Entenza, J. M., Francioli, P., Mcdevitt, D., Foster, T. J., Francois, P. & Vaudaux, P. 1995. Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infection and Immunity,* 63**,** 4738-4743.

Morfeldt, E., Taylor, D., Von Gabain, A. & Arvidson, S. 1995. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *The EMBO Journal,* 14**,** 4569.

Murray, D. K. & Bremer, H. 1996. Control ofspoT-dependent ppGpp Synthesis and Degradation in *Escherichia coli*. *Journal of Molecular Biology,* 259**,** 41-57.

Nakamura, Y. & Ito, K. 2003. Making sense of mimic in translation termination. *Trends in Biochemical Sciences,* 28**,** 99-105.

Nanamiya, H., Kasai, K., Nozawa, A., Yun, C. S., Narisawa, T., Murakami, K., Natori, Y., Kawamura, F. & Tozawa, Y. 2008. Identification and functional analysis of novel (p) ppGpp synthetase genes in *Bacillus subtilis*. *Molecular Microbiology,* 67**,** 291-304.

Nardi, G., Di Silvestre, A., De Monte, A., Massarutti, D., Proietti, A., Grazia Troncon, M., *et al*. 2001. Reduction in Gram-positive pneumonia and antibiotic consumption following the use of a SDD protocol including nasal and oral mupirocin. *European Journal of Emergency Medicine,* 8**,** 203–214.

Ní Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Höök, M. & Foster, T. J. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Molecular Microbiology,* 30**,** 245-257.

Novick, R., Projan, S., Kornblum, J., Ross, H., Ji, G., Kreiswirth, B., Vandenesch, F. & Moghazeh, S. 1995. Theagr P2 operon: An autocatalytic sensory transduction system in *Staphylococcus aureus*. *Molecular and General Genetics MGG,* 248**,** 446-458.

Novick, R. P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology,* 48**,** 1429-1449.

Novick, R. P., Ross, H., Projan, S., Kornblum, J., Kreiswirth, B. & Moghazeh, S. 1993. Synthesis of *staphylococcal* virulence factors is controlled by a regulatory RNA molecule. *The EMBO Journal,* 12**,** 3967.

Nudler, E. & Gottesman, M. E. 2002. Transcription termination and anti-termination in *E. coli*. *Genes to Cells,* 7**,** 755-768.

Nyström, T. 2004. Stationary-phase physiology. *Annual review of microbiology,* 58**,** 161-181.

Ohlsen, K., Koller, K.-P. & Hacker, J. 1997. Analysis of expression of the alpha-toxin gene (*hla*) of *Staphylococcus aureus* by using a chromosomally encoded *hla: lacZ* gene fusion. *Infection and Immunity,* 65**,** 3606-3614.

Ohlsen, K., Ziebuhr, W., Koller, K.-P., Hell, W., Wichelhaus, T. A. & Hacker, J. 1998. Effects of Subinhibitory Concentrations of Antibiotics on Alpha-Toxin (hla) Gene Expression of Methicillin-Sensitive and Methicillin-Resistant *Staphylococcus aureus* Isolates. *Antimicrobial Agents and Chemotherapy,* 42**,** 2817-2823.

Olson, M. E., Nygaard, T. K., Ackermann, L., Watkins, R. L., Zurek, O. W., Pallister, K. B., Griffith, S., Kiedrowski, M. R., Flack, C. E. & Kavanaugh, J. S. 2013. *Staphylococcus aureus* nuclease is an SaeRS-dependent virulence factor. *Infection and Immunity,* 81**,** 1316-1324.

O'Neill, A. J. 2010. *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Letters in Applied Microbiology*, 51, 358–361.

Pamp, S. J., Frees, D., Engelmann, S., Hecker, M. & Ingmer, H. 2006. Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*. *Journal of Bacteriology,* 188**,** 4861-4870.

Parenti, M., Hatfield, S. & Leyden, J. 1987. Mupirocin: a topical antibiotic with a unique structure and mechanism of action. *Clinical Pharmacy*, 6**,** 761-770.

Patel, J. B., Gorwitz, R. J. & Jernigan, J. A. 2009. Mupirocin Resistance. *Clinical Infectious Diseases*, 49**,** 935-941.

Paul, B. J., Berkmen, M. B. & Gourse, R. L. 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. *Proceedings of the National Academy of Sciences,* 102**,** 7823-7828.

Pfaffi ,M. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research,* 29,2003-2007.

