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Studies on regulatory networks governing virulence gene transcription in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus pathogenicity is dependent on the coordinated action of a number of virulence factors and the expression of these virulence factors is determined by several global regulators. The main regulator seems to be *agr* but there are several additional regulators (mostly *sarA* homologues) involved that mainly act downstream of *agr*. Some of these regulators control virulence gene expression directly but they also regulate each other forming complex regulatory networks. The work described in this thesis aims at better understanding of the function of the *agr* system and how different regulators act together in controlling transcription of virulence genes.

Most virulence factors in *S. aureus* are expressed in a growth phase-dependent manner governed by the auto-inducible quorum sensing system *agr*. Activation of *agr* results in rapid increase of the regulator RNAIII and occurs in response to accumulation of the auto-inducing peptide (AIP). In order to activate the *agr* system a low basal transcription of the *agr* operon must be assumed. This basal activity of the operon is stimulated by *sarA*. To be able to study how SarA would affect activation of *agr*, a mathematical model of the *agr* system was set up. The model predicted that the *agr* system is hysteretic, meaning that activation of *agr* occurs in a switch-like manner at a specific concentration of AIP, whereas it is inactivated at a specific lower concentration of AIP. According to the model, SarA does not seem essential for the function of the *agr* switch but alters the concentration of AIP (cell density) at which *agr* is activated. This was supported by Northern blot analysis of RNAIII in *S. aureus* mutants with different levels of *sarA* expression.

To determine how *agr* and the other regulators act together in controlling transcription of virulence genes, we studied the regulation of one gene (*spa*) that is negatively regulated by *agr* and the genes encoding extracellular proteases (*aur* and *sspA*), which are positively regulated by *agr*. To analyze the general principles of how each component in a regulatory system contributes to expression of a virulence gene, a mathematical model of the regulation of *spa* (protein A) transcription was developed.

Parameter values in this mathematical model were determined by fitting the output of the model to quantitative Northern blot data from various *S. aureus* regulatory mutants using a gradient search method. The model was validated by correctly predicted *spa* expression levels in different regulatory mutants not included in the parameter value search. The mathematical model revealed that Rot and SarS act synergistically to stimulate *spa* expression and that *sarA* and *sarS* seem to balance each other in a way that when the activating impact of *sarS* is small, e.g. in the wild type, the repressive impact of *sarA* is small, while in a *agr*-deficient background, when the impact of *sarS* is maximal, the repressive effect of *sarA* is close to its maximum.

Previous studies have shown that SarR down-regulates transcription of *sarA*, which is a repressor of the *aur* and *sspA* transcription. This means that inactivation of *sar*R would result in decreased transcription of *aur* and *sspA*, which was confirmed by mRNA analysis using quantitative real-time PCR. However, we also found that *sar*R acted as a direct stimulator of *aur* and *sspA* transcription and that *sar*R is required for maximal transcription of *aur* and *sspA*.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Erik Gustafsson**, Patric Nilsson, Stefan Karlsson and Staffan Arvidson (2004) Characterizing the dynamics of the quorum-sensing system in *Staphylococcus aureus*. J Mol Microbiol Biotechnol. 8(4):232-242

II. **Erik Gustafsson**, Stefan Karlsson, Jan Oscarsson, Peter Sögård, Patric Nilsson and Staffan Arvidson (2009) Mathematical modelling of the regulation of *spa* (protein A) transcription in *Staphylococcus aureus*. Int J Med Microbiol. 299(1):65-74

III. **Erik Gustafsson** and Jan Oscarsson (2008) Maximal transcription of *aur* (aureolysin) and *sspA* (serine protease) in *Staphylococcus aureus* requires staphylococcal accessory regulator R (*sarR*) activity. FEMS Microbiol Lett. 284(2):158-164

"Gråa dagar skyndar aldrig på"

Lars Winnerbäck

To my parents

The best ever!

CONTENTS

1 INTRODUCTION

1.1 REGULATION OF VIRULENCE FACTORS IN BACTERIA

Bacterial infections are usually a consequence of successful colonization of the host as well as breaching the host's immune defence-system. To colonize the host and subsequently cause an infection, the bacteria must be able to enter the host and attach to different tissues and organs. During the infectious process the bacteria will experience vastly different environments and must be able to quickly adapt to these in order to survive. Bacterial traits necessary for their ability to cause infection are referred to as virulence factors and different sets of virulence factors are used in various types of infections and at different stages during the infectious process. It seems reasonable to assume that virulence factors must be expressed at the right time to be beneficial for the bacteria and not just waste of energy and time. Many virulence genes are regulated by global regulators in response to the different environmental conditions. The regulators also regulate each other forming complex regulatory networks or systems where the level of expression of individual or a set of virulence genes is determined by the activity of several regulators. Studying the function of such regulatory systems governing expression of the virulence genes is today an active field of research aiming at the development of new methods for therapy against infectious diseases.

1.2 BACTERIAL RESPONSE TO ENVIRONMENTAL CHANGES USING SIGNAL TRANSDUCTION SYSTEMS

One type of system that has evolved to help bacteria to sense and respond to changes in environmental conditions is called two-component signal transduction systems. They typically consist of a membrane-located sensor, usually a histidine protein kinase, and an intracellular response regulator, that generally act as a transcription factor. The sensor is autophosphorylated at a conserved histidine residue in response to a specific stimulus. The phosphate group is then transferred to a conserved aspartate residue in the response regulator. One special type of two-component system is called quorum sensing system and bacteria having such a system can sense and respond to their own bacterial cell density. That bacteria can respond to their own cell density was discovered more than 35 years ago in bioluminescent marine bacteria, *Vibrio fischeri* (Eberhard, 1972; Nealson *et al.*, 1970) that form a symbiotic relationship with certain species of squids, which provide nourishment for the bacteria to grow. The bioluminescence generated by *V. fischeri* may mimic shimmering moonlight in the sea, thereby enabling the squids to evade attacks by predators. Bioluminescence requires a lot of energy and it is only worthwhile for the bacteria to produce light at high population densities. At low population densities the light would be to dim to promote symbiosis. It has been shown that at higher cell densities, all bacterial cells in a population start to transcribe the genes coding for bioluminescence. The understanding of such density-dependent regulation was greatly advanced when the crucial signalling molecule, an N-acyl homoserine lactone (AHL), was identified in *V. fischeri* (Eberhard *et al.*, 1981).

In Gram-positive bacteria the cell-to-cell signalling molecule is a small secreted peptide (usually called pheromone) that is produced and secreted by the bacteria.

The pheromone then interacts with the membrane-bound sensor of the two-component system. Some examples of mechanisms regulated through quorum sensing systems are competence in *Streptococcus pneumoniae* (Pestova *et al.*, 1996) and *Bacillus subtilis* (Magnuson *et al.*, 1994), bactericine production in *Lactobacillus sakei* (Brurberg *et al.*, 1997; Eijsink *et al.*, 1996), conjugal transfer of plasmids in *Enterococcus faecalis* (Clewell, 1993) and virulence gene transcription in *Staphylococcus aureus* (see section 1.9.1). Gram-negative bacteria instead use AHLs as cell-to-cell signalling molecules, which are small diffusible molecules that act on intracellular receptors in the bacteria. AHL has, except for regulating light emission in *V. fischeri* and *Vibrio harveyi* (reviewed in Miller and Bassler, 2001), for-example been shown to regulate biofilm formation in *Pseudomonas aeruginosa* (Davies *et al.*, 1998) and coordinate a variety of adaptive processes in *Escherichia coli* and Salmonella (Walters and Sperandio, 2006).

1.3 SYSTEMS BIOLOGY AND MATHEMATICAL MODELLING

A system can be defined as a set of interacting or interdependent entities forming an integrated whole and traditional molecular biology research usually follows Descartes' reductionism, where an understanding of the whole system is based on investigating how different components work separately. During the last decade a research field called systems biology has emerged that instead aims to focus on the whole system, on its biological function, and how it arises from the underlying interacting components. Systems biology research thus follows holism, which implies that the properties of a given system cannot be determined or explained by its components alone, instead the system as a whole determines in an important way how the individual parts behave. However, the desire to understand biological systems on a systems-level is not new for systems biology, e.g. both cybernetics, founded by Norbert Wiener (1894-1964), and general systems theory, founded by Ludwig von Bertalanffy (1910-1972), basically aims at such understanding and originate from the early second half of the 20th century. An essential and inseparable part of all scientific activity, especially in systems biology, is the process of generating models, which are simplified representations or descriptions mainly created to explain the workings of real-world systems or concepts. The statistician George E. P. Box once claimed that all models are essentially wrong since they are just simplifications of the reality (Box, 1979) but, because of its simplicity, a model is often understandable and hopefully still representative for the real-world system.

"Essentially, all models are wrong, but some of them can be very useful"
George E P Box (born 1919)

Models can be divided into descriptive models and predictive models. Descriptive models are usually graphical models, created by scientists to collect and structure current knowledge about the system, and can be used to reason about the system but without comparing the output of the model as a whole with corresponding experimental data. A graphical model is thus just a graphical representation of what the different components are and some information of how these components interact and affect each other.

Studies on regulatory systems, e.g. the systems governing expression of the virulence genes in *S. aureus*, have during last decades been frequently focused on creating graphical models of the networks (Frees *et al.*, 2005; Manna and Cheung, 2003, 2006a;

Oscarsson *et al.*, 2005; Oscarsson *et al.*, 2006a; Oscarsson *et al.*, 2006b; Schmidt *et al.*, 2001; Schmidt *et al.*, 2003), but very often the complexity of the systems makes it impossible to intuitively understand whether a system as a whole can work in a way that explains available experimental data, i.e. if the components and biological mechanisms crucial for the systems behaviour are included in the model. To test this and also be able to extract new information about the system, not obvious from the graphical representation, a predictive model must be created. This is usually made by translating the graphical representation into some sort of mathematical model. Being arguably the most widespread formalism to model dynamical systems in science, ordinary differential equations have been widely used to analyze gene regulatory systems (de Jong, 2002). Such a mathematical model can then be used to identify the components crucial for the behaviour of the system but also to evaluate which components that have most impact on systems properties, or identifying mechanisms not obvious from the experimental data such as synergistic and antagonistic effects between different components.

1.4 THE PROCESS OF MODELLING DYNAMICAL SYSTEMS

In the present study, the following modelling workflow has been suggested for the process of developing a model for a dynamical system:

1. Create a descriptive model on the ideas about how the system works. This descriptive model is visually expressed in a flowchart, typically involving boxes representing state-variables and arrows representing material flows or causal effects. The most important task in modelling is to identify which components and processes that are important for the purpose of the model and therefore must be included. The descriptive model may be derived from known properties about the system but usually includes a set of hypotheses suggested by current knowledge about the system, in which case the hypotheses can be tested by comparing output from the model to experimental data.
2. The descriptive model is translated to a mathematical model consisting of a differential equation for each state variable. The mathematical rate expressions may be deducted from fundamental physical and chemical mechanics or from phenomenological experience. The resulting differential equations are usually non-linear.
3. Some parameters in the mathematical model may be deduced from measurements on isolated components while others have to be estimated by fitting the output from the model to experimental data. A problem is that the complexity of the parameter space usually is too high to be fully determined by observable data.
4. The mathematical model is then experimentally evaluated, i.e. predictions are generated with the model and compared to experimental data not used when fitting parameter values. If the model cannot predict new experimental data an iterative refinement of the model is necessary.
5. Finally, the validated model can be used for predictions. If possible, the predictions should finally be verified experimentally.

