A Closer Look at the Transcriptome of *Staphylococcus aureus*

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PhD Thesis University of Utrecht

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A Closer Look at the Transcriptome of *Staphylococcus aureus*

Staphylococcus aureus onder de loep

(met een samenvatting in het Nederlands)

Proefschrift

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door

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician; he is also a child placed before natural phenomena which impress him like a fairy tale

Marie Curie

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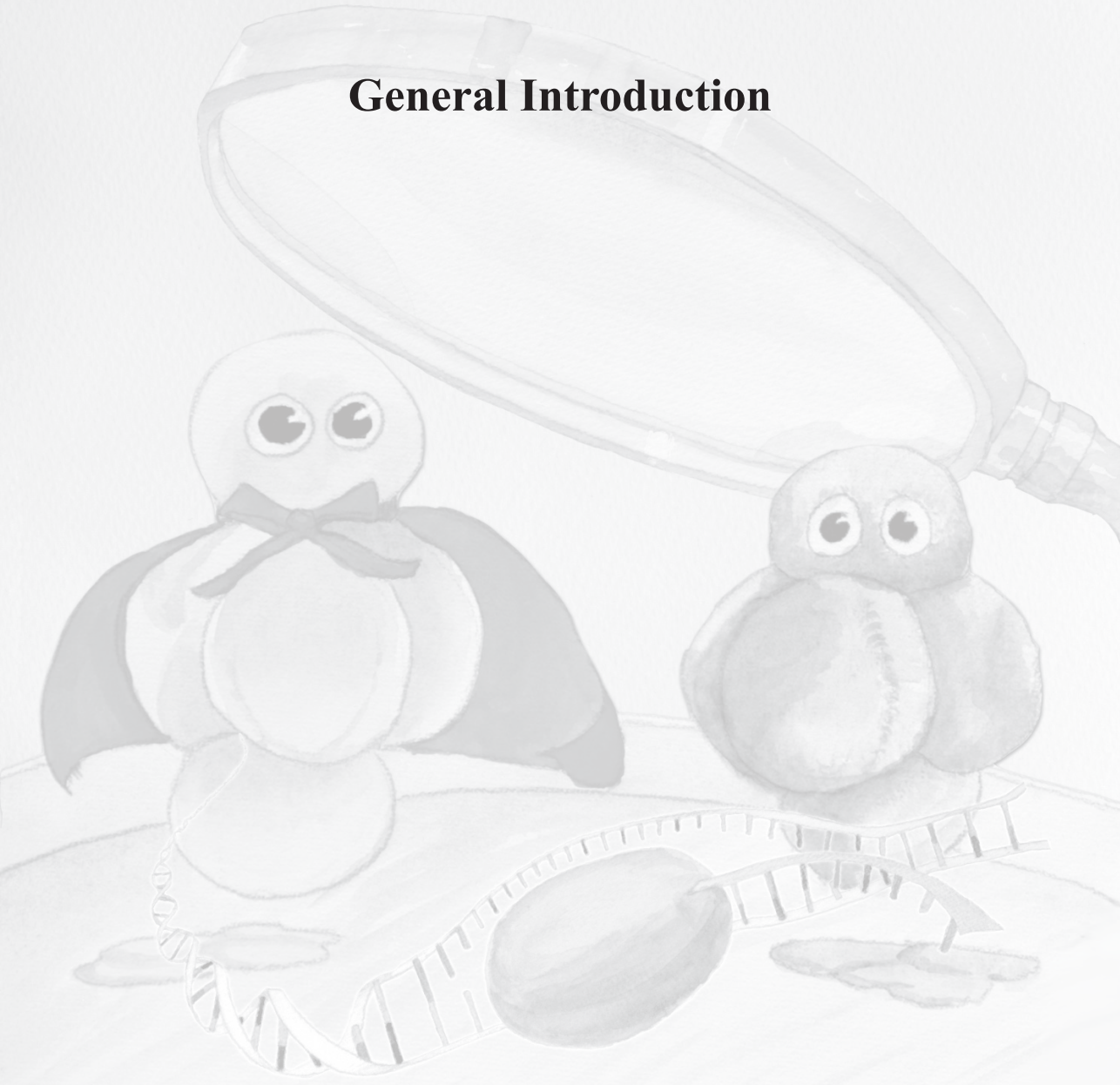

1.1 *Staphylococcus aureus*

Staphylococci belong to the class *Bacilli*, order *Bacillales* and family *Staphylococcae* (1). Under the microscope the bacteria appear as round (cocci) growing in grape-like clusters. *Staphylococcus aureus* can be distinguished from other staphylococcal species by the gold pigmentation of colonies and positive results of coagulase tests. The *S. aureus* genome is approximately 2.8 Mbp with a ~37% G+C content and contains plasmids, prophages and transposons, usually harboring virulence genes and antibiotic resistance genes which may easily be transmitted on these mobile elements (1). The combination of variable elements and virulence factors varies from strain to strain and seems to be reflective of the disease manifestation in humans or animals (2,3).

1.2 *S. aureus* colonization and infection

S. aureus is asymptomatically carried in the human population (4,5). Approximately 20% of the human population is persistent carrier of one particular strain, an additional 60% is intermittent carrier of varying strains and around 20% is non-carrier. In humans, multiple body sites can be colonized, usually the mucosal surface of the anterior nares, the pharynx, armpits, perineum and the skin (Fig. 1) (6-8). The high colonization rate can postulate an increased risk for surgical site and blood-borne infections. For this reason, nasal and pharyngeal carriers can be decolonized in the hospital setting by treatment twice daily over a period of 5 days with mupirocin use

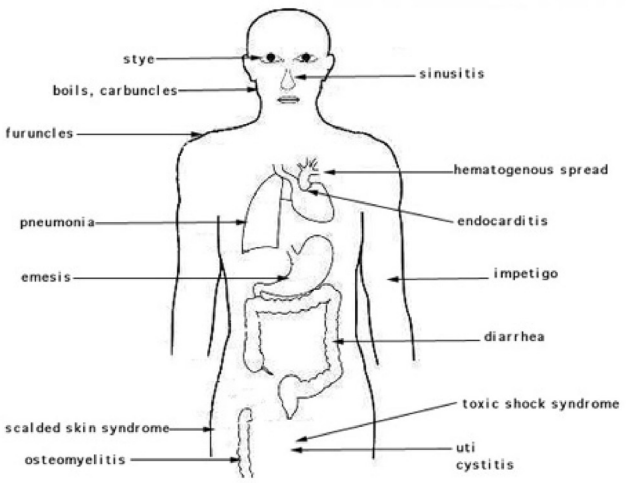

Figure 1. Colonization of human body.
S. aureus sites of infection and the associated diseases (Todar's Online Textbook of Bacteriology).

ointment (9,10). However, 5-30% of these patients remain colonized after treatment (6,11,12). Remarkably, decolonization of the nose with mupirocin ointment usually also has a decolonizing effect on the pharynx, indicating that the nose is the most important colonization and seeding place of the human body. Due to risk of developing resistance, clinicians are reluctant to mupirocin in all patients (13).

Staphylococcal infections occur when the mucosal or skin barrier breaches, e.g. by scratching, mechanical stress or surgery, thereby allowing access to the adjoining tissues or the bloodstream (1). Clinical manifestations range from skin infections to severe infections like endocarditis or osteomyelitis. The infections can be acute (1,14) or chronic (15).

A pathogen needs to adhere to cells, invade cells and circumvent the immune system to survive within the host to be able to establish an infection. For this, it requires a variety of different genes that are tightly regulated to quickly respond to changing environments. The *S. aureus* genome contains approximately 2600 to 2800 genes, including adhesins, toxins, enzymes and other secreted components important to establish infections (Table 1). Surface proteins that bind extracellular-matrix molecules, microbial-surface components recognizing adhesive matrix molecules (MSCRAMMs), are considered important for colonization of the host (1,16). Other surface proteins, like Protein A, have been recognized as having antiphagocytic properties (1). Tissue spread is facilitated by the production of large amounts of toxins. In addition, enzymes contribute in facilitating the spread of infection to adjoining tissues by destroying the tissue. The remaining secreted components are mainly involved in immune evasion.

1.3 The innate immune system

The human immune system comprises two protection mechanisms against invading organisms, the innate and the adaptive immune system. While the adaptive immune system is relatively slow and takes several days to become fully active, the innate immune system acts rapidly (17). The innate immune system consists of physical barriers, like the skin and mucous membrane as well as secretory elements, like tears and saliva. When the barrier breaches, bacteria can invade the human body and trigger the human immune system by activating the complement system. The complement system is composed of a variety of proteins and protein fragments and its role is to recruit effector molecules that label (bacterial) cells and target them for killing by immune effector cells like neutrophils (18). The complement pathway can be activated via three separate routes (Fig. 2) (19): a) The Classical Pathway, activated by antibodies; b) the Lectin Pathway, activated by recognition of conserved

microbial sugars by a mannan binding lectin; c) the Alternative Pathway, that can be spontaneous activated via hydrolyzed C3, but usually acts as an amplification loop after C3b is formed.
After activation via different targets, all three pathways join at cleavage of C3 in C3a and C3b, the latter being deposited on the target surface (20). All three pathways end with the generation of the anaphylatoxin C5a which leads to pore formation in the target cell membrane and direct killing in the case of gram-negative bacteria via the membrane attack complex. The release of chemo attractant molecules like C3a and C5a attract phagocytes to the site of infection (21). Phagocytic cells have specific receptors for complement molecules bound to the target membrane that enhance phagocytosis. Recognition of the Fc- region of IgG and complement proteins like C3b deposited on the bacterial surface is important for opsonization by neutrophils.

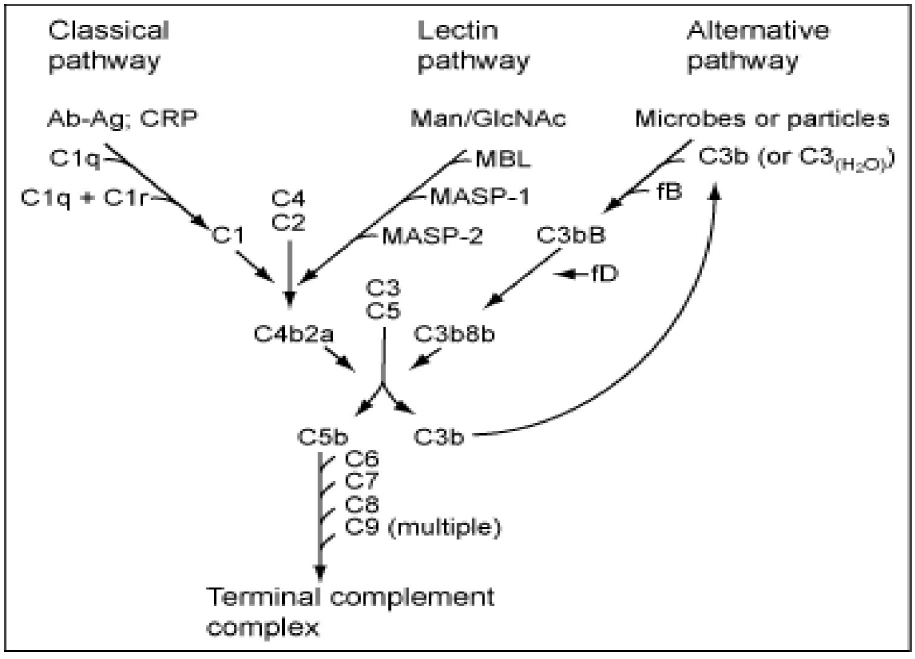

Figure 2. Separate routes of complement activation.
The Classical pathway is activated by antibodies, the Lectin pathway by recognition of conserved microbial sugars by a mannan binding lectin and the Alternative pathway is activated spontaneously by hydrolysed C3, but usually acts as amplification route after C3b deposition. All pathways come together at the C3 convertase.

Table 1. Virulence genes with function and regulation.

Product	Gene	function	Gene type	place of expression	Ref
extracellular adherence protein	*eap*	Inhibition of phagocytic engulfment	virulence gene	extra-cellular	(22,23)
adhesin	*atl*	colonization of host tissues	virulence gene	surface	(24)
bone binding protein	*bbp*	Colonization of host tissues	virulence gene	surface	(25)
collagen binding protein	*cna*	Colonization of host tissues	virulence gene	surface	(26)
fibronectin binding protein A	*fnbA*	Colonization of host tissues	virulence gene	surface	(27)
fibronectin binding protein B	*fnbB*	Colonization of host tissues	virulence gene	surface	(27)
NPQTN cell wall surface anchor protein	*isdA*	Colonization of host tissues	virulence gene	surface	(28)
serine-aspartate repeat	*sdrC*	Colonization of host tissues	virulence gene	surface	(29)
serine-aspartate repeat	*sdrD*	Colonization of host tissues	virulence gene	surface	(29)
clumping factor A	*clfA*	Colonization of host tissues/immunological disguise and modulation	virulence gene	surface	(30)
clumping factor B	*clfB*	Colonization of host tissues/immunological disguise and modulation	virulence gene	surface	(31,32)
exfoliative/epidermolytic toxin A	*eta*	Contribution to symptoms of septic shock/ scalded skin syndrome	superantigen	secreted	(33)
enterotoxin A and J	*sea*	Food poisoning/TSS	superantigen	secreted	(34,35)
enterotoxin H	*seh*	Food poisoning/TSS	superantigen	secreted	(36)
coagulase D	*coaD*	Immunological disguise and modulation	enzyme	extra-cellular	(30,32,37)
coagulase E	*coaE*	Immunological disguise and modulation	enzyme	extra-cellular	(30,32,37)
Von Willebrand binding protein	*vWbp*	Immunological disguise and modulation	enzyme	secreted	(38)
protein A	*spa*	Immunological disguise and modulation/ inhibition of phagocytic engulfment	virulence gene	surface	(32,37,39,40)
inhibitory protein	*scn*	Immunological disguise and modulation/ survival in phagocytes	virulence gene	secreted	(41)
capsular polysaccharide synthesis enzyme 5	*cap5*	Inhibition of phagocytic engulfment	virulence gene	surface	(42)
extracellular fibrinogen binding protein	*efb*	Inhibition of phagocytic engulfment	virulence gene	secreted	(43,44)

extracellular complement-binding protein	*ecb*	Inhibition of phagocytic engulfment	virulence gene	secreted	(43)
extracellular matrix binding protein	*emp*	Inhibition of phagocytic engulfment	virulence gene	extra-cellular	(45,46)
MHC class II analog	*map*	Inhibition of phagocytic engulfment	virulence gene	extra-cellular	
glycerol ester hydrolase	*geh*	Lysis of eukaryotic cell membranes and bacterial spread	enzyme	extra-cellular	(32,47)
alpha hemolysin precursor	*hla*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	extra-cellular	(32,35,37, 40,48)
beta hemolysin	*hlb*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	extra-cellular	(32,37,40)
delta hemolysin	*hld*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	extra-cellular	(37,40,48)
gamma hemolysin	*hlgA*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	secreted	(32,39)
gamma hemolysin	*hlgB*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	secreted	(32)
gamma hemolysin	*hlgC*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	secreted	(32)
hyaluronate lyase	*hysA*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	secreted	-
lipase/esterase	*Lip*	Lysis of eukaryotic cell membranes and bacterial spread	toxin	secreted	(49)
nuclease/thermonuclease	*nuc*	Lysis of eukaryotic cell membranes and bacterial spread	enzyme	surface	(47)
intercellular adhesion protein C	*icaC*	Pathogenesis	virulence gene	surface	(50)
IgG-binding protein	*sbi*	Pathogenesis	virulence gene		
staphylokinase	*sak*	Plasminogen activator	virulence gene	secreted	(40,51)
V8 protease	*sspA*	spreading factor	enzyme		(35,52)}
Staphopain (protease II)	*scp*	spreading, nutrition	virulence gene	secreted	(52)
catalase	*katA*	Survival in phagocytes	enzyme	secreted	(53)
secretory antigen precursor	*ssaA*	Survival in phagocytes	enzyme	secreted	(54)
staphyloxanthin	*staphy-loxanthin*	Survival in phagocytes	enzyme	secreted	(55)

Staphylococcal complement inhibitor B	*scn-B*	Immunological disguise and modulation/ survival in phagocytes	virulence gene	secreted	(56,57)
formyl peptide receptor (-like)	*fIpR-like*	Immunological disguise and modulation/ survival in phagocytes	virulence gene	secreted	(58,59)
Staphylococcal superantigen-like protein	*ssl7*	Immunological disguise and modulation/ survival in phagocytes	virulence gene	secreted	(60)

1.4 Staphylococcal innate immune evasion

S. aureus has developed multiple factors to overcome the fulminant attack of the human immune system and help in the invasion, the ability to cause disease and for survival in the host. It produces many extracellular factors that enable colonization of the host and toxins that damage host tissue and help in immune evasion (21,61).

I) Staphylococcal Protein A (SpA) is a surface protein that was the first anti-opsonic molecule identified in *S. aureus*. SpA binds the Fc part of IgG molecules, thereby covering the bacterial surface with IgG molecules and preventing recognition by Fc-receptors on phagocytes (62).

II) Staphylococcal Complement INhibitor (SCIN) targets C3 convertases of the Classical, Alternative and Lectin Pathway of the complement system, thereby effectively inhibiting C3b deposition on the bacterial surface and thus phagocytosis. In addition, C5a generation via all pathways does not take place thereby preventing attraction of neutrophils by C5a (41,57).

III) Extracellular fibrinogen binding protein (Efb) was first found to bind fibrinogen with its N-terminal part (45,63,64). Recent studies have shown that Efb can bind to C3b with its C-terminal part, thereby inhibiting the Classical and Alternative Pathway (65). Additionally, it was described that it inhibits platelet activation, as such inhibiting release of inflammatory mediators, and modulates platelet function (66).

IV) Phagocytosis can also be inhibited via the production of Staphylokinase (SAK) (51). SAK is able to target plasminogen to the bacterial surface and activate it into plasmin. Plasmin in turn can cleave IgG and C3b, leading to removal of IgG and C3b on the bacterial surface (67).

V) Extracellular complement-binding protein (Ecb) blocks C3b-containing convertases and down-regulates phagocytic and neutrophil responses (43). Furthermore, Ecb is able to block C5a-dependent neutrophil recruitment into the peritoneal cavity in a mouse model of immune complex peritonitis.

In addition to these factors, a number of other proteins have been described to interfere with the innate immune system. These include Formyl Peptide Receptor(-Like) (FLIPR(-like)) (58,59), Clumping factor A and B (ClfA, ClfB) (68,69) and von Willebrand-factor Binding protein (vWBp) (70).

1.5 Regulation of virulence genes

The presence or absence of a certain gene is not sufficient to explain a role in pathogenesis. Regulation is crucial to ascertain that the right amount of a gene product is produced at the right phase of bacterial growth and the infection process. Therefore virulence gene expression in many pathogens, e.g. *S. aureus* and Group A Streptococci, is required to evade the innate immune

system and establish microbial survival in the host (21,61).
In vivo virulence gene regulation differs from *in vitro* regulation by a multitude of factors. During *in vitro* growth, three distinct phases of growth can be distinguished; lag phase, exponential phase and stationary phase, influencing most of the gene expression patterns and regulation. In laboratory cultures of *S. aureus*, cell wall proteins that contribute to evasion of the immune system or act as adhesins are expressed during the exponential phase, while the production of toxins and enzymes for tissue invasion is mainly in the stationary phase (Fig. 3) (71). *In vivo* growth and gene expression however, is dependent on host factors found in blood and tissues and the factors of the innate immune system that appear during an infection (72). Most studies on staphylococcal virulence have focused on *in vitro* virulence gene regulation using rich media (73). Infection related conditions have been simulated *in vitro* using rabbit serum (74) or pig serum (75). The *in vivo* related studies mainly relied on knock-out constructs of regulator genes which showed significantly less development of infections compared to the wild-type strain (34,39,76-78). Only few studies have used patient material isolated from wounds, both deep and superficial, to study gene expression of usually low numbers of genes (79,80). These studies showed that gene expression differed marginally depending on the conditions of the infection. Another *in vivo* study described gene expression of several genes in the nose of persistent carriers (81). This study showed an elevated expression of adhesins as well as innate immune evasion proteins and is of importance for further research on gene expressions and regulation in early infections.
Differential regulation of unlinked genes encoding virulence proteins, toxins and adhesins is accomplished by global regulators, like two-component systems (of which 16 are present in the genome) (72,82-84), quorum-sensing systems, DNA-binding proteins (85,86) and the recently identified sRNAs (87-91) (Table 2).
The most extensively studied quorum sensing system is the *agr* operon, which modulates the expression of virulence genes in response to autoinducing peptides (AIP) (85). The operon combines a two-component system (a*grA* and *C*) and a density-sensing cassette (*agrB* and *D*). The Agr system controls the switch from exponential to stationary growth in standard laboratory cultures. Moreover, the *agr* locus encodes the first and the most described regulatory RNA involved in pathogenesis, RNAIII, the intracellular effector of the *agr* operon. RNAIII is a relatively large regulatory sRNA (514 nt) encoding a 26 amino acid protein, delta toxin (Hld), at the 5'-end and folds in a complex secondary structure (90,92,93). RNAIII acts as *trans*-acting sRNA and represses the expression of surface proteins in the exponential phase and activates the expression of extracellular toxins and enzymes in the post-

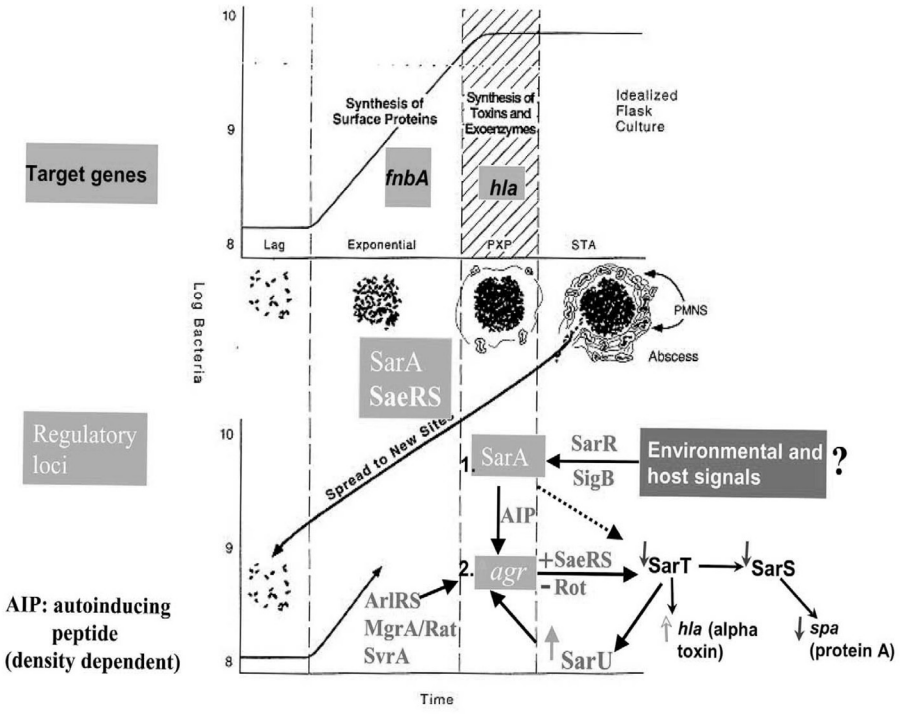

Figure 3. Regulation of virulence genes during culture laboratory growth.
Cell wall proteins are expressed during the exponential phase, while the production of toxins and enzymes is mainly in the stationary phase. Different two-component systems are active during the phases of growth, thereby regulating the genes essential in that phase (adapted from Cheung et al. (72)).

exponential phase (94,95). The 5'-end of RNAIII positively regulates the expression of *hla* by preventing intramolecular mRNA structure formation which blocks the RBS (96), while the 3'-end negatively regulates the expression of *spa*, *rot*, *efb* and *coa* by blocking the RBS for translation (92,93,97).
Novel techniques in the recent past have allowed to study regulation in even more detail. Ever since Staphylococcal RNAIII was described as the first regulatory RNA increasingly non-transcribed RNA elements, functioning as regulator, catalyzer or controller of vital cell functions and virulence, are being discovered in various species.
Genomes and transcriptomes have been and are being studied via two different technologies, namely microarray technology and whole genome sequencing. DNA microarray technology, via large scale DNA mapping (98) and sequencing (99) was used for genome-wide quantification of specific genes. Also expression patterns of specific genes were determined (100) and with the use of probes that cover the entire genome (including intergenic regions), it is

now relatively simple to study complete transcriptomes (101). In addition to the development of the microarray technology, sequencing methods have been improved and have become more cost effective. This opens the possibility to perform large scale transcriptome experiments in short periods of time with a high reproducibility, sensitivity, specificity and accuracy. Especially transcriptome sequences of published whole genome sequences are highly informative since reads can be precisely mapped on the genome to extract the maximum amount of information from the sequencing data.

Table 2. Regulators of virulence genes

Name	Gene	regulator (pos)	regulator (neg)	Ref
extracellular adherence protein	*eap*			
adhesin	*atl*			
bone binding protein	*bbp*			
collagen binding protein	*cna*			
fibronectin binding protein A	*fnbA*	SarA	Agr	(27)
fibronectin binding protein B	*fnbB*	SarA	Agr	(27)
NPQTN cell wall surface anchor protein	*isdA*			
serine-aspartate repeat	*sdrC*			
serine-aspartate repeat	*sdrD*			
clumping factor A	*clfA*			(30)
clumping factor B	*clfB*	Rot/MgrA		(31,32)
exfoliative/epidermolytic toxin A	*eta*	Agr		(102)
enterotoxin A and J	*sea*			(34,35)
enterotoxin H	*seh*			(36)
coagulase D	*coaD*	SarA/Sae/Rot	MgrA	(30,32,37)
coagulase E	*coaE*			(30,32,37)
von Willebrand binding protein	*vWbp*			
protein A	*spa*	Rot/SarH1	MgrA/ArlS	(32,37, 39,40)
inhibitory protein	*scn*	Agr		
capsular polysaccharide synthesis enzyme 5	*capA*	Agr/SarA		(42)
extracellular fibrinogen binding protein	*efb*			
extracellular complement-binding protein	*ecb*			
extracellular matrix binding protein	*emp*			
MHC class II analog	*map*			
glycerol ester hydrolase	*geh*	Agr	Rot	(32,47)
alpha hemolysin precursor	*hla*	Agr /Sae	SarH1/ MgrA/ArlS/ Rot	(32,35,37, 40,48)
beta hemolysin	*hlb*	Agr/Sae	Rot	(32,37,40)

delta hemolysin	*hld*	Agr/SarA/ MgrA/SarHI	SarT	(32,37, 40,48)
gamma hemolysin	*hlgA*	Agr	Rot	(32,39)
gamma hemolysin	*hlgB*	Agr	Rot	(32,39)
gamma hemolysin	*hlgC*	Agr	Rot	(32,39)
hyaluronate lyase	*hysA*			
lipase/esterase	*lip*	Agr/MgrA		(49)
nuclease/thermonuclease	*Nuc*	Agr/Sae/MgrA		(47)
intercellular adhesion protein C	*icaC*			
IgG-binding protein	*sbi*			
staphylokinase	*sak*	Agr		(40,51)
V8 protease	*sspA*	Agr	SarA	(35,52)
segregation and condensation protein A	*scpA*	Agr	SarA	(52)
catalase	*katA*			
secretory antigen precursor	*ssaA*			
staphyloxanthin	*staphylo-xanthin*			

1.6 Regulatory RNAs

RNA regulators of plasmid copy numbers (103,104) were already described even before the first short interfering RNAs (siRNAs) or microRNAs (miRNAs) were discovered (105). Nowadays, hundreds of candidate sRNAs in numerous bacteria have been predicted, by means of computational searches (106,107), microarray (108,109) and deep sequencing or RNA-seq approaches (88,89,110,111).