Pohl, K., Francois, P., Stenz, L., Schlink, F., Geiger, T., Herbert, S., Goerke, C., Schrenzel, J. & Wolz, C. 2009. CodY in *Staphylococcus aureus:* a regulatory link between metabolism and virulence gene expression. *Journal of Bacteriology,* 191**,** 2953-2963.

Potrykus, K. & Cashel, M. 2008. (p) ppGpp: Still Magical?*. *Annu. Rev. Microbiol.,* 62**,** 35-51.

Pragman, A. A., Ji, Y. & Schlievert, P. M. 2007. Repression of *Staphylococcus aureus* SrrAB using inducible antisense srrA alters growth and virulence factor transcript levels. *Biochemistry,* 46**,** 314-321.

Pragman, A. A. & Schlievert, P. M. 2004. Virulence regulation in *Staphylococcus aureus*: the need for in vivo analysis of virulence factor regulation. *FEMS Immunology & Medical Microbiology,* 42**,** 147-154.

Pragman, A. A., Yarwood, J. M., Tripp, T. J. & Schlievert, P. M. 2004. Characterization of virulence factor regulation by *SrrAB*, a two-component system in *Staphylococcus aureus*. *Journal of Bacteriology,* 186**,** 2430-2438.

Prevost, G., Cribier, B., Couppie, P., Petiau, P., Supersac, G., Finck-Barbancon, V., Monteil, H. & Piemont, Y. 1995. Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infection and Immunity,* 63**,** 4121-4129.

Proft, T. & Fraser, J. D. 2003. Bacterial superantigens. *Clinical & Experimental Immunology,* 133**,** 299-306.

Rahman, M., W. C. Noble, and B. Cookson. 1987. Mupirocin resistant S*taphylococcus aureus*. Lancet, 2, 387-388

Ramakrishnan, V. 2002. Ribosome Structure and the Mechanism of Translation. *Cell,* 108**,** 557-572.

Ramsey, M. A., Bradley, S. F., Kauffman, C. A. & Morton, T. M. 1996. Identification of chromosomal location of *mupA* gene, encoding low-level mupirocin resistance in *staphylococcal* isolates. *Antimicrobial Agents and Chemotherapy,* 40**,** 2820-2823.

Ratnayake-Lecamwasam, M., Serror, P., Wong, K.-W. & Sonenshein, A. L. 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes & Development,* 15**,** 1093-1103.

Rechtin, T. M., Gillaspy, A. F., Schumacher, M. A., Brennan, R. G., Smeltzer, M. S. & Hurlburt, B. K. 1999. Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*. *Molecular Microbiology,* 33**,** 307-316.

Recsei, P., Kreiswirth, B., O'reilly, M., Schlievert, P., Gruss, A. & Novick, R. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. *Molecular and General Genetics MGG,* 202**,** 58-61.

Reed, S. B., Wesson, C. A., Liou, L. E., Trumble, W. R., Schlievert, P. M., Bohach, G. A. & Bayles, K. W. 2001. Molecular Characterization of a Novel *Staphylococcus aureus* Serine Protease Operon. *Infection and Immunity,* 69**,** 1521-1527.

Reiß, S., Pané-Farré, J., Fuchs, S., François, P., Liebeke, M., Schrenzel, J., Lindequist, U., Lalk, M., Wolz, C. & Hecker, M. 2011. Global analysis of the *Staphylococcus aureus* response to mupirocin. *Antimicrobial Agents and Chemotherapy***,** 56, 787-804.

Reznikoff, W. S., Siegele, D. A., Cowing, D. W. & Gross, C. A. 1985. The regulation of transcription initiation in bacteria. *Annual Review of Genetics,* 19**,** 355-387.

Rivera, F. E., Miller, H. K., Kolar, S. L., Stevens, S. M. & Shaw, L. N. 2012. The impact of CodY on virulence determinant production in community-associated methicillin-resistant *Staphylococcus aureus*. *Proteomics,* 12**,** 263-268.

Rodnina, M., Savelsbergh, A., Katunin, V. & Wintermeyer, W. 1997. Hydrolysis of GTP by elongation factor G drives RNA movement on the ribosome. *Nature,* 385**,** 37-41.

Rodnina, M. V. & Wintermeyer, W. 2003. Peptide bond formation on the ribosome: structure and mechanism. *Current Opinion in Structural Biology,* 13**,** 334-340.

Rollof, J., Hedström, S. & Nilsson-Ehle, P. 1987. Lipolytic activity of *Staphylococcus aureus* strains from disseminated and localized infections. *Acta Pathologica Microbiologica Scandinavica Series B: Microbiology,* 95**,** 109-113.