1.5 MATHEMATICAL MODELLING OF QUORUM SENSING SYSTEMS

Bioluminescence in *V. fischeri* is regulated in a cell density dependent manner by the LuxI/LuxR quorum sensing circuit (for a description see Miller and Bassler, 2001) and a mathematical model based on the fundamental properties of the control system formed by

the *lux* genes and their products have been published (James *et al.*, 2000). These authors studied the response of a single cell to external concentrations of signalling molecules and derived a system of ordinary differential equations. Their model predicted that the system is bistable, i.e. two stable metabolic states exist corresponding to a non-bioluminescent state and a bioluminescent state, respectively. Later a mathematical model of the primary quorum sensing system in *P. aeruginosa* (the *las* system) was presented (Dockery and Keener, 2001). The authors proposed that quorum sensing works by "switching" between two stable steady-states, reflecting relatively low and high rates of production of *las* components, whereby increasing population density (treated as a parameter in the model) triggers the switching from low to high production rates. Such behaviour is referred to as hysteresis (Fig. 1) meaning that for each concentration of a signalling substance (*S*) below level *x* or above level *y*, there is just one single steady-state concentration of the response molecule (*R*). At concentrations of *S* between level *x* and *y* the system is bistable and two different steady-state levels of *R* can be achieved (solid sections in figure 1). This means that high levels of *R* (switching the system to an ON state) will not be reached until the concentration of *S* exceeds level *y*. Starting in an activated state, at concentrations of *S* above level *y*, low levels of *R* (switching the system to an OFF state) will not be reached until the concentration of *S* has decreased below level *x*. Interestingly, switches seem to control well-studied mechanisms among prokaryotes, e.g. the λ phage lysis/lysogeny switch (Arkin *et al.*, 1998; Isaacs *et al.*, 2003) and the *lac* operon in *Escherichia coli* (Ozbudak *et al.*, 2004).

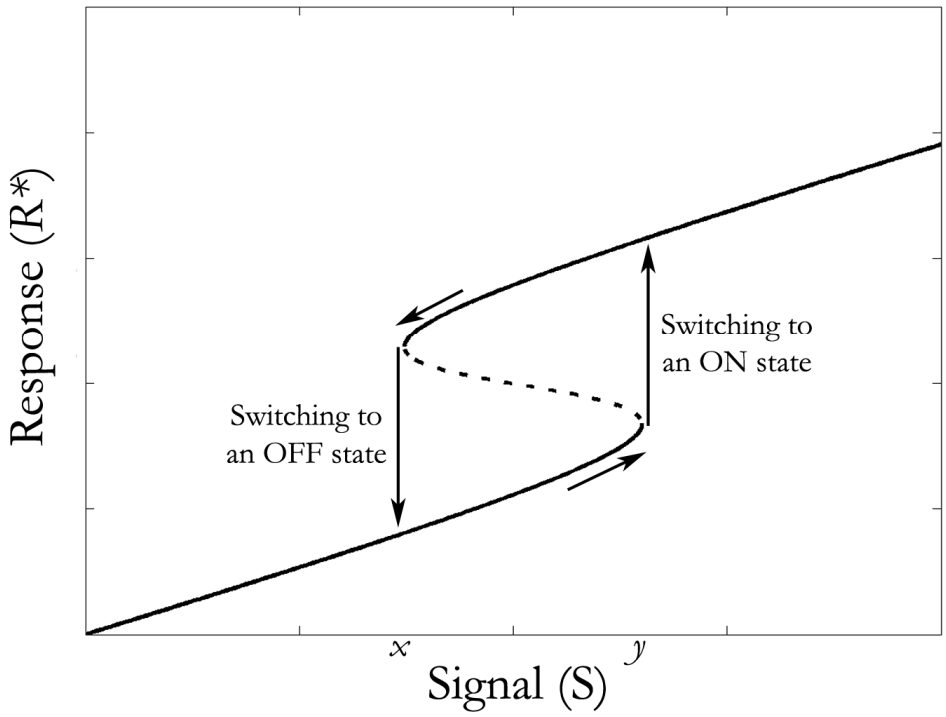

Figure 1. A sketch of a hysteresis curve in bistable signalling circuits. The steady-state level of the response molecule (denoted R^*) is plotted against the concentration of signalling substance (*S*). Activation of the system will not occur until the concentration of the signal exceeds level *y*, whereas inactivation of the system occurs when the signal concentration declines below level *x*.

1.6 *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus is a Gram-positive, non-motile, facultative anaerobic coccus that was discovered in the 1880s in pus from surgical abscesses by the surgeon and microbiologist Sir Alexander Ogston. He named the bacteria after the Greek's *staphyle* meaning "a bunch of grapes" and *kokkus* meaning berry because the bacteria looked like a bunch of berries under the microscope. The Latin's *aureum* for gold refers to the golden pigmentation of many *S. aureus* strains. *S. aureus* can be distinguished from other members in the staphylococcal genus by its ability to produce the extracellular enzyme coagulase and is therefore often referred to as coagulase-positive staphylococci.

S. aureus colonizes the anterior nares, the axillae and perineum of at least one third of the human population (asymptomatic carriers), but can also cause a wide range of infections (Table 1). The primary site of infection is often the skin where the bacteria usually cause minor lesions such as skin-abscesses, impetigo and furunculosis. However, the bacteria can spread into the bloodstream and further on to other tissues and organs, causing deep invasive infections such as osteomyelitis, endocarditis and septic arthritis. Patients with long-lasting breaches in the skin, e.g. as a result of haemodialysis, catheters and central venous lines, as well as patients suffering from diabetes mellitus, rheumatoid arthritis, cancer and HIV-infection, are most prone to deep infection (Jacobsson *et al.*, 2007; Laupland *et al.*, 2003).

Table 1. Examples of diseases caused by *S. aureus*.

Skin and wound infections
Folliculitis
Abscesses in all tissue
Septicaemia
Endocarditis
Septic arthritis
Pneumonia
Osteomyelitis
Scalded skin syndrome
Toxic shock syndrome
Food-poisoning

S. aureus can also cause toxin-mediated diseases such as food-poisoning, toxic shock syndrome (TSS) and staphylococcal scalded skin syndrome (SSSS), of which TSS is most feared because of its high mortality. The bacterium has an enormous ability to survive outside the host and can survive for weeks in non-optimal environments such as bed-clothing. Due to its ability to survive outside the host, and because the bacteria emerge resistance to multiple antibiotics, *S. aureus* is a major problem both in community- and hospital settings.

1.7 VIRULENCE FACTORS EXPRESSED BY *S. AUREUS*

The broad range of infections (Table 1) caused by *S. aureus* is, at least in part, a consequence of its many virulence factors. The virulence is generally considered to be multifactorial and due to the combined action of several virulence determinants. One

exception is the toxinoses, i.e. toxic shock syndrome, staphylococcal scalded skin syndrome and staphylococcal food-poisoning, which are caused by toxic shock syndrome toxin-1, exfoliative toxins A and B, and different staphylococcal enterotoxins, respectively (reviewed in Iandolo, 1989; Lowy, 1998).

Today, more than 40 different virulence factors have been described (Table 2) and based on their biological activity the virulence factors can be divided into three categories: those that mediate adhesion to cells and tissue, those that contribute to tissue damage and spread, and those that protect the bacteria from the host immune system. It is generally assumed that virulence factors that mediate adhesion are of main importance early in the infectious process whereas factors that contribute to tissue damage and spread, as well as protection from the immune system are more important during later stages of infection. The virulence factors of *S. aureus* are briefly discussed in the following sections.

1.7.1 Attachment of bacteria

One crucial factor in the colonization process is the ability of *S. aureus* cells to adhere to components of human extracellular matrix. Adherence to host extracellular matrix proteins is probably essential in the early phases of wound infections and is mediated by several surface proteins (Table 2). These virulence factors are called MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) (Foster and Hook, 1998) and the host matrix proteins recognized by these include fibronectin (Flock *et al.*, 1987; Kuusela, 1978; Kuusela *et al.*, 1984), collagen (Flock *et al.*, 1987; Patti *et al.*, 1992; Speziale *et al.*, 1986), elastin (Park *et al.*, 1991; Park *et al.*, 1996), vitronectin (Chhatwal *et al.*, 1987; Liang *et al.*, 1995), thrombospondin (Kawabata *et al.*, 1985) and bone sialo protein (Ryden *et al.*, 1989). Most MSCRAMMs are covalently bound to the cell-wall peptidoglycan via the threonine residue in the sorting signal motif, LPXTG, the C-terminus of the MSCRAMM protein (Fischetti *et al.*, 1990). The LPXTG motif is cleaved by sortase, which is an enzyme with transpeptidase activity (Mazmanian *et al.*, 1999; Schneewind *et al.*, 1995; Ton-That *et al.*, 1997) and an amide bound is formed between the carboxyl-group of threonine and the amino-group of cell wall cross-bridges. Most of the surface proteins are secreted through the Sec pathway guided by the N-terminal signal peptide (Lee and Schneewind, 2001; Navarre and Schneewind, 1999; Schneewind *et al.*, 1992; Schneewind *et al.*, 1993).

S. aureus also binds to tissue by adhesion to deposited plasma proteins such as fibrinogen (Boden and Flock, 1989), prothrombin (Kawabata *et al.*, 1985), and plasminogen (Kuusela and Saksela, 1990). Binding to host soluble plasma proteins might also mask the bacteria to look like self thus hiding from the immune system. To denote all microbial binding proteins interacting with mammalian target proteins the term receptins has been suggested (Kronvall and Jonsson, 1999). One typical receptin in *S. aureus* is protein A, which binds to the Fc-domain of immunoglobulin G (Forsgren and Sjoquist, 1966) and von Willebrand factor, which is a large serum glycoprotein that mediate platelets adhesion at sites of endothelial damage (Hartleib *et al.*, 2000).

Table 2. Virulence factors produced by *S. aureus*.