Four main classes of sRNAs can be distinguished (112): I) sRNAs acting as antisense RNAs by base pairing with target mRNA. Two different mechanisms of action have been identified. The first comprises *cis*-acting sRNAs that are characterized by the appearance of sRNA and the target mRNA on the same DNA locus, but transcribed in opposite directions. These sRNAs have an extensive binding to the target mRNA. The second group of *trans*-acting RNAs is transcribed from a different locus on the genome than the genes that are regulated and have only partial base pairing complementarities (typically 6 to 12 contiguous nucleotides) to the target mRNA. These sRNAs are able to regulate multiple targets. *Trans*-acting sRNAs are characterized by promoter and terminator sequences; II) RNAs that can sequester regulatory proteins, thus disturbing their activity; III) CRISPR RNAs regulating DNA maintenance; IV) Riboswitches and RNA thermometers found in the 5'-UTR of the mRNA they regulate.

sRNAs have functions varying from modulation of mRNA stability, DNA maintenance, transcription or translation (Fig. 4). Binding of an sRNA to the 5'-end of an mRNA transcript can repress translation

when the ribosome binding site (RBS) is blocked. However, when the RNA structure of the mRNA transcript is very complex, binding of the sRNA can make the RBS accessible and as such activate translation. In addition, sRNA binding can lead to degradation of the mRNA by ribonucleases or stabilize the mRNA. Binding can also occur at the 3'-end or in the coding region, thereby influencing translation efficiency, translation elongation or target stability. A variety of mechanisms, like protein binding, RNA conformation, RNA base pairing or interactions with DNA, is used to reach these outcomes (105). Regulation via sRNAs has several advantages compared to protein-mediated transcriptional control (113): I) Rapid responses are ensured since mRNA is the direct target; II) Only low numbers of base pairing will already ensure specific and fast recognition of mRNA; III) Rapid clearance of the sRNAs when they are no longer needed; 4) Through modulation of the conformation of RNAs, the number of contacts can be increased making recognition of multiple targets easier.

Microarray technology with (tiling) probes covering the complete genome in combination with genome and transcriptome sequencing (RNA-seq) have successfully been used to study a variety of pathogens under different conditions (88,109,111,114,115). In these studies, untranslated regions (UTRs), gene structures, operons, small RNAs and antisense transcripts have been described, all of which influencing the regulation of virulence gene expression. For example, in *Listeria monocytogenes* long 5'- and 3'-UTRs could have regulatory functions on genes antisense of these long UTRs, or 5'-UTRs could harbor riboswitches that can act as transcriptional terminator for the downstream gene (109,115). The majority of antisense RNAs will likely

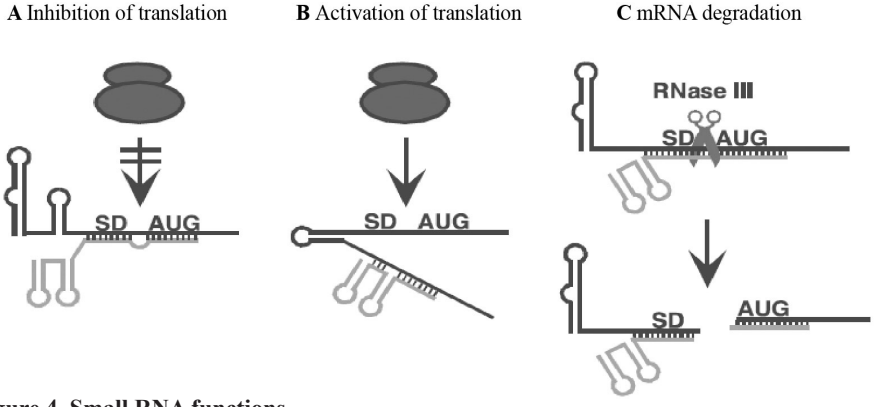

Figure 4. Small RNA functions.
(A) sRNA interaction can inhibit binding of the ribosome and block translation. (B) Binding of an sRNA can open a complex mRNA structure and release the SD-sequence for binding of the ribosome. (C) sRNA-mRNA interaction can create a molecule that is recognized by RNase III which leads to mRNA degradation. Modified from Wagner and Darfeuille, 2005.

be noncoding due to limitations imposed by the overlapping protein-coding sequence (116).
Additional sRNAs are expressed by *S. aureus* (87-91), but the functions are still largely unknown. However, a C-rich box conserved regulatory motif has been described for *S. aureus* sRNAs suggesting a shared mechanism for gene regulation (89,90). Regulatory RNAs are particularly suitable for controlling the bacterial virulence. Regulation of biofilm formation has been described for two pathogens: 1) down-regulation of PrfA, an important regulator in biofilm formation and mediator in the transition from extracellular to intracellular pathogen, in *Listeria monocytogenesis* by sRNA SreA (117). 2) Biofilm formation and the type III secretion system in *Pseudomonas aeruginosa* is governed by RsmZ (118). Recently two sRNAs regulating immune evasion proteins have been described, firstly, regulation of streptokinase activity in Group A Streptococci via FasX (119). Secondly, SprD regulates the *S. aureus* binder of IgG (*sbi*) by blocking the Shine–Dalgarno sequence and the start codon (120).

1.7 Regulation through operons

An operon is a string of open reading frames transcribed as a single mRNA, typically identified by short intergenic distances and the presence of a single promoter in front of the first open reading frame and a terminator at the end, but more complex structures have been described (49,121-124). Regulation of expression is highly dependent on the organization of an operon (123). Different theories have been proposed to describe the evolution of operon structures. Firstly, genes transcribed in an operon are usually functionally related and are often involved in the same metabolic pathway (123). Secondly, operons ensure co-transfer of genes to other genomes via horizontal transfer thereby increasing fitness and preservation of constituent genes (125). In prokaryotes, an estimated 50% of the genes is part of an operon (123). However, the regulation of especially virulence genes at the operon level not studied extensively. A reason for this could be that many virulence genes are located on mobile elements to ensure rapid exchange between different strains and are therefore not thought to be regulated at an operon level.
The complete understanding of pathogenicity is dependent on knowledge of the entire genome and transcriptome of human pathogens, including virulence and regulatory genes, regulating RNAs and operons. Nonetheless, complete information on operon structure based on experimental data are scarce and many operon predictions are based on *Escherichia coli* genomes and to lesser extent *Bacillus subtilis* genomes. These predictions mainly consider the conservation of gene clusters, intergenic distances, functional relations and

the limited available experimental evidence (121,126). For *S. aureus*, *in silico* operon predictions are available, based on the intergenic distances, conserved gene clusters, *rho*-independent terminators and the few experimentally validated operons (121,127). The combination of *in silico* data with co-expression data from high density microarrays have successfully been used for *E. coli* and have yielded accurate results (128,129).
Other key features in gene regulation are UTRs located at the 5'- or 3'-end of genes or operons. Especially 5'-UTRs can harbor important regulatory functions, like riboswitches, that regulate their own activity in response to concentrations of its target molecule and control many metabolic pathways. Stabilizing effects on the mRNA transcript, including regulation of the half-life, mainly occur via the 3'-UTR (130,131). In addition, many *trans*-encoded small non-coding RNAs (sRNAs) bind to the 5'-UTR to regulate translation and/or stability of the mRNA transcript (130-132).

1.8 Aim and outline of this thesis

The aim of this thesis was to increase the knowledge of the interaction of *S. aureus* with its host to further complete the comprehension of the pathogen's success rate; first of all, by determining the exact niche of *S. aureus* during carrier-ship. Secondly, through an improved understanding of the diverse regulatory mechanisms at transcript level of this major human pathogen.

Colonization is an important step in infection. Nasal carriage of *S. aureus* is well known and the nose is considered the main seeding place for infections. However, the exact niche in the nose is still unknown. Knowledge of this niche is the first step in understanding *S. aureus* colonization. Therefore, the location of *S. aureus* in nasal tissue was determined (**Chapter 2**).
Gene regulation is the main focus of this thesis. For this purpose, gene expression levels under standard growth conditions for all genes, the reverse complement of these genes as well as probes covering the intergenic regions on both strands were determined using microarray technology. The experimental identification of the operon structure and the 5'- and 3'-UTRs is an important step in understanding gene regulation of *S. aureus* and the microarray data were used to study and unravel the operon structures of *S. aureus* and identify 5'- and 3'-UTRs (**Chapter 3**). Small noncoding RNAs (sRNAs) are increasingly recognized to contribute to regulation of prokaryotic gene expression and the presence of these regulating elements was also explored during standard laboratory growth with the focus on interactions with virulence genes (**Chapter 4**). Since regulation of virulence genes is of particular interest, knock-out and overexpression strains were used to explore

the *in vivo* relevance of putative sRNAs to their predicted mRNA targets coding for proteins involved in virulence. In **Chapter 5**, gene regulation was studied further by exposing two genetically similar *S. aureus* strains to human blood and IMDM both at 5% CO_2. The gene expression of each strain upon exposure to these conditions was determined and comparison of the altered gene expression revealed remarkable differences between the strains, but also between the conditions. Identification and understanding of these differences between similar strains, is an important step for improved comprehension of the *S. aureus* infections. Finally in **Chapter 6**, the studies described in this thesis are summarized, discussed and put in perspective.

References

1. Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N.Engl.J.Med.*, **339**, 520-532.
2. Baba, T., Bae, T., Schneewind, O., Takeuchi, F. and Hiramatsu, K. (2008) Genome sequence of Staphylococcus aureus strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *J.Bacteriol.*, **190**, 300-310.
3. Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T. *et al.* (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, **359**, 1819-1827.
4. Bode, L.G.M., Kluytmans, J.A.J.W., Wertheim, H.F.L., Bogaers, D., Vandenbroucke-Grauls, C.M.J.E., Roosendaal, R., Troelstra, A., Box, A.T.A., Voss, A., van der Tweel, I. *et al.* (2010) Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. *N Engl J Med*, **362**, 9-17.
5. Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin.Microbiol.Rev.*, **10**, 505-520.
6. Perl, T.M., Cullen, J.J., Wenzel, R.P., Zimmerman, M.B., Pfaller, M.A., Sheppard, D., Twombley, J., French, P.P. and Herwaldt, L.A. (2002) Intranasal mupirocin to prevent postoperative *Staphylococcus aureus* infections. *N Engl J Med*, **346**, 1871-1877.
7. Wertheim, H.F.L., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A. and Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis*, **5**, 751-762.
8. Williams, R.E. (1963) Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriol Rev*, **27**, 56-71.
9. Kallen, A.J., Wilson, C.T. and Larson, R.J. (2005) Perioperative intranasal mupirocin for the prevention of surgical-site infections: systematic review of the literature and meta-analysis. *Infect Control Hosp Epidemiol*, **26**, 916-922.
10. van Rijen, M., Bonten, M., Wenzel, R. and Kluytmans, J. (2008) Mupirocin ointment for preventing *Staphylococcus aureus* infections in nasal carriers. *Cochrane Database Syst Rev*, CD006216.
11. Ammerlaan, H.S., Kluytmans, J.A., Wertheim, H.F., Nouwen, J.L. and Bonten, M.J. (2009) Eradication of methicillin-resistant *Staphylococcus aureus* carriage: a systematic review. *Clin Infect Dis*, **48**, 922-930.

12. Wertheim, H.F.L., Verveer, J., Boelens, H.A.M., van Belkum, A., Verbrugh, H.A. and Vos, M.C. (2005) Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. *Antimicrob Agents Chemother*, **49**, 1465-1467.
13. Humphreys, H. (2009) Preventing surgical site infection. Where now? *J Hosp Infect*, **73**, 316-322.
14. Vojtov, N., Ross, H.F. and Novick, R.P. (2002) Global repression of exotoxin synthesis by staphylococcal superantigens. *Proc.Natl.Acad.Sci.U.S.A*, **99**, 10102-10107.
15. Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., Renzoni, A., Lucchini, S., Lew, D.P., Vaudaux, P. *et al.* (2007) A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC.Genomics*, **8**, 171-185.
16. Patti, J.M., Allen, B.L., McGavin, M.J. and Hook, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol*, **48**, 585-617.
17. Beutler, B. (2004) Innate immunity: an overview. *Mol Immunol*, **40**, 845-859.
18. Rooijakkers, S.H. and van Strijp, J.A. (2007) Bacterial complement evasion. *Mol. Immunol.*, **44**, 23-32.
19. Gasque, P. (2004) Complement: a unique innate immune sensor for danger signals. *Mol.Immunol.*, **41**, 1089-1098.
20. Serruto, D., Rappuoli, R., Scarselli, M., Gros, P. and van Strijp, J.A. (2010) Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat Rev Microbiol*, **8**, 393-399.
21. Foster, T.J. (2005) Immune evasion by *staphylococci*. *Nat.Rev.Microbiol.*, **3**, 948-958.
22. Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.I., Herrmann, M. and Preissner, K.T. (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med*, **8**, 687-693.
23. Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. and Flock, J.I. (2003) The adhesive and immunomodulating properties of the multifunctional *Staphylococcus aureus* protein Eap. *Microbiology*, **149**, 2701-2707.
24. Takahashi, J., Komatsuzawa, H., Yamada, S., Nishida, T., Labischinski, H., Fujiwara, T., Ohara, M., Yamagishi, J. and Sugai, M. (2002) Molecular characterization of an atl null mutant of Staphylococcus aureus. *Microbiol Immunol*, **46**, 601-612.
25. Tung, H., Guss, B., Hellman, U., Persson, L., Rubin, K. and Ryden, C. (2000) A bone sialoprotein-binding protein from Staphylococcus aureus: a member of the staphylococcal Sdr family. *Biochem J*, **345 Pt 3**, 611-619.
26. Benito, Y., Lina, G., Greenland, T., Etienne, J. and Vandenesch, F. (1998) trans-complementation of a *Staphylococcus aureus agr* mutant by *Staphylococcus lugdunensis agr* RNAIII. *J Bacteriol*, **180**, 5780-5783.
27. Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J. and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *Rna*, **6**, 668-679.
28. Clarke, S.R., Mohamed, R., Bian, L., Routh, A.F., Kokai-Kun, J.F., Mond, J.J., Tarkowski, A. and Foster, S.J. (2007) The Staphylococcus aureus surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe*, **1**, 199-212.
29. O'Brien, L., Kerrigan, S.W., Kaw, G., Hogan, M., Penades, J., Litt, D., Fitzgerald, D.J., Foster, T.J. and 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. *Mol Microbiol*, **44**, 1033-1044.
30. Birkey, S.M., Liu, W., Zhang, X., Duggan, M.F. and Hulett, F.M. (1998) Pho signal transduction network reveals direct transcriptional regulation of one two-component system by another two-component regulator: *Bacillus subtilis* PhoP directly regulates production of ResD. *Mol Microbiol*, **30**, 943-953.
31. Chamberlain, N.R. and Imanoel, B. (1996) Genetic regulation of fatty acid modifying enzyme from *Staphylococcus aureus*. *J Med Microbiol*, **44**, 125-129.
32. Chan, P.F. and Foster, S.J. (1998) The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. *Microbiology*, **144 (Pt 9)**, 2469-2479.
33. Balaban, N., Goldkorn, T., Gov, Y., Hirshberg, M., Koyfman, N., Matthews, H.R., Nhan, R.T., Singh, B. and Uziel, O. (2001) Regulation of Staphylococcus aureus pathogenesis via target of RNAIII-activating Protein (TRAP). *J Biol Chem*, **276**, 2658-2667.
34. Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C. and Tarkowski, A. (1993) The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect Immun*, **61**, 3879-3885.
35. Alekshun, M.N., Levy, S.B., Mealy, T.R., Seaton, B.A. and Head, J.F. (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. *Nat Struct Biol*, **8**, 710-714.
36. Kravitz, G.R., Dries, D.J., Peterson, M.L. and Schlievert, P.M. (2005) Purpura fulminans due to *Staphylococcus aureus*. *Clin Infect Dis*, **40**, 941-947.
37. de Beer, T., Fang, J., Ortega, M., Yang, Q., Maes, L., Duffy, C., Berton, N., Sippy, J., Overduin, M., Feiss, M. *et al.* (2002) Insights into specific DNA recognition during the assembly of a viral genome packaging machine. *Mol Cell*, **9**, 981-991.
38. Bjerketorp, J., Jacobsson, K. and Frykberg, L. (2004) The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. *FEMS Microbiol Lett*, **234**, 309-314.
39. Blevins, J.S., Elasri, M.O., Allmendinger, S.D., Beenken, K.E., Skinner, R.A., Thomas, J.R. and Smeltzer, M.S. (2003) Role of sarA in the pathogenesis of *Staphylococcus aureus* musculoskeletal infection. *Infect Immun*, **71**, 516-523.
40. Bunce, C., Wheeler, L., Reed, G., Musser, J. and Barg, N. (1992) Murine model of cutaneous infection with gram-positive cocci. *Infect Immun*, **60**, 2636-2640.
41. Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M. and van Strijp, J.A.G. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat.Immunol.*, **6**, 920-927.
42. Chakrabarti, S.K. and Misra, T.K. (2000) SarA represses agr operon expression in a purified in vitro Staphylococcus aureus transcription system. *J Bacteriol*, **182**, 5893-5897.
43. Jongerius, I., Kohl, J., Pandey, M.K., Ruyken, M., van Kessel, K.P.M., van Strijp, J.A.G. and Rooijakkers, S.H.M. (2007) Staphylococcal complement evasion by various convertase-blocking molecules. *J Exp Med*, **204**, 2461-2471.
44. Palma, M., Shannon, O., Quezada, H.C., Berg, A. and Flock, J.I. (2001) Extracellular fibrinogen-binding protein, Efb, from *Staphylococcus aureus* blocks platelet aggregation due to its binding to the alpha-chain. *J Biol Chem*, **276**, 31691-31697.
45. Boden, M.K. and Flock, J.I. (1992) Evidence for three different fibrinogen-binding proteins with unique properties from *Staphylococcus aureus* strain Newman. *Microb*

Pathog, **12**, 289-298.
46. Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., Qazi, S., Hill, P. and Herrmann, M. (2005) sae is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology*, **151**, 1789-1800.
47. Bischoff, M., Entenza, J.M. and Giachino, P. (2001) Influence of a functional sigB operon on the global regulators sar and agr in Staphylococcus aureus. *J Bacteriol*, **183**, 5171-5179.
48. Bayer, M.G., Heinrichs, J.H. and Cheung, A.L. (1996) The molecular architecture of the *sar* locus in *Staphylococcus aureus*. *J Bacteriol*, **178**, 4563-4570.
49. Brunskill, E.W. and Bayles, K.W. (1996) Identification of LytSR-regulated genes from *Staphylococcus aureus*. *J.Bacteriol.*, **178**, 5810-5812.
50. Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W. and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. *Infect Immun*, **67**, 5427-5433.
51. Rooijakkers, S.H., van Wamel, W.J., Ruyken, M., van Kessel, K.P. and van Strijp, J.A. (2005) Anti-opsonic properties of staphylokinase. *Microbes.Infect.*, **7**, 476-484.
52. Bronner, S., Stoessel, P., Gravet, A., Monteil, H. and Prevost, G. (2000) Variable expressions of Staphylococcus aureus bicomponent leucotoxins semiquantified by competitive reverse transcription-PCR. *Appl Environ Microbiol*, **66**, 3931-3938.
53. Over, U., Tuc, Y. and Soyletir, G. (2000) Catalase-negative Staphylococcus aureus: a rare isolate of human infection. *Clin Microbiol Infect*, **6**, 681-682.
54. Martin, P.K., Bao, Y., Boyer, E., Winterberg, K.M., McDowell, L., Schmid, M.B. and Buysse, J.M. (2002) Novel locus required for expression of high-level macrolide-lincosamide-streptogramin B resistance in Staphylococcus aureus. *J Bacteriol*, **184**, 5810-5813.
55. Clauditz, A., Resch, A., Wieland, K.P., Peschel, A. and Gotz, F. (2006) Staphyloxanthin plays a role in the fitness of Staphylococcus aureus and its ability to cope with oxidative stress. *Infect Immun*, **74**, 4950-4953.
56. Rooijakkers, S.H.M., Ruyken, M., van, R.J., van Kessel, K.P.M., van Strijp, J.A.G. and van Wamel, W.J.B. (2006) Early expression of SCIN and CHIPS drives instant immune evasion by Staphylococcus aureus. *Cell Microbiol.*, **8**, 1282-1293.
57. Rooijakkers, S.H.M., Wu, J., Ruyken, M., van Domselaar, R., Planken, K.L., Tzekou, A., Ricklin, D., Lambris, J.D., Janssen, B.J., van Strijp, J.A.G. *et al.* (2009) Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nat Immunol*, **10**, 721-727.
58. Prat, C., Bestebroer, J., de Haas, C.J., van Strijp, J.A. and van Kessel, K.P. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol*, **177**, 8017-8026.
59. Prat, C., Haas, P.J., Bestebroer, J., de Haas, C.J., van Strijp, J.A. and van Kessel, K.P. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from *Staphylococcus aureus* (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J Immunol*, **183**, 6569-6578.
60. Bestebroer, J., Aerts, P.C., Rooijakkers, S.H., Pandey, M.K., Kohl, J., van Strijp, J.A. and de Haas, C.J. (2010) Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell Microbiol*, **12**, 1506-1516.
61. Rooijakkers, S.H.M., van Kessel, K.P.M. and van Strijp, J.A.G. (2005) Staphylococcal innate immune evasion. *Trends Microbiol.*, **13**, 596-601.
62. Forsgren, A. and Sjoquist, J. (1966) "Protein A" from S. aureus. I. Pseudo-immune reaction with human gamma-globulin. *J Immunol*, **97**, 822-827.
63. Boden, M.K. and Flock, J.I. (1989) Fibrinogen-binding protein/clumping factor from

Staphylococcus aureus. *Infect Immun*, **57**, 2358-2363.
64. Boden, M.K. and Flock, J.I. (1994) Cloning and characterization of a gene for a 19 kDa fibrinogen-binding protein from Staphylococcus aureus. *Mol Microbiol*, **12**, 599-606.
65. Lee, L.Y., Liang, X., Hook, M. and Brown, E.L. (2004) Identification and characterization of the C3 binding domain of the Staphylococcus aureus extracellular fibrinogen-binding protein (Efb). *J Biol Chem*, **279**, 50710-50716.
66. Palma, M., Nozohoor, S., Schennings, T., Heimdahl, A. and Flock, J.I. (1996) Lack of the extracellular 19-kilodalton fibrinogen-binding protein from Staphylococcus aureus decreases virulence in experimental wound infection. *Infect Immun*, **64**, 5284-5289.
67. Harpel, P.C., Sullivan, R. and Chang, T.S. (1989) Binding and activation of plasminogen on immobilized immunoglobulin G. Identification of the plasmin-derived Fab as the plasminogen-binding fragment. *J Biol Chem*, **264**, 616-624.
68. Entenza, J.M., Foster, T.J., Ni, E.D., Vaudaux, P., Francioli, P. and Moreillon, P. (2000) Contribution of clumping factor B to pathogenesis of experimental endocarditis due to Staphylococcus aureus. *Infect.Immun.*, **68**, 5443-5446.
69. Heying, R., van de, G.J., Que, Y.A., Moreillon, P. and Beekhuizen, H. (2007) Fibronectin-binding proteins and clumping factor A in Staphylococcus aureus experimental endocarditis: FnBPA is sufficient to activate human endothelial cells. *Thromb.Haemost.*, **97**, 617-626.
70. McCarthy, A.J. and Lindsay, J.A. (2010) Genetic variation in Staphylococcus aureus surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. *BMC Microbiol*, **10**, 173-188.
71. Cheung, A.L., Projan, S.J. and Gresham, H. (2002) The Genomic Aspect of Virulence, Sepsis, and Resistance to Killing Mechanisms in Staphylococcus aureus. *Curr Infect Dis Rep*, **4**, 400-410.
72. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol.Med.Microbiol.*, **40**, 1-9.
73. Ray, B., Ballal, A. and Manna, A.C. (2009) Transcriptional variation of regulatory and virulence genes due to different media in *Staphylococcus aureus*. *Microb Pathog*, **47**, 94-100.
74. Yarwood, J.M., McCormick, J.K., Paustian, M.L., Kapur, V. and Schlievert, P.M. (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. *J.Bacteriol.*, **184**, 1095-1101.
75. Wiltshire, M.D. and Foster, S.J. (2001) Identification and analysis of *Staphylococcus aureus* components expressed by a model system of growth in serum. *Infect.Immun.*, **69**, 5198.
76. Cheung, A.L., Eberhardt, K.J., Chung, E., Yeaman, M.R., Sullam, P.M., Ramos, M. and Bayer, A.S. (1994) Diminished virulence of a sar-/agr- mutant of Staphylococcus aureus in the rabbit model of endocarditis. *J.Clin.Invest*, **94**, 1815-1822.
77. Cheung, A.L., Yeaman, M.R., Sullam, P.M., Witt, M.D. and Bayer, A.S. (1994) Role of the sar locus of Staphylococcus aureus in induction of endocarditis in rabbits. *Infect.Immun.*, **62**, 1719-1725.
78. Nilsson, I.M., Bremell, T., Ryden, C., Cheung, A.L. and Tarkowski, A. (1996) Role of the staphylococcal accessory gene regulator (sar) in septic arthritis. *Infect Immun*, **64**, 4438-4443.
79. Joost, I., Blass, D., Burian, M., Goerke, C., Wolz, C., von Muller, L., Becker, K., Preissner, K., Herrmann, M. and Bischoff, M. (2009) Transcription analysis of the

extracellular adherence protein from *Staphylococcus aureus* in authentic human infection and *in vitro*. *J Infect Dis*, **199**, 1471-1478.
80. Loughman, J.A., Fritz, S.A., Storch, G.A. and Hunstad, D.A. (2009) Virulence gene expression in human community-acquired *Staphylococcus aureus* infection. *J.Infect. Dis.*, **199**, 294-301.
81. Burian, M., Wolz, C. and Goerke, C. (2010) Regulatory adaptation of *Staphylococcus aureus* during nasal colonization of humans. *PLoS One*, **5**, e10040.
82. Arvidson, S. and Tegmark, K. (2001) Regulation of virulence determinants in *Staphylococcus aureus*. *Int J Med Microbiol*, **291**, 159-170.
83. Cheung, A.L. and Zhang, G. (2002) Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Front Biosci*, **7**, d1825-1842.
84. Novick, R.P. and Jiang, D. (2003) The staphylococcal *saeRS* system coordinates environmental signals with *agr* quorum sensing. *Microbiology*, **149**, 2709.
85. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
86. Pragman, A.A. and Schlievert, P.M. (2004) Virulence regulation in *Staphylococcus aureus*: the need for *in vivo* analysis of virulence factor regulation. *FEMS Immunol. Med.Microbiol.*, **42**, 147-154.
87. Abu-Qatouseh, L.F., Chinni, S.V., Seggewiss, J., Proctor, R.A., Brosius, J., Rozhdestvensky, T.S., Peters, G., von Eiff, C. and Becker, K. (2010) Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. *J Mol Med*, **88**, 565-575.
88. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J. and Francois, P. (2010) Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One*, **5**, e10725.
89. Bohn, C., Rigoulay, C., Chabelskaya, S., Sharma, C.M., Marchais, A., Skorski, P., Borezee-Durant, E., Barbet, R., Jacquet, E., Jacq, A. *et al.* (2010) Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. *Nucleic Acids Res*, **38**, 6620-6636.
90. Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C. *et al.* (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res*, **37**, 7239-7257.
91. Scott, M.G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., Wang, A., Lee, K., Doria, S., Hamill, P. *et al.* (2007) An anti-infective peptide that selectively modulates the innate immune response. *Nat.Biotechnol.*, **25**, 465-472.
92. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**, 1353-1366.
93. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.
94. Janzon, L. and Arvidson, S. (1990) The role of the delta-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Embo J*, **9**, 1391-1399.
95. Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B. and Moghazeh,