Romagnani, S., Giudizi, M. G., Del Prete, G., Maggi, E., Biagiotti, R., Almerigogna, F. & Ricci, M. 1982. Demonstration on protein A of two distinct immunoglobulin-binding sites and their role in the mitogenic activity of *Staphylococcus aureus* Cowan I on human B cells. *The Journal of Immunology,* 129**,** 596-602.

Rosenstein, R. & Götz, F. 2000. *Staphylococcal* lipases: biochemical and molecular characterization. *Biochimie,* 82**,** 1005-1014.

Saravia-Otten, P., Müller, H. & Arvidson, S. 1997. Transcription of *Staphylococcus aureus* fibronectin binding protein genes is negatively regulated by *agr* and an agr-independent mechanism. *Journal of Bacteriology,* 179**,** 5259-5263.

Schelin, J., Wallin-Carlquist, N., Cohn, M. T., Lindqvist, R., Barker, G. C. & Rådström, P. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence,* 2**,** 580.

Schlax, P. J. & Worhunsky, D. J. 2003. Translational repression mechanisms in prokaryotes. *Molecular Microbiology,* 48**,** 1157-1169.

Schlievert, P. M. & Kelly, J. A. 1984. Clindamycin-induced suppression of toxic-shock syndrome-associated exotoxin production. *Journal of Infectious Diseases,* 149**,** 471-471.

Schreiber, G., Ron, E. & Glaser, G. 1995a. ppGpp-mediated regulation of DNA replication and cell division in *Escherichia coli*. *Current Microbiology,* 30**,** 27-32.

Schreiber, G., Ron, E. Z. & Glaser, G. 1995b. ppGpp-mediated regulation of DNA replication and cell division in *Escherichia coli*. *Current Microbiology,* 30**,** 27-32.

Seah, C., Alexander, D. C., Louie, L., Simor, A., Low, D. E., Longtin, J. & Melano, R. G. 2012. MupB, a new high-level mupirocin resistance mechanism in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 56**,** 1916-1920.

Seidl, K., Bischoff, M. & Berger-Bächi, B. 2008a. CcpA mediates the catabolite repression of tst in *Staphylococcus aureus*. *Infection and Immunity,* 76**,** 5093-5099.

Seidl, K., Goerke, C., Wolz, C., Mack, D., Berger-Bächi, B. & Bischoff, M. 2008b. *Staphylococcus aureus* CcpA affects biofilm formation. *Infection and Immunity,* 76**,** 2044-2050.

Seidl, K., Stucki, M., Ruegg, M., Goerke, C., Wolz, C., Harris, L., Berger-Bächi, B. & Bischoff, M. 2006. *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. *Antimicrobial Agents and Chemotherapy,* 50**,** 1183-1194.

Shands, K. N., Schmid, G. P., Dan, B. B., Blum, D., Guidotti, R. J., Hargrett, N. T., Anderson, R. L., Hill, D. L., Broome, C. V. & Band, J. D. 1980. Toxic-shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases. *New England Journal of Medicine,* 303**,** 1436-1442.

Shaw, L., Golonka, E., Potempa, J. & Foster, S. J. 2004. The role and regulation of the extracellular proteases of *Staphylococcus aureus*. *Microbiology,* 150**,** 217-228.

Sheehan, B. J., Foster, T. J., Dorman, C. J., Park, S. & Stewart, G. S. 1992. Osmotic and growth-phase dependent regulation of the eta gene of *Staphylococcus aureus*: a role for DNA supercoiling. *Molecular and General Genetics MGG,* 232**,** 49-57.

Shimizu, Y., Shimizu, N. & Hayashi, M. 1973. In Vitro Repression of Transcription of the Trytophan Operon by trp Repressor. *Proceedings of the National Academy of Sciences,* 70**,** 1990-1994.

Shine, J. & Dalgarno, L. 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences,* 71**,** 1342-1346.

Shivers, R. P., Dineen, S. S. & Sonenshein, A. L. 2006. Positive regulation of *Bacillus subtilis* ackA by CodY and CcpA: establishing a potential hierarchy in carbon flow. *Molecular Microbiology,* 62**,** 811-822.

Showsh, S. A., De Boever, E. H. & Clewell, D. B. 2001. Vancomycin Resistance Plasmid in *Enterococcus faecalis* That Encodes Sensitivity to a Sex Pheromone Also Produced by *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 45**,** 2177-2178.