Virulence factor	**Gene**
Toxins	
Alpha (α)-hemolysin	*hla*
Beta (β)-hemolysin	*hlb*
Delta (δ)-hemolysin	*hld*
Enterotoxins A-J	*entA-J*
Exfoliative toxin A/B	*entA/B*
Gamma-hemolysin	*hlgA-C*
Panton-Valentine leukocidine	*lukS/F-PV*
Toxic shock syndrome toxin-1	*tst*
Leukocidin	*lukE/D*
Enzymes	
Catalase	*kat*
Cystein protease	*sspB*
V8 serine protease	*sspA*
Aureolysin	*aur*
Staphopain	*scp*
Lipase/esterase	*lip/geh*
Nuclease	*nuc*
PI-phospholipase C	*plc*
Hyaluronidase	*hysA*
Serine-like proteins	*splA-F*
Coagulase	*coa*
Staphylokinase	*sak*
Surface proteins	
Clumping factor A/B	*clfA/B*
Collagen binding proteins	*cna*
Fibrinogen binding protein	*fbpA*
Fibronectin binding protein A/B	*fnBPA/B*
Lactoferrin binding protein	*hLf-BP*
Protein A	*spa*
Laminin binding protein	*eno*
Extracellular matrix binding protein homologue	*emp*
Extracellular matrix binding homologue	*ebh*
Vitronectin binding protein	*vnBP*
Elastin bindning protein	*ebpS*
Bone sialoprotein binding protein	*bbp*
Serine-aspartate repeat protein	*sdrC, D* and *E*

1.7.2 Invasion and spread

Several secreted virulence factors (exotoxins) seem to promote invasion and spread of *S. aureus*. One particular group is the hemolysins, e.g. alpha (α)-, beta (β)-, gamma (γ)-, and delta (δ)-hemolysin, which all damage the cytoplasmic membrane and lyse a variety of eukaryotic cells (reviewed in Dinges *et al.*, 2000). Another group includes different enzymes capable of degrading various proteins, lipids and hyaluronic acid, a component of the extracellular matrix (Farrell *et al.*, 1995).

The α-toxin is the most studied hemolysin and the gene encoding the toxin, *hla*, is carried by most *S. aureus* strains, but their expression of α-toxin seems to vary considerable (Li *et al.*, 1997). The toxin has an affinity for several different cell-types and among humans, monocytes and platelets seems to be most susceptible (Bhakdi and Tranum-Jensen, 1991). Several animal models of infection have revealed the importance of α-toxin in staphylococcal infection (Bayer *et al.*, 1997; Callegan *et al.*, 1994; Gemmell *et al.*, 1997). The toxin also plays an integral role in biofilm formation (Caiazza and O'Toole, 2003).

Another important toxin is the Panton Valentine leukocidine (PVL), which almost exclusively forms pores in leukocytes through a mechanism similar to that used by the α-toxin (Joubert *et al.*, 2007). Strains carrying the gene for PVL have been associated with lethal necrotising pneumonia in clinical studies (Gillet *et al.*, 2002) but based on the observation that PVL did not contribute to lethal pneumonia in mice (Bubeck Wardenburg *et al.*, 2007), the role of PVL in pulmonary diseases have been questioned (Diep and Otto, 2008).

1.7.3 Protection from host defence mechanisms

S. aureus resist host immune defenses by several different mechanisms where most are directed against phagocytosis and antibody response. Phagocytosis is prevented by capsular polysaccharides, which are expressed by more than 90% of clinical *S. aureus* strains (Karakawa and Vann, 1982), and by protein A (see section 1.8.1). Another way to protect from host-defences is achieved by producing coagulase, which is a prothrombin activator, and is protective by forming a fibrin clot around the bacterial cells. Coating with plasma proteins and other soluble proteins (see section 1.7.1) might protect the bacteria from various host defence mechanisms including phagocytosis. The bacteria also produce proteases (see section 1.8.2), which have the ability to cleave and thereby inactivate all known immunoglobulin classes (Prokesova *et al.*, 1992). Other factors that might protect the bacteria from host defense mechanisms are superantigens and PVL (see section 1.7.2). Superantigens bind to the class II major histocompatibility complex on macrophages and the β-receptor subunit of T-lymphocytes, leading to unspecific activation and proliferation of the T-cells and subsequently a systemic release of cytokines. The superantigens of *S. aureus* include the toxic shock syndrome toxin (TSST-1) and the staphylococcal enterotoxins (SE), which causes toxic shock syndrome and food-poisoning, respectively. However, their contribution to survival of bacteria in the host remains unknown. *S. aureus* also express exfoliative toxins, ETA and ETB, which are involved in staphylococcal scalded skin syndrome (SSSS) (Melish and Glasgow, 1970). It has been recognised for long time that the exfoliative toxins induce potent T-cell proliferation (Morlock *et al.*, 1980) but whether they should be implicated as superantigens is still controversial.

1.8 THE VIRULENCE FACTORS INVESTIGATED IN THIS STUDY

1.8.1 Protein A

Protein A is one of the major cell-surface proteins on *S. aureus* cells and is found in essentially all *S. aureus* strains (Forsgren, 1969). It is structurally similar to MSCRAMMs but instead of binding to matrix molecules, protein A has an affinity for the Fc-domain of the immunoglobulin G subclasses (Forsgren and Sjoquist, 1966). This means that *S. aureus* cells expressing protein A are covered by wrong-directed antibodies, which are thought to prevent phagocytosis (Dossett *et al.*, 1969; Forsgren, 1969). Studies have also shown that protein A induces inflammatory responses in human airway and corneal epithelial cells (Gomez *et al.*, 2004; Kumar *et al.*, 2007), and also triggers T cell-independent B cell proliferation (Bekeredjian-Ding *et al.*, 2007). Since protein A binds to von Willebrand factor (Hartleib *et al.*, 2000) (see section 1.7.1) it also might play a role in adhesion during the initiation of intravascular infection or wound..

The N-terminal signalling sequence of protein A is followed by four or five immunoglobulin-binding domains (E, D, A, B and C), each consisting of approximately 60 amino acid residues. X-ray crystallography studies of the B-domain in complex with the Fc-region of IgG subclass I showed that 11 amino acid residues in the B-domain of protein A and nine amino-acid residues in the IgG fragment were involved in the binding surface (Deisenhofer, 1981).

The importance of protein A has been demonstrated in a murine septic arthritis model (Palmqvist *et al.*, 2002) and in subcutaneous infections in mice (Patel *et al.*, 1987), where mutant strains deficient in the *spa* gene, encoding protein A, were slightly less virulent than the parental strains. On the other hand, *spa* mutants were just as virulent as parental strains in a rabbit model of keratitis (Callegan *et al.*, 1994), which is not surprisingly considering the immune status of the eye.

1.8.2 Extracellular proteases

S. aureus produce several extracellular proteases that are able to interact with host defense mechanisms and tissue components, and degrade several important host proteins including the heavy chains of all immunoglobulin classes, plasma proteinase inhibitor and elastin (Potempa *et al.*, 1986; Potempa *et al.*, 1988; Potempa *et al.*, 1991; Prokesova *et al.*, 1992). The proteases also degrade bacterial cell surface molecules such as fibronectin binding proteins and clumping factor B (Karlsson *et al.*, 2001) and thus potentially play a role in the bacterial switch from adhesive to invasive phase.

S. aureus produce four different proteases; serine protease (*sspA*), a cystein protease called staphopain B (*sspB*), a metalloprotease called aureolysin (*aur*) and a second cystein protease called staphopain A (*scpA*). Serine protease (*sspA*) preferentially cleaves glutamoyl peptide bounds and is often referred to as V8 protease after the strain from which it was isolated (Drapeau *et al.*, 1972). Because of its restricted substrate specificity, SspA generates mostly large peptides and it seems reasonable to believe that SspA would have limited action in providing nutrients to the bacteria. However, during infection SspA might inactivate important host molecules such as immunoglobulins. SspA is also involved in releasing surface proteins such as fibronectin-binding proteins (FnBPs) and protein A from the bacterial surface (Karlsson *et al.*, 2001).

V8 protease is encoded in an operon together with the gene encoding staphopain B, while aureolysin and staphopain A are encoded by separate gene loci. The cystein proteases (*sspB* and *scp*) have broad substrate specificity and catalyze the hydrolysis of peptide-, thiol ester- and amide ester-bounds (reviewed in Arvidson, 2000). Aureolysin is a zink-dependent metalloprotease, which cleaves peptide bounds on the N-terminal side of hydrophobic amino acid residues (Bjoorklind and Jornvall, 1974; Drapeau, 1978).

All four proteases are synthesized as pre-proenzymes. The pre-fragment is a typical signaling peptide, which is cleaved during secretion, yielding an inactive proenzyme. The proenzyme must then be proteolytically cleaved in order to be active. Studies have shown that the main proteolytic activation of V8 protease occurs via aureolysin through dichotomy and mutants deficient in *aur* also lack the activity of serine protease (Drapeau, 1978). Activation of serine protease can also occur in an *aur*-independent way but at decreased efficiency (Shaw *et al.*, 2004). In the case of SspB, which is processed by SspA, the proenzyme appears to be enzymatically active (Rice *et al.*, 2001). The enzymes responsible for activation of aureolysin and staphopain remain to be determined.

Although the exact function of the extracellular proteases is not known Signature-tagged mutagenesis (STM) indicated that they are important virulence factors in mouse abscess, bacteraemia and wound infections (Coulter *et al.*, 1998). Another study showed that inactivation of *sspA* and/or ssp*B* resulted in virulence attenuation in a mouse abscess model whereas inactivation of *aur* or *scpA* had no effect (Shaw *et al.*, 2004). Furthermore, a vast majority of *S. aureus* strains isolated from colonized skin of patients with acute-phase atopic dermatitis produced high levels of proteases (especially aureolysin and serine protease) (Miedzobrodzki *et al.*, 2002).

1.9 REGULATORS CONTROLLING VIRULENCE GENE EXPRESSION

It was early shown that most soluble extracellular proteins were expressed mainly during post-exponential phase of growth (Abbas-ali and Coleman, 1977) indicating a global growth phase-dependent regulatory mechanism. A number of pleiotropic mutants with altered production of proteins were also reported further indicating the existence of global virulence gene regulators (Björklind and Arvidson, 1980; Forsgren *et al.*, 1971; Kondo and Katsuno, 1973; Omenn and Friedman, 1970). One such mutant, *exp*$^{-}$, which accumulated in continuous culture showed an increased production of protein A and coagulase, and a decreased production of most secreted toxins and enzymes (Björklind and Arvidson, 1980). Later, the locus responsible for the phenotype was identified with transposon insertion (Recsei *et al.*, 1986) and named *agr* (accessory gene regulator). Strains deficient in *agr* showed an up-regulation of cell-wall associated proteins, and down-regulation of secreted toxins and enzymes (Morfeldt *et al.*, 1988; Peng *et al.*, 1988; Recsei *et al.*, 1986). In addition, several other global regulators, e.g. *sarA* (Cheung *et al.*, 1992) and a number of *sarA*-homologues (reviewed in Arvidson and Tegmark, 2001; Cheung and Zhang, 2002), have been identified to be involved in virulence gene regulation in *S. aureus*.

1.9.1 The *agr* system

The *agr* system was originally identified as a Tn551 insertion resulting in up-regulation of coagulase and the cell-wall associated protein A, and down-regulation of secreted toxins and enzymes such as α-hemolysin, proteases and toxic shock syndrome toxin (Morfeldt *et al.*, 1988; Peng *et al.*, 1988; Recsei *et al.*, 1986).