S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.*, **12**, 3967-3975.
96. Morfeldt, E., Taylor, D., von Gabain, A. and Arvidson, S. (1995) Activation of alpha-toxin translation in Staphylococcus aureus by the trans-encoded antisense RNA, RNAIII. *Embo J*, **14**, 4569-4577.
97. Geisinger, E., Adhikari, R.P., Jin, R., Ross, H.F. and Novick, R.P. (2006) Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol.Microbiol.*, **61**, 1038-1048.
98. Poustka, A., Pohl, T., Barlow, D.P., Zehetner, G., Craig, A., Michiels, F., Ehrich, E., Frischauf, A.M. and Lehrach, H. (1986) Molecular approaches to mammalian genetics. *Cold Spring Harb Symp Quant Biol*, **51 Pt 1**, 131-139.
99. Cantor, C.R., Mirzabekov, A. and Southern, E. (1992) Report on the sequencing by hybridization workshop. *Genomics*, **13**, 1378-1383.
100. Kato-Maeda, M., Gao, Q. and Small, P.M. (2001) Microarray analysis of pathogens and their interaction with hosts. *Cell Microbiol.*, **3**, 713-719.
101. Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467-470.
102. Balaban, N. and Novick, R.P. (1995) Translation of RNAIII, the *Staphylococcus aureus agr* regulatory RNA molecule, can be activated by a 3'-end deletion. *FEMS Microbiol.Lett.*, **133**, 155-161.
103. Stougaard, P., Molin, S. and Nordstrom, K. (1981) RNAs involved in copy-number control and incompatibility of plasmid R1. *Proc Natl Acad Sci U S A*, **78**, 6008-6012.
104. Tomizawa, J. and Itoh, T. (1981) Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. *Proc Natl Acad Sci U S A*, **78**, 6096-6100.
105. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615-628.
106. Livny, J., Teonadi, H., Livny, M. and Waldor, M.K. (2008) High-throughput, kingdom-wide prediction and annotation of bacterial non-coding RNAs. *PLoS.ONE.*, **3**, e3197.
107. Weinberg, Z., Barrick, J.E., Yao, Z., Roth, A., Kim, J.N., Gore, J., Wang, J.X., Lee, E.R., Block, K.F., Sudarsan, N. *et al.* (2007) Identification of 22 candidate structured RNAs in bacteria using the CMfinder comparative genomics pipeline. *Nucleic Acids Res*, **35**, 4809-4819.
108. Landt, S.G., Abeliuk, E., McGrath, P.T., Lesley, J.A., McAdams, H.H. and Shapiro, L. (2008) Small non-coding RNAs in Caulobacter crescentus. *Mol Microbiol*, **68**, 600-614.
109. Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K. *et al.* (2009) The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature*, **459**, 950-956.
110. Guell, M., van Noort, V., Yus, E., Chen, W.H., Leigh-Bell, J., Michalodimitrakis, K., Yamada, T., Arumugam, M., Doerks, T., Kuhner, S. *et al.* (2009) Transcriptome complexity in a genome-reduced bacterium. *Science*, **326**, 1268-1271.
111. Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermuller, J., Reinhardt, R. *et al.* (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature*, **464**, 250-255.
112. Romby, P. and Charpentier, E. (2010) An overview of RNAs with regulatory functions in gram-positive bacteria. *Cell Mol Life Sci*, **67**, 217-237.
113. Lioliou, E., Romilly, C., Romby, P. and Fechter, P. (2010) RNA-mediated regulation

in bacteria: from natural to artificial systems. *N Biotechnol*, **27**, 222-235.
113. Lioliou, E., Romilly, C., Romby, P. and Fechter, P. (2010) RNA-mediated regulation in bacteria: from natural to artificial systems. *N Biotechnol*, **27**, 222-235.
114. Sharma, C.M. and Vogel, J. (2009) Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol*, **12**, 536-546.
115. Sorek, R. and Cossart, P. (2010) Prokaryotic transcriptomics: a new view on regulation, physiology and pathogenicity. *Nat Rev Genet*, **11**, 9-16.
116. Dornenburg, J.E., Devita, A.M., Palumbo, M.J. and Wade, J.T. (2010) Widespread antisense transcription in *Escherichia coli*. *MBio*, **1**, e00024-00010.
117. Loh, E., Dussurget, O., Gripenland, J., Vaitkevicius, K., Tiensuu, T., Mandin, P., Repoila, F., Buchrieser, C., Cossart, P. and Johansson, J. (2009) A trans-acting riboswitch controls expression of the virulence regulator PrfA in Listeria monocytogenes. *Cell*, **139**, 770-779.
118. Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. and Filloux, A. (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A*, **103**, 171-176.
119. Ramirez-Pena, E., Trevino, J., Liu, Z., Perez, N. and Sumby, P. (2010) The group A Streptococcus small regulatory RNA FasX enhances streptokinase activity by increasing the stability of the *ska* mRNA transcript. *Mol Microbiol*, **78**, 1332-1347.
120. Chabelskaya, S., Gaillot, O. and Felden, B. (2010) A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog*, **6**, e1000927.
121. Brouwer, R.W.W., Kuipers, O.P. and Hijum, S.A.F.T. (2008) The relative value of operon predictions. *Brief.Bioinform.*, **9**, 367-375 Epub.
122. Helmann, J.D., Wu, M.F., Kobel, P.A., Gamo, F.J., Wilson, M., Morshedi, M.M., Navre, M. and Paddon, C. (2001) Global transcriptional response of *Bacillus subtilis* to heat shock. *J Bacteriol*, **183**, 7318-7328.
123. Okuda, S., Kawashima, S., Kobayashi, K., Ogasawara, N., Kanehisa, M. and Goto, S. (2007) Characterization of relationships between transcriptional units and operon structures in *Bacillus subtilis* and *Escherichia coli*. *BMC.Genomics*, **8**, 48-60.
124. Sun, G., Sharkova, E., Chesnut, R., Birkey, S., Duggan, M.F., Sorokin, A., Pujic, P., Ehrlich, S.D. and Hulett, F.M. (1996) Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. *J Bacteriol*, **178**, 1374-1385.
125. Lawrence, J.G. and Roth, J.R. (1996) Selfish operons: horizontal transfer may drive the evolution of gene clusters. *Genetics*, **143**, 1843-1860.
126. Salgado, H., Moreno-Hagelsieb, G., Smith, T.F. and Collado-Vides, J. (2000) Operons in *Escherichia coli*: genomic analyses and predictions. *Proc.Natl.Acad.Sci.U.S.A*, **97**, 6652-6657.
127. Wang, L., Trawick, J.D., Yamamoto, R. and Zamudio, C. (2004) Genome-wide operon prediction in *Staphylococcus aureus*. *Nucleic Acids Res.*, **32**, 3689-3702.
128. Sabatti, C., Rohlin, L., Oh, M.K. and Liao, J.C. (2002) Co-expression pattern from DNA microarray experiments as a tool for operon prediction. *Nucleic Acids Res.*, **30**, 2886-2893.
129. Tjaden, B., Saxena, R.M., Stolyar, S., Haynor, D.R., Kolker, E. and Rosenow, C. (2002) Transcriptome analysis of *Escherichia coli* using high-density oligonucleotide probe arrays. *Nucleic Acids Res.*, **30**, 3732-3738.
130. Agaisse, H. and Lereclus, D. (1996) STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. *Mol.Microbiol.*, **20**, 633-643.
131. Chen, L.H., Emory, S.A., Bricker, A.L., Bouvet, P. and Belasco, J.G. (1991)

Structure and function of a bacterial mRNA stabilizer: analysis of the 5' untranslated region of *ompA* mRNA. *J.Bacteriol.*, **173**, 4578-4586.
132. Vogel, J. (2009) A rough guide to the non-coding RNA world of Salmonella. *Mol. Microbiol.*, **71**, 1-11.

Chapter 2

Hair follicles as a niche of *Staphylococcus aureus* in the nose; is a more effective decolonization strategy needed?

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Abstract

Staphylococcus aureus is the major cause of surgical site infections and especially methicillin resistant *S. aureus* (MRSA) is increasingly accounting for infections worldwide. Preventing surgical site infections by screening and decolonizing positive patients only reduces the number of infections, but does not completely eradicate the risk. A balance between prevention, costs and the chance of mupirocin resistant *S. aureus* will have to be created or decolonization strategies will have to be optimized. Therefore, it is essential to know the exact location of *S. aureus* during colonization.

In this study, for the first time the exact location of *S. aureus* in the human nose was determined using a histological approach. We showed the presence of *S. aureus* in the cornified layer of the squamous epithelium, the associated keratin and mucous debris and in hair follicles in the vestibulum nasi. The presence of *S. aureus* in hair follicles suggests that this could be the niche from which relapses occur after decolonization. In order to improve decolonization strategies, the use of an ointment might have to be reconsidered.

Introduction

Staphylococcus aureus is the causative agent of many hospital and community-acquired infections. Nasal carriage of *S. aureus* is the main risk factor for blood-borne and surgical site infections (1,2). Around 20% of the human population is persistent carrier of one particular strain and an additional 60% is intermittent carrier of varying strains, increasing the risk of nosocomial infections (3). In addition to the vestibulum nasi of the nose, the pharynx, armpits, perineum and the skin can be colonized by *S. aureus* (4,5). Previous studies have shown that nasal and pharyngeal carriers can be decolonized when treated twice daily over a period of 5 days with mupirocin ointment. Nonetheless, 5-30% remain colonized after treatment (4,6,7). The perineum can be decolonized by mupirocin ointment and stringent washing with antimicrobial soap, armpits and skin can be decolonized with chlorhexidine. Remarkably, decolonization of the nose usually has a decolonizing effect on the pharynx, perineum and armpits as well (8), suggesting that the nose is the most important colonization and seeding place of the human body.

Nasal colonization is thought to occur in the vestibulum nasi (2,5,9). This part of the nose is covered with keratinized epidermis containing hair follicles, sebaceous glands and apocrine sweat glands. It has been described that *S. aureus* primarily colonizes the moist squamous epithelium on the septum adjacent to the nasal ostium (10). Furthermore, it has been suggested that in intermittent carriers *S. aureus* resides on the mucosa only, while in persistent carriers *S. aureus* might additionally reside in a niche, possibly the apocrine

sweat glands, from where the mucosa can be (re)colonized (9). However, all these studies rely on nasal swabs taken from different parts of the nose and therefore the exact niche for *S. aureus* to colonize the nose remains uncertain. Presently, we decided to precisely determine the location of *S. aureus* in the human nose using a histological approach. *S. aureus* could be visualized in the cornified layer of the stratified squamous epithelium, in the associated keratin and mucous debris within the vestibulum nasi and in addition in the outer and inner portions of hair follicles. This association with hair follicles and the predilection of colonization by *S. aureus* with regions that are covered with hairs, like armpit, scalp and perineum, would fit the assumption that the nose would provide a niche for *S. aureus* from which relapses occur after decolonization.

Methods

Study material

In 2007 swabs were taken from the noses of 37 human cadavers that were donated for scientific purposes in our hospital. In total, 23 female and 14 male bodies ranging from 59 to 101 years old were used. After the nose swab was taken the nose was subsequently removed and vertically dissected in three or four parts, fixed in 4% buffered formaldehyde and embedded in paraffin. Tissue sections of 4 μm thick were cut and mounted on a glass microscope slide.

Immunohistochemistry

After deparaffinization and blocking endogenous peroxidase activity, the tissue sections were subjected to antigen retrieval by boiling in 10 mM sodium-citrate buffer (pH 6.0) for 20 min. Sections were slowly cooled to 37°C and rinsed with PBS. Primary antibody, mouse monoclonal anti-SpA (1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA), was diluted 600x in PBS-0.05% Tween-20, 150 μl was added to the sections and incubated for 1 h at room temperature (RT). Sections were washed with PBS for 5 min at RT and incubated with 150 μl Powervision Goat anti-Mouse/Rabbit/Rat IgG (Immunologic, Duiven, The Netherlands) for 30 min at RT. Hereafter, sections were washed with PBS for 5 min at RT, rinsed in 10 mM citrate buffer (pH 6.0) for 5 min at RT and incubated with 200 μl 3',3'-diaminobenzidine (DAB) for 10 min at RT. Sections were washed with PBS and put in water prior to staining with haematoxylin. Sections were dehydrated, mounted and visualized using light microscopy.

Results

S. aureus is present in the vestibulum nasi in mucous debris and hair follicles

Positive *S. aureus* cultures were found in nine out of 37 nose swabs, no difference was seen in male and female derived swabs. *Staphylococcus aureus* protein A (SpA)-specific antibodies were used to visualize *S. aureus* in the vestibulum nasi and internal nares (Fig. 1).

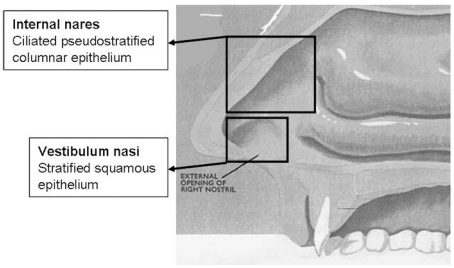

Figure 1. Dissection of the human nose. Representation of the human nose; sections covered the vestibulum nasi as well as the internal nares.

In eight out of nine culture-positive noses *S. aureus* was found in the vestibulum nasi. No bacteria were detected in the ciliated mucosa covering the major part of the nose or in its associated serous glands. The majority of the bacteria were found within the cornified layer of the stratified squamous epithelium and in the associated keratin and mucous debris within the vestibulum (Fig. 2A).
To our surprise, in six out of nine culture-positive noses the bacteria were also detected in the outer portion of the hair follicle shafts (Fig. 2B). In addition, in two out of six hair follicle-positive noses bacteria were detected in deeper parts of the hair follicle (Fig. 2C).
The specificity of the anti-SpA antibody was tested in chicken livers injected with either *S. epidermidis* or *S. aureus*. The anti-SpA showed a clear positive staining in the sections containing *S. aureus*, while no reactivity was observed with sections containing *S. epidermidis* (results not shown). Specificity of the primary antibody was further confirmed by staining negatively-cultured noses containing many gram-positive bacteria but not *S. aureus* (Fig. 2D) and *S. aureus* positive noses with mock primary antibodies (results not shown). Both did not show any stained bacteria.

Discussion

The nasal flora covering the mucosa of the vestibulum nasi can contain various potential pathogens. Protection against infection occurs via the hair and mucous covered stratified squamous epithelium which entraps airborne particles and prevents them from passing deeper into the respiratory tract. This region is followed by the ciliated and mucosal membrane of the internal

nares which transports mucous and micro organisms to the nasopharynx. The vestibulum nasi is frequently colonized by different gram-positive and gram-negative bacteria and competition for this niche is likely to occur (11).

This is the first study to detect *S. aureus in situ* in human nasal tissue and to prove that the presence of *S. aureus* is indeed restricted to the vestibulum nasi. The membrane associated protein SpA was chosen as a target for the primary antibody because it has been shown to be an important protein in maintaining colonization, given that ΔSpA mutants are able to colonize, but are not able to persist for prolonged periods of time (3). The histological approach presently used is based on the assumption that SpA is expressed during all stages of nasal colonization. However, expression of SpA during nasal colonization has never been studied. Therefore, although we were able to specifically stain *S. aureus* in the nose, it could be that we did not stain all *S. aureus* present in the culture-positive noses.

A previous study (9) speculated on the possibility of different colonization locations in persistent and intermittent carriers, in which *S. aureus* could colonize the mucous membrane in intermittent and persistent carriers, but would use an additional niche in persistent carriers. We found *S. aureus* in the deeper parts of the hair follicles in two out of nine of the culture-positive noses. This might indeed suggest a niche apart from the mucosa in persistent carriers. We do not have any information on carrier status of the donors, so we can draw no conclusions about a possible association between persistent carriership and the presence of *S. aureus* in hair follocles. A more in-depth study will have to be performed for a more definitive answer concerning colonization differences.

The occurrence of relapses after decolonization and the difficulties to decolonize some individuals might be related to the hiding of *S. aureus* in hair follicles. It might be worthwhile to reconsider the use of ointment to deliver mupirocin, as it is questionable if hairs and in particular the follicles will be efficiently reached.

In conclusion, this study revealed the presence of *S. aureus* in the cornified layer of the stratified squamous epithelium, the associated keratin and debris of the vestbulum nasi and in addition in the outer and inner portions of hair follicles of a subset of the culture-positive donors.

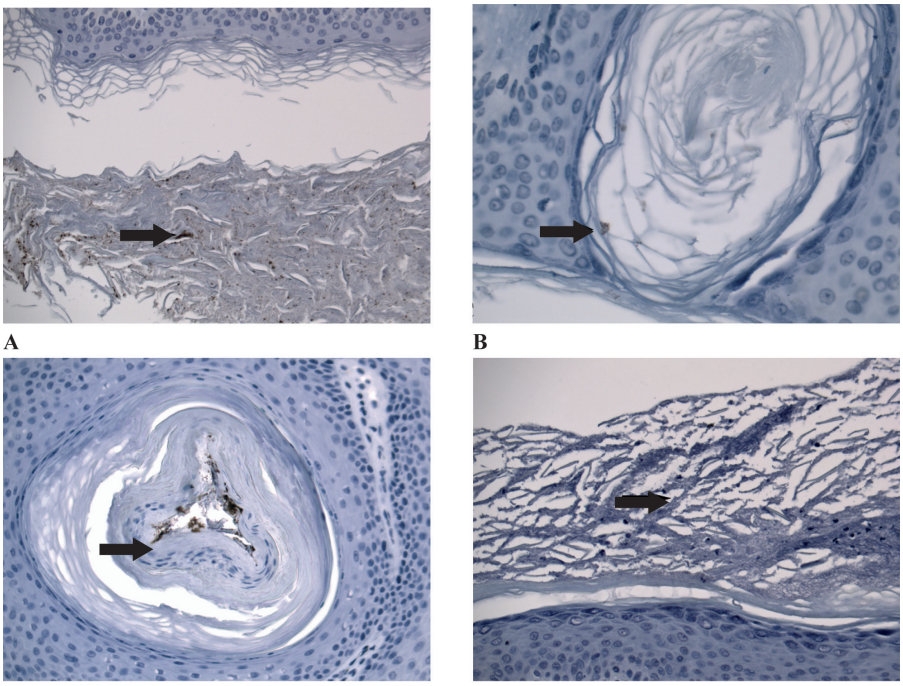

Figure 2. *S. aureus* in the vestibulum nasi.
(A) *S. aureus* in the cornified layer of the stratified squamous epithelium and in the associated keratin and mucous debris within the vestibulum. (B) *S. aureus* in the outer portion of the hair follicle shafts. (C) *S. aureus* in the inner portion of the hair follicle shafts. (D) Negatively cultured nose containing many gram-positive bacteria with no anti-SpA stained bacteria indicating specificity of the anti-SpA antibody.

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References

1. Bode, L.G.M., Kluytmans, J.A.J.W., Wertheim, H.F.L., Bogaers, D., Vandenbroucke-Grauls, C.M.J.E., Roosendaal, R., Troelstra, A., Box, A.T.A., Voss, A., van der Tweel, I. *et al.* (2010) Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. *N Engl J Med*, **362**, 9-17.
2. Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin.Microbiol.Rev.*, **10**, 505.
3. Nouwen, J., Boelens, H., van Belkum, A. and Verbrugh, H. (2004) Human factor in *Staphylococcus aureus* nasal carriage. *Infect Immun*, **72**, 6685-6688.
4. Wertheim, H.F.L., Verveer, J., Boelens, H.A.M., van Belkum, A., Verbrugh, H.A. and

Vos, M.C. (2005) Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. *Antimicrob Agents Chemother*, **49**, 1465-1467.

5. Williams, R.E. (1963) Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriol Rev*, **27**, 56-71.
6. Ammerlaan, H.S., Kluytmans, J.A., Wertheim, H.F., Nouwen, J.L. and Bonten, M.J. (2009) Eradication of methicillin-resistant *Staphylococcus aureus* carriage: a systematic review. *Clin Infect Dis*, **48**, 922-930.
7. Perl, T.M., Cullen, J.J., Wenzel, R.P., Zimmerman, M.B., Pfaller, M.A., Sheppard, D., Twombley, J., French, P.P. and Herwaldt, L.A. (2002) Intranasal mupirocin to prevent postoperative *Staphylococcus aureus* infections. *N Engl J Med*, **346**, 1871-1877.
8. Kluytmans, J.A.J.W. and Wertheim, H.F.L. (2005) Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection*, **33**, 3-8.
9. Wertheim, H.F.L., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A. and Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis*, **5**, 751-762.
10. Cole, A.M., Tahk, S., Oren, A., Yoshioka, D., Kim, Y.H., Park, A. and Ganz, T. (2001) Determinants of *Staphylococcus aureus* nasal carriage. *Clin Diagn Lab Immunol*, **8**, 1064-1069.
11. Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R., Rumke, H.C., Verbrugh, H.A. and Hermans, P.W. (2004) Colonisation by *Streptococcus pneumoniae* and Staphylococcus aureus in healthy children. *Lancet*, **363**, 1871-1872.

Chapter 3

Operon Structure of *Staphylococcus aureus*

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Abstract

In bacteria, gene regulation is one of the fundamental characteristics in survival, colonization and pathogenesis. Operons play a key role in regulating expression of diverse genes both in metabolism and virulence. However, operon structures in pathogenic bacteria are determined by *in silico* approaches only. These approaches are dependent on e.g. intergenic distances and terminator and promoter sequences. Knowledge of operon structures is of crucial importance in understanding the pathophysiology of infections. Transcriptome data obtained from growth curves in defined medium were used to predict operons in *Staphylococcus aureus*. This unbiased approach and the use of five highly reproducible biological replicates resulted in 93.5% significantly regulated genes. These data combined with Pearson correlation coefficients of the transcriptional profiles, enabled us to accurately compile 93% of the genome in operon structures. A total of 1.640 genes of different functional classes were identified in operons. Interestingly, we found several operons containing virulence genes and showed synergistic effects for two complement convertase inhibitors transcribed in one operon. This is the first experimental approach to fully identify operon structures in *S. aureus*. It forms the basis for further *in vitro* regulation studies and will have a profound impact on understanding the bacterial pathophysiology *in vivo*.

Introduction

Staphylococcus aureus is the major cause of intravascular and systemic infections such as bacteremia, endocarditis and sepsis (1,2). Nonetheless, the knowledge of the regulation of the response of *S. aureus* upon interaction with the human host, but also during growth in culture media, is limited (3). Prokaryotic gene expression is tightly regulated under different conditions, depending on cell density (quorum sensing), energy availability and environmental signals (4,5).

Microbial growth under laboratory conditions can be divided in three phases: (1) lag phase, when nutrients are abundant and cell density is low; (2) log phase, when cells grow exponentially; (3) stationary phase, when nutrients are scarce or absent and cell density is high. In general, in log phase many ribosomal proteins are abundantly expressed, while in stationary phase stress response genes and quorum sensing genes are up-regulated (6-9). The expression of genes related to virulence is of special interest in the interaction with the host. Virulence gene expression in many pathogens, e.g. *S. aureus* and Group A Streptococci, is required to evade the innate immune system and establish microbial survival in the host (10,11). Usually, virulence genes encoding surface proteins are up-regulated during log phase, while toxins are

up-regulated during stationary phase (12).
An operon is a series of genes transcribed as a single mRNA, mostly identified by short intergenic distances and the presence of a single promoter in front of the first gene and a terminator at the end, but more complex structures have been described (13-18). Several theories have been postulated to explain the formation of operons. Firstly, genes transcribed in an operon are usually functionally related and are often involved in the same metabolic pathway (16). Secondly, operons ensure cotransfer of genes to other genomes via horizontal transfer thereby increasing fitness and preservation of constituent genes (19). Operons have an important role in regulated gene expression and an estimated 50% of the genes in prokaryotes are part of an operon (16). However, hardly any operon structures are experimentally identified for important pathogenic gram-positive bacteria. In addition, the role of operons in the regulation of virulence genes is hardly known. Operon predictions have mainly been based on *Escherichia coli* genomes and to lesser extent *Bacillus subtilis* genomes. These predictions take into account the intergenic distances, conservation of gene clusters, functional relations and the limited available experimental evidence (13,20). For *S. aureus* mainly *in silico* operon predictions are available, based on the intergenic distances, conserved gene clusters and to a lesser extent, *rho*-independent terminators and the few experimentally validated operons (13,18). Co-expression patterns from microarray experiments and high-density oligonucleotide probe arrays in combination with *in silico* predictions have already successfully been used as an operon prediction tool in *E. coli* and are considered as accurate (21,22).
Another important feature for understanding gene regulation is the presence or absence of 5'-and 3'-untranslated regions (UTRs). The 5'-UTRs in prokaryotes can have important regulatory functions since riboswitches, which are known to regulate metabolic pathways, are located within the 5'-UTR and many *trans*-encoded small non-coding RNAs (sRNAs) bind to the 5'-UTR to regulate translation and/or stability of the mRNA (23-25). The 3'-UTRs have a stabilizing effect and prolong the half-life of the mRNA transcript (23,24).
In the present study, we performed a growth-dependent RNA expression analysis of the highly virulent, community-acquired methicillin-susceptible *S. aureus* strain MSSA476 to determine operon structures in the staphylococcal genome. We found 62% of the genes located within an operon. Data were compared to and combined with an online *in silico* prediction method which is to our knowledge the most complete available operon prediction for *S. aureus* as well as to a computational operon prediction by Wang *et al*.(18) This study features the fundamentals for further gene regulation studies of *S. aureus* both *in vitro* and *in vivo*.

Materials and methods

Bacterial strain

In this study the sequenced, highly virulent, community-acquired methicillin-susceptible *S. aureus* strain MSSA476 was used for all experiments (26).

Growth conditions

MSSA476 was grown overnight in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA, USA). These overnight cultures were diluted (1:7) in fresh prewarmed IMDM and grown twice to mid-log phase culture (A_{660} ~0.5) prior to the growth experiment. The second midlog phase culture was diluted to an A_{660} of 0.3 with prewarmed IMDM and directly transferred to fresh prewarmed IMDM to obtain an A_{660} of 0.03. Samples were taken at 1, 2, 3, 4, 5, 6 and 9 h post inoculation. A_{660} was measured and dilutions were plated on sheep blood-agar plates to determine colony forming units (CFUs). Cultures were incubated at 37°C and 180 rpm.

RNA extraction

RNA extraction was performed at room temperature unless stated otherwise. RNA was purified using the NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol with some adjustments. Bacteria were spun 30 sec at 13000 rpm, immediately resuspended in 350 µl RA1 buffer supplemented with 3.5 µl β-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands) and vortexed vigorously. Resuspended bacteria were added to 0.5 ml 0.1 mm silica beads (Merlin, Breda, The Netherlands), disrupted using a mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 30 sec at 5.000 rpm and samples were frozen at -80°C overnight. The samples were thawed slowly and purified. Total RNA was eluted in 60 µl RNase-free MilliQ water. RNA yield was measured using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and quality was measured using a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Both the RNA integrity number (RIN) and the presence of degradation products were checked.