Shryock, T., Dye, E. & Kapral, F. 1992. The accumulation of bactericidal lipids in staphylococcal abscesses. *Journal of Medical Microbiology,* 36**,** 332-336.

Shyp, V., Tankov, S., Ermakov, A., Kudrin, P., English, B. P., Ehrenberg, M., Tenson, T., Elf, J. & Hauryliuk, V. 2012. Positive allosteric feedback regulation of the stringent response enzyme RelA by its product. *EMBO Reports,* 13**,** 835-839.

Siboo, I. R., Cheung, A. L., Bayer, A. S. & Sullam, P. M. 2001. Clumping Factor A Mediates Binding of *Staphylococcus aureus* to Human Platelets. *Infection and Immunity,* 69**,** 3120-3127.

Simor, A. E., Stuart, T. L., Louie, L., Watt, C., Ofner-Agostini, M., Gravel, D., Mulvey, M., Loeb, M., Mcgeer, A. & Bryce, E. 2007. Mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* strains in Canadian hospitals. *Antimicrobial Agents and Chemotherapy,* 51**,** 3880-3886.

Sivaraman, K., Venkataraman, N. & Cole, A. M. 2009. *Staphylococcus aureus* nasal carriage and its contributing factors. *Future Microbiology,* 4**,** 999-1008.

Smeltzer, M. S., Hart, M. E. & Iandolo, J. J. 1993. Phenotypic characterization of xpr, a global regulator of extracellular virulence factors in *Staphylococcus aureus*. *Infection and Immunity,* 61**,** 919-925.

Somerville, G. A., Morgan, C. I., Fitzgerald, J. R., Dorward, D. W., Reitzer, L. J. & Musser, J. M. 2002. *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. *Infection and Immunity,* 70**,** 6373-6382.

Sonenshein, A. L. 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Current Opinion in Microbiology,* 8**,** 203-207.

Sonenshein, A. L. 2007. Control of key metabolic intersections in *Bacillus subtilis*. *Nature Reviews Microbiology,* 5**,** 917-927.

Srivatsan, A. & Wang, J. D. 2008. Control of bacterial transcription, translation and replication by (p)ppGpp. *Current Opinion in Microbiology,* 11**,** 100-105.

Steiner, K. & Malke, H. 2001. relA-Independent Amino Acid Starvation Response Network of *Streptococcus pyogenes*. *Journal of Bacteriology,* 183**,** 7354-7364.

Stevens, D. L., Ma, Y., Salmi, D. B., Mcindoo, E., Wallace, R. J. & Bryant, A. E. 2007. Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. *Journal of Infectious Diseases,* 195**,** 202-211.

Stevens, D. L., Maier, K. A., Laine, B. M. & Mitten, J. E. 1987. Comparison of clindamycin, rifampin, tetracycline, metronidazole, and penicillin for efficacy in prevention of experimental gas gangrene due to *Clostridium perfringens*. *Journal of Infectious Diseases,* 155**,** 220-228.

Stewart, P. S. & William Costerton, J. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet,* 358**,** 135-138.

Storz, G., Opdyke, J. & Wassarman, K. 2006. Regulating bacterial transcription with small RNAs. Cold Spring Harbor symposia on quantitative biology. *Cold Spring Harbor Laboratory Press*, 71,269-273.

Subrt, N., Mesak, L. R. & Davies, J. 2011. Modulation of virulence gene expression by cell wall active antibiotics in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy,* 66**,** 979-984.

Sunohara, T., Jojima, K., Tagami, H., Inada, T. & Aiba, H. 2004. Ribosome stalling during translation elongation induces cleavage of mRNA being translated in *Escherichia coli*. *Journal of Biological Chemistry,* 279**,** 15368-15375.

Sutherland, R., Boon, R. J., Griffin, K. E., Masters, P. J., Slocombe, B. & White, A. R. 1985. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. *Antimicrobial Agents and Chemotherapy,* 27**,** 495-498.

Takahashi, K., Kasai, K. & Ochi, K. 2004. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. *Proceedings of the National Academy of Sciences,* 101**,** 4320-4324.

Taylor, C. M., Beresford, M., Epton, H. A., Sigee, D. C., Shama, G., Andrew, P. W. & Roberts, I. S. 2002. *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. *Journal of Bacteriology,* 184**,** 621-628.

Tojo, S., Kumamoto, K., Hirooka, K. & Fujita, Y. 2010. Heavy Involvement of Stringent Transcription Control Depending on the Adenine or Guanine Species of the Transcription Initiation Site in Glucose and Pyruvate Metabolism in *Bacillus subtilis*. *Journal of Bacteriology,* 192**,** 1573-1585.