The *agr* system, which is a quorum sensing system, is encoded within the *agr* locus (depicted in Fig. 2) consisting of two divergent transcriptional units, driven by the *agr* P2 and *agr* P3 promoters, respectively (Janzon and Arvidson, 1990; Kornblum *et al.*, 1990; Novick *et al.*, 1993). The *agr* P2 operon contains four different genes, *agrB*, *D*, *C* and *A* where AgrC and AgrA are homologous to proteins belonging to classical two-component signal transduction systems and constitute the sensor and response regulator, respectively (Novick *et al.*, 1995). AgrC is activated by an octapeptide pheromone, designated AIP for auto-inducing peptide, which is encoded within the *agrD* gene, (Ji *et al.*, 1995; Lina *et al.*, 1998). The post-translational modification and secretion of AgrD requires mechanisms involving AgrB (Ji *et al.*, 1995; Mayville *et al.*, 1999; Otto *et al.*, 1998). More precisely, the AIP is cleaved out from the 45 amino acid peptide synthesized by *agrD* and a ring structure is formed by a thiolactone linkage between a central conserved cystein and the C-terminal amino acid residue (Zhang *et al.*, 2002). The *agr* system is autocatalytic in such a way that the AIP binds to the sensor, AgrC, which is presumably autophosphorylated at a conserved histidine residue (Lina *et al.*, 1998). The phosphate is then transferred to the response regulator, AgrA, which binds to (Koenig *et al.*, 2004) and stimulates the *agr* P2 and *agr* P3 promoters, respectively. The *agr* P2 operon is transcribed at a low basal level during early exponential phase of growth (Ji *et al.*, 1995), to ensure production of the auto-inducing components.

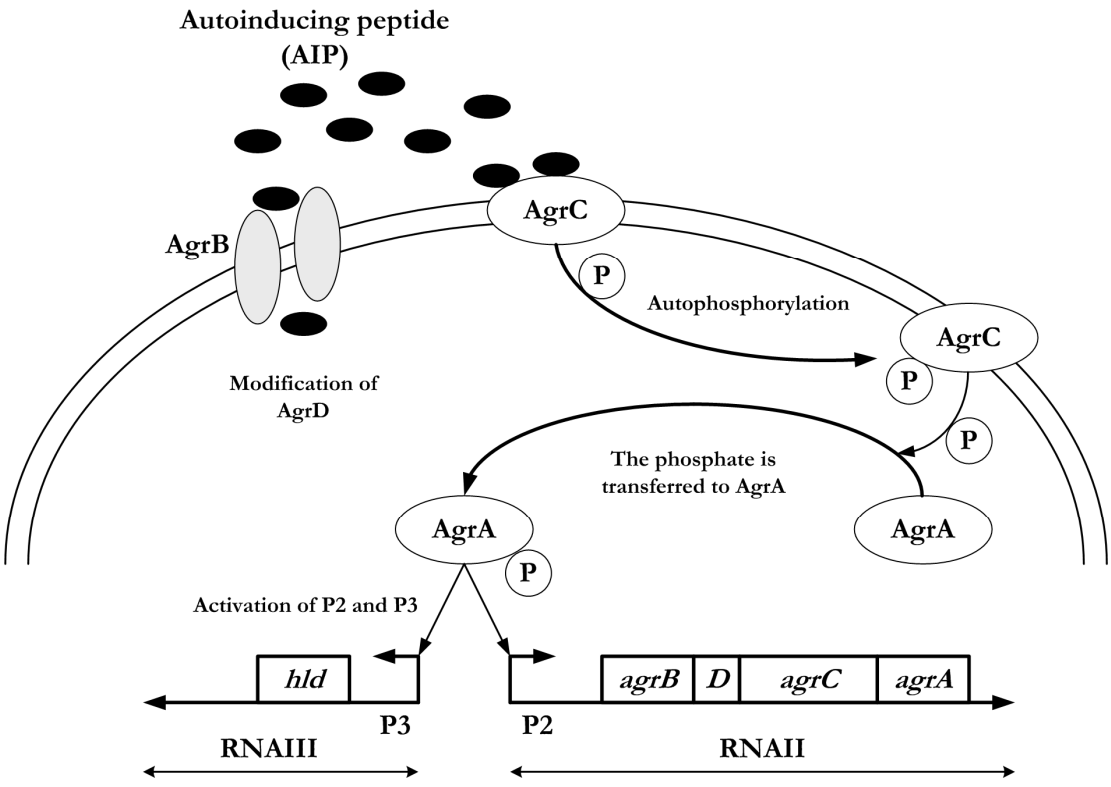

Figure 2. Schematic illustration of the *agr* system. (Adapted from Arvidson and Tegmark, 2001)

The effector molecule of the *agr* system is the 512 nucleotide (RNAIII) *agr* P3 transcript (Janzon and Arvidson, 1990; Novick *et al.*, 1993). RNAIII contains a small (48 nt) gene encoding δ-hemolysin (*hld*) (Janzon *et al.*, 1989) and even though δ-hemolysin is not involved in the regulation of virulence genes, translation of δ-hemolysin appear to influence the regulatory function of RNAIII (Janzon and Arvidson, 1990; Novick *et al.*, 1993). RNAIII is synthesised in mid- to post-exponential phase of growth as a result of accumulation of AIP, which at a specific concentration triggers the *agr* response. It has been proposed that the *agr* system acts as a bistable switch (see Fig. 1) meaning that activation of *agr*, and subsequently synthesis of RNAIII, occurs at a specific concentration of AIP, whereas inactivation of the system occurs at a specific lower AIP concentration (**paper I**), a phenomenon that results in certain inertia in the AIP-response and probably prevents small adjustments in cellular levels of RNAIII. This is consistent with the observation that RNAIII is a long-lived transcript with a half-life of 15 minutes (Janzon *et al.*, 1989) indicating that optimal functioning of the *agr* system requires high concentrations of RNAIII. In a study using gene-chip technology it was shown that more than 100 genes were up-regulated by *agr*, whereas 34 were down-regulated (Dunman *et al.*, 2001).

It was for a long time unclear by which mechanisms RNAIII regulates virulence gene transcription but it was speculated that RNAIII might act via regulatory proteins. Based on the observation that inactivation of the regulatory gene locus *rot* only had a phenotype in *agr* mutant strains it was postulated that RNAIII acted be sequestering the Rot protein (McNamara *et al.*, 2000). However, evidence has now been presented showing that RNAIII regulates virulence gene transcription by preventing *rot* mRNA translation (Geisinger *et al.*, 2006). A predicted secondary structure of RNAIII, which has been confirmed by biochemical probing methods, revealed that the molecule is organized in a 14 stem loop structure where the hairpins are structurally independent, meaning that deletions or unfolding in one of the hairpins do not affect any of the others (Benito *et al.*, 2000). Secondary structure predications of *rot* mRNA showed that loops of the RNAIII molecule are complementary with loops of the *rot* mRNA translation initiation region (Geisinger *et al.*, 2006), suggesting that interaction might stop *rot* mRNA translation. Indeed the interaction between RNAIII and *rot* mRNA has been demonstrated using gel retardation assays (Boisset *et al.*, 2007).

It has also been reported that the *agr* system can be activated by a 38-kDa cytoplasmic protein called RAP (RNAIII-activating protein) (Balaban and Novick, 1995; Balaban *et al.*, 1998) via phosphorylation of another cytoplasmic protein called TRAP (Target of RNAIII-activating protein) (Balaban *et al.*, 2001; Gov *et al.*, 2004). However, recent studies have taken the edge of RAP by showing that the *trap* mutant used in previous studies had a stop codon in *agrA* resulting in the *agr* mutant phenotype, and that inactivation of *trap* had no impact on expression of *agr* (Adhikari *et al.*, 2007; Shaw *et al.*, 2007; Tsang *et al.*, 2007).

In addition to its effect on *rot* mRNA translation, RNAIII also has a direct effect on the translation of certain virulence gene mRNAs (Huntzinger *et al.*, 2005; Morfeldt *et al.*, 1995). For example, the 5' part of RNAIII interacts with the untranslated leader sequence of *hla* mRNA thereby resolving an intramolecular base-pairing that otherwise block the ribosome binding site of the *hla* transcript, leading to enhanced translation of α-hemolysin (Morfeldt *et al.*, 1995). In addition, the 3' end domain of RNAIII interacts with the 5' part of *spa* mRNA and inhibits formation of the translation initiation complex (Huntzinger *et al.*, 2005).

S. aureus strains can be divided into four different *agr* groups based on the sequence of *agrB*, *agrC* and *agrD* (Jarraud *et al.*, 2000; Ji *et al.*, 1997). Each AIP can only activate *agr* in strains belonging to same group, while it inhibits *agr* in strains belonging to other groups. This observation is interesting and has been suggested for therapeutic purposes (Ji *et al.*, 1997), which is supported by studies showing that simultaneous inoculation of group-II AIP with a virulent group-I *S. aureus* strain in a murine abscess model of infection significantly attenuated virulence (Mayville *et al.*, 1999). On the other hand, we have shown that a relatively high concentration of inhibitory AIP is probably required to turn off an already activated *agr* system, meaning that it seems unlikely that an intruding *S. aureus* strain could outrival an already established strain by means of the *agr* system (**paper I**).

Some correlation between *agr* group and certain types of infections has been demonstrated, e.g. endocarditis is mainly caused by strains belonging to *agr* group I and II (Jarraud *et al.*, 2002), toxic shock syndrome by strains belonging to *agr* group III (Jarraud *et al.*, 2002; Ji *et al.*, 1997) and staphylococcal scalded skin syndrome (SSSS) by strains belonging to *agr* group IV (Jarraud *et al.*, 2000). However, no significant correlation between types of invasive infections and *agr* type have been determined (Jacobsson *et al.*, 2008) and the relevance of *agr* groups in infections can be questioned.

That the *agr* system is important during infection is strongly supported by the observations that *agr* mutants are greatly attenuated in virulence in different animal models of infection including arthritis, subcutaneous abscesses, mastitis, endocarditis and osteomyelitis (reviewed in Collins and Tarkowski, 2000). However, even though *agr* is important during different types of infection, *agr*-defective mutants still occur in clinical materials and these mutants arise and persist during infections (Traber *et al.*, 2008).

1.9.2 The *sarA* locus

The *sarA* locus (staphylococcal accessory regulator) was identified as a Tn917 insertion in the clinical strain DB leading to increased production of α-hemolysin, serine protease and lipase and decreased synthesis of coagulase and fibronectin binding proteins, a phenotype opposite to that of an *agr* mutant, indicative of a role as a transcriptional repressor (Cheung *et al.*, 1992). However, when the mutation was transferred to the prototype *S. aureus* strain NTCC8325-4 a different phenotype was observed, which resembled that of an *agr* mutant with increased synthesis of protein A and decreased production of α-hemolysin (Cheung and Projan, 1994). It was recently demonstrated (Oscarsson *et al.*, 2006a) that *sarA* seemingly acted as an activator of *hla* transcription in 8325-4 due to reduced expression of a *sarA* homologue, *sarS* (see 1.10.3). The phenotype of the 8325-4 *sarA* mutant might to some extent also be explained by reduced RNAIII levels in the *sarA* mutant (Cheung and Ying, 1994; Cheung *et al.*, 1997b; Chien and Cheung, 1998; Chien *et al.*, 1998) even though a *sarA* mutation in 8325-4 only results in slightly lower levels of RNAIII and mostly a delay in onset of RNAIII synthesis (**paper I**; Tegmark *et al.*, 2000). It should be pointed out that the effect of *sarA* on *agr* expression is more prominent in other *S. aureus* strains (Karlsson and Arvidson, 2002). The minor effect of *sarA* on *agr* expression in strain 8325-4 might be a result of low SarA levels in this strain due to a mutation in *rsbU*, which affects the activity of σB (Kullik *et al.*, 1998).