Microarray design

A whole genome Agilent microarray (8x15k) was designed using the MSSA476 sequence with the Agilent Technologies eArray microarray design software (https://earray.chem.agilent.com/earray/). The complete design was performed in a two-step procedure. Firstly, 60-mer probes were designed to target all protein-coding genes, as well as rRNA, tRNA, sRNA of MSSA476 and the naturally occurring plasmid pSAS. Probes were mainly designed at the 3'-end of the genes. One probe per target was designed and tested for

cross-hybridization. Intergenic regions were defined as non-coding regions between adjacent genes irrespective of their orientation with no gene present on the opposite strand. Probes were designed to cover the complete, specified intergenic regions on both strands with at least one probe per hundred nucleotides, where possible. Secondly, probes were validated. BLAST was used to exclude from the analysis all probes which, besides the target, match the genome over a length of 20 nucleotides or more. Furthermore, all probes which did not give a signal in a comparative genomic hybridization experiment using the same array design were excluded from the analysis.

Labeling, hybridization and scanning

Total RNA was labeled in a one-step labeling with fluorescent dyes by direct labeling. A total of 10 µg RNA was randomly primed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (12.5 ng/ µl), in a total volume of 30 µl, for 2 h at 42°C with the incorporation of Cy5- or Cy3-dUTP (Agilent Technologies) with a ratio dUTP/dTTP of 3/1, yielding approximately 4 µg labeled cDNA. RNA template was removed by hydrolysis with 3 µl 2.5 M NaOH (Sigma-Aldrich) for 15 min at 70°C. Hydrolysis was stopped by neutralization with 15 µl 2 M MOPS (Sigma-Aldrich) and put on ice. Labeled cDNA was purified using Qia-quick PCR purification kit (Qiagen, Valencia, CA, USA). Incorporation of Cy3 or Cy5 was determined using a NanoDrop ND-1000.

The common reference was created by pooling 400 ng Cy5-labeled RNA sample of each time point of each growth curve, 35 points in total. Labeled cDNA was hybridized according to manufacturer's protocol (Agilent Technologies). A total of 300 ng Cy3-labeled cDNA and 300 ng Cy5-labeled common reference was mixed, 10x Blocking agent was added to a total volume of 25 µl. The mixture was heated to 95°C for 3 min, followed by addition of 2x hybridization buffer to a volume of 50 µl. A total of 40 µl was loaded onto an 8x15k array and hybridized for 18 hours at 65°C and 20 rpm in a dedicated hybridization oven (Agilent Technologies).

After the hybridization the arrays were washed in buffer 1 for 1 min at room temperature, then 5 min in wash buffer 1 at room temperature and finally 1 min in wash buffer 2 at 37°C (Agilent Technologies). Slides were spun for 3 min at 300 rpm to dry and scanned immediately. Data was extracted and processed using Feature Extraction^{tm} software (version 9.5.1, Agilent Technologies). Median spot intensities were extracted by Feature Extraction software (version 9.5.1, Agilent Technologies).

Data analysis and statistical tests

Processing of the data was performed using R (version 2.7.0) and the

Bioconductor MAANOVA package (version 1.10.0). All slides were subjected to a set of quality control checks, i.e. visual inspection of the scans, examination of the consistency among the replicated samples by principal component analysis, testing against criteria for signal to noise ratios, testing for consistent performance of the labelling dyes and visual inspection of pre- and post-normalized data with box and ratio-intensity plots. When the data was checked for effects of (random) experimental factors, slide and sample effects were observed. Slide effects were detected because eight arrays were printed on one glass slide and sample effects occurred as a consequence of the repeated measure design. After $\log_2$ transformation, the data were normalized by a LOWESS smoothing procedure to correct for dye bias effects. The resulting data were analyzed using a two-stage mixed ANOVA model (27,28). The gene specific model included terms for Array, Slide and Sample effects (random), and Time and Reference (fixed). Genes that were differentially expressed between any of the time points were identified by a permutation test. Resulting p-values were corrected for multiple testing by calculating the false discovery discovery rate (FDR) (29). The significance threshold was set at 0.05 FDR.

Operon prediction

Correlation coefficients of transcriptional profiles were determined for all adjacent probes, including the profiles of probes in intergenic regions. Pearson correlation coefficients of the transcriptional profiles for all adjacent probes were calculated over all time points and replicas: $\rho = Cov(x,y)/(\sigma x.\sigma y)$ (with $-1 \leq \rho \leq 1$).

A correlation coefficient of ≥ 0.80 was used for the prediction. Operons were determined by correlating the time-dependent transcriptional profiles of adjacent probes with all five replicas included. Correlation was thus calculated over 35 points and visualized. The distribution of non-correlated probes was determined for probes that were either 50 probes separated from each other or for probes that were adjacent to each other but on opposite strands. Both analyses resulted in similar distributions. Based on this distribution, 6.2% of the probes would indicate false positive operon predictions at a correlation coefficient of 0.8. Predicted operons were compared to an online *in silico* prediction (http://bioinformatics.biol.rug.nl/websoftware/operon/operon_start.php) and to a computational analysis based on strain Mu50 (18). These predictions are based on intergenic distances and *rho*-independent terminators or gene orientation, intergenic distances, conserved gene clusters, terminators and the confidence score of adjacent genes to be in an operon, respectively.

Reverse transcription PCR

A subset of predicted operons was validated using reverse transcription (RT)-PCR. Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used in combination with specific primers (listed in Table 1) to reverse transcribe RNA. cDNA synthesis was performed with 1 µg total RNA according to manufacturer's protocol for specific primers. The reaction was incubated at 55°C for 50 min and stopped by incubating at 85°C for 5 min. RNA was removed by adding 2 U RNase H and incubation at 37°C for 20 min. The cDNA products were subsequently detected by PCR using primers listed in Table 1.

PCRs were carried out in 25 µl reactions and consisted of 1 µl cDNA, 0.25 µM of each primer, 1x Phusion HF buffer, 0.5 mM $MgCl_2$, 2 mM dNTPs and 0.2 U Phusion high-fidelity polymerase (Bioké, Leiden, The Netherlands). Amplification was performed with an initial denaturation of 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 1 min 30 sec, followed by a final extension at 72°C for 5 min. Resulting DNA fragments were separated on a 0.8% agarose gel in TBE buffer with a 1 kb marker (Invitrogen) and visualized with ethidium bromide staining.

Alternative pathway hemolytic assay

Preparation of recombinant Efb and SCIN-B was previously described (30). Alternative pathway hemolytic assays were performed as described earlier (31). Briefly, 10% human serum was pre-incubated with Efb or SCIN-B (both at 0.6 µg/ml or 1 µg/ml) alone or with Efb and SCIN-B together at a total inhibitor concentration of 0.6 µg/ml (0.3 µg/ml Efb plus 0.3 µg/ml SCIN-B) or 1 µg/ml (0.5 µg/ml plus 0.5 µg/ml SCIN-B). Rabbit erythrocytes were added, incubated for 1 hour at 37ºC and lysis was measured. Data was analyzed by a two-tailed unpaired student's t-test.

Northern blot

A total of 7 µg RNA was separated on a 0.8% agarose-0.66 M formaldehyde gel (Sigma-Aldrich). RNA was transferred to a Brightstar®-plus positively charged membrane (Applied Biosystems, Foster City, CA, USA) overnight by capillary transfer and fixed to the membrane at 80°C for 2 h. The probe was created by amplification of the gene of interest and purified with Qiaquick PCR purification kit (Qiagen). DNA probe was labeled with [α-^{32}P]-dATP using the nick-translation kit (Invitrogen). Blot was hybridized overnight with the probe in ULTRAhyb hybridization buffer (Applied Biosystems,) at 42°C, subsequently washed 2x 5 min at 42°C with 2x SSC/0.1% SDS and 2x 15 min at 42°C with 0.1x SSC/0.1% SDS. Blot was overnight autoradiographed on a Bio-Rad phosphoimager (Applied Biosystems).

Table 1. Primers used in this study.

Primer location	Name	Sequence
SAS0670-SAS0673	0673_F	AGTTGGTGCTGTTGCCTCT
	0670_R	TTGTTGCGCGAGTTCATTAG
SAS1172-SAS1175	1175_R	TTGGTGTGTGTAATGGGAATG
	1172_F	TTCGTTTAACACGTTTAGGTTCAA
SAS1431-SAS1435	1435_F	ACGCAATACGAGGTAGATATTA
	1431_R	GTTCTGGTGCAATGCCTGTA
SAS0057-SAS0058	0058_R	CTTCTACGTTCTTTGGCCTGA
	0057_F	TGGGTTGTCAACGTACAGGA
	0056_F	TAGCCAAGCAAGGGCAATTA
SAS1765	1763_R	TTTTATCTGTAAACTGACCCTTGTC
	1765_R	TGGTCGAATGTTCCATAATCG
	1765_F	TTCATTGTTCGGATTTACATTTAG
SAS1091-SAS1092	1091_F	CGAAGGATACGGTCCAAGAG
	1092_R	GCATCAGCCATTGATACGAA
SAS1739-SAS1746	1739_RV	ACCACGAATGATCTCCAAGC
	1740_FW	AATCCACATCCGGTTAATGC
	1740_RV	GCATTAACCGGATGTGGATT
	1741_F	CAAGTTAATAAATCAAAGGAGTT
	1741_RV	AACTCCTTTGATTTATTAACTTG
	1742_FW	TGTGATGAAAAACCATGACGA
	1742_RV	TCGTCATGGTTTTTCATCACA
	1743_FW	TGTAATCGCGTCAACAAACG
	1743_RV	TCAAATGAATTCCAGAACTTTATA
	1744_FW	TTTGTTTGGTGGACTTTCAGG
	1744_RV	TTGCGATGCTAAATCCATTG
	1745_FW1	CCGAAATCGAAATTCCAAAA
	1745_RV1	TTCGGGTCCTCGATAAGATG
	1745_FW2	AACAGGTTTCGGGACAACAA
	1745_RV2	AATTGTTGTCCCGAAACCTG
	1745_RV3	GGATTGATTCTTTCATCTGAGCA
	1746_FW	TGATTCAGCAGGTGACGAAC

Results

High reproducibility of five independent growth curves

Bacterial growth in defined medium was optimally synchronized. The resulting growth curves were highly reproducible (Fig. 1A). Three growth phases could be distinguished, log phase (1-3 h post inoculation (p.i.)), late log phase (4 h p.i.) and stationary phase (5-9 h p.i.) respectively. A lag phase, in which bacteria adjust to new circumstances and start dividing in a nutrient rich environment, was not observed in our experiments. The reproducibility of the five replicates was further assessed by Principal Component Analysis (PCA) of the normalized microarray data (Fig. 1B), which showed clustering of the five biological replicas at the sampled time points.

Four basic gene expression profiles during growth

Many of the genes (2,473 of 2,644, corresponding to 93.5% of the genome)

were significantly regulated somewhere during growth (False Discovery Rate <0.05). The gene expression data were visually represented by hierarchical clustering using Ward's method on a heatmap, where the Z score normalized averaged signal intensities from the five independent growth curves were shown (Fig. 2).

The Z score normalization expresses each gene expression profile as a deviation from the mean in standard-deviation units and allows the comparison of gene expression patterns whose absolute expression levels may differ by orders of magnitude (32). The individual significantly regulated genes were grouped with other genes based on shared expression profiles. Four basic expression profiles could be distinguished (Fig. 2A). Cluster related function analyses were based on main functions, JCVI subroles and Gene Ontology (GO) functions (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) and mapped to all genes (See Supplemental Table S1 for complete lists of regulated genes). Cluster 1: Genes down-regulated in log phase and up-regulated in stationary phase represented by 122 genes (4.9% of significantly regulated genes). These genes encode for energy and DNA metabolism, but also for several virulence factors, like drug transporters and drug resistance. Cluster 2: Genes down-regulated over time, in total 1147 genes (46.3%). This cluster mainly contained ribosomal proteins. In addition, many cell envelope genes (*murC/D*) and genes encoding proteins involved in cellular processes were represented. Cluster 3: Genes up-regulated in log phase, then down-regulated in stationary phase represented by 463 genes (17.8%). This cluster mainly consisted of genes involved in iron binding and transport, like *srtB* and genes encoding iron compound ABC transporters. Moreover, *agrABCD* genes belong to this group as well. Cluster 4: Genes up-regulated over time. This cluster contained 746 genes (30.1%). These genes mainly encode proteins with metabolic functions and stress responses. In addition, RNAIII and the quorum sensing genes *luxS* and *traP* were up-regulated over time. Furthermore, Ward's clustering of virulence genes showed expression profiles similar to the profiles observed for the complete gene set (Fig. 2B). Remarkably, the group of up-regulated virulence genes during log phase was relatively much larger than was observed in the overall gene expression analysis, 31.9% compared to 17.8%. In this group, genes encoding immune evasion proteins, like complement inhibitors but also genes encoding proteins for eukaryotic cell lysis and bacterial transmission were highly represented. As expected, genes encoding proteins for toxin production and resistance were mostly up-regulated during stationary phase. Genes encoding surface proteins for colonization of the host did not group together but were found in all four clusters (See Supplemental Table S2 for complete lists of regulated virulence genes). The functionally related virulence genes probably have overlapping, but not

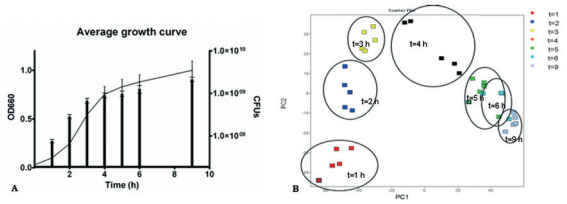

Figure 1. Growth of MSSA476 in IMDM and quality control.
(A) Three growth phases could be identified, log, late log and stationary phase. Lines represent average of five growth curves (A_{660}) and bars represent total CFUs with error bars. (B) Principal Component Analysis (PCA) of microarray data showing all time points of five independent growth curves. Replicates cluster together indicating high reproducibility of the growth curves.

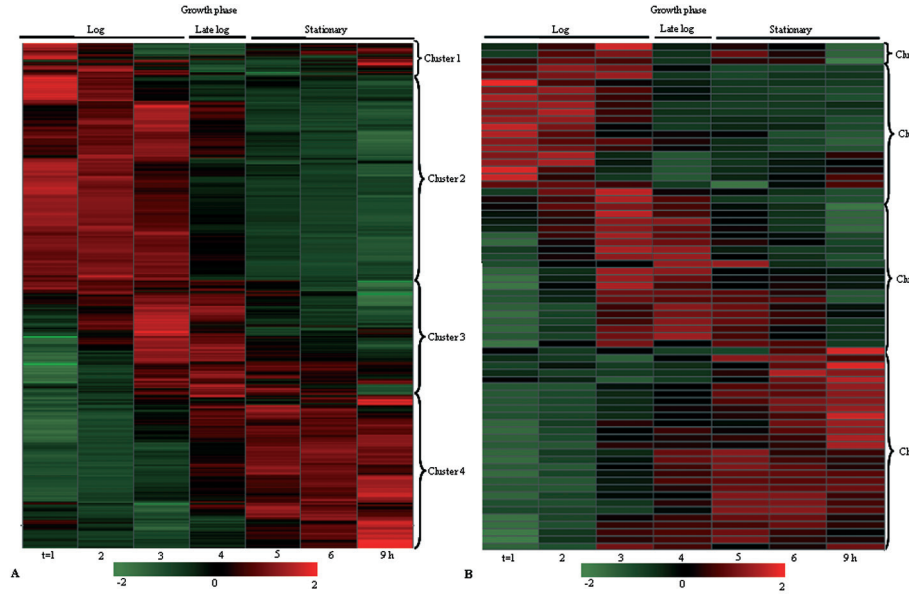

Figure 2. Heatmap of significantly regulated genes divided over three growth phases.
Rows represent individual gene probes, columns represent individual time points. The scale is represented by red (Z > 0), green (Z < 0), and black (Z = 0). Cluster 1: Down-regulated during log phase. Cluster 2: Down-regulated over time. Cluster 3: Up-regulated during log phase. Cluster 4: Up-regulated over time. (A) Heatmap containing 2.473 genes. (B) Heatmap of virulence genes.

identical functions, which could explain the differences in regulation during growth. This is in accordance with the fact that bacteria tend to loose non-functional or redundant genes (33).

Operons and UTRs determined by adjacent probe correlation

Genes transcribed in an operon or containing a 5'- or 3'-UTR were determined

using correlations between adjacent probes, considering both the probes in coding regions and in intergenic regions. With correlation coefficient cut-off set to 0.80, we found 483 operons containing two or more genes and 1,004 single genes, omitting tRNAs and rRNAs (See Supplemental Table S3 for the complete list of predicted operons). In total, 1,640 of 2,644 genes were transcribed in an operon, corresponding to 62% of the total genome (Fig. 3A). We found no prevalence of operons on the original half or terminus half nor on the leading or lagging strand. The data in this study and the *in silico* prediction were 68.8% concordant for both operons and single genes (http://bioinformatics.biol.rug.nl/websoftware/operon/operon_start.php). We predicted 139 operons to be larger, 88 operons to be smaller, 11 to be potentially differentially regulated and 52 to be completely different compared to the *in silico* data. Compared to the computational prediction, 60% of the operons were predicted concordantly. The *in silico* and computational predictions were for 76% concordant. The percentages can be explained due to gene content differences between strains Mu50 and MSSA476 and due to similar parameters for the *in silico* and computational prediction. We found 176 (6.6%) genes with a correlation coefficient between 0.65 and 0.80, which we assigned uncertain. The computational prediction assigned several of these genes to an operon and the others as single genes, indicating the uncertainty in this range of correlation coefficients. Wang *et al.* (18) referred to 36 operons described in literature as a validation for their prediction. We found eight differences, three of which (*splABCDEF*, *pheST* and *egc*) can be explained by gene content differences between MSSA476 and Mu50 (34-36). The remaining five operons (*mnh*, *femAB*, *lac*, *sigB* and *sirABC*) differ from both the computational prediction as well as the previously described operons (37-41). The operon containing *mnh* genes and the *lac* operon were not experimentally validated and were described as operons based on the functional relation of the genes and promoter and terminator sequences found (37,38). The *femAB* operon and the *sigB* operon have been experimentally validated (41,42). *FemAB* was validated as a two gene operon (42), while we predicted two single genes with a correlation coefficient of 0.51. The *sigB* operon was described in *S. aureus* as a four gene operon (41) and in *B. subtilis* as an eight gene operon (43).

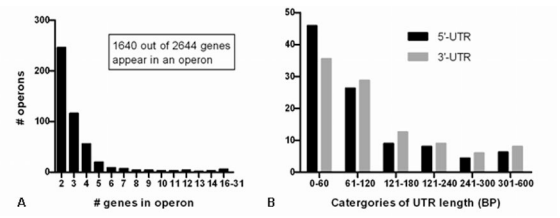

Figure 3. Predicted operons and UTR length.
(A) Number of operons containing two or more genes. (B) The percentage of 5'- and 3'-UTR in length categories.

We assigned this potentially eight gene operon as uncertain since the correlation coefficients were between 0.65 and 0.80. The *sirABC* operon has been described previously as a three gene operon (39,44). However, the operon was not experimentally validated and a knock-out inactivation of *sirA* described no downstream effect on *sirB* (44), while this would be expected if the genes would be transcribed in an operon. We predicted a single gene and a two gene operon. One operon not described by Wang *et al*. is the *sae* operon. This operon was predicted in both the computational and the *in silico* prediction as a three gene operon and one single gene. Steinhuber *et al.* (45) however, already published this a four gene operon and experimentally validated the operon with RT-PCR and northern blotting. According to our prediction the *sae* operon is a four gene operon (Fig. 4A).
Correlation coefficients were also used to predict 5'- and 3'-UTRs. Of the 1.487 operons and single genes 435 (29%) contained a 5'-UTR and 456 (31%) contained a 3'-UTR; 177 (12%) of these operons or single genes contained both a 5'- and 3'-UTRs. Genes or operons containing UTRs had mostly 5'-UTRs smaller than 100 bp. Large UTRs (>100 bp) were more frequently found on the 3'-end (Fig. 3B). UTRs were associated with genes of all different functional classes.

Operon structure of *S. aureus*
In Supplemental table S4 the operon structure of *S. aureus* is described. The prediction based upon the expression data in this study, the *in silico* prediction, the computational prediction based on Mu50 and the conclusion based on the combination of three predictions are presented.

RT-PCR confirmed presence of operons
A subset of operons was validated with RT-PCR. A reaction without Superscript III was added as control for absence of DNA contamination (results not shown). In Figure 4, five operons are shown, of which three operons were predicted to be larger and two operons were predicted to be smaller compared to the *in silico* data. Genes and the *in silico* predictions are visualized together with the expression based predictions. The *sae* operon was predicted *in silico* to be expressed as a three gene operon and a single gene, while we predicted a four gene operon. RT-PCR confirmed a four gene operon (Fig. 4A: ~2.9 kb), in concordance with a previous study (45). RT-PCR for the operons predicted to be larger, showed for SAS1172-SAS1175 an amplification product at ~2.3 kb and for SAS1431-SAS1435 an amplification product at ~2.1 kb and one at 2.5 kb, corresponding to the size of the complete operons (Fig.4B/C). For the operons predicted to be smaller an amplification product was present at ~2.6 kb for SAS0056-SAS0057 and at ~1.1 kb for SAS1765, while no amplification

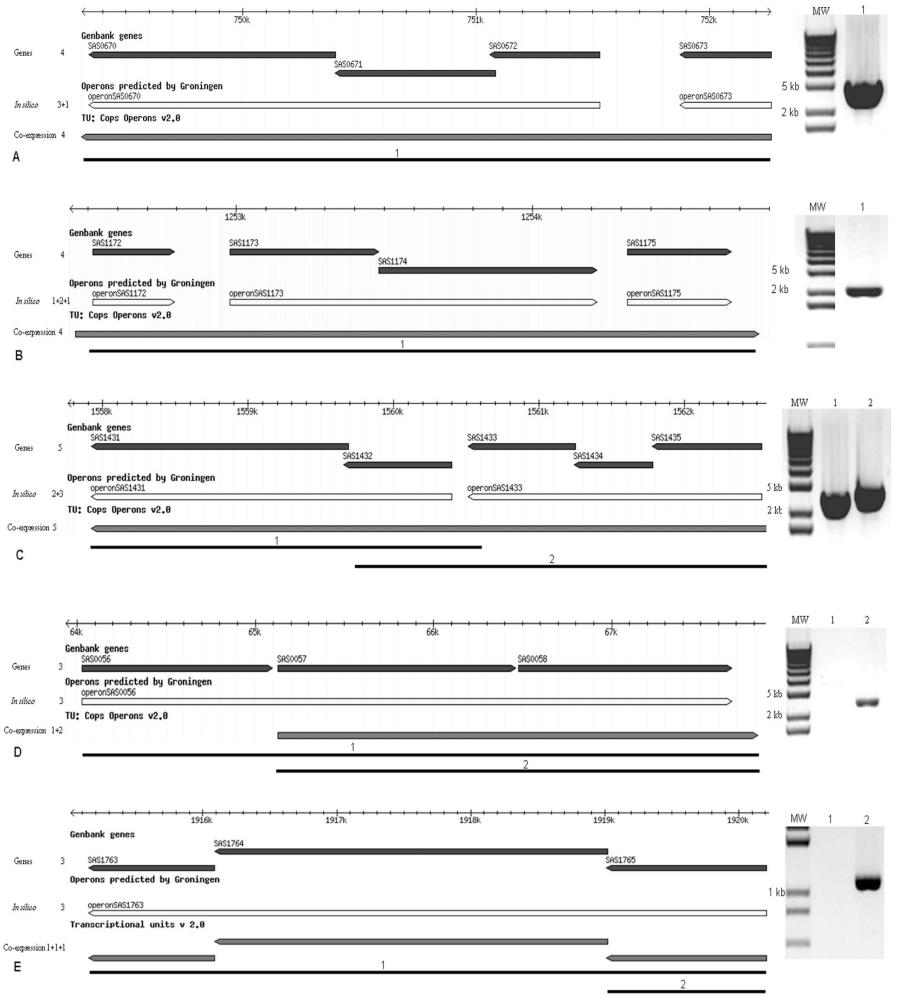

Figure 4. Subset of genes validated with RT-PCR.
Genes (black), *in silico* predictions (white) and co-expression predictions (dark grey) are visualized. Co-expression predictions were based on correlation coefficients between gene probes as well as intergenic probes. RT-PCR products were separated on a 1% TBE agarose gel with a 1 kb ladder. (A) *sae* operon containing four genes. (B, C) Operons predicted to be larger compared to *in silico* data. RT-PCR showed correct size of bands as predicted experimentally. (D, E) Operons predicted to be smaller compared to *in silico* data. RT-PCR indicated absence of complete operon and presence of smaller operon. Lane 1: *in silico* predicted operon, lane 2: co-expression based predicted operon.

product was present at the size of the *in silico* predicted operons SAS0056-SAS0058 and SAS1763-SAS1765, respectively. Finally, two operons consisting of virulence genes were validated. One operon consisted of two

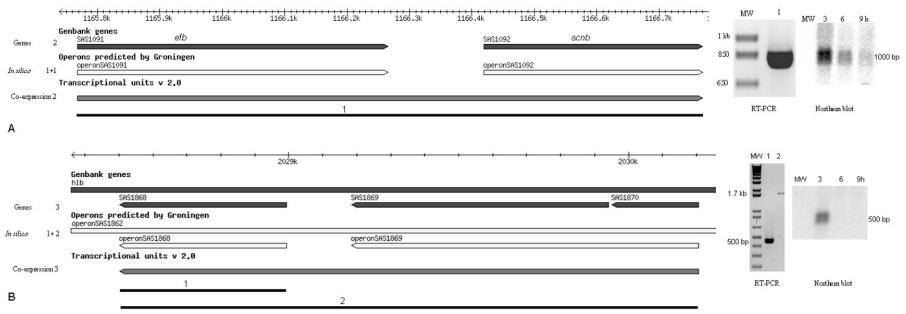

Figure 5. Two operons containing virulence genes.
(A) RT-PCR and northern blot showed *efb* located within an operon with *scnb*. (B) RT-PCR showed *sak* transcribed in an operon with autolysin and holin encoding genes. Northern blot analysis only showed a 500 bp transcript identifying *sak*.

genes, *efb* and *scnb* (30), the other of three genes, *sak*, an autolysin and a holin encoding gene (46) (Fig. 5). Both operons were analyzed with RT-PCR and showed an amplification product of the expected sizes of ~1 kb and ~1.8 kb, respectively. Northern blot analysis of the *efb*-*scnb* operon confirmed the presence of a ~1 kb transcript, analysis of the *sak*-autolysin-holin operon only showed a transcript of ~500 bp corresponding to the length of *sak*, indicating that *sak* is probably transcribed as a single gene as well as in an operon. The operon is only transcribed at low expression levels according to the microarray data.

In the expression profile analysis, 11 operons were found that seemed to be differentially regulated in a growth phase-dependent manner (see Supplemental Table S5 for the complete list). For example, *epiABCDPFEG,* an eight gene operon containing lantibiotic genes, was previously identified in *Staphylococcus epidermidis* as *epiABCD*, *epiPQ* and *epiFEG* with a transcription start site in front of *epiF* (Fig. 6). The epidermin operon in *S. aureus* was expressed as one operon in log phase, but split into two operons in the stationary phase according to the expression data (Fig. 7). The complete operon was up-regulated in log-phase until 4 h p.i., then four genes were down-regulated and four genes were up-regulated in the stationary phase.