Torvaldsen, S., Roberts, C. & Riley, T. V. 1999. The continuing evolution of methicillin-resistant *Staphylococcus aureus* in Western Australia. *Infection Control and Hospital Epidemiology,* 20**,** 133-135.

Trotochaud, A. E. & Wassarman, K. M. 2004. 6S RNA function enhances long-term cell survival. *Journal of Bacteriology,* 186**,** 4978-4985.

Tucker, P. W., Hazen Jr, E. E. & Cotton, F. A. 1978. *Staphylococcal* nuclease reviewed: A prototypic study in contemporary enzymology. I isolation; physical and enzymatic properties. *Molecular and Cellular Biochemistry,* 22**,** 67-78.

Ugrappa, Nagalakshmi., Karl, Waern., and Michael, Snyder. 2010. RNA-Seq: A Method for Comprehensive transcriptome Analysis. *Current Protocols in Molecular Biology*, 9,1,e78644

Upton, A., Lang, S. & Heffernan, H. 2003. Mupirocin and *Staphylococcus aureus:* a recent paradigm of emerging antibiotic resistance. *Journal of Antimicrobial Chemotherapy,* 51**,** 613-617.

Valeva, A., Palmer, M. & Bhakdi, S. 1997. *Staphylococcal* α-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. *Biochemistry,* 36**,** 13298-13304.

Vaudaux, P., Francois, P., Bisognano, C., Kelley, W. L., Lew, D. P., Schrenzel, J., Proctor, R. A., Mcnamara, P. J., Peters, G. & Von Eiff, C. 2002. Increased expression of clumping factor and fibronectin-binding proteins by hemB mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Infection and Immunity,* 70**,** 5428-5437.

Vojtov, N., Ross, H. F. & Novick, R. P. 2002. Global repression of exotoxin synthesis by *staphylococcal* superantigens. *Proceedings of the National Academy of Sciences,* 99**,** 10102-10107.

Wang, J. D., Sanders, G. M. & Grossman, A. D. 2007. Nutritional control of elongation of DNA replication by (p) ppGpp. *Cell,* 128**,** 865-875.

Wendrich, T. M. & Marahiel, M. A. 1997. Cloning and characterization of a relA/spoT homologue from *Bacillus subtilis*. *Molecular Microbiology,* 26**,** 65-79.

Woese, C. R., Olsen, G. J., Ibba, M. & Söll, D. 2000. Aminoacyl-tRNA synthetases, the genetic code, and the evolutionary process. *Microbiology and Molecular Biology Reviews,* 64**,** 202-236.

Wolz, C., Geiger, T. & Goerke, C. 2010. The synthesis and function of the alarmone (p)ppGpp in firmicutes. *International Journal of Medical Microbiology,* 300**,** 142-147.

Wolz, C., Pöhlmann-Dietze, P., Steinhuber, A., Chien, Y. T., Manna, A., Van Wamel, W. & Cheung, A. 2000. Agr-independent regulation of fibronectin-binding protein (s) by the regulatory locus sar in *Staphylococcus aureus*. *Molecular Microbiology,* 36**,** 230-243.

Wu, J. & Xie, J. 2009. Magic spot:(p) ppGpp. *Journal of Cellular Physiology,* 220**,** 297-302.

Yarwood, J. M., Mccormick, J. K. & Schlievert, P. M. 2001. Identification of a Novel Two-Component Regulatory System That Acts in Global Regulation of Virulence Factors of *Staphylococcus aureus*. *Journal of Bacteriology,* 183**,** 1113-1123.

Yoshizumi, S., Zhang, Y., Yamaguchi, Y., Chen, L., Kreiswirth, B. N. & Inouye, M. 2009. *Staphylococcus aureus* YoeB homologues inhibit translation initiation. *Journal of Bacteriology,* 191**,** 5868-5872.

Zhong, Wang., Mark, Gerstein., and Michael , Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics,* 10, 57-63.

Zimbelman, J., Palmer, A. & Todd, J. 1999. Improved outcome of clindamycin compared with beta-lactam antibiotic treatment for invasive *Streptococcus pyogenes* infection. *The Pediatric Infectious Disease Journal,* 18**,** 1096-1100.

Zubay, G., Morse, D. E., Schrenk, W. J. & Miller, J. 1972. Detection and isolation of the repressor protein for the tryptophan operon of *Escherichia coli*. *Proceedings of the National Academy of Sciences,* 69**,** 1100-1103.