The *sarA* gene, depicted in Fig. 3, is transcribed from three different promoters (*sar* P1, *sar* P2 and *sar* P3), which terminates at a common 3' end resulting in three different transcripts of 560, 800 and 1200 nt in size (Bayer *et al.*, 1996). The *sar* P1

and *sar* P2 promoters are recognized by a vegetative sigma factor (σA) and are active during early exponential phase of growth, while the *sar P3*-promoter is recognized by an alternative sigma factor (σB) and is induced in post-exponential phase cells (Bayer *et al.*, 1996; Deora *et al.*, 1997; Manna *et al.*, 1998). Two other open reading frames are present upstream of the *sar* P1 and *sar* P3 promoters, respectively, but translation of these has not been demonstrated (Manna *et al.*, 1998). However, these coding sequences might modulate SarA expression and/or activity at the post-transcriptional level (Wolz *et al.*, 2000).

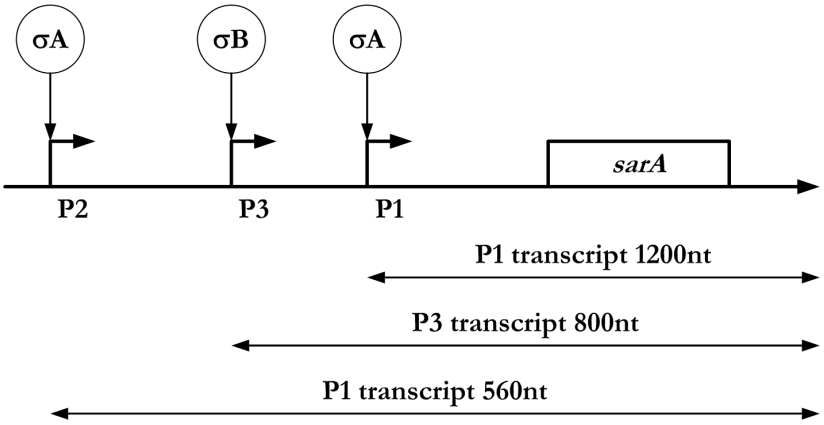

Figure 3. Schematic illustration of the *sarA* locus. (Adapted from Tegmark, 2000)

The product of the *sarA* locus is a small (14.7 kDa) basic protein (pI 9.2) with predominantly α-helical structure (Cheung and Projan, 1994; Rechtin *et al.*, 1999) and the crystal structure of SarA-DNA complex revealed that SarA binds to DNA as a dimer (Schumacher *et al.*, 2001). Foot-printing and DNA-binding experiments have suggested several SarA-binding elements (Sar boxes). In a study by Rechtin and co-workers multiple SarA-binding sites were identified in the intergenic region between *agr* P2 and *agr* P3 (Rechtin *et al.*, 1999), whereas Cheung and co-workers only identified one single site (Chien and Cheung, 1998). Similar SarA-binding sites have been identified upstream of -35 promoter boxes of several target genes regulated by *sarA* including *hla*, *spa*, *fnbA*, *fnbB* and *sec* (Chien *et al.*, 1999). However, a comparison of all sequences reported to bind SarA revealed no consensus binding sequence and that SarA binds to AT-rich sequences in an unspecific way (Tegmark, 2000).

SarA seems to regulate several target genes in a direct way independent of *agr*. Interestingly, binding of SarA seems to activate some target genes, such as the staphylococcal proteases (*aur*, *sspA* and *scp*) (**paper III**; Karlsson and Arvidson, 2002; Lindsay and Foster, 1999; Tegmark *et al.*, 2000), *cna* (Gillaspy *et al.*, 1998) and *spa* (Chien *et al.*, 1999; Sterba *et al.*, 2003), and repress others such as *tst* (Chan and Foster, 1998) and *fnbA* (Wolz *et al.*, 2000). However, it has been suggested that *sarA* is basically a repressor and the apparent activation of some genes occurs via other global regulators (Arvidson and Tegmark, 2001). This is in agreement with studies showing that transcription of *agr*, *hla* and *spa* is regulated by *sarA* via the *sarA*-homologues *sarS*, *sarT* and *sarU* (Manna and Cheung, 2003; Oscarsson *et al.*, 2006a; Schmidt *et al.*, 2001; Tegmark *et al.*, 2000).

The importance of *sarA* in virulence has been demonstrated in several animal models of infections (Booth *et al.*, 1997; Cheung *et al.*, 1994; Nilsson *et al.*, 1996). Notably, the *sarA* mutant was completely avirulent in an endocarditis model (Cheung *et al.*,

1994), where bacterial adhesins are believed to be of major importance, indicating that *sarA* is partly redundant.

1.9.3 The *sarA*-homologues

In addition to *sarA* several *sarA*-homologues have been identified, e.g. *sar*R (Manna and Cheung, 2001), *sarS* (Tegmark *et al.*, 2000), *sarT* (Schmidt *et al.*, 2001), *sarU* (Manna and Cheung, 2003), *sarV* (Manna *et al.*, 2004), *sarX* (Manna and Cheung, 2006a), *sarY* (Cheung *et al.*, 2004), *sarZ* (Tamber and Cheung, 2009) and *rot* (McNamara *et al.*, 2000). With exception for *rot* (pI 5.0) all *sarA*-homologous gene loci encode basic proteins (pI values between 8.5 and 10.7) that share high degree of similarity with SarA. All SarA-homologues have a highly conserved amino acid motif located in the C-terminal half of the proteins, which indicates a common function. Three of the SarA-homologues, SarS, SarU and SarY, are composed of two similar halves where each half is homologues to SarA (Cheung *et al.*, 2004).

SarR was found to bind the *sarA* promoter region and repress *sarA* expression (Manna and Cheung, 2001). Because of increased SarA levels in the *sar*R mutant inactivation of *sar*R also resulted in slightly increased *agr* expression but SarR also seems to directly regulate *agr* by binding to its promoter region (Manna and Cheung, 2006b). We have shown that *sar*R activity is required for maximal *aur* and *sspA* transcription (**paper III**).

SarS (previously named SarH1) was isolated when searching for proteins with affinity for the promoter regions of the RNAIII gene, *hla*, *ssp* and *spa*, respectively (Tegmark *et al.*, 2000). Northern blot analysis revealed that transcription of *sarS* was repressed by *agr* and *sarA*, and inactivation of *sarS* in *agr* and/or *sarA* mutant strains resulted in increased *hla* and decreased *spa* transcription (Tegmark *et al.*, 2000). The transcription of *spa* is low in strain 8325-4 as compared to other strains (Li *et al.*, 1997), which can be explained by low levels of SarS. This is in agreement with the observation that 8325-4 is deficient in the teicoplanin-associated locus regulator, *tca*R, that is an activator of *sarS* transcription (McCallum *et al.*, 2004).

SarT was found by searching the *S. aureus* genome for regulators with homology to *sarA* and, using Northern and Western blot analyses, it was shown that *sarT* reduces the expression of *hla* (Schmidt *et al.*, 2001). The same study also revealed that transcription of *hla* was enhanced in a *sarA sarT* double mutant as compared to the *sarA* single mutant, indicating that *sarA* partly induces *hla* transcription by repressing *sarT*. Recent studies support that *sarT* (and *rot*) is required to remove the repressor SarA from the *sarS* promoter resulting in increased SarS levels, which subsequently results in down-regulation of *hla* and up-regulation of *spa* transcription (Oscarsson *et al.*, 2005; Oscarsson *et al.*, 2006a; Schmidt *et al.*, 2003).

SarU is adjacent to *sarT* but divergently transcribed (Manna and Cheung, 2003). SarT binds to and represses *sarU* transcription, and since RNAII and RNAIII transcription was decreased in a *sarU* mutant and increased in a *sarT* mutant it was hypothesized that *sarT* down-regulates *agr* expression by repressing *sarU* (Manna and Cheung, 2003).

SarV was found to be a regulator mainly involved in autolysis. Transcription of *sarV* is repressed by SarA and MgrA, both of which bind to *sarV* promoter fragments (Manna *et al.*, 2004). Interestingly, *sarV* did not seem to regulate *sarA*, *sarR*, *sarS*, *sarT*, *sarU*, *rot* or *sae* but had a minor effect on expression of RNAII and RNAIII.

Rot was identified by screening a Tn917 transposon library for mutations that restored the expression of α-hemolysin and proteases in a genetically defined *agr*-null derivative of *S. aureus* (McNamara *et al.*, 2000). Because of its repressive effect on α-hemolysin the gene was named *rot* for repressor of toxins. Transcriptional analysis revealed that the effect of *rot* on virulence gene expression is generally opposite to that of *agr* (McNamara *et al.*, 2000; Said-Salim *et al.*, 2003) and since the regulatory function of *rot* was only observed in *agr* deficient strains, i.e. strains lacking RNAIII, it was hypothesized that RNAIII sequesters Rot, thereby preventing it from interacting with target gene promoters (McNamara *et al.*, 2000). An alternative hypothesis suggested that RNAIII is required for degradation of Rot by the ClpXP protease (Frees *et al.*, 2005). However, by using transcriptional and translational fusions, and Northern and Western hybridizations, evidence have now been presented suggesting that RNAIII blocks the translation of the *rot* mRNA through binding (Boisset *et al.*, 2007; Geisinger *et al.*, 2006).

MgrA (multiple gene regulator, also named NorR or Rat) is a *sarA*-homologue within the MarR family and is a regulator of *S. aureus* autolysis (Ingavale *et al.*, 2003; Luong *et al.*, 2003; Truong-Bolduc *et al.*, 2003). Since the discovery of *mgrA*, a number of reports have been published regarding its role in regulating virulence gene transcription and biofilm formation (Ingavale *et al.*, 2005; Ingavale *et al.*, 2003; Luong *et al.*, 2003; Luong *et al.*, 2006; Trotonda *et al.*, 2008). However, the results obtained in the different studies are contradictory to each other, not least the effect of *mgrA* on transcription of RNAIII (Ingavale *et al.*, 2005; Ingavale *et al.*, 2003), and the role of *mgrA* in virulence gene transcription is therefore difficult to assess. One reason for the contradictory results might be that *mgrA* mutants are profoundly altered in growth as compared to wild-type strains (Ingavale *et al.*, 2003; Oscarsson *et al.*, 2005; Truong-Bolduc *et al.*, 2003).