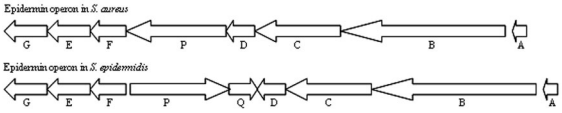

Figure 6. Epidermin operon in *S. aureus* and *S. epidermidis*.
In *S. aureus* all genes are located on the same strand, while in *S. epidermidis epiPQ* are located on the other strand of *epiABCD* and *epiFEG*.

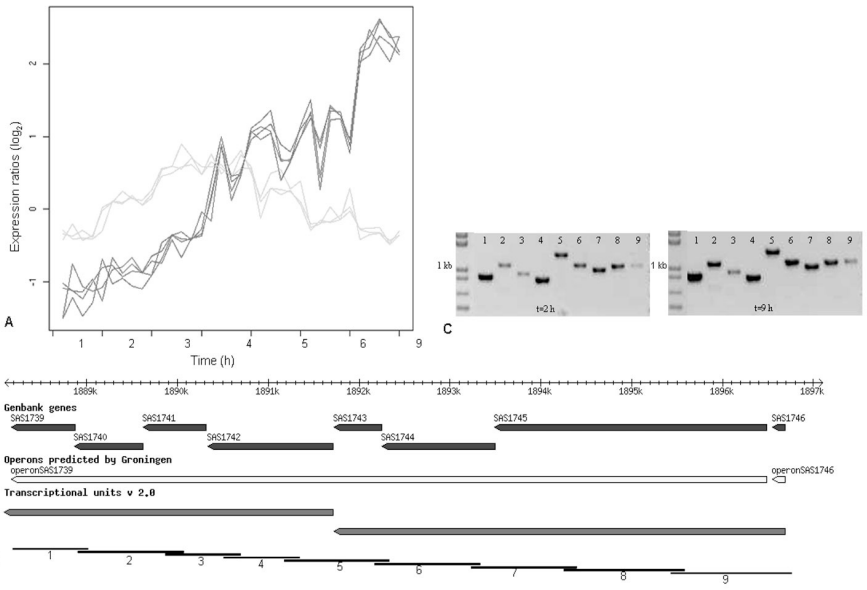

Figure 7. Operon *epiABCDPFEG*.
(A) Expression profiles showing equal expression patterns for eight genes in the log phase and different patterns in the stationary phase. (B) *In silico* predictions compared to expression predictions. At the bottom the tiling of the RT-PCR amplifications is indicated. (C) Tiling RT-PCR showed presence of eight genes expressed as an operon at t=2 and t=9.

RT-PCR showed the presence of the complete transcript throughout the growth curve. 5'-RACE experiments showed a potential transcription initiation site in front of *epiF*, in accordance with the co-expression based prediction (data not shown). Previous studies described internal promoters or terminators in operons for several bacteria. For example, in *B. subtilis* two operons, *sigB* (15) and *resABCDE* (17) had internal promoters. In *S. aureus* 3 examples of differentially regulated operons have been described, *cidABC* and *lrgAB* with an internal promoter site (14,47) and *srrAB* with no additional transcription initiation site (48).

Efb and SCIN-B have a synergistic effect on complement inhibition

The microarray data show that the genes encoding Extracellular fibrinogen binding protein (Efb) and Staphylococcal Complement INhibitor (SCIN)-B are transcribed in one operon. Efb and SCIN-B both inhibit the complement system but use different mechanisms (Fig. 9).

SCIN-B targets C3 convertases of the Classical, Alternative and Lectin Pathway of the complement system, thereby effectively inhibiting C3b deposition on the bacterial surface and thus phagocytosis and C5a generation

via all pathways.
Since the genes encoding for Efb and SCIN-B are in one operon and inhibit the complement system via different mechanisms (31,49-51) and interaction sites we hypothesized that Efb and SCIN-B might have synergistic effects. Therefore, we used an Alternative pathway (AP) dependent hemolytic assay where MAC-dependent killing of rabbit erythrocytes was used as a read-out for complement activity. Complement mediated lysis of rabbit erythrocytes was only inhibited for 10% by Efb or SCIN-B alone (0.6 µg/ml) but addition of Efb and SCIN-B together (0.3 µg/ml Efb and 0.3 µg/ml SCIN-B) significantly increased this inhibition up to 30% (Fig. 8). Similar results were obtained for Efb and SCIN-B at a concentration of 1 µg/ml.

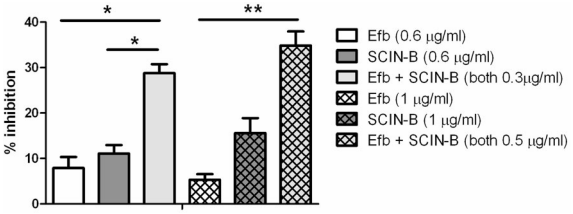

Figure 8. Synergistic effect between Efb and SCIN-B in complement inhibition
AP-dependent hemolytic assay. Rabbit erythrocytes were incubated with 10% human serum in the presence of Efb (0.6 µg/ml or 1 µg/ml), SCIN-B (0.6 µg/ml or 1 µg/ml) or Efb and SCIN-B together (0.3 µg/ml +0.3 µg/ml or 0.5 µg/ml + 0.5 µg/ml), * $p < 0.05$, ** $p < 0.01$. Figure represents the mean ± SEM of four separate experiments.

Discussion

Using data from our transcriptome analysis through time, we were able to identify the operon structure from the entire *Staphylococcus aureus* genome, covering the majority of open reading frames. A reliable prediction based on the transcriptome is only possible when many genes are significantly regulated and the reproducibility is high. Therefore, we used five independent, highly reproducible growth curves with seven time points each. This resulted in discrimination of significantly regulated genes at a fold change as small as 0.28 and differential regulation of 93.5% of all genes. This is extremely sensitive as compared to other studies and crucial to the prediction of the complete operon structure (6,7,9). Operons were predicted by calculating the correlation coefficients of transcriptome data of all adjacent probes at all seven time points. We used a relatively high correlation cut-off value of ≥0.80 to reduce the number of false-positive operons. Validation of this cut-off was achieved by RT-PCR of several operons and showed an accurate prediction above this cut-off, regardless of the size of the intergenic region. Below a correlation coefficient

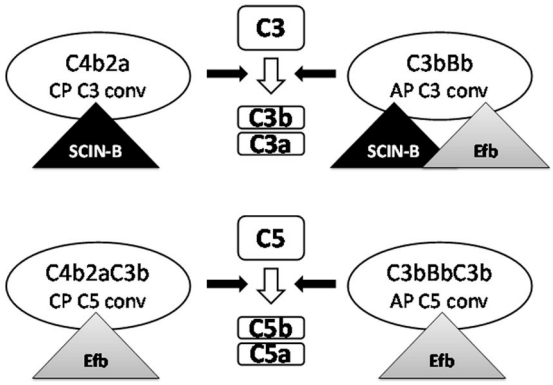

Figure 9. Predicted synergism from modes of action
Depiction of the two C3 convertases and two C5 convertases of the complement system and the targets of the Staphylococcal complement inhibitors Efb and SCIN-B. The Classical Pathway C3 convertase (CP C3 conv) is only inhibited by SCIN-B. The Alternative Pathway C3 convertase (AP C3 conv) can be inhibited by both Efb and SCIN-B. The Classical Pathway C5 convertase (CP C5 conv) is only inhibited by Efb. The Alternative Pathway C3 convertase (AP C5 conv) is only inhibited by Efb. The two molecules together can inhibit all complement convertases.

of 0.65, absence of operons was predicted and validated. Even though intergenic regions were small, visual inspection of the expression patterns showed large differences in this correlation coefficient range. Therefore, we conclude that predictions based on expression data are more accurate than the *in silico* prediction for these correlation coefficients. Genes showing low correlation coefficients for expression were also shown to be transcribed in an operon in studies in *E. coli* (52,53). Between correlation coefficients 0.65 and 0.80 several operons were detected that were not predicted with the cut-off we used. The computational and *in silico* predictions for these operons did not give a conclusive answer either. This indicates that validation is essential for predictions with correlation coefficients between 0.65 and 0.80, representing only 6.6% of the genome.

A comparison of studies in *E. coli* and *B. subtilis* showed that among the different prediction methods the intergenic distance was the most valuable single prediction variable (54). However, the combination of intergenic distance with functional information or gene expression data proved to be even more accurate (20,21,54-56). We compared and combined the operons predicted, using expression data with *in silico* predictions based on intergenic distances. We conclude that operon predictions using highly reproducible and large numbers of expression data are more accurate than predictions based on intergenic distances only. Bacterial pathogenesis is dependent on the presence of virulence genes, but

also on the expression regulation of these genes. To be able to understand the transcriptional regulation, *in vitro* and *in vivo*, knowledge of the operon structure of *S. aureus* is essential (16). Virulence genes are usually located on mobile elements and are exchanged regularly. Regulation of virulence genes at an operon level is in general not expected to exist, because of the regular exchange. Nonetheless, we found several virulence genes transcribed in an operon with other virulence genes like *efb* in combination with *scnb* and *sak* in combination with autolysin and holin encoding genes (Fig. 5), but also virulence genes that were transcribed in an operon with genes encoding hypothetical proteins or acetyltransferases. We showed that addition of Efb and SCIN-B together enhanced complement inhibition significantly, indicating that *S. aureus* has evolved this operon to counterattack complement activation even more efficiently as with the single inhibitors alone.

In conclusion, the high number of significantly regulated genes in combination with the statistical power of seven time points sampled in five biological replicas used to calculate correlation coefficients enabled us to accurately predict operons in the genome of *S. aureus* in an unbiased approach. It identified the presence of virulence genes within an operon and the synergistic action of the translated proteins was proven. Herewith, a basis has been set for future studies on gene regulation and host-pathogen interactions both *in vitro* and *in vivo*.

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Accession numbers

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References

1. Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N.Engl.J.Med.*, **339**, 520-532.
2. Sibbald, M.J.J.B., Ziebandt, A.K., Engelmann, S., Hecker, M., de, J.A., Harmsen, H.J.M., Raangs, G.C., Stokroos, I., Arends, J.P., Dubois, J.Y.F. *et al.* (2006) Mapping

the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol.Biol.Rev.*, **70**, 755-788.

3. Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., Renzoni, A., Lucchini, S., Lew, D.P., Vaudaux, P. *et al.* (2007) A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC.Genomics*, **8**, 171.
4. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.
5. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
6. Bergman, N.H., Anderson, E.C., Swenson, E.E., Niemeyer, M.M., Miyoshi, A.D. and Hanna, P.C. (2006) Transcriptional profiling of the *Bacillus anthracis* life cycle *in vitro* and an implied model for regulation of spore formation. *J.Bacteriol.*, **188**, 6092-6100.
7. Rodrigues, F., Sarkar-Tyson, M., Harding, S.V., Sim, S.H., Chua, H.H., Lin, C.H., Han, X., Karuturi, R.K., Sung, K., Yu, K. *et al.* (2006) Global map of growth-regulated gene expression in *Burkholderia pseudomallei*, the causative agent of melioidosis. *J.Bacteriol.*, **188**, 8178-8188.
8. Selinger, D.W., Cheung, K.J., Mei, R., Johansson, E.M., Richmond, C.S., Blattner, F.R., Lockhart, D.J. and Church, G.M. (2000) RNA expression analysis using a 30 base pair resolution *Escherichia coli* genome array. *Nat.Biotechnol.*, **18**, 1262-1268.
9. Thompson, L.J., Merrell, D.S., Neilan, B.A., Mitchell, H., Lee, A. and Falkow, S. (2003) Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infect Immun*, **71**, 2643-2655.
10. Foster, T.J. (2005) Immune evasion by staphylococci. *Nat.Rev.Microbiol.*, **3**, 948-958.
11. Rooijakkers, S.H.M., van Kessel, K.P.M. and van Strijp, J.A.G. (2005) Staphylococcal innate immune evasion. *Trends Microbiol.*, **13**, 596-601.
12. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol.Med.Microbiol.*, **40**, 1-9.
13. Brouwer, R.W.W., Kuipers, O.P. and Hijum, S.A.F.T. (2008) The relative value of operon predictions. *Brief.Bioinform.*
14. Brunskill, E.W. and Bayles, K.W. (1996) Identification of LytSR-regulated genes from *Staphylococcus aureus*. *J.Bacteriol.*, **178**, 5810-5812.
15. Helmann, J.D., Wu, M.F., Kobel, P.A., Gamo, F.J., Wilson, M., Morshedi, M.M., Navre, M. and Paddon, C. (2001) Global transcriptional response of *Bacillus subtilis* to heat shock. *J Bacteriol*, **183**, 7318-7328.
16. Okuda, S., Kawashima, S., Kobayashi, K., Ogasawara, N., Kanehisa, M. and Goto, S. (2007) Characterization of relationships between transcriptional units and operon structures in *Bacillus subtilis* and *Escherichia coli*. *BMC.Genomics*, **8**, 48.
17. Sun, G., Sharkova, E., Chesnut, R., Birkey, S., Duggan, M.F., Sorokin, A., Pujic, P., Ehrlich, S.D. and Hulett, F.M. (1996) Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. *J Bacteriol*, **178**, 1374-1385.
18. Wang, L., Trawick, J.D., Yamamoto, R. and Zamudio, C. (2004) Genome-wide operon prediction in *Staphylococcus aureus*. *Nucleic Acids Res.*, **32**, 3689-3702.
19. Lawrence, J.G. and Roth, J.R. (1996) Selfish operons: horizontal transfer may drive the evolution of gene clusters. *Genetics*, **143**, 1843-1860.

20. Salgado, H., Moreno-Hagelsieb, G., Smith, T.F. and Collado-Vides, J. (2000) Operons in *Escherichia coli*: genomic analyses and predictions. *Proc.Natl.Acad.Sci.U.S.A*, **97**, 6652-6657.
21. Sabatti, C., Rohlin, L., Oh, M.K. and Liao, J.C. (2002) Co-expression pattern from DNA microarray experiments as a tool for operon prediction. *Nucleic Acids Res.*, **30**, 2886-2893.
22. Tjaden, B., Saxena, R.M., Stolyar, S., Haynor, D.R., Kolker, E. and Rosenow, C. (2002) Transcriptome analysis of *Escherichia coli* using high-density oligonucleotide probe arrays. *Nucleic Acids Res.*, **30**, 3732-3738.
23. Agaisse, H. and Lereclus, D. (1996) STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. *Mol.Microbiol.*, **20**, 633-643.
24. Chen, L.H., Emory, S.A., Bricker, A.L., Bouvet, P. and Belasco, J.G. (1991) Structure and function of a bacterial mRNA stabilizer: analysis of the 5' untranslated region of *ompA* mRNA. *J.Bacteriol.*, **173**, 4578-4586.
25. Vogel, J. (2009) A rough guide to the non-coding RNA world of Salmonella. *Mol. Microbiol.*, **71**, 1-11.
26. Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R. *et al.* (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc.Natl.Acad.Sci.U.S.A*, **101**, 9786-9791.
27. Kerr, M.K., Martin, M. and Churchill, G.A. (2000) Analysis of variance for gene expression microarray data. *J Comput Biol*, **7**, 819-837.
28. Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C. and Paules, R.S. (2001) Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol*, **8**, 625-637.
29. Hochberg, Y. and Benjamini, Y. (1990) More powerful procedures for multiple significance testing. *Stat Med*, **9**, 811-818.
30. Jongerius, I., Kohl, J., Pandey, M.K., Ruyken, M., van Kessel, K.P.M., van Strijp, J.A.G. and Rooijakkers, S.H.M. (2007) Staphylococcal complement evasion by various convertase-blocking molecules. *J Exp Med*, **204**, 2461-2471.
31. Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M. and van Strijp, J.A.G. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat.Immunol.*, **6**, 920-927.
32. Cheadle, C., Vawter, M.P., Freed, W.J. and Becker, K.G. (2003) Analysis of microarray data using Z score transformation. *J Mol Diagn*, **5**, 73-81.
33. Savill, N.J. and Higgs, P.G. (2000) Redundant and non-functional guide RNA genes in *Trypanosoma brucei* are a consequence of multiple genes per minicircle. *Gene*, **256**, 245-252.
34. Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M. and Lina, G. (2001) *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol*, **166**, 669-677.
35. Reed, S.B., Wesson, C.A., Liou, L.E., Trumble, W.R., Schlievert, P.M., Bohach, G.A. and Bayles, K.W. (2001) Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. *Infect Immun*, **69**, 1521-1527.
36. Savopoulos, J.W., Hibbs, M., Jones, E.J., Mensah, L., Richardson, C., Fosberry, A., Downes, R., Fox, S.G., Brown, J.R. and Jenkins, O. (2001) Identification, cloning, and expression of a functional phenylalanyl-tRNA synthetase (*pheRS*) from *Staphylococcus aureus*. *Protein Expr Purif*, **21**, 470-484.

37. Breidt, F., Jr., Hengstenberg, W., Finkeldei, U. and Stewart, G.C. (1987) Identification of the genes for the lactose-specific components of the phosphotransferase system in the *lac* operon of *Staphylococcus aureus*. *J Biol Chem*, **262**, 16444-16449.
38. Hiramatsu, T., Kodama, K., Kuroda, T., Mizushima, T. and Tsuchiya, T. (1998) A putative multisubunit Na+/H+ antiporter from *Staphylococcus aureus*. *J Bacteriol*, **180**, 6642-6648.
39. Horsburgh, M.J., Ingham, E. and Foster, S.J. (2001) In *Staphylococcus aureus*, *fur* is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J Bacteriol*, **183**, 468-475.
40. Kopp, U., Roos, M., Wecke, J. and Labischinski, H. (1996) Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? *Microb Drug Resist*, **2**, 29-41.
41. Kullik, I.I. and Giachino, P. (1997) The alternative sigma factor *sigmaB* in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. *Arch Microbiol*, **167**, 151-159.
42. Berger-Bachi, B., Barberis-Maino, L., Strassle, A. and Kayser, F.H. (1989) FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. *Mol Gen Genet*, **219**, 263-269.
43. Wise, A.A. and Price, C.W. (1995) Four additional genes in the *sigB* operon of Bacillus subtilis that control activity of the general stress factor *sigma B* in response to environmental signals. *J Bacteriol*, **177**, 123-133.
44. Dale, S.E., Sebulsky, M.T. and Heinrichs, D.E. (2004) Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*. *J Bacteriol*, **186**, 8356-8362.
45. Steinhuber, A., Goerke, C., Bayer, M.G., Doring, G. and Wolz, C. (2003) Molecular architecture of the regulatory Locus *sae* of *Staphylococcus aureus* and its impact on expression of virulence factors. *J.Bacteriol.*, **185**, 6278-6286.
46. Rooijakkers, S.H., van Wamel, W.J., Ruyken, M., van Kessel, K.P. and van Strijp, J.A. (2005) Anti-opsonic properties of staphylokinase. *Microbes.Infect.*, **7**, 476.
47. Rice, K.C., Patton, T., Yang, S.J., Dumoulin, A., Bischoff, M. and Bayles, K.W. (2004) Transcription of the *Staphylococcus aureus cid* and *lrg* murein hydrolase regulators is affected by sigma factor B. *J.Bacteriol.*, **186**, 3029-3037.
48. Pragman, A.A., Yarwood, J.M., Tripp, T.J. and Schlievert, P.M. (2004) Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. *J.Bacteriol.*, **186**, 2430-2438.
49. Hammel, M., Sfyroera, G., Ricklin, D., Magotti, P., Lambris, J.D. and Geisbrecht, B.V. (2007) A structural basis for complement inhibition by *Staphylococcus aureus*. *Nat Immunol*, **8**, 430-437.
50. Rooijakkers, S.H. and van Strijp, J.A. (2007) Bacterial complement evasion. *Mol. Immunol.*, **44**, 23.
51. Rooijakkers, S.H.M., Wu, J., Ruyken, M., van Domselaar, R., Planken, K.L., Tzekou, A., Ricklin, D., Lambris, J.D., Janssen, B.J., van Strijp, J.A.G. *et al.* (2009) Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nat Immunol*, **10**, 721-727.
52. Price, M.N., Huang, K.H., Alm, E.J. and Arkin, A.P. (2005) A novel method for accurate operon predictions in all sequenced prokaryotes. *Nucleic Acids Res.*, **33**, 880-892.
53. Xiao, G., Martinez-Vaz, B., Pan, W. and Khodursky, A.B. (2006) Operon information improves gene expression estimation for cDNA microarrays. *BMC.Genomics*, **7**, 87.
54. Karimpour-Fard, A., Leach, S.M., Gill, R.T. and Hunter, L.E. (2008) Predicting

protein linkages in bacteria: which method is best depends on task. *BMC. Bioinformatics.*, **9**, 397.
55. Bockhorst, J., Qiu, Y., Glasner, J., Liu, M., Blattner, F. and Craven, M. (2003) Predicting bacterial transcription units using sequence and expression data. *Bioinformatics.*, **19**, i34-i43.
56. de Hoon, M.J.L., Imoto, S., Kobayashi, K., Ogasawara, N. and Miyano, S. (2004) Predicting the operon structure of *Bacillus subtilis* using operon length, intergene distance, and gene expression information. *Pac.Symp.Biocomput.*, **9**, 276-287.

Additional data files can be accessed via Nucl. Acids Res. (2010) doi: 0.1093/nar/gkq05

Figure S1. Expression profiles of RT-PCR validated operons.
Green: expression of non-coding probes, red expression of gene probes. (A) *sae* operon, four gene operon with intergenic region and 3'-UTR. (B) SAS1172-SAS1175, four gene operon with two intergenic regions and 5'-and 3'-UTR. (C) SAS1431-SAS1435, five gene operon with intergenic region and 5'-UTR. (D) SAS0056-SAS0058, two gene operon with 3'-UTR. (E) SAS1763-SAS1765, single genes. (F) SAS1091-SAS1092, two gene operon with intergenic region containing *efb* and *scnb*.

Table S1. Complete lists of regulated genes.

Table S2. Complete lists of regulated virulence genes.

Table S3. Complete list of experimentally predicted operons.

Table S4. Comparison of expression based and *in silico* predictions.

Table S5. Potential differentially regulated operons.

Chapter 4

Two small RNAs in *Staphylococcus aureus* with a potential role in virulence regulation

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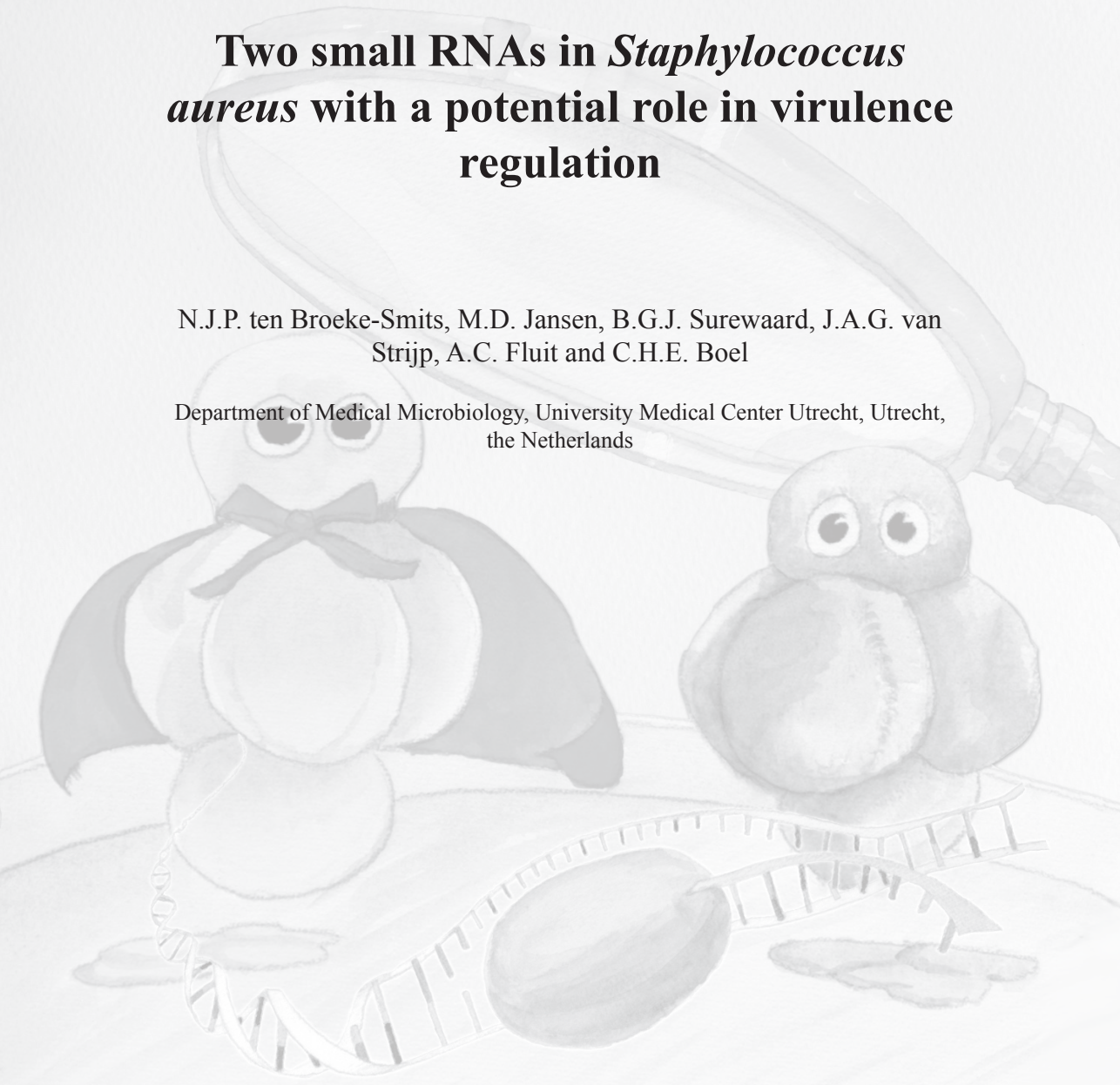

Abstract

To survive under harsh and changing environments, a microorganism needs to be able to quickly adapt gene expression. In the last decade, the influential role of small RNAs (sRNAs) in bacterial gene regulation has been demonstrated for a diverse array of genes and various prokaryotes. In *Staphylococcus aureus*, hundreds of intergenic regions have been identified that could encode a sRNA candidate. However, the functional role in gene regulation is still largely unknown. More comprehensive knowledge of regulatory RNAs will be essential to fully understand staphylococcal colonization and pathogenicity. Here, we have used transcriptome data acquired from microarray analysis on five independent highly-reproducible growth curves in defined medium to predict sRNA candidates in the intergenic regions of *S. aureus*. A total of 115 putative sRNAs were identified. IntaRNA was used to predict potential mRNA targets involved in virulence for these putative sRNAs. Five sRNA candidates remained for further characterization. *In vivo* analysis of two knock-out strains, Msa079 and Msa004 constructed in MSSA476, showed a trend in post-transcriptional regulation of the predicted targets, Efb and Hld. However, *in vitro* band shift assays with the sRNA and its mRNA target, did not confirm the *in vivo* trends of regulation. The *in vitro* sRNA/mRNA interactions were weaker than was expected from the *in vivo* data. The results of the sRNA regulation experiments described here show the difficulties of studying sRNA regulation and may contribute to better understanding virulence gene regulation via sRNAs in *S. aureus*.