1.9.4 Sigma B

In order to cope with unfavorable conditions such as starvation, exposure to heat, high salt or extreme pH many bacteria induce specific stress programs. Some of these stresses induce activation of specific sigma factors, which are required for transcription of specific genes needed for survival (reviewed in van Schaik and Abee, 2005). In *S. aureus* there is only one stress sigma factor, σB, in addition to the vegetative sigma factor, σA (Kullik and Giachino, 1997; Wu *et al.*, 1996). Sigma factor B is encoded by *sigB*, in an operon together with the additional genes, *rsbU*, *rsbV* and *rsbW*. During normal conditions σB is bound by the anti-sigma factor RsbW and can therefore not bind to the RNA polymerase core enzyme. When the bacteria experience stress, RsbV is activated by RsbU through dephosphorylation and induces release of σB from RsbW (Palma and Cheung, 2001). The signaling pathway that activates RsbU is still unknown.

Sigma factor B controls the transcription of several genes directly, e.g. *katA* (katalase) (Horsburgh *et al.*, 2002), *asp23* (alkaline shock protein) (Gertz *et al.*, 1999; Kullik *et al.*, 1998), *clfA* and *coa* (Nicholas *et al.*, 1999), but also indirectly via the σB-dependent promoters in front of *sarA* (Bayer *et al.*, 1996; Deora *et al.*, 1997; Palma and Cheung, 2001)

and *sarS* (Tegmark *et al.*, 2000). The expression of *agr* is also repressed by σB by a yet unknown mechanism (Bischoff *et al.*, 2001). Most likely this effect is indirect via other global regulators.

Most studies on virulence gene regulation have been carried out in the prototype *S. aureus* strain 8325-4. Interestingly, this strain is σB-deficient due to an 11 bp deletion in the *rsbU* gene (Kullik *et al.*, 1998). Repairing *rsbU* (generating strain SH1000) resulted in decreased RNAIII levels and down-regulation of genes coding for exoproteins including *hla* and proteases (Giachino *et al.*, 2001; Horsburgh *et al.*, 2002). However, the reduced protease production could also be an effect of increased σB-dependent expression of *sarA* in this strain (Karlsson and Arvidson, 2002). It could be argued that because 8325-5 is σB-deficient it is not a good prototype strain to use when studying virulence gene regulation. However, in this thesis work, it was shown that the σB-deficiency in 8325-4 does not seem to have any major effect on the principal regulation of virulence factors (**paper II**).

A role of σB in infection has been shown in a murine model of septic arthritis (Jonsson *et al.*, 2004). In this animal model of infection the *rsbU*-repaired isogenic SH1000 strain caused significantly more severe arthritis as compared to 8325-4, indicating that σB might be important in certain types of infections. In addition, σB-deficient mutants were significantly less virulent in a central venous catheter-related model of multiorgan infection (Lorenz *et al.*, 2008). However, σB did not have any impact in mouse subcutaneous abscess- (Chan *et al.*, 1998), murine wound-, hematogenous pyelonephritis-, or rat osteomyelitis models of infection (Nicholas *et al.*, 1999).

1.9.5 The *saeRS*, *arlRS*, and *srrAB* two-component systems

Another regulatory locus was identified by a Tn551 insertion and was named *saeRS*, for *S. aureus* exoprotein expression, because the insertion affected the expression of many extracellular proteins (Giraudo *et al.*, 1994). The *saeRS* locus is a classical two-component signal transduction system where SaeS is the receptor protein kinase and SaeR the response regulator, respectively (Giraudo *et al.*, 1999). Transcription of *saeRS* was decreased in *agr* and *sarA* mutants during post-exponential phase of growth indicating that *saeRS* is downstream of both RNAIII and *sarA* in the regulatory pathway (Novick and Jiang, 2003). It has been verified that *sae* has no effect on RNAIII or *sarA* transcription (Giraudo *et al.*, 1997) and the regulator is therefore excluded from the mathematical models developed in this thesis.

Interestingly, a recent study showed that the effect of RNAIII on *saeRS* might be *rot*-dependent since transcription of *saeRS* (and *hla*) was increased in a *rot* single mutant derived from the σB-positive strain COL as compared to parental strain (Li and Cheung, 2008). These results are in contrast to other studies showing that *rot*-mutations has no effect on *hla* expression in *agr*-positive strains (McNamara *et al.*, 2000; Said-Salim *et al.*, 2003). One possible explanation might be that RNAIII levels in strain COL are much lower than in 8325-4 because of the σB-deficiency in 8325-4 but this hypothesis needs to be tested. The importance of *saeRS* during infections has been demonstrated in a mouse-model of infection (Giraudo *et al.*, 1994).

Another two-component system named *arlRS* was identified on the basis of its control of autolysis and the multidrug efflux pump NorA in *S. aureus* (Fournier and Hooper, 2000). Inactivation of either *arlR* or *arlS* resulted in increased transcription of *hla*,

hlb, *lip*, *coa*, *ssp* and *spa* production (Fournier *et al.*, 2001; Fournier and Klier, 2004). It was also reported that *agr*-null mutants were defective in *arlRS* expression. On the other hand, *arlS* mutant expresses higher levels of RNAII and RNAIII indicating that *agr* and *arlRS* represents an auto-repression circuit. It was also shown that *arlRS* mutations resulted in decreased *sarA* transcription suggesting that *arlRS* modulates virulence gene transcription by interacting with both *agr* and *sarA* regulatory loci. This is consistent with the reported global up-regulation of exoprotein synthesis by *arlRS* mutation, which is probably an effect of increased RNAIII levels in the *arlRS*-mutant (Fournier *et al.*, 2001). It has been shown that *arlRS* also represses the expression of *spa* (Fournier et al., 2001), probably by up-regulating *sarA* expression.

The fourth two-component system in *S. aureus* involved in expression of virulence factors, especially under microaerobic conditions, is the *srrAB* locus (staphylococcal respiratory response) (Yarwood *et al.*, 2001), previously named *srhSR* (Throup *et al.*, 2000). Northern blot analyses revealed that RNAIII expression was increased in *ssrB* mutants as compared to parental strain particularly in microaerobic conditions (Yarwood *et al.*, 2001). On the same time, transcription of *tst* was increased in the *ssrB* mutant under microaerobic conditions, and to a lesser extent, under aerobic conditions as well. Under aerobic conditions, production of protein A was down-regulated in the *ssrB* mutant as compared to the parental strain, which is in agreement with the up-regulation of RNAIII in this strain. However, under microaerobic conditions protein A expression was up-regulated in the *ssrB* mutant as compared to the parental strain. Interestingly, a recent study using strain RN4220 demonstrated that *srrAB* enhances *tst* and *spa* transcription under aerobic conditions, while under low-oxygen conditions, *srrAB* decreases transcription of these genes (Pragman *et al.*, 2007), in agreement with the earlier study (Yarwood *et al.*, 2001).

1.10 NETWORKS REGULATING VIRULENCE GENE EXPRESSION

1.10.1 Regulation of protein A

Protein A is one example of a virulence factor that is negatively regulated by RNAIII (Janzon *et al.*, 1986; Recsei *et al.*, 1986). Transcription of *spa* requires SarS, which is the main regulator of *spa* mRNA synthesis (Tegmark *et al.*, 2000). As transcription of *sarS* is inhibited by RNAIII via Rot (and *sarT*) (Oscarsson *et al.*, 2005; Said-Salim *et al.*, 2003; Schmidt *et al.*, 2001), *agr* mutants of *S. aureus* produce high levels of protein A. It has been suggested that Rot and SarT together stimulate transcription of *sarS* by removing SarA, which binds to the *sarS* promoter region and inhibits *spa* transcription (Oscarsson *et al.*, 2005; Said-Salim *et al.*, 2003; Schmidt *et al.*, 2003; Tegmark *et al.*, 2000). Rot also binds to the *spa* promoter and activates *spa* transcription in a *sarS*-independent way (Oscarsson *et al.*, 2005).

Transcription of *spa* is also repressed by *sarA* (Cheung *et al.*, 1997b), partly in a direct way (Chien *et al.*, 1999; Sterba *et al.*, 2003). Evidence have been presented supporting that SarA competes with SarS for the same binding sites within the *spa* promoter region (Gao and Stewart, 2004; Oscarsson *et al.*, 2005) but *sarA* also represses *spa* transcription in the absence of *sarS* (Tegmark *et al.*, 2000). In addition, *sarA* is a repressor of *sarT* transcription (Schmidt *et al.*, 2001).

Protein A expression is also regulated at the post-transcriptional level by RNAIII that down-regulates protein A synthesis by base-pairing with the ribosome

binding site of the *spa* mRNA, thereby recruiting endoribonuclease III, which subsequently degrades the *spa* messenger (Huntzinger *et al.*, 2005). A schematic illustration of the regulatory network governing *spa* transcription is shown in **paper II** and this network will be further discussed later in this thesis (see section 2.2.2).

1.10.2 Regulation of extracellular proteases

Expression of extracellular proteases is activated by *agr* (Björklind and Arvidson, 1980; Janzon *et al.*, 1986; Lindsay and Foster, 1999) and repressed by *sarA* (Chan and Foster, 1998; Lindsay and Foster, 1999) in such a way that production takes place during the late exponential and post-exponential phase of growth. Activation of *aur* and *sspA* by RNAIII seems to be mediated by *rot* (Oscarsson *et al.*, 2006b; Said-Salim *et al.*, 2003), which is a major repressor of proteases. It has also been shown that maximal transcription of *aur* and *sspA* requires *sar*R activity (**paper III**).

1.10.3 Regulation of α-hemolysin expression

Another virulence factor that is up-regulated by *agr* is α-hemolysin. Transcription of *hla* is initiated during exponential phase of growth when RNAIII has started to accumulate (Janzon *et al.*, 1986; Morfeldt *et al.*, 1988; Recsei *et al.*, 1986). RNAIII appears to increase expression of α-hemolysin both at the transcriptional and post-transcriptional level. Firstly, RNAIII enhances *hla* transcription by inhibiting translation of *rot* mRNA (Geisinger *et al.*, 2006; McNamara *et al.*, 2000; Oscarsson *et al.*, 2005). The lack of Rot (and *sarT*) results in reduced levels of the *hla* repressor SarS (Oscarsson *et al.*, 2005; Said-Salim *et al.*, 2003; Schmidt *et al.*, 2001; Tegmark *et al.*, 2000). It has also been suggested that *sarA* enhances *hla* expression via up-regulation of *agr* transcription. This effect could either be direct as SarA has been shown to bind to the *agr* promoter region (Cheung *et al.*, 1997a; Chien *et al.*, 1998; Heinrichs *et al.*, 1996; Sterba *et al.*, 2003) or indirect via down-regulation of *sarT*, which represses the *agr* stimulator *sarU* (Manna and Cheung, 2003; Schmidt *et al.*, 2001). Secondly, RNAIII also interacts with the *hla* transcript in a way that promotes translation (Morfeldt *et al.*, 1995). A schematic illustration of the regulatory network governing *hla* transcription is shown in figure 4.

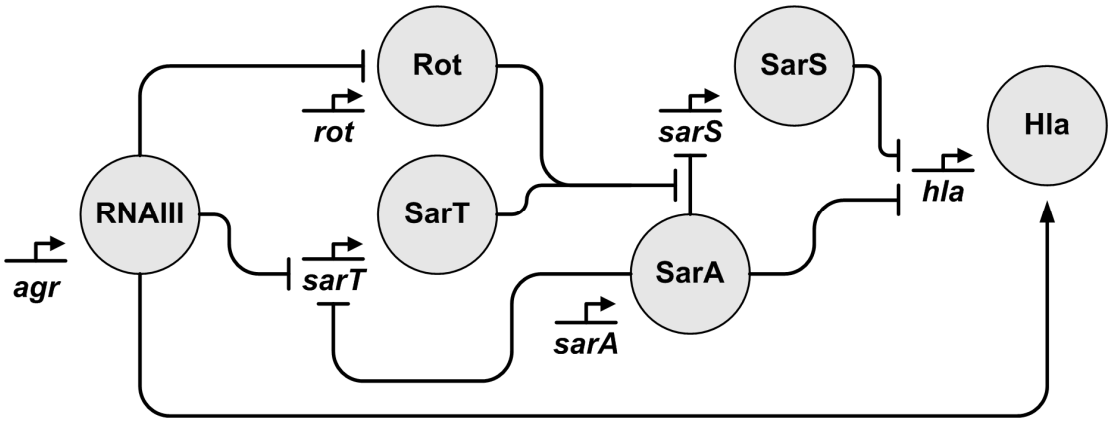

Figure 4. Schematic illustration of the regulation of α-hemolysin. (Adapted from Oscarsson *et al.*, 2006a)

In strain DB (and other clinical strains) production of α-hemolysin was increased in the *sarA* mutant as compared to the parental strain, indicating that *sarA* acted as a repressor of *hla* expression (Blevins *et al.*, 2002; Cheung *et al.*, 1992; Karlsson and Arvidson, 2002). However, when the *sarA* mutation was transferred to the prototype strain 8325-4 α-hemolysin production was decreased, instead indicating that *sarA* is an activator of *hla* transcription (Blevins *et al.*, 2002; Cheung and Ying, 1994; Tegmark *et al.*, 2000). These results seem conflicting but could be explained by differences in levels of SarS among strains (Oscarsson *et al.*, 2006a) together with the fact that *sarA* partly activates *hla* transcription by repressing *sarS* (Tegmark *et al.*, 2000).

2 THE PRESENT STUDY

2.1 AIMS OF STUDY

The *agr*-system seems to regulate virulence gene transcription via other regulators (mostly *sarA*-homologues) and this regulation is complex. The current view of the regulatory networks governing virulence gene transcription is mainly based on the analysis of strain 8325-4 harbouring different combinations of regulatory mutations. So far, few studies have been undertaken to analyze how the regulators act together in controlling the transcription of virulence genes. **The first aim** of this thesis was to study the dynamics of the *agr* system to get further insight in the function of the *agr* system.

To determine how *agr* and the other regulators work together, we have also studied the regulation of one gene (*spa*) that is negatively regulated by *agr* and the genes encoding extracellular proteases (*aur* and *sspA*), which are positively regulated by *agr*. **The second aim** of this thesis was to analyze the general principles of how each component in a regulatory system contributes to expression of a virulence gene. For this, a mathematical model of the regulation of *spa* was developed. In addition, functional analysis of the regulation of *aur* and *sspA* was also carried out. Understanding how the regulatory networks govern the expression of virulence factors is of vital importance for full comprehension of *S. aureus* pathogenesis.

2.2 RESULTS AND DISCUSSION

2.2.1 Dynamics of the *agr* system

The *agr* system in *S. aureus* consists of a positive feed-back loop (see Fig. 2) suggesting that it might be bistable, similar to the quorum sensing systems in *V. fischeri* (James *et al.*, 2000) and *P. aeruginosa* (Dockery and Keener, 2001). To test this and to get further insight in the function of the *agr* system a mathematical model was developed (**paper I**).

The mathematical model describes how the concentration of AIP influences the activity of the *agr* system, i.e. the intracellular concentration of RNAIII, in a non-growing homogeneous bacterial population. More precisely it describes the interaction between AIP and AgrC, the subsequent phosphorylation of AgrA and the following activation of the *agrC* and *agrA* genes, and *agr* P3 (RNAIII) promoter. The endogenous synthesis of AIP was disregarded in the model as we wanted to analyze RNAIII synthesis in response to a fixed AIP concentration. To be able to study how SarA would affect activation of the *agr* system by modulating the basal *agr* P2 activity, and the effect of adding inhibitory AIP to the system, these components were also included. The mathematical model was formulated using ordinary differential equations, based on fundamental kinetic principles.

As expected, plotting the steady-state level of RNAIII against AIP concentrations revealed hysteresis behaviour (**paper I**), which is characteristic of quorum sensing systems (Dockery and Keener, 2001; Fagerlind *et al.*, 2004; Karlsson *et al.*, 2007). Bistability in quorum sensing systems is a consequence of positive feed-back within the systems and because the feed-back circuits in these models were described non-linearly (reviewed in Ferrell, 2002). It should be pointed out that whether bistability occurs in our model or not is dependent on parameter values. For example, we have assumed that *sarA* mainly affects the AgrA-independent transcription of the *agr* P2 promoter. This

assumption was based on two observations; (i) RNAIII appears one hour later, at a much higher cell density, in the *sarA* mutant (PC1839) as compared to parental strain (8325-4) but reaches the same final levels in the *sarA* mutant as in wild-type (**paper I**; Dunman *et al.*, 2001; Tegmark *et al.*, 2000), and (ii) the level of the *agr* P2 transcript during early exponential phase of growth is lower in the *sarA* mutant than in parental strain (Dunman *et al.*, 2001). When simulating over-expression of *sarA* the magnitude of the hysteresis gradually decreased and finally bistability disappeared (**paper I**). It has been shown in *P. aeruginosa* that hysteresis only occurs if there is a sufficient up-regulation of the transcriptional activator LasR (Anguige *et al.*, 2004). This means that the lack of bistability in our model, when *sarA* was over-expressed, is probably an effect of decreased auto-catalytic impact of AgrA in proportion to the impact of SarA on the *agr* P2 promoter. Interestingly, in the model, inactivation of *sarA* had little impact on the final steady-state levels of RNAIII, which is in agreement with experimental studies (**paper I**; Tegmark *et al.*, 2000). On the other hand, the model predicted that inactivation of *sarA* would lead to decreased sensitivity of the system to AIP because of reduced cellular steady-state concentrations of AgrC and AgrA. This means that in a growing culture, activation of the *agr* system would occur later (at a higher cell density) due to reduced *agr* P2 activity, and therefore a lower rate of AIP accumulation in the culture, and because of decreased sensitive of the cells to AIP.

To validate the model, levels of RNAIII were analyzed in *S. aureus* strains expressing different levels of SarA (8325-4 *sarA* knockout mutant and in 8325-4 containing the inducible *sarA* gene on a plasmid) (**paper I**). The experimental data show that RNAIII synthesis started at a three-fold higher cell density in the *sarA* mutants as compared to the parental strain. When *sarA* was over-expressed RNAIII synthesis occurred after an increment in cell density half of that in the wild-type, regardless of the level of *sarA* over-expression suggesting that SarA cannot stimulate *agr* P2 activity over a certain level.

The hysteretic behaviour resulting in certain inertia of the switch probably ensures that once the system has been activated it should not be turned off in response to local fluctuations in AIP concentration. It has been shown that biofilm formation in *S. aureus* is negatively regulated by *agr* (Vuong *et al.*, 2000) meaning the bacterial cells cannot grow as a biofilm and in a planctonic state at the same time. In has also been shown that *S. aureus* cells can be internalized by epithelial and endothelial cells and that *agr* is important for the bacterial cells to survive inside the eukaryotic cell (Kahl *et al.*, 2000; Qazi *et al.*, 2001). Once the bacteria have been trapped inside the endosome AIP starts to accumulate. The switch-like activation the *agr* would result in rapid onset of exoprotein synthesis, which might be important for quick escape into the cytoplasm of the eukaryotic cell.

Natural inhibitory AIP have almost the same affinity for heterogeneous and homogeneous AgrC molecules (Mayville *et al.*, 1999). Using identical affinity constants for activating and inhibitory AIP in the model revealed that the concentration of inhibitory AIP required to turn off the *agr* system is relatively high compared to the concentration of AIP required for *agr* activation (**paper I**). Whether a high enough concentration of inhibitory AIP can be administrated to turn off the *agr* system in an established *S. aureus* infection needs to be determined. However, it seems unlikely that an intruding *S. aureus* strain could outrival an already established strain by means of its *agr* system.

2.2.2 Mathematical modelling of the regulation of *spa* transcription

Recently, a study was published describing the provisional mechanisms by which *agr* (RNAIII), *sarA*, *sarS*, *sarT*, *rot* and *mgrA* regulate *spa* transcription in the *S. aureus* prototype strain 8325-4 (Oscarsson *et al.*, 2005). In **paper II** a mathematical model of this network was formulated and analyzed to predict the role and impact of each regulator on *spa* transcription.

The mathematical model was based on the descriptive model of the network (Oscarsson *et al.*, 2005) and was formulated using ordinary differential equations. It should be pointed out that *mgrA* was excluded from the model because its role in *sarS* and *spa* expression is difficult to asses from published studies (see chapter 1.9.3). As the *agr* system acts as a bistable switch (**paper I**), we choose to model a cell in which *agr* is fully activated and RNAIII levels are maximal, which simplified the model considerably.

To be able to make quantitative predictions with the model, parameter values were determined that gave the best correlation between *sarS*, *sarT* and *spa* transcription data generated *in vitro* and *in silico* (output data from the mathematical model). More precisely, we defined a penalty-function describing how much mRNA levels, predicted with the model, differed from quantitative mRNA levels *in vitro* (derived from Northern blot experiments) in a set of regulatory mutants defining the interactions in the regulatory network. One potential global minimum was identified by searching iteratively for minima to the penalty-function using a numerical quasi-Newton method starting from randomly generated values of all parameters (**paper II**). The parameter values belonging to this minimum generated a very good match between *in silico* and experimental *in vitro* data showing that the proposed network structure could describe the *in vitro* data.

To evaluate the mathematical model we (i) simulated a cell in the late exponential phase of growth by adjusting the levels of RNAIII and SarA according to experimental mRNA levels, (ii) predicted *spa* transcription in a number of regulatory mutants, which were not previously analyzed and therefore not included in the parameter value searches and, (iii) applied published RNAIII, *sarA* and *sarS* transcription data from the clinical osteomyelitis isolate UAMS-1 to the mathematical model (**paper II**). The predictions made with the model in case (i) and (ii) were experimentally verified by Northern hybridization (**paper II**) and although these predictions did not exactly agree with experimental data, we considered the model to be accurate enough to be used for further studies. The mathematical model predicted 15-fold higher *spa* mRNA levels in strain UAMS-1 compared to RN6390 (laboratory isolate of 8325-4), which is in good agreement with published experimental data showing eight-fold higher *spa* mRNA levels in UAMS-1 compared to RN6390 (Cassat *et al.*, 2006) further strengthening the validity of the model.