Introduction

Staphylococcus aureus harbors a diverse set of virulence factors, ranging from adhesins and invasins to toxins and immune evasion proteins. The expression of these virulence factors is controlled by regulatory systems, like two-component and quorum sensing systems, that enable *S. aureus* to quickly alter the gene expression upon changes in the environment (1,2). In addition, increasing numbers of small RNAs (sRNAs) are being discovered in all three domains of life (3,4) and a fundamental role for virulence regulation has been identified (5,6).

Small RNAs have been described as important regulators of many genes by base pairing with target mRNA to modulate transcription, translation or stability (4,7) or by binding to proteins to adjust their function (7-9). *Trans*-acting sRNAs inhibit or activate translation of multiple targets via binding to the 5'-untranslated region (UTR) of an mRNA and thereby blocking or unmasking the ribosome binding site (RBS). Binding can also occur at the 3'-end or in the coding region, thereby influencing translation efficiency,

translation elongation or target stability. *Trans*-acting sRNAs can regulate multiple targets. Less common are c*is*-acting sRNAs, located on the DNA strand opposite of the target gene, with large completely complementary regions. Most of these sRNAs are found on mobile genetic elements, like plasmids, but chromosomally encoded types have been identified lately (10,11).

Controlling bacterial virulence via sRNAs is highly convenient since I) responses are rapid, II) only low numbers of nucleotides are involved in base pairing which allows fast and specific recognition and III) through modulation of the conformation of RNAs the number of contacts between sRNA and target mRNA can be increased, making recognition of multiple targets or interaction sites easier (12). Up to date, most characterized sRNAs are involved in regulatory pathways and allow the bacteria to respond to stress situations, sense cell density and adjust metabolism during growth (4,13). Also, sRNAs involved in virulence regulation are recognized in increasing numbers of bacterial species. So far, ~20 sRNAs have been described to be involved in virulence regulation in diverse pathogens (6), like *Listeria monocytogenes* (14)*, Pseudomonas aeruginosa* (15) and Group A Streptococci (16).

The most extensively studied and best described virulence regulating sRNA is RNAIII which is located within the *agr* quorum sensing system of *S. aureus* (17). Agr is the center of an important regulatory network in *S. aureus* and RNAIII is the effector molecule in this system. RNAIII is 514 nucleotides long, folds into a complex secondary structure and is an important regulator of virulence (18-23). RNAIII acts as *trans*-acting sRNA and represses the expression of surface proteins in the exponential phase and activates the expression of extracellular toxins and enzymes in the post-exponential phase (24,25). The 5'-end of RNAIII also encodes delta toxin (Hld), a small polypeptide of 26 amino acids, secreted without a signal peptide (26). Delta toxin is lytic for a diverse set of cells and cellular organelles (24) and is considered a phenol soluble modulin (PSM) (27). Another virulence regulating sRNA in *S. aureus* is SprD. It is located on a pathogenicity island and has been found to regulate *S. aureus* binder of IgG (Sbi) *in vivo* (28,29). With the knowledge about the regulatory importance of sRNAs for virulence genes, the identification and characterization of sRNAs becomes more important.

This study aims to provide further insight in the regulation of virulence genes in *S. aureus*. Therefore, we predicted sRNA candidates in intergenic regions (IGRs) in the community-acquired and highly virulent MSSA476 strain (30). To this end we used expression data obtained from microarray analysis of five highly reproducible biological replicates of standard laboratory growth curves in defined medium (IMDM). We focused on sRNA candidates with the potential to regulate virulence genes and identified five sRNAs that were

predicted *in silico* to interact with the 5'-end of mRNA transcripts encoding virulence factors. *In vivo* analysis of two sRNA knock-out strains (Msa004 and Msa079) showed a trend for sRNA mediated up-regulation of Hld. In addition, down-regulation of an important protein involved in immune evasion, extracellular fibrinogen binding protein (Efb) (31) was suggested for both sRNAs. However, further *in vitro* characterization of sRNA interaction with the 5'-end of the predicted mRNA did not result in additional evidence for the observed trends of regulation of Efb or Hld by either sRNA.

Material and methods

RNA preparation and prediction of RNA transcripts

In this study the data of a previously described, highly reproducible time course experiment with five biological replicas (32) was used to predict RNA transcripts in intergenic regions. RNA was isolated using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany), labeled and hybridized to the microarrays. Arrays were subsequently scanned, data extracted and analyzed. Operons were predicted by calculating pearson correlation coefficients of the transcriptional profiles for all adjacent probes over all time points and replicas: $\rho = Cov(x,y)/(\sigma x.\sigma y)$ (with $-1 \leq \rho \leq 1$). A correlation coefficient of ≥ 0.80 was used for the prediction of operons. The same strategy was used in this study to identify putative sRNAs located in the IGRs.

Potential sRNAs were accepted for further analysis if two or more probes showed a correlation coefficient ≥ 0.80 and a minimum expression level (≥ 7) of the normalized data. Predicted sRNAs were subjected to further analysis:

I) Transcriptional profiles of predicted sRNAs were manually compared to profiles of adjacent genes located on the same strand. Predicted sRNAs with similar transcription profiles were assigned as untranslated region (UTR) and omitted.

II) Predicted sRNA sequences were BLAST searched and non-conserved sequences within staphylococcal genomes were excluded.

III) Predicted sRNAs were checked for the presence of open reading frames (ORFs) using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and BLAST searched. Transcripts harboring potential ORFs were excluded for further analysis.

IV) Riboswitches were identified using the Rfam database (http://www.sanger.ac.uk/Software/Rfam/index.shtml) (33) and Mfold (34) and omitted.

Target mRNA prediction

Target mRNAs of the potential sRNAs were predicted using IntaRNA version

1.2.5 (http://rna.informatik.uni-freiburg.de:8080/IntaRNA.jsp), which uses a combined energy score of the free energy of hybridization and the free energy required for making the interaction sites available with seed parameters set at seven (35). RNA interaction sites were identified in the 5'-regions of the mRNA spanning 180 nucleotides upstream and 200 nucleotides downstream of the translation start codon of 20 virulence genes (Table 1).

Predicted interactions were searched for possible sRNA-mRNA interfaces taking into account several preset standards: I) the interaction of RNA molecules usually does not stretch 9-60 bp, II) highly entangled structures are not commonly found and III) binding occurs usually around the Shine-

Table 1. Virulence genes used in target predictions.

Product	Gene	function	Ref
extracellular adherence protein	*eap*	Inhibition of phagocytic engulfment	(36,37)
clumping factor A	*clfA*	Colonization of host tissues/immunological disguise and modulation	(38)
clumping factor B	*clfB*	Colonization of host tissues/immunological disguise and modulation	(39,40)
enterotoxin A and J	*sea*	Food poisoning/TSS	(41,42)
exfoliative/ epidermolytic toxin A	*eta*	Contribution to symptoms of septic shock/ scalded skin syndrome	(43)
enterotoxin H	*seh*	Food poisoning/TSS	(44)
Von Willebrand binding protein	*vWbp*	Immunological disguise and modulation	(45)
protein A	*spa*	Immunological disguise and modulation/ inhibition of phagocytic engulfment	(40,46-48)
inhibitory protein	*scn*	Immunological disguise and modulation/ survival in phagocytes	(49)
extracellular fibrinogen binding protein	*efb*	Inhibition of phagocytic engulfment	(31,50)
staphylokinase	*sak*	Plasminogen activator	(47,51)
extracellular complement-binding protein	*ecb*	Inhibition of phagocytic engulfment	(31)
extracellular matrix binding protein	*emp*	Inhibition of phagocytic engulfment	(37,52)
MHC class II analog	*map*	Inhibition of phagocytic engulfment	
alpha hemolysin precursor	*hla*	Lysis of eukaryotic cell membranes and bacterial spread	(40,42,47, 48,53)
delta hemolysin	*hld*	Lysis of eukaryotic cell membranes and bacterial spread	(47,48,53)
IgG-binding protein	*sbi*	Pathogenesis	
Staphylococcal complement inhibitor B	*scn-B*	Immunological disguise and modulation/ survival in phagocytes	(49,54)
formyl peptide receptor (-like)	*fIpR-like*	Immunological disguise and modulation/ survival in phagocytes	(55,56)
Staphylococcal superantigen-like protein	*ssl7*	Immunological disguise and modulation/ survival in phagocytes	(57)

Dalgarno (SD) sequence (58). Interactions were scored positive when the interaction seed site was located around the SD-sequence, thereby blocking or unmasking the site for ribosome binding and possibly block or activate translation.

5'- and 3'-RACE

5'-RACE (Rapid Amplification of cDNA Ends) assays were carried out as described by Argaman et al. (59) with minor modifications (primers used are listed in Supplementary Table S1). Briefly, 5'-triphosphates were converted to monophosphates by treating 5 µg total RNA with 10 units of tobacco acid pyrophosphatase, 50 µM sodium acetate (pH 6.0), 1 mM EDTA, 0.1% β-mercaptoethanol and 0.01% Triton X-100 (Tebu-bio, Le-Perray-en-Yvelines, France) at 37°C for 60 min in a total reaction volume of 20 µl. Control RNA was incubated under the same conditions in the absence of the enzyme. Reactions were stopped by phenol:chloroform extraction, followed by ethanol sodium acetate precipitation. Precipitated RNAs were dissolved in RNase free water and mixed with 20 µM 5' RNA adapter in a total volume of 20 µl (A3 5'-GAU AUG CGC GAA UUC CUG UAG AAC GAA CAC UAG AAG AAA-3': Biosynthesis, Lewisville, TX, USA), 10 units T4 RNA ligase (New England Biolabs, Ipswich, MA, USA), 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP and 10 mM dithiothreitol. The mixture was incubated at 37°C for 90 min and directly used for cDNA synthesis. Superscript III First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA, USA) was used in combination with random hexamers to reverse transcribe RNA. cDNA synthesis was performed with 4 µl adapter ligation mix according to manufacturer's protocol for random hexamers. The reaction was incubated at 55°C for 50 min and stopped by incubating at 85°C for 5 min. RNA was removed by adding 2 units RNase H and incubation at 37°C for 20 min. The reverse transcription products were amplified by the use of 1 µl RT mix, 10 µM of each gene specific and adapter-specific primer (B6 5'-CGC GAA TTC CTG TAG A-3'), 1.5 mM $MgCl_2$, 250 µM dNTPs and 1 unit of Hotstar *Taq*-polymerase (Qiagen, Valencia, CA, USA). Cycling conditions were as follows: 94°C/15 min; 35 cycles of 94°C/40 sec, 58°C/40 sec, 72°C/40 sec; 72°C/15 min. Products were directly cloned into pCR 2.1 TOPO-vector (Invitrogen). Bacterial colonies were screened for the presence of an insert by colony PCR with universal M13 primers. The PCR fragments were purified on QIAquick spin columns (Qiagen) and sequenced.

3'-RACE experiments were performed with RNA that had been dephosphorylated with calf intestine alkaline phosphatase (CIAP) (New England Biolabs). Ligation was done as described above with a 3' RNA adapter (E1 5'-phosphate-UUC ACU GUU CUU AGC GGC CGC AUG CUC-idT-3';

idT, 3' inverted deoxythymidine: Biosynthesis). Reverse transcription was carried out as described, but with 30 µM adapter specific primer E4 (E4 5'-GAG CAT GCG GCC GCT AAG AAC AGT GAA-3'). PCR amplification with gene specific primers and adapter specific primer E6 (E6 5'-GCC GCT AAG AAC AGT GA-3'), cloning and sequence analysis was done as described above. SUPERaseIN (10 units) (Applied Biosystems, Foster City, CA, USA) was added to all enzymatic RNA treatments.

Construction of sRNA overexpression and knock-out strains

DNA coding for the putative sRNAs was cloned in the +1 site of the SCIN promoter in the pSK236 shuttle vector, in a PCR with an overlap to the SCIN promoter (PCR1). PCRs were carried out in 40 µl reactions and consisted of 1 µl DNA, 0.25 µM of each primer, 1x Phusion HF buffer, 2 mM dNTPs and 0.4 U Phusion high-fidelity polymerase (Bioké, Leiden, The Netherlands). Cycling conditions were as follows 98°C/30 sec; 30 cycles of 98°C/10 sec, 55°C/30 sec, 72°C/30 sec, 72°C/5 min. Then the SCIN promoter was created by PCR with an overlap to the sRNA (PCR2) using the same mix as described above without the addition of DNA. Amplification was as follows 98°C/30 sec; 10 cycles of 98°C/10 sec, 60°C/25 min, 72°C/5 min, 72°C/5 min. PCR1 and PCR2 were mixed (PRC3), 5 µl each, with 1x Phusion HF buffer, 2 mM dNTPs and 0.4 U Phusion high-fidelity polymerase (Bioké). Amplification was performed as described for PCR2. Five µl of PCR3 was used to amplify the SCIN-sRNA construct, using the amplification scheme of PCR1. The DNA fragment containing promoter and sRNA was sequenced and subsequently ligated into the pSK236 shuttle vector. The ligation product was transformed to chemically competent *Escherichia. coli* TOP10 cells and grown on LB agar containing 50 µg/ml carbenicillin. Purified plasmid containing the right insert was transformed to electro competent RN4220 *S. aureus* cells, using 500 ng plasmid, 60 µl competent cells, settings: 200 Ω, 25 µF and 1.5 kV. After electro shock 160 µl Todd Hewitt broth (TH) was added and cells were allowed to recover by incubation at 37°C for 1 hour. Cells were plated on TH agar (THA) containing 7.5 µg/ml chloramphenicol and grown overnight at 37°C. Plasmids were purified from fresh overnight cultures, checked for the insert and subsequently transformed to electro competent MSSA476 *S. aureus* cells as described above.

Small RNA knock-out strains for the putative sRNAs were created in MSSA476 using PCRs as described for creating the overexpression strains. Around 1000 bp upstream and 1000 bp downstream of the sRNA were amplified with primers listed in Supplementary Table S1, as described for PCR1. Resulting DNA fragments were purified with PCR purification columns (Qiagen) and 5 µl was ligated as described in PCR2. Resulting DNA (5 µl) was directly

used in PCR3. Resulting DNA fragments were purified with PCR purification columns (Qiagen). PCR fragment and vector pKOR were digested, after which the vector was dephosphorylated and both were purified (Qiagen). Ligation was done overnight at 16°C with a vector insert ratio of 1:5 in a total volume of 15 µl. 7.5 µl of the ligation mix was transformed to DH5α chemical competent *E. coli*. The purified plasmid was sequenced for the insert and transformed to electro competent RN4220 *S. aureus* cell as described above. Cells were plated on TH agar (THA) with 7.5 µg/ml chloramphenicol and grown overnight at 30°C. The resulting plasmid was then transferred to electro competent MSSA476 and grown overnight on THA with 7.5 µg/ml chloramphenicol at 30°C. Single colonies were grown in TH for 6 h-overnight at 30°C with vigorous shaking. Plasmid integration was checked by streaking out culture on THA with 7.5 µg/ml chloramphenicol and growing them at 43°C overnight. Single colonies were picked and streaked on fresh THA with 7.5 µg/ml chloramphenicol and grown overnight at 43°C. Colonies were PCR screened. Single cross-over colonies were grown in TH without antibiotics at 30°C overnight, diluted 1:100.000 in sterile water and 100 µl was spread on THA containing 50 µg/ml anhydrotetracycline and incubated at 37°C overnight. Big colonies were picked and streaked on THA with 10 µg/ml chloramphenicol or plain THA and grown overnight at 37°C. Colonies growing on plain THA were presumed knock-out strains and sequenced. Confirmed knock-out strains were used in subsequent experiments.

Characterization of overexpression and knock-out strains

Medium and plates for growing MSSA476 cells containing the pSK vector with or without sRNA construct contained 7.5 µg/ml chloramphenicol. Knock-out strains were grown without antibiotics.

Overexpression and knock-out strains and their control strains were grown overnight at 37°C and 180 rpm. Cultures were 1:10 diluted in fresh medium and allowed to grow to midlog A_{660} ~0.5. The cells were again transferred to fresh medium to A_{660} ~0.03 and samples were taken at three and six hours post inoculation. Both bacterial pellet and supernatant were stored for further analysis.

The bacterial pellet was used for RNA isolation and cDNA synthesis using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in combination with random hexamers. cDNA synthesis was performed with 1 µg total RNA according to manufacturer's protocol for random hexamers. The absence and overexpression of sRNAs was tested in Quantitative real-time PCRs (qRT-PCR) using SYBR Green with primers listed in Supplementary Table S1. The settings were as follows; 50°C/2 min, 95°C/10 min; 40 cycles of 95°C/15 sec, 60°C/1 min. For SYBR Green samples

a dissociation curve was added to the PCR. The levels of mRNA transcripts were tested in RT-PCR with 200 nM FAM-TAMRA-labeled probes for *efb* and *spa* (Supplementary Table S1) in a mix with 900 nM primers (Supplementary Table S1) and Taqman Universal PCR Mastermix (Applied Biosystems). Levels of *hld* were measured using SYBR Green as described above.

Protein levels in the collected supernatants were determined using Luminex (Efb) and high-performance liquid chromatography (HPLC) (Hld). Samples for Luminex were serially diluted and incubated with 600x diluted human pooled serum. Samples were incubated while shaking for 35 min. at 37°C on a thermomixer plate shaker (Eppendorf, Hamburg, Germany) to allow complex formation between protein present in supernatant and antibodies present in serum. Beads coated with Efb as described in (60) were added to the mix and incubated while shaking for 30 min at 37°C on the plate shaker to allow complex formation between remaining antibodies in serum and protein coated to the beads. Bead-bound antibodies were detected with anti-human IgG-PE and measured using Luminex (Millipore, Austin, TX, USA).

Analytical HPLC was performed using an automatic HPLC system (Shimadzu, Duisburg, Germany) with an analytical reversed-phase column, an UV detector operating at 214 nm with a flow rate of 0.75 mL/min. A Phenomenex Gemini C18 (110 Å**,** 5 µm, 250 x 4.6 mm) column was used with trifluoroacetic acid (TFA) buffers (buffer A: H_2O:MeOH, 95:05, v:v; buffer B: MeOH:H_2O, 95:5, v:v, both containing 0.1% TFA). Elution was effected with either a linear gradient from 100% A to 100% B over 48 min or a linear gradient from 40% A to 100% B. The retention time of delta toxin was determined by applying synthetic N-formylated delta toxin (Genscript, Piscataway, NJ, USA). The peptides were characterized using electrospray mass spectrometry (ESI-MS) performed on a Thermo Finnigan LCQ DECA XP MAX ion trap mass spectrometer or a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer.

In vitro transcription and band shift assays

In vitro transcription was performed using the MEGAshortscript™ T7 kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. Small RNA fragments were created according to 5'- and 3'-mapped ends with a T7 promoter sequence at the 5'-end (primers are listed in Supplementary Table S1). Target mRNA fragments were created 180 nucleotides upstream to 200 nucleotides downstream of transcription start codon with a T7 promoter sequence at the 5'-end.

The *in vitro* transcribed sRNAs were dephosphorylated with 0.1 U/µl CIAP (New England Biolabs, Ipswich, MA, USA) in 10 µl end volume. End-labeling with [γ-^{32}P]-dATP using the kinaseMax™ kit (Applied Biosystems)

was performed according to manufacturer's protocol. Labeled RNA was purified with NucAway Spin columns (Applied Biosystems) and renatured by incubation at 90°C for 2 min and 4°C for 1 min in RNase free water, followed by at least 15 min incubation at 20°C in TMN buffer (20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl) (22).
5'-end-labeled sRNA (0.1 nM) was incubated with an increasing amount of target mRNA (0, 1, 5, 10, 20, 50, 100, 200, 250 nM) in a total volume of 10 µl for 15 min at 37°C in TMN buffer. Reactions were stopped by adding gel loading buffer. Samples were separated on a native 6% TBE-polyacrylamide gel in 1x Tris-borate buffer at 4°C. Specificity of the binding was tested with increasing amounts (0, 0.05, 0.1, 1, 0 nM) of an unlabeled DNA oligonucleotide with the same sequence and length as the sRNA, incubated with 0.1 nM [γ-^{32}P]-dATP labeled sRNA and 50 nM target mRNA. Quantification was done with a PhosphoImager analyzer.

Results

Microarray based identification of sRNAs

Previously, we compiled the operon structure of *S. aureus* from five independent growth curve experiments using microarray (32). In the present study, the same strategy was used to predict RNA transcripts in IGRs. We identified 212 IGRs consisting of two or more probes with a Pearson correlation of 0.80 or higher. Manually identified UTRs were excluded (73 in total), as well as three non-conserved sequences and transcripts harboring potential small proteins or peptides (21 in total). The remaining 115 sRNA candidates were subsequently used for further characterization (Supplementary Table S2). In previous studies 32 of these 115 sRNAs were identified in *S. aureus* N315 (Supplementary Table S3) (22,29,61-63).

In silico predictions for RNA interactions

IntaRNA was used to predict mRNA targets of the 115 sRNA candidates and five putative sRNAs were identified with the seed interaction site around the SD-sequence of one or more mRNA targets involved in virulence (Table 2). Two of the predicted sRNAs (Msa079 and Msa100) were previously identified by *in silico* predictions (22). RNA sequencing previously identified the IGR that encodes candidate Msa016 however it is not clear whether the predicted sRNAs are the same (61). These three sRNA candidates have not been characterized further.

Characteristics of sRNAs

The 5'- and 3'-ends of the sRNA candidates were determined in 5'- and

Table 2. IntaRNA predicted sRNAs and mRNA complexes.
Predicted sRNAs with their *in silico* predicted potential mRNA target and the start and stop site as identified by microarray analysis. Orientation of the sRNA compared to the adjacent genes was determined.

Name	Array_start	Array_stop	Orientation	mRNA target
Msa004	115709	115828	> < >	*efb*/*hld*
Msa016[b]	453895	454192	> > >	*spa*/*efb*
Msa079[a]	2071356	2071595	< > >	*efb*/*hld*
Msa100[a]	2429777	2429896	> > >	*ssl7*
Msa103	2484692	2484838	< > >	*sbi*/*sak*

[a] Previously identified by *in silico* predictions (22)
[b] Previously identified region by RNA sequencing (61)

Table 3. 5'- and 3'-ends of sRNAs determined with 5'- and 3'-RACE experiments

Name	5'-end	3'-end
Msa004	ND	ND
Msa016	453960	454210
Msa079	2071319	2071526
Msa100	2429667	2429895
Msa103	2484692	2484883

ND Not identified

3'-RACE experiments. The transcriptional start sites and the 3'-ends of the sRNAs were in general accordance with the microarray data (Table 3). For Msa004 the 5'- and 3'-ends could not be identified. Consequently, the transcriptional start site and 3'-end from the microarray data were used for cloning.

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Expression patterns of the five sRNAs during standard laboratory growth in IMDM were determined from the microarray data. Stable expression during

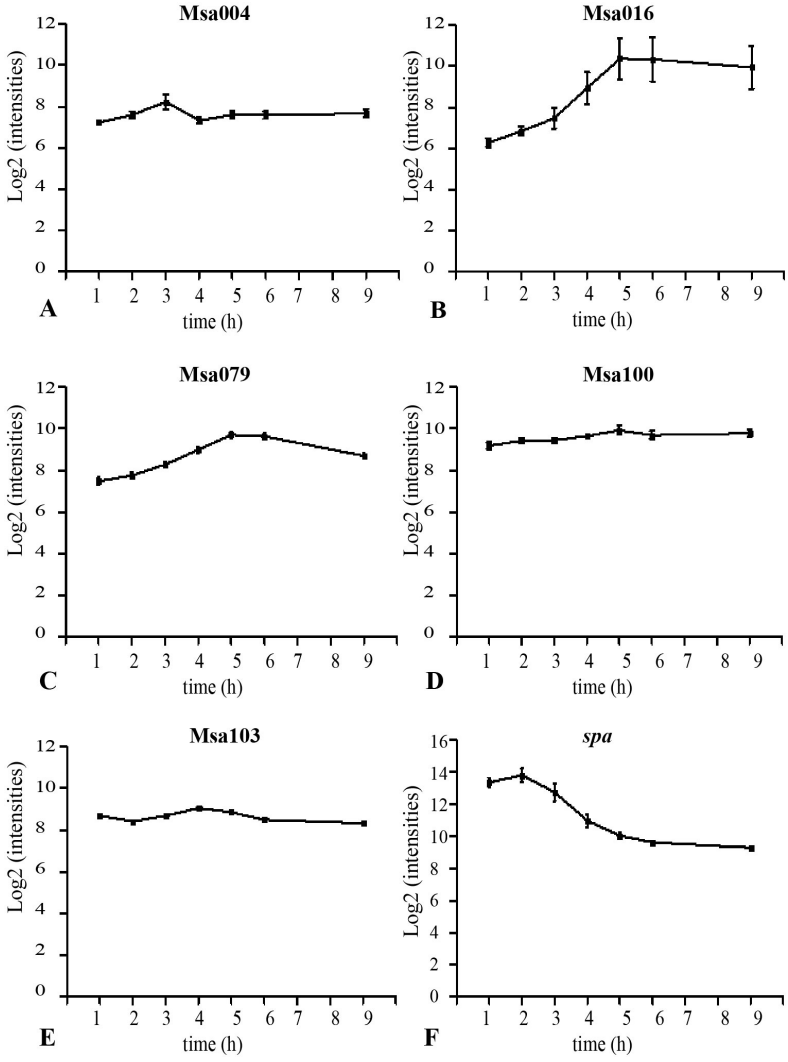

Figure 1. Expression patterns of putative sRNAs.

Log2 fold-change of average expression levels over five biological replicas as determined from microarray data (A) Msa004; (B) Msa016; (C) Msa079, (D) Msa100, (E) Msa103 and (F) *spa* mRNA.

growth was observed for Msa004, Msa100 and Msa103 (Fig. 1A, 1D and E). Msa079 is up-regulated during exponential phase and down-regulated during stationary phase (Fig. 1C) and Msa016 is up-regulated throughout growth (Fig. 1B). Compared to the expression patterns of the potential mRNA targets, we could only identify an inverse correlation for Msa016 and *spa* mRNA (Fig. 1F), which could indicate regulation under these conditions. The expression levels of the sRNAs were much lower (100-1000x) in comparison to the expression levels of the mRNA targets, as observed from the microarray data as well as in RT-PCR.

Characteristics of knock-out strains

The sequences of the five sRNAs, using the 5'- and 3'-ends as identified in RACE experiments and for Msa004 as identified from the microarray data, were used to construct knock-out strains. Construction of knock-out strains succeeded for Msa004 and Msa079, but failed for Msa016, Msa100 and Msa103.

Quantitative real-time (RT)-PCR was used to check strains ΔMsa004 and Δ Msa079. As expected, sRNA transcript could be detected. Next, expression levels of the predicted *efb* and *hld* mRNA targets were identified. This showed no differences in ΔMsa004 and ΔMsa079 compared to wild-type MSSA476, indicating that the mRNA targets were not degraded during regulation.

Interaction of Msa004 and Msa079 with Hld

IntaRNA predictions for sRNA candidates, Msa004 and Msa079, showed interaction of the sRNA with the SD-sequence of *hld* mRNA (Fig. 2C/D), suggesting a regulatory effect. Supernatant from mid-exponential phase growing cells of strains ΔMsa004, ΔMsa079 and wild-type MSSA476 were used to further characterize these *in silico* predictions in *in vivo* situations. A trend of up-regulation of Hld, confirmed with a C8 column, was observed for both knock-out strains compared to the wild-type MSSA476. Both deformylated and formylated Hld were identified (Fig. 2A). The area under the curve for the formylated Hld was determined to quantify levels of Hld (Fig. 2B). The trends were not statistically significant because of large variations between two biological replicas. Nevertheless, these results may suggest an inhibitory effect of Msa004 and Msa079 on Hld.

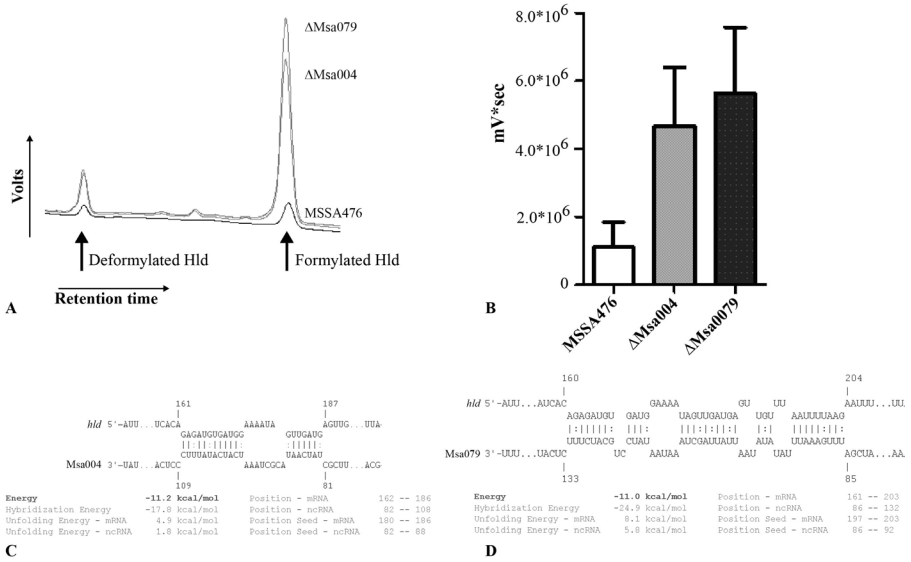

Figure 2. Delta toxin levels of knock-out strains compared to wild-type MSSA476.
Delta toxin levels measured using HPLC. (A) Hld level in MSSA476 is represented by the low peak, while Hld levels in ΔMsa004 and ΔMsa079 are represented by the higher peaks. (B) Area under the curve of knock-out strains compared to MSSA476 in two biological replicates. (C) IntaRNA prediction for Msa004 and (D) for Msa079, both covering the SD-sequence of the target mRNA.

Interaction of Msa004 and Msa079 with Efb

IntaRNA predictions for both sRNA candidates also showed interaction at the SD-sequence of *efb* mRNA (Fig. 3B/C). Therefore, Luminex assays were used to determine levels of Efb in supernatants of ΔMsa004 and ΔMsa079 strains compared to wild-type MSSA476. The percentage of Efb antibodies present in the human serum that were captured by the proteins present in the supernatant was determined in biological duplicates and plotted. A trend of down-regulation was observed for ΔMsa079 and ΔMsa004 (Fig. 3A). The observed trends were not statistically significant due to large variations between the two biological replicates. Nonetheless, these data might imply up-regulation of Efb by Msa004 and Msa079.

Band shift assays show *in vitro* sRNA-mRNA interaction

Band shift assays were performed to test sRNA-mRNA interactions *in vitro* (19,22,64,65), to support the observed *in vivo* trends of Efb and Hld regulation and to further evaluate the *in silico* predicted interactions between sRNA and the mRNA target. All five sRNAs that were predicted *in silico* to potentially regulate virulence protein encoding genes were tested, even though mutants were obtained

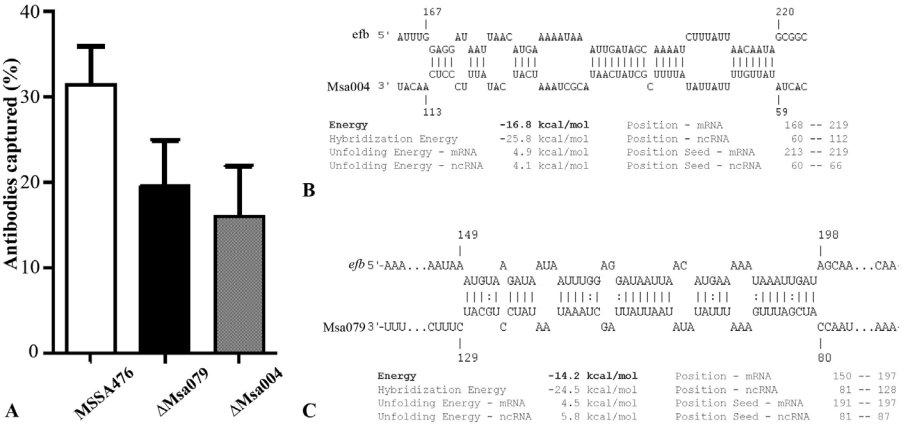

Figure 3. Efb levels of knock-out strains compared to wild-type MSSA476.
Efb levels determined with Luminex. (A) Efb levels measured in supernatants of knock-out strains ΔMsa079 and ΔMsa004 appeared lower than for wild-type MSSA476. (B) IntaRNA prediction for Msa004 and (C) for Msa079, both covering the SD-sequence of the target mRNA.

and tested for Msa004 and Msa079 only. *In vitro* ^{32}P-labeled sRNA (0.1 nM) was incubated with increasing amounts of *in vitro* generated 5'-end of the target mRNA and separated on a 6% TBE-PAGE gel (Fig. 4). Dissociation constants (K_d) were estimated as the concentration target mRNA allowing 50% of sRNA binding (62). A dissociation constant of ~40 nM was obtained for Msa004 with *efb* mRNA (Fig. 4A). A weaker interaction with *hld* mRNA was obtained for Msa004 (K_d>250 nM) (Fig. 4B). Msa079 with *efb* mRNA showed a dissociation constant of ~250 nM (Fig. 4C), the band shift assay with *hld* mRNA failed. Weak interactions were observed for Msa103 with *sak* and *sbi* mRNA (K_d>250 nM) (Fig. 4D and E). A strong interaction was identified for Msa016 and *spa* mRNA (~30 nM) and slightly weaker with *efb* mRNA (~40 nM) (Fig. 4F and G). Msa100 displayed a slight interaction with the predicted target *ssl7* mRNA (Fig. 4H). Control experiments with increasing concentrations of unlabeled DNA oligonucleotide with the same sequence and length as the sRNA and 0.1 nM *in vitro* transcribed labeled sRNA were added to 50 nM *in vitro* transcribed mRNA and showed a decreasing shift (Fig 4I). Taken together, these *in vitro* data do not show the anticipated interactions of Msa004 and Msa079 with the predicted target mRNAs and as such do not support the *in vivo* trends in regulation.

Discussion

Small RNAs are being appreciated as important regulatory elements, with advantages over protein-based regulation. Benefits include additional levels

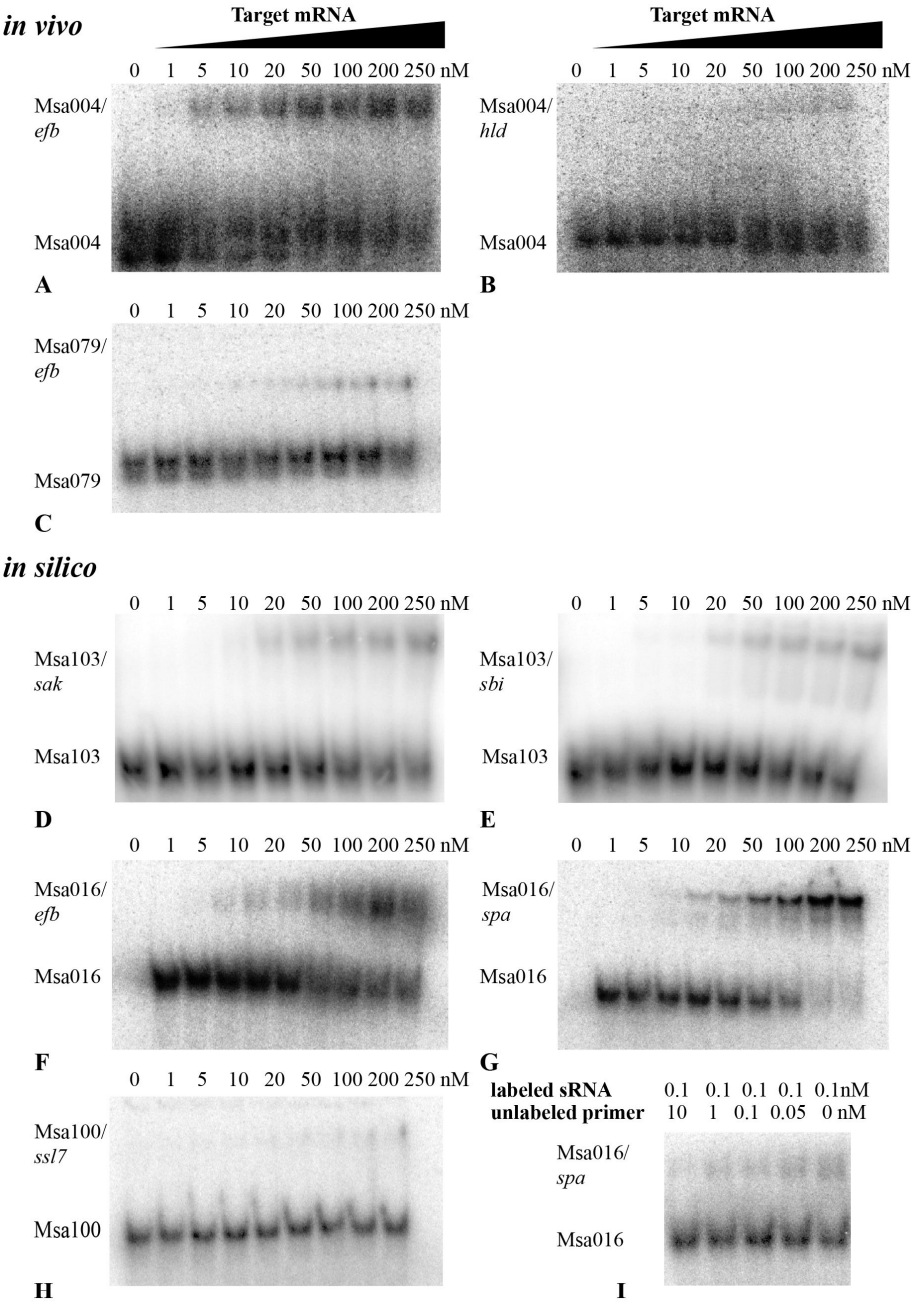
in vivo
Target mRNA
0 1 5 10 20 50 100 200 250 nM
Msa004/
efb
Msa004
A
Target mRNA
0 1 5 10 20 50 100 200 250 nM
Msa004/
hld
Msa004
B
0 1 5 10 20 50 100 200 250 nM
Msa079/
efb
Msa079
C
in silico
0 1 5 10 20 50 100 200 250 nM
Msa103/
sak
Msa103
D
0 1 5 10 20 50 100 200 250 nM
Msa103/
sbi
Msa103
E
0 1 5 10 20 50 100 200 250 nM
Msa016/
efb
Msa016
F
0 1 5 10 20 50 100 200 250 nM
Msa016/
spa
Msa016
G
0 1 5 10 20 50 100 200 250 nM
Msa100/
ssl7
Msa100
H
labeled sRNA 0.1 0.1 0.1 0.1 0.1nM
unlabeled primer 10 1 0.1 0.05 0 nM
Msa016/
spa
Msa016
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Figure 4. Band shift assays of sRNAs with predicted target mRNA.
0.1 nM ^{32}P-labeled sRNA was incubated for 15 min in TMN buffer with increasing concentrations of target mRNA (0-250 nM). Interaction of sRNA with the target mRNA is visualized by the shift of label. Msa004 is able to form a complex with *efb* mRNA (A) and only slightly with *hld* mRNA (B). Msa079 showed interaction with *efb* (C) Msa103 can form a complex with *sak* (D) and *sbi* (E) and Msa016 forms a complex with *efb* (F) and *spa* (G) mRNA. Msa100 shows a mild interaction with *ssl7* mRNA (H). (I) Increasing amounts of unlabeled primer showed a decrease in shift of labeled Msa016 (0.1 nM) with *spa* mRNA (50 nM).

of regulation, fast regulation, reduced metabolic cost, reduced leakiness of targets regulated at two levels (66) and rapid clearance of regulatory RNAs when they are no longer needed (5). Hundreds of sRNA candidates have been identified by means of computational predictions, microarray analysis or whole transcriptome sequencing in various prokaryotes (14,22,29,61-63,67). However, functional analysis of putative sRNAs is cumbersome, labor intensive and progresses only slowly. In this work we examined the function of sRNAs as regulatory elements in pathogenicity of *S. aureus*.

Out of a set of 115 putative sRNAs, five were identified with IntaRNA to have the potential to influence the regulation of virulence genes and were used for further analysis. Several programs can be used to predict sRNA-mRNA interactions, but only IntaRNA predicted all targets for the previously described RsaE sRNA in *S. aureus* (22), hence this program was chosen.

The functionality of the sRNAs was tested *in vivo* with knock-out strains created in MSSA476. A trend in regulation of the predicted targets, Efb and Hld, was observed for ΔMsa004 and ΔMsa079 compared with wild-type MSSA476 (Fig. 2 and 3). However, further *in vitro* characterization of the binding interactions between sRNA and 5'-end of the target mRNA showed interaction capacities that were weaker than anticipated from the *in vivo* results. This might be explained by the fact that the *in silico* predictions and the experiments concerning *in vitro* interaction between sRNA and mRNA have some drawbacks. First of all, *in silico* predictions are based on a seed interaction site, representing the interaction site of sRNA with target mRNA, in which the optimal length and the nucleotide composition have not yet been fully defined and these prediction programs still need to be optimized (68). Secondly, band shift assays have some clear disadvantages and results should be interpreted with care; I) Band shift assays are carried out using optimized binding conditions for *in vitro* transcribed RNAs that have partial complementary nucleotides, which are likely to bind when incubated together. As a consequence, these experiments are not representative for *in vivo* situations. II) An arbitrary number of nucleotides upstream and downstream of the start codon of the mRNA is used to determine RNA interactions. The RNA structure is likely to be different when the number of nucleotides upstream

and downstream of the start codon is changed, which can lead to a more stable interaction or no interaction at all. The presence and the length of a 5'-UTR might be of importance for mRNA target predictions. III) Gram-negative bacteria use a chaperone protein, Hfq, to stabilize sRNA-mRNA interactions. Even though the Hfq homologue in *S. aureus* seems not to be involved in RNA complex formation (69), a Hfq-like or comparable stabilizing protein might still be necessary for some sRNA-mRNA interactions in Gram-positive bacteria (23,70,71). Lastly, there might be binding of the sRNA to the mRNA or the 3'-end of the mRNA to establish an interaction or there might be a direct interaction of the sRNAs with the protein instead of regulation via binding to the 5'-end of the mRNA and thereby blocking translation (7). Further experiments should be conducted to determine the controversy between *in vitro* and *in vivo* results presented here, e.g. band shift assays with the *S. aureus* Hfq homologue, toe printing assays to determine whether ribosome blocking occurs, determine half-life of sRNAs and examine expression levels of sRNAs upon exposure to different conditions, like temperature or pH change (19,22,64,65).

We also attempted to investigate the effect of the sRNA candidates by constructing overexpression mutants in MSSA476. The five constructed overexpression strains contained the correct insert, but showed large variabilities of sRNA overexpression in biological replicates or overexpression could not even be detected. In the analysis of these unexpected results, it turned out that the minimum inhibitory concentration (MIC) for chloramphenicol of wild-type MSSA476 was comparable to the concentration used for creating and growing the overexpression strains. Therefore these strains may have lost the plasmid during growth. In addition, it has been proposed that unintended consequences, like regulation of other mRNAs or proteins, appear in prokaryotes when an sRNA is overexpressed in the cell (68) and regulation of the sRNA by the target mRNA has been argued for eukaryotic miRNAs (72). Thus, the use of double cross-over knock-out strains to test effects of sRNAs is probably more reliable than using overexpression strains.

Major concentration differences between sRNA and mRNA were observed in this study. Band shift assays only showed interaction of sRNA with the target mRNA at concentrations of the target mRNA >100x the concentration of the sRNA, resulting in k_d values found for other functional sRNA-mRNA interactions (19). This concentration difference was also observed in our *in vivo* experiments. For the potential sRNA mediated up-regulation of Efb, the low concentration sRNAs in comparison to the high concentration of mRNA is not an issue. However, Hld showed trends of down-regulation by both sRNAs, even though the sRNA concentrations were much lower than the mRNA concentrations. This regulatory effect is

more difficult to explain. Possibly multiple layers of regulatory actions are involved in regulation of the described targets, e.g. more sRNAs regulating the same target or even mRNAs regulating the target (68). This could be of relevance in studies using knock-out strains, where the knocked-out sRNA action might be substituted by other sRNAs. In fact, regulation take-over by other sRNAs or mRNAs could occur when knocking-out an sRNA (personal communication Dr. Geissmann). Also, the fast and specific interaction of sRNAs with only low numbers of base pairs, thereby acting rapidly in regulation of the target mRNA (12), may create the opportunity to regulate high levels of target mRNA, while only low numbers of sRNA are present in the cell. A long half-life of the sRNAs could be an indication for reuse of sRNAs for regulation. Additionally, Hld is located on RNAIII, which is shown to have complex secondary and tertiary structures (73). Therefore, the biological role of these complex RNA structures might be of importance for Hld regulation (74).

A remarkably strong band shift was obtained for Msa016 and *spa* mRNA, which was also identified with IntaRNA to have strong interaction capacities with the 5'-UTR of *spa* mRNA, thereby covering the SD-sequence. During standard laboratory growth, *spa* is highly down-regulated (32), while Msa016 is strongly up-regulated. This could be an indication for sRNA mediated regulation of *spa* mRNA. However, creation of a knock-out and/or overexpression strain of Msa016 failed. This might implicate the relevance of Msa016 or SpA for survival of the bacterial cell, sustained by the absence of strains which lack SpA. Alternatively, as supported by the observed regulation during growth, Msa016 could be critical for another function during growth, e.g. starvation stress or cell density response.

In summary, despite that technical difficulties and experimental limitations restrict our ability to draw firm conclusions, we have been able to show trends of *in vivo* regulation of Hld and Efb by two independent sRNAs, Msa004 and Msa079. However, additional experiments are needed to fully characterize and comprehend the function of these sRNA candidates, since *in vitro* experiments did not fully support the *in vivo* trends of regulation.

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References

1. Boyce, J.M. (1992) Methicillin-resistant *Staphylococcus aureus* in hospitals and long-term care facilities: microbiology, epidemiology, and preventive measures. *Infect Control Hosp Epidemiol*, **13**, 725-737.
2. Toledo-Arana, A., Repoila, F. and Cossart, P. (2007) Small noncoding RNAs controlling pathogenesis. *Curr Opin Microbiol*, **10**, 182-188.
3. Sharp, P.A. (2009) The centrality of RNA. *Cell*, **136**, 577-580.
4. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615-628.
5. Gripenland, J., Netterling, S., Loh, E., Tiensuu, T., Toledo-Arana, A. and Johansson, J. (2010) RNAs: regulators of bacterial virulence. *Nat Rev Microbiol*, **8**, 857-866.
6. Papenfort, K. and Vogel, J. (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe*, **8**, 116-127.
7. Storz, G., Altuvia, S. and Wassarman, K.M. (2005) An abundance of RNA regulators. *Annu.Rev.Biochem.*, **74**, 199-217.
8. Majdalani, N., Vanderpool, C.K. and Gottesman, S. (2005) Bacterial small RNA regulators. *Crit Rev Biochem Mol Biol*, **40**, 93-113.
9. Pichon, C. and Felden, B. (2007) Proteins that interact with bacterial small RNA regulators. *FEMS Microbiol Rev*, **31**, 614-625.
10. Brantl, S. (2007) Regulatory mechanisms employed by cis-encoded antisense RNAs. *Curr Opin Microbiol*, **10**, 102-109.
11. Georg, J. and Hess, W.R. (2011) cis-antisense RNA, another level of gene regulation in bacteria. *Microbiol Mol Biol Rev*, **75**, 286-300.
12. Lioliou, E., Romilly, C., Romby, P. and Fechter, P. (2010) RNA-mediated regulation in bacteria: from natural to artificial systems. *N Biotechnol*, **27**, 222-235.
13. Vogel, J. and Wagner, E.G. (2007) Target identification of small noncoding RNAs in bacteria. *Curr.Opin.Microbiol.*, **10**, 262-270.
14. Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K. *et al.* (2009) The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature*, **459**, 950-956.
15. Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. and Filloux, A. (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A*, **103**, 171-176.
16. Ramirez-Pena, E., Trevino, J., Liu, Z., Perez, N. and Sumby, P. (2010) The group A Streptococcus small regulatory RNA FasX enhances streptokinase activity by increasing the stability of the *ska* mRNA transcript. *Mol Microbiol*, **78**, 1332-1347.
17. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
18. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**, 1353-1366.
19. Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T.,

Vandenesch, F. and Romby, P. (2010) Staphylococcus aureus RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. *PLoS Pathog*, **6**, e1000809.

20. Geisinger, E., Adhikari, R.P., Jin, R., Ross, H.F. and Novick, R.P. (2006) Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol.Microbiol.*, **61**, 1038-1048.
21. Geisinger, E., George, E.A., Muir, T.W. and Novick, R.P. (2008) Identification of ligand specificity determinants in AgrC, the *Staphylococcus aureus* quorum-sensing receptor. *J.Biol.Chem.*, **283**, 8930-8938.
22. Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C. *et al.* (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res*, **37**, 7239-7257.
23. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.
24. Janzon, L. and Arvidson, S. (1990) The role of the delta-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Embo J*, **9**, 1391-1399.
25. Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B. and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.*, **12**, 3967-3975.
26. Fitton, J.E., Dell, A. and Shaw, W.V. (1980) The amino acid sequence of the delta haemolysin of *Staphylococcus aureus*. *FEBS Lett*, **115**, 209-212.
27. Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A. *et al.* (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med*, **13**, 1510-1514.
28. Chabelskaya, S., Gaillot, O. and Felden, B. (2010) A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog*, **6**, e1000927.
29. Pichon, C. and Felden, B. (2005) Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc.Natl.Acad.Sci.U.S.A*, **102**, 14249-14254.
30. Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R. *et al.* (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc.Natl.Acad.Sci.U.S.A*, **101**, 9786-9791.
31. Jongerius, I., Kohl, J., Pandey, M.K., Ruyken, M., van Kessel, K.P.M., van Strijp, J.A.G. and Rooijakkers, S.H.M. (2007) Staphylococcal complement evasion by various convertase-blocking molecules. *J Exp Med*, **204**, 2461-2471.
32. ten Broeke-Smits, N.J.P., Pronk, T.E., Jongerius, I., Bruning, O., Wittink, F.R., Breit, T.M., van Strijp, J.A.G., Fluit, A.C. and Boel, C.H.E. (2010) Operon structure of *Staphylococcus aureus*. *Nucleic Acids Res*, **38**, 3263-3274.
33. Griffiths-Jones, S., Moxon, S., Marshall, M., Khanna, A., Eddy, S.R. and Bateman, A. (2005) Rfam: annotating non-coding RNAs in complete genomes. *Nucleic Acids Res*, **33**, D121-124.
34. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, **31**, 3406-3415.

35. Busch, A., Richter, A.S. and Backofen, R. (2008) IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics*, **24**, 2849-2856.
36. Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.I., Herrmann, M. and Preissner, K.T. (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med*, **8**, 687-693.
37. Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. and Flock, J.I. (2003) The adhesive and immunomodulating properties of the multifunctional Staphylococcus aureus protein Eap. *Microbiology*, **149**, 2701.
38. Birkey, S.M., Liu, W., Zhang, X., Duggan, M.F. and Hulett, F.M. (1998) Pho signal transduction network reveals direct transcriptional regulation of one two-component system by another two-component regulator: *Bacillus subtilis* PhoP directly regulates production of ResD. *Mol Microbiol*, **30**, 943-953.
39. Chamberlain, N.R. and Imanoel, B. (1996) Genetic regulation of fatty acid modifying enzyme from *Staphylococcus aureus*. *J Med Microbiol*, **44**, 125-129.
40. Chan, P.F. and Foster, S.J. (1998) The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. *Microbiology*, **144 (Pt 9)**, 2469-2479.
41. Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C. and Tarkowski, A. (1993) The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect Immun*, **61**, 3879-3885.
42. Alekshun, M.N., Levy, S.B., Mealy, T.R., Seaton, B.A. and Head, J.F. (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. *Nat Struct Biol*, **8**, 710-714.
43. Balaban, N. and Novick, R.P. (1995) Translation of RNAIII, the *Staphylococcus aureus agr* regulatory RNA molecule, can be activated by a 3'-end deletion. *FEMS Microbiol.Lett.*, **133**, 155-161.
44. Kravitz, G.R., Dries, D.J., Peterson, M.L. and Schlievert, P.M. (2005) Purpura fulminans due to *Staphylococcus aureus*. *Clin Infect Dis*, **40**, 941-947.
45. Bjerketorp, J., Jacobsson, K. and Frykberg, L. (2004) The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. *FEMS Microbiol Lett*, **234**, 309-314.
46. Blevins, J.S., Elasri, M.O., Allmendinger, S.D., Beenken, K.E., Skinner, R.A., Thomas, J.R. and Smeltzer, M.S. (2003) Role of sarA in the pathogenesis of *Staphylococcus aureus* musculoskeletal infection. *Infect Immun*, **71**, 516-523.
47. Bunce, C., Wheeler, L., Reed, G., Musser, J. and Barg, N. (1992) Murine model of cutaneous infection with gram-positive cocci. *Infect Immun*, **60**, 2636-2640.
48. de Beer, T., Fang, J., Ortega, M., Yang, Q., Maes, L., Duffy, C., Berton, N., Sippy, J., Overduin, M., Feiss, M. *et al.* (2002) Insights into specific DNA recognition during the assembly of a viral genome packaging machine. *Mol Cell*, **9**, 981-991.
49. Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M. and van Strijp, J.A.G. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat.Immunol.*, **6**, 920-927.
50. Palma, M., Shannon, O., Quezada, H.C., Berg, A. and Flock, J.I. (2001) Extracellular fibrinogen-binding protein, Efb, from *Staphylococcus aureus* blocks platelet aggregation due to its binding to the alpha-chain. *J Biol Chem*, **276**, 31691-31697.
51. Rooijakkers, S.H., van Wamel, W.J., Ruyken, M., van Kessel, K.P. and van Strijp, J.A. (2005) Anti-opsonic properties of staphylokinase. *Microbes.Infect.*, **7**, 476-484.

52. Boden, M.K. and Flock, J.I. (1992) Evidence for three different fibrinogen-binding proteins with unique properties from *Staphylococcus aureus* strain Newman. *Microb Pathog*, **12**, 289-298.
53. Bayer, M.G., Heinrichs, J.H. and Cheung, A.L. (1996) The molecular architecture of the *sar* locus in *Staphylococcus aureus*. *J Bacteriol*, **178**, 4563-4570.
54. Rooijakkers, S.H.M., Wu, J., Ruyken, M., van Domselaar, R., Planken, K.L., Tzekou, A., Ricklin, D., Lambris, J.D., Janssen, B.J., van Strijp, J.A.G. *et al.* (2009) Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nat Immunol*, **10**, 721-727.
55. Prat, C., Bestebroer, J., de Haas, C.J., van Strijp, J.A. and van Kessel, K.P. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol*, **177**, 8017-8026.
56. Prat, C., Haas, P.J., Bestebroer, J., de Haas, C.J., van Strijp, J.A. and van Kessel, K.P. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from *Staphylococcus aureus* (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J Immunol*, **183**, 6569-6578.
57. Bestebroer, J., Aerts, P.C., Rooijakkers, S.H., Pandey, M.K., Kohl, J., van Strijp, J.A. and de Haas, C.J. (2010) Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell Microbiol*, **12**, 1506-1516.
58. Muckstein, U., Tafer, H., Hackermuller, J., Bernhart, S.H., Stadler, P.F. and Hofacker, I.L. (2006) Thermodynamics of RNA-RNA binding. *Bioinformatics*, **22**, 1177-1182.
59. Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H. and Altuvia, S. (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol*, **11**, 941-950.
60. Verkaik, N., Brouwer, E., Hooijkaas, H., van Belkum, A. and van Wamel, W. (2008) Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins. *J Immunol Methods*, **335**, 121-125.
61. Abu-Qatouseh, L.F., Chinni, S.V., Seggewiss, J., Proctor, R.A., Brosius, J., Rozhdestvensky, T.S., Peters, G., von Eiff, C. and Becker, K. (2010) Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. *J Mol Med*, **88**, 565-575.
62. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J. and Francois, P. (2010) Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One*, **5**, e10725.
63. Bohn, C., Rigoulay, C., Chabelskaya, S., Sharma, C.M., Marchais, A., Skorski, P., Borezee-Durant, E., Barbet, R., Jacquet, E., Jacq, A. *et al.* (2010) Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. *Nucleic Acids Res*, **38**, 6620-6636.
64. Sonnleitner, E., Sorger-Domenigg, T., Madej, M.J., Findeiss, S., Hackermuller, J., Huttenhofer, A., Stadler, P.F., Blasi, U. and Moll, I. (2008) Detection of small RNAs in Pseudomonas aeruginosa by RNomics and structure-based bioinformatic tools. *Microbiology*, **154**, 3175-3187.
65. Wilms, I., Voss, B., Hess, W.R., Leichert, L.I. and Narberhaus, F. (2011) Small RNA-mediated control of the Agrobacterium tumefaciens GABA binding protein. *Mol Microbiol*, **80**, 492-506.
66. Beisel, C.L. and Storz, G. (2011) The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in *Escherichia coli*.

Mol Cell, **41**, 286-297.
67. Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermuller, J., Reinhardt, R. *et al.* (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature*, **464**, 250-255.
68. Storz, G., Vogel, J. and Wassarman, K.M. (2011) Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Mol Cell*, **43**, 880-891.
69. Bohn, C., Rigoulay, C. and Bouloc, P. (2007) No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. *BMC.Microbiol.*, **7**, 10-19.
70. Nielsen, J.S., Larsen, M.H., Lillebaek, E.M., Bergholz, T.M., Christiansen, M.H., Boor, K.J., Wiedmann, M. and Kallipolitis, B.H. (2011) A small RNA controls expression of the chitinase ChiA in *Listeria monocytogenes*. *PLoS One*, **6**, e19019.
71. Nielsen, J.S., Lei, L.K., Ebersbach, T., Olsen, A.S., Klitgaard, J.K., Valentin-Hansen, P. and Kallipolitis, B.H. (2010) Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. *Nucleic Acids Res*, **38**, 907-919.
72. Seitz, H. (2009) Redefining microRNA targets. *Curr Biol*, **19**, 870-873.
73. Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J. and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *Rna*, **6**, 668-679.
74. Wan, Y., Kertesz, M., Spitale, R.C., Segal, E. and Chang, H.Y. (2011) Understanding the transriptome through RNA structure. *Nat Rev Genet*, **12**

Supplementary Tables

Supplementary Table S1. Primers used in this study.

Primer	Sequence	Function
Msa100pF	gcgttttcctaggagttttca	3'-RACE
Msa100pR	tgaaaactcctaggaaaacgc	5'-RACE
Msa079pF	tcctgcatctttctcatacaattt	3'-RACE
Msa079pR	aaattgtatgagaaagatgcagga	5'-RACE
Msa103pF	cctctcatccttatacgccatt	3'-RACE
Msa103pR	aatggcgtataaggatgagagg	5'-RACE
Msa004mF	ttcgctatcaatacgctaaatca	3'-RACE
Msa004mR	tgatttagcgtattgatagcgaa	5'-RACE
Msa016pF	ccatcgttttgtcctcctg	3'-RACE
Msa016pR	acaggaggacaaaacgatgg	5'-RACE
Spa_F	TAATACGACTCACTATAGGG aaatagcgtgattttgcggtt	*In vitro* transcription
Spa_R	gctttggataaaaccattacgt	*In vitro* transcription
Sak_F	TAATACGACTCACTATAGGG aaatgttaaatatttgttaattattttt	*In vitro* transcription
Sak_R	tagcaattgatttcctttaccat	*In vitro* transcription
efb_F	TAATACGACTCACTATAGGG aaaagtagaggcatgaatttttg	*In vitro* transcription
efb_R	tgatatttaaaagtaccatcattg	*In vitro* transcription
Sbi_F	TAATACGACTCACTATAGGG gtacttccttacttaaaatacg	*In vitro* transcription
Sbi_R	ttcttctgtgatacctttagat	*In vitro* transcription
Hld_f	TAATACGACTCACTATAGGG ttatattgcctaactgtaggaa	*In vitro* transcription
Hld_R	actataaaatacttttttgtgcat	*In vitro* transcription
Msa103_F	TAATACGACTCACTATAGGG gtgccaatccgcttca	*In vitro* transcription
Msa1035_R	ctcattactgttgtcgctct	*In vitro* transcription
Msa100_F	TAATACGACTCACTATAGGG aaatacataggtttgaaatctatat	*In vitro* transcription
Msa100_R	ttttttaaaatcatggatagtatacc	*In vitro* transcription
Msa079_F	TAATACGACTCACTATAGGG aaatactttatcctctttaaaaataa	*In vitro* transcription
Msa079_R	aaattaactgtctagtatcaacg	*In vitro* transcription
Msa016_F	TAATACGACTCACTATAGGG gaaaatgtcgataattgctttg	*In vitro* transcription
Msa016_R	tcactggtaagtaagttataaaaa	*In vitro* transcription
Msa004_F	TAATACGACTCACTATAGGG gcaaaatacaaaaatggagaact	*In vitro* transcription
Msa004_R	atacttggtggagcaatagttt	*In vitro* transcription
Scin_F_EcoRI	aacacaagaattcattaatgtactt	Overexpression construction

Msa004_ Scin_F_EcoRI	aacacaagaattc*attaatgtacttttttttagtta gtcattaaaataaattagta*gcaaaatacaaaaat	Overexpression construction
Msa004_ Scin_R_EcoRI	atttttgtattttgc*tactaatttatttttaatgactaac taaaaaaaagtacattaat*gaattcttgtgtt	Overexpression construction
SCMsa004_F	*taaaataaattagta*gcaaaatacaaaaatggagaact	Overexpression construction
SCMsa004_R_ BamHI	aacacggatccatacttggtggagcaatagttt	Overexpression construction
Msa079_ Scin_F_EcoRI	aacacaagaattc*attaatgtacttttttttagttagt cattaaaataaattagta*aaatactttatcctcttta	Overexpression construction
Msa079_ Scin_R_EcoRI	taaagaggataaagtattt*tactaatttatttttaatgac taactaaaaaaaagtacattaat*gaattcttgtgtt	Overexpression construction
SCMsa079_F	*taaaataaattagta*aaatactttatcctctttaaaaat	Overexpression construction
SCMsa079_R_ BamHI	aacacggatccaaattaactgtgtctagtatcaacgat	Overexpression construction
Scin_F_KpnI	aacacaaggtaccattaatgtactt	Overexpression construction
Msa016_ Scin_F_KpnI	aacacaaggtacc*attaatgtacttttttttagttagtc attaaaataaattagta*gaaaatgtcgataat	Overexpression construction
Msa016_ Scin_R_KpnI	attatcgacattttc*tactaatttatttttaatgactaact aaaaaaaagtacattaat*ggtaccttgtgtt	Overexpression construction
SCMsa016_F	taaaataaattagtagaaaatgtcgataattgctttgatga	Overexpression construction
SCMsa016_R_ BamHI	aacacggatcctcactggtaagtaagttataaaaa	Overexpression construction
rt_Spa_F	ccagctaataacgctgcaccta	Real-time PCR
rt_Spa_R	gaaactggtgaagaaaatccattca	Real-time PCR
rt_Spa_probe	FAM-cgctaatgataatccaccaaatacagttg-TAMRA	Real-time PCR
rt_hld_F	ggagtgatttcaatggcaca	Real-time PCR
rt_hld_R	agtgaatttgttcactgtgtcg	Real-time PCR
rt_efb_F	ctacaattgcgtcaacagcagat	Real-time PCR
rt_efb_R	accatcattgtactctacgatattgtga	Real-time PCR
rt_efb_probe	FAM-cgagcgaaggatacggtccaagagaaa-TAMRA	Real-time PCR

Capital letters: T7-promoter sequence
Italic: SCIN promoter
Underlined: restriction site

Supplementary Table S2. Predicted sRNAs
Small RNAs with the array predicted start and stop sites and the orientation of the sRNA to the adjacent genes.

Name	start	stop	Orientation
Msa001	74741	74871	> < >
Msa002	93215	93394	< > <
Msa003	95235	95580	< < >
Msa004	115709	115828	> < >
Msa005	124918	125037	ND
Msa006	133658	133780	< > <
Msa007	164026	164145	> < >
Msa008	172213	172469	> < >
Msa009	174030	174179	> < >
Msa010	182208	182377	> > <
Msa011	220313	220096	< < >
Msa012	280750	280869	> > >
Msa013	402916	403095	< < >
Msa014	409060	409179	< < >
Msa015	417999	418118	ND
Msa016*	453895	454192	> > >
Msa017*	453895	454683	> < >
Msa018	489568	489958	> > >
Msa019	507845	508111	> > >
Msa020*	559382	559612	> > >
Msa021	629412	629531	> > >
Msa022	661970	662168	< > <
Msa023*	662345	662657	< < <
Msa024*	674008	674484	> > <
Msa025	680713	680832	< < >
Msa026*	688184	688303	> > >
Msa027	694116	694295	> > >
Msa028*	709354	709473	> < >
Msa029	716224	716319	> > >
Msa030	725933	726052	< > >
Msa031	736764	736883	< > <
Msa032	766216	766397	> > >

Msa033	776720	776810	< > >
Msa034	776720	776810	< < >
Msa035	785074	785193	> > <
Msa036	788713	788832	> > >
Msa037	794230	794469	< < >
Msa038	803344	803479	> > >
Msa039*	806939	807058	> > >
Msa040	849428	849667	> > <
Msa041	888579	888744	< > >
Msa042	909686	909805	< < <
Msa043	954521	954640	< < >
Msa044*	959300	959419	> > <
Msa045	968806	968938	> > <
Msa046	976249	976428	< > >
Msa047*	1119391	1119619	> > >
Msa048	1121016	1121153	> > >
Msa049*	1121155	1121399	> > >
Msa050*	1168619	1168738	> > >
Msa051*	1168619	1168738	< < <
Msa052	1195407	1195567	> > >
Msa053	1219234	1219448	< > >
Msa054	1257909	1258088	< > >
Msa055	1282386	1282505	> > >
Msa056	1307661	1307780	> > >
Msa057	1319638	1319901	> > >
Msa058*	1327806	1327925	> > >
Msa059	1340685	1340845	> > >
Msa060	1383206	1383394	> > <
Msa061*	1409491	1409627	> > >
Msa062	1527185	1527374	< < <
Msa063	1541043	1541222	< < <
Msa064	1569579	1569698	< > >
Msa065	1631093	1631332	< < <
Msa066	1646015	1646134	< < >
Msa067	1646135	1646314	< < >

Msa068*	1674421	1674548	< < <
Msa069*	1715298	1715433	< < <
Msa070*	1715434	1715553	< < <
Msa071*	1818114	1818381	< < <
Msa072	1920261	1920440	< < <
Msa073	1970710	1970829	< > >
Msa074	1995446	1995785	< < >
Msa075	2012690	2012809	< > >
Msa076	2027042	2027161	< < <
Msa077*	2027867	2028046	< < <
Msa078*	2028086	2028226	< < <
Msa079	2071356	2071595	< > >
Msa080	2078452	2078661	< < <
Msa081	2084255	2084434	< > >
Msa082	2089942	2090241	> > <
Msa083	2089942	2090301	> < <
Msa084	2146276	2146395	< < >
Msa085	2147860	2147979	> < <
Msa086	2160494	2160668	< > <
Msa087*	2179482	2179687	< < <
Msa088	2187022	2187324	< < >
Msa089	2191988	2192107	< > >
Msa090	2202375	2202494	< < >
Msa091	2206683	2206813	> < >
Msa092*	2285086	2285205	< > <
Msa093	2314147	2314266	< < >
Msa094	2338867	2339161	< < <
Msa095	2350651	2351357	< < <
Msa096	2362893	2363038	> < <
Msa097	2409898	2410017	> < >
Msa098	2412635	2412776	< > <
Msa099*	2428873	2429656	> < >
Msa100	2429777	2429896	> > >
Msa101*	2465929	2466147	> < >
Msa102*	2465929	2466087	< < <

Msa103	2484692	2484838	< > >
Msa104	2515690	2515947	< > >
Msa105	2533576	2533755	< > <
Msa106*	2537885	2538051	< < <
Msa107	2569061	2569269	< < <
Msa108	2574261	2574380	< > >
Msa109	2587552	2587787	< < >
Msa110*	2609517	2609696	< < >
Msa111	2617238	2617357	< > >
Msa112	2663007	2663132	< < <
Msa113*	2690267	2691388	< < <
Msa114*	2751592	2751771	< < <

ND: Not Determined

*Overlapping predictions with previous studies (Supplementary Table S4)

Supplementary Table S3. Predicted sRNAs compared to previous studies.
Previously identified sRNAs in N315 compared to the predicted sRNAs in this study. Orientation indicates position of the sRNA to the adjacent genes.

Name	Start_N315	Stop_N315	Start_MSSA476	Stop_MSSA476	Orientation	This study	References
Teg45	570162	570452	559355	559645	> >	Msa020	(62)
RsaC	673628	674172	673628	674172	> <	Msa024	(22)
Teg99	694341	694422	688194	688273	> >	Msa026	(62)
Teg20as	715434	716442	709289	710297	NA	Msa028	(62)
Teg109	812740	812794	806895	806949	> >	Msa039	(62)
Teg68	1649444	1649294	1674536	1674686	< <	Msa068	(62)
Teg70	1696907	1696634	1715307	1715580	> <	Msa069/Msa070	(62)
RsaE	959321	959420	959321	959420	> <	Msa044	(22)
RsaF	959399	959502	959399	959502	> <	Msa044	(22)
Teg92	975399	975459	959337	959397	> <	Msa044	(62)
RsaX14	1801062	1801380	1818063	1818382	NA	Msa071	(22)
RsaOP	1089149	1089566	1119365	1119782	> >	Msa047	(22)
Teg55	1090985	1091109	1121201	1121325	> >	Msa049	(62)
RsaX10	1138640	1138848	1168646	1168854	NA	Msa050/Msa051	(22)
Teg14	2007062	2007164	2027188	2027290	< <	Msa058	(62)
Teg104	1299165	1299246	1327797	1327878	> >	Msa058	(62)
Teg15	2008692	2008781	2028103	2028197	< <	Msa078	(62)
RsaOR	2008572	2009085	2027983	2028501	< <	Msa077/Msa078	(22)
Teg113	1378945	1378990	1409488	1409533	> >	Msa061	(62)
Teg63	1379119	1379184	1409662	1409727	> >	Msa087	(62)

Teg27	2437048	2435872	2428490	2429666	> < >	Msa099	(62)
Sau-30	2294537	2294600	2285047	2285110	NA	Msa092	(61)
RsaX21	2544796	2545113	2537808	2538125	NA	Msa106	(22)
RsaX18	2436200	2436800	2428818	2429418	NA	Msa099/Msa110	(22)
Teg130	2473489	2473552	2466064	2466127	< > <	Msa101	(62)
RsaX20	2473450	2473566	2466025	2466141	NA	Msa101/Msa102	(22)
Teg32	2709941	2709839	2691294	2691396	< < <	Msa113	(62)
RsaX25	2769241	2769725	2751358	2751842	NA	Msa114	(22)
Teg141	2769772	2769394	2751511	2751889	< < <	Msa114	(62)
RsaX05	470600	470900	454554	454853	NA	Msa017	(22)
Sau-41	469933	470088	453886	454041	NA	Msa016/Msa017	(61)
Teg49	667114	666952	662191	662353	< < <	Msa023	(62)

NA: Not Available

Chapter 5

Transcriptome profile in human blood of genetically similar S*taphylococcus aureus* strains

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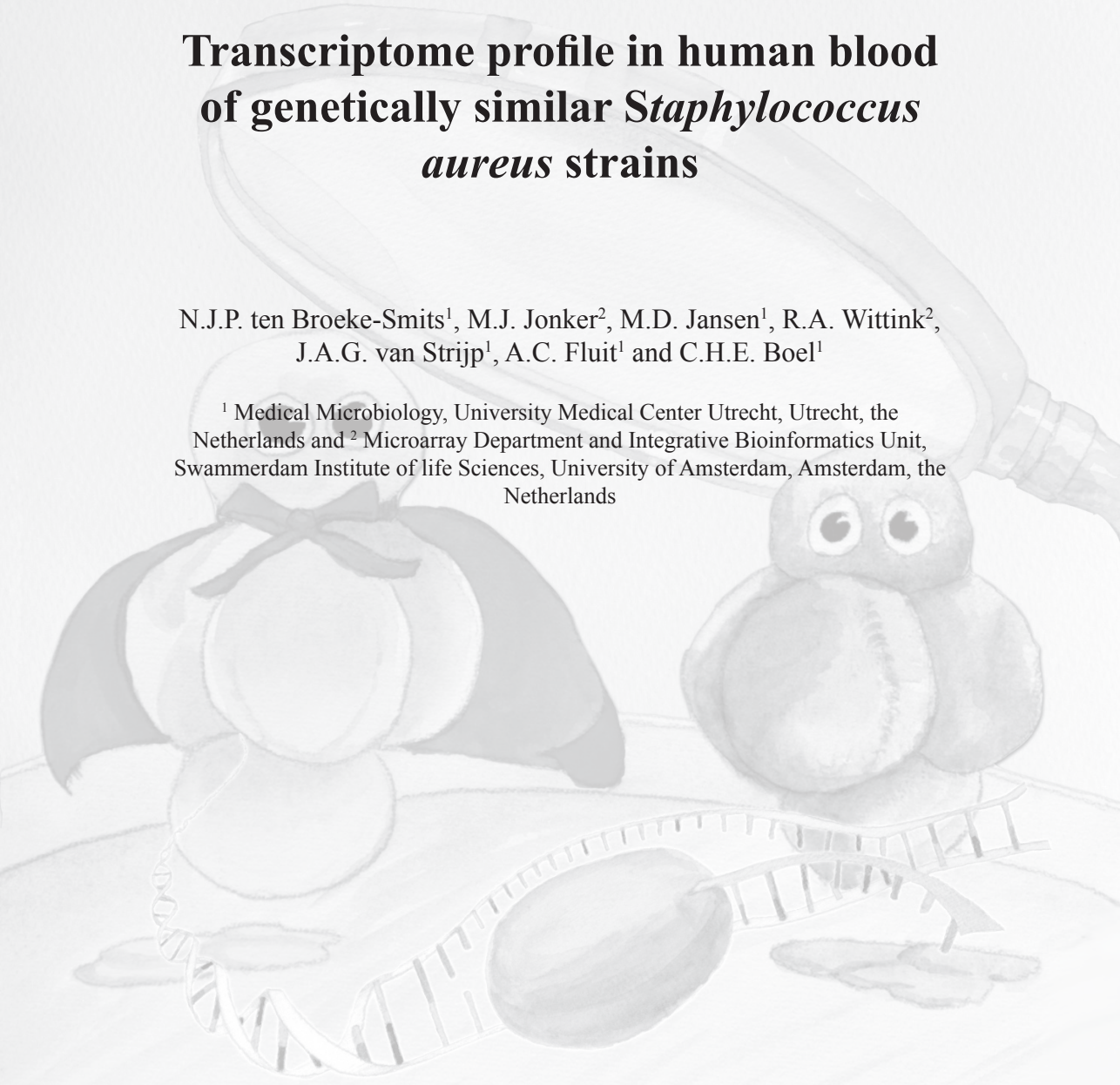

Abstract

Staphylococcus aureus is able to cause a wide variety of infections with an increasing threat in the community. A fundamental role of gene regulation for survival, proliferation and colonization in the host is proposed. Nonetheless, studies on gene regulation after exposure to host related factors, like serum or blood, are limited. Here, a comparative pilot study between two genetically similar, completely sequenced strains has been conducted to determine differential regulation after exposure to human blood and IMDM both at 5% CO_2. Remarkable differences were identified between the two strains as well as between the two conditions. Genes encoding for proteases and toxins were up-regulated higher in MW2 relative to MSSA476, whereas genes encoding immune evasion proteins were more pronounced up-regulated in MSSA476 compared to MW2. A different strategy of surviving the encounter of neutrophils might be applied by the two strains. A comparison between the two conditions showed the up-regulation of genes encoding staphylococcal superantigen-like proteins (Ssls) in blood, while no differential regulation in IMDM was observed. These genes might have an important role in survival of the pathogens in human blood once they are phagocytosed. The results in this pilot experiment show large differences in gene regulation of two genetically similar *S. aureus* strains after exposure to human blood and might explain the different disease manifestation of the two *S. aureus* strains.

Introduction

Staphylococcus aureus is the causative agent of many hospital and community-acquired infections. It is a leading cause of intravascular and systemic infections such as bacteremia, endocarditis and sepsis (1,2). The genome of *S. aureus* is approximately 2.8 Mbp with a ~37% G+C content. It may incorporate a number of mobile genetic elements, like pathogenicity islands, plasmids, prophages and transposons, which often harbor virulence genes and antibiotic resistance genes (1). The combination and content of these mobile elements is variable which leads to variation in virulence factors between strains (3,4). Nevertheless, genetically similar strains can cause different infections. As such, no association has yet been made between gene content and disease manifestation, except for clonal complex 121 which shows a relation to impetigo (4,5). Therefore, it might well be that not the gene content as such, but the regulation of virulence factors in different environments is essential in establishing a particular type of infection.

Despite the fact that *S. aureus* is an important

pathogen, knowledge about the adaptive process required to survive and multiply within hostile environments within the human host is limited (6,7). In controlled standard laboratory growth conditions, gene expression is growth-phase and medium-dependent (8,9) and tightly regulated. It is depending on energy availability, cell density (quorum sensing) and environmental signals (10,11). Up-regulation of virulence genes encoding surface proteins during log phase and up-regulation of toxins during stationary phase is controlled by global regulatory elements such as *sae*, *agr*, *sarA* and *rot* (12-14). Agr is a major regulator of virulence genes and is involved in both up-regulation of genes encoding exoproteins as well as down-regulation of genes encoding cell wall proteins in the stationary growth phase (13,15,16). Another important regulator involved in the regulatory cascade of virulence gene expression is Rot (repressor of toxins). Rot negatively regulates genes encoding secreted proteins, like hemolysins and proteases (14). It furthermore positively regulates a number of genes encoding cell surface adhesins. Recently, the positive regulation of genes encoding staphylococcal superantigen-like proteins (SSLs) was shown to be Rot-dependent (17). Genes regulated by Rot are all assumed to play a role in tissue invasion (14). SarA up-regulates genes encoding cell wall proteins and down-regulates toxins in the log phase (18). The two-component system SaeRS controls the regulation of major virulence genes in the exponential growth phase, including hemolysin A and B (*hla* and *hlb*), coagulase (*coa*), extracellular adherence protein (*eap*) and fibronectin binding protein A (*fnbA*) (19,20). In addition to the global regulators, bacterial small RNAs (sRNAs) have been shown to regulate expression of virulence genes in a variety of bacteria, including *S. aureus* (21). Of these, RNAIII, the effector molecule of the *agr* quorum sensing system, is the best-described. It activates the expression of *hla* and represses the expression of staphylococcal protein A (*spa*), extracellular fibrinogen binding protein (*efb*) and *coa* (10,22). Also the virulence regulator *rot* is repressed by RNAIII (22). SprD is another sRNA involved in virulence regulation as it regulates *S. aureus* binder of IgG (*sbi*) (23). Host signals, e.g. those related to innate immune defenses or those present in target tissues, are probably involved in triggering regulation of genes involved in virulence. However, the role of regulators upon entering the host environment is still largely unknown.

Studies on the molecular basis to comprehend the response of *S. aureus* to host signals have been performed in serum (24,25), purified neutrophils (26) or conditions mimicking phagocytosis (27). In addition, two studies have been directed to characterization of expression of a limited set of virulence genes in infected tissues using quantitative real-time (RT-)PCR. This has shown the important role of Agr-regulated expression of virulence factors (28) and more specifically for *eap* in the impairment of healing of deep wounds (29). Recently, Malachowa and colleagues (7) have analyzed the altered gene expression of USA300 upon exposure to human blood, compared to the expression levels in the inoculation culture (in TSB) set as t=0 min. A strong up-regulation of the gamma-hemolysin subunits *hlgA*, *hlgB* and *hlgC* was identified upon exposure to blood and these genes were demonstrated in mouse models to be relevant in infection. With the knowledge of the ability of *S. aureus* to cause a variety of diseases, which could be related to different gene regulation, the importance of studying multiple strains on exposure to changing environments becomes apparent.

Here we describe a pilot experiment in which we compared two genetically highly similar strains, MW2 and MSS476, during culture in human blood and in IMDM both at 5% CO_2. The aim of the study was to identify genes involved in virulence that are potentially regulated after exposure to human blood. The assumption was that any consistent difference in expression profiles in blood as compared to IMDM might give an indication for specific regulation upon contact with blood. Secondly, we wanted to investigate whether genetically similar stains showed similar expression patterns of (virulence) genes. A difference between the strains could imply different adaptation processes upon changing environments and might give an indication for the ability of causing various types of infection. Both strains were community-acquired, belonged to sequence type (ST)1 and were initially isolated from blood. MSSA476 caused osteomyelitis (30) and MW2 caused a fatal septicemia and septic arthritis (3). Large differences in expression patterns were observed between blood and IMDM both at 5% CO_2, but remarkably also between the two strains in the same environment. In blood but not in IMDM the *ssl* genes were highly up-regulated, which might indicate a major role for these genes in survival in human blood. Furthermore, the data indicate that genetically similar *S. aureus* strains

can show considerable differences in gene expression after exposure to the same environment. As such, different infection or survival strategies might be used by genetically similar *S. aureus* strains.

Materials and methods

Bacterial strains

In this study, the sequenced, highly virulent, community-acquired methicillin-susceptible *S. aureus* strain MSSA476 (30) and the sequenced, community-acquired MRSA strain MW2 (3,30) were used. The genetic differences between the strains can be mainly attributed to mobile genetic elements (Table 1).

Table 1. Mobile genetic elements in MW2 and MSSA476 (3,26)

	MW2