Using the model, we then analyzed the direct effect of each regulator on transcription of its target gene/s in a wild-type and in an *agr*-deficient gene background (**paper II**). Interestingly, the impact of *sarS*, which is believed to be the main regulator of *spa* transcription in the wild-type (Tegmark *et al.*, 2000), seems to be small, i.e. *sarS* could only enhance *spa* transcription in a direct way up to 30 % of its maximum. Similarly, *rot* could only stimulate *spa* activity up to 15 % of its maximum. Analyzing the parameters describing *spa* mRNA synthesis in different regulatory mutants revealed that Rot alone could increase *spa* promoter activity by 70 %, while SarS alone could stimulate the promoter activity six-fold, whereas Rot and SarS together enhanced *spa* promoter activity 24-fold, suggesting that Rot and SarS might act synergistically to activate *spa*. This result

remains to be verified experimentally. Predictions with the model also showed that the ability of *sarS* to stimulate *spa* transcription in an *agr* deficient strain, in which *sarS* transcription is up-regulated and *rot* mRNA is translated, is prominently increased because of higher levels of Rot protein. However, the regulatory potential of *sarA* to repress *spa* transcription is also elevated in the *agr* mutant indicating that *sarA* and *sarS* seem to balance each other in a way that, when the activating impact of *sarS* is small, i.e. in the wild-type, the repressive impact of *sarA* is small, while in an *agr*-deficient background, when the impact of *sarS* is maximal, the repressive effect of *sarA* is close to its maximum.

Further analyses with the model (**paper II**) showed that expression of *spa* is most sensitive to changes in concentration of *sarA* followed by *sarS* > *agr* and *rot* > *sarT*, i.e. sensitivity of *spa* expression to *sarA* was two-times higher than that to *sarS* and five-times higher than that of *agr*/*rot*. Furthermore, *sarA* had the greatest impact (20-fold range of regulation) on *spa* transcription, followed by *sarS* (10-fold range of regulation). These data indicates that even though *agr* has been considered to be the overall main regulator of *spa* expression, *sarA* and *sarS* appear to be more important.

Finally, we analyzed the importance of σB in regulation of *spa* transcription since strain 8325-4 is σB-deficient (Kullik *et al.*, 1998) and it could therefore be argued that our model is not representative for σB^+ *S. aureus* strains (**paper II**). The RNAIII, *sarA* and sa*rS* mRNA levels in the *rsbU*$^+$ strain SH1000 were determined using Northern hybridization and quantitative real-time PCR. Using these data in the model, *spa* expression was predicted to be 50 % higher in SH1000 as compared to 8325-4, which was consistent with Northern hybridization. Interestingly, dose-response curves generated with the model revealed that the impact of the different regulators was essentially unaffected by the *rsbU* mutation showing that the σB-deficiency of strain 8325-4 does not affect the principal regulation of *spa* transcription.

2.2.3 The role of *sarR* in *aur* and *sspA* transcription

Production of extracellular proteases is mainly regulated by *agr*, *rot* and *sarA* (see chapter 1.10.2). Furthermore, the global regulator *sar*R represses *sarA* expression (Manna and Cheung, 2001) indicating that *sar*R might regulate *aur* and *sspA* transcription via *sarA*. In **paper III** the role of *sar*R in *aur* and *sspA* transcription was studied.

It has been shown that production of *aur* and *sspA* is controlled by a regulatory network, in which *agr*, *sarA* and *rot* seem to be most important, i.e. transcription of *aur* and *sspA* is mainly regulated through repression by *sarA* and *rot*, and RNAIII stimulates protease production by inhibiting translation of *rot* mRNA (see chapter 1.10.2). Inactivation of *sar*R in 8325-4 resulted in up-regulation of *sarA* in agreement to earlier studies (Manna and Cheung, 2001) and down-regulation of *aur* and *sspA* expression in agreement with *sarA* being a repressor of *aur* and *sspA* mRNA synthesis (Karlsson and Arvidson, 2002; Tegmark *et al.*, 2000). However, inactivation of *sar*R in a *sarA* knockout mutant revealed that *sar*R also stimulated *aur* and *sspA* transcription in a *sarA*-independent way, which is in agreement with studies showing that SarR binds to *aur* and *sspA* promoter regions (Oscarsson *et al.*, 2006b; Tegmark *et al.*, 2000). The same results were obtained in SH1000 (*rsbU*$^+$) and in one clinical strain, excluding that this direct regulatory effect of *sar*R was restricted to 8325-4 (**paper III**). We could also show that maximal *aur* and *sspA* transcription required *sar*R activity (**paper III**).

To test whether *rot* could be a possible link between *sarR* and transcriptional regulation of *aur* and *sspA* we compared *aur* and *sspA* mRNA levels in an *agr rot* double and *agr rot sarR* triple mutant (**paper III**). Our findings revealed that *aur* and *sspA* mRNA levels were lower in the *agr rot sarR* triple mutant as compared to the *agr rot* double mutant indicating that *sarR* enhanced *aur* and *sspA* transcription even in the absence of *rot*. Interestingly, inactivation of *sarR* in strain 8325-4 decreased the levels of *rot* mRNA, which might explain why the relative reduction in *aur* and *sspA* transcription was greater in the *rot sarR* double mutant than in the *sarR* single mutant.

By comparing *aur* and *sspA* mRNA levels in *sarA* single and *sarA sarR* double mutants we also concluded that repression of *aur* by *sarA* is *sarR*-dependent (**paper III**). This could perhaps be a result of SarA and SarR having cooperative regulatory effects on *aur* and *sspA* promoters. Similarly, SarR could compete with SarA (and Rot) for binding sites on the *aur* and *sspA* promoter elements. However, these hypotheses remain to be tested.

3 GENERAL CONCLUSIONS

The regulation of virulence genes in *S. aureus* is very complex and several global regulators are involved in determining their expression. The *agr* system seems to be a main regulator for controlling virulence gene expression but there are several additional regulators (mostly *sarA* homologues) involved that mainly act downstream of *agr*. Some of these regulators control virulence gene expression directly but they also regulate each other forming complex regulatory networks. In this thesis I have mainly focused on how these regulators act together in controlling transcription of virulence genes.

It has been suggested that the *agr* system control the bacterial switch from adhesive to a more invasive phenotype (Arvidson and Tegmark, 2001). Due to the positive feed-back loop within the *agr* system, RNAIII is regulated in a switch-like manner as the population density (AIP concentration) alters (**paper I**). The hysteretic behaviour results in certain inertia of the switch and probably ensures that once the system has been activated it should not be turned off in response to local fluctuations in AIP concentration. SarA does not seem essential for function of the *agr* switch but it alters the concentration of AIP (bacterial cell density) at which the *agr* system is activated.

In this thesis I have also studied one gene (*spa*) that is positively regulated by *agr* (**paper II**) and the genes encoding extracellular proteases (*aur* and *sspA*) (**paper III**), which are negatively regulated by *agr*. RNAIII regulates transcription of *spa* and the genes encoding extracellular proteases via inhibiting *rot* mRNA translation (Geisinger *et al.*, 2006). In the case of *aur* and *sspA* transcription, Rot acts as a repressor (Oscarsson *et al.*, 2006b; Said-Salim *et al.*, 2003), while in the case of *spa* transcription Rot stimulates transcription of *sarS* (Oscarsson *et al.*, 2005; Said-Salim *et al.*, 2003), which is an activator of *spa* (Tegmark *et al.*, 2000). Surprisingly, Rot also has a direct positive effect on *spa* transcription (Oscarsson *et al.*, 2005). Stimulation of *spa* transcription by SarS is mainly due to competition with the *spa* repressor SarA for binding sites at the *spa* promoter (Gao and Stewart, 2004; Oscarsson *et al.*, 2005). In contrast to its effect on *spa* transcription, SarS does not compete with SarA on binding sites at the *aur* and *sspA* promoters (Oscarsson *et al.*, 2006b). Interestingly, *sarR* has no effect on *spa* transcription (Oscarsson *et al.*, 2005) but is required for maximal transcription of *aur* and *sspA* (**paper III**).

The mathematical model developed in **paper II** revealed that Rot and SarS act synergistically to stimulate *spa* expression. As Rot and SarS do not seem to bind to the same site on the *spa* promoter region (Oscarsson *et al.*, 2005) the present study suggests that (i) association of either Rot or SarS to the *spa* promoter increases the affinity of the other, or (ii) association of both Rot and SarS increases the affinity for the RNA-polymerase or the sigma-factor to the *spa* promoter. These hypotheses could be tested either by experimental studies or by using the mathematical model developed (**paper II**). However, to test this with the model would require additional transcriptional data from strains harbouring different regulatory mutations. Using the model we also found that *sarA* has a higher regulatory potential on *spa* transcription in an *agr* mutant where the levels of Rot and SarS are high than in a wild-type where Rot and SarS are at low levels.

The effect of *agr* on transcription of the genes encoding the extracellular proteases is also indirect via *rot* (Oscarsson *et al.*, 2006b; Said-Salim *et al.*, 2003) but in contrast to regulation of *spa* this effect is *sarS*-independent. We have shown that *sarR* is required for maximal *aur* and *sspA* transcription and activates *aur* and *sspA* in a direct way (**paper III**), but also via *sarA* in agreement with earlier studies (Manna and Cheung, 2001).

Similar to *spa* regulation transcription of *aur* and *sspA* is also repressed by SarA (Chan and Foster, 1998; Lindsay and Foster, 1999). It seems that repression of *aur* by *sarA* is *sar*R-dependent (**paper III**). This could perhaps be a result of SarA and SarR having cooperatively effects on *aur* and *sspA* promoters. SarR could also compete with SarA (and Rot) for binding sites on the *aur* and *sspA* promoter elements. However, these hypotheses remain to be tested.

Interestingly, *sarS*, which is a key regulator of *spa* transcription (**paper II**; Tegmark *et al.*, 2000), has no apparent effect on transcription of *aur* and *sspA*, whereas *sar*R seems to be important in regulating transcription of *aur* and *sspA* (**paper III**) and has no effect on *spa* transcription (Oscarsson *et al.*, 2005). On the other hand, SarA is a strong repressor of transcription of both *spa* and the genes encoding the extracellular proteases (Chan and Foster, 1998; Cheung *et al.*, 1997b; Lindsay and Foster, 1999).

Based on these observations the following general model for virulence gene regulation in *S. aureus* can be hypothesised. Each specific target virulence gene is regulated by a number of *sarA*-homologues forming a module. The *sarA*-homologues within such a module acts together in both a synergistic and antagonistic way to regulate transcription of the virulence gene. To simultaneously regulate all virulence genes the modules are then connected with each other through *sarA*-homologues that are present in more than one module.

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Malin and Me
(Agnes, 2009)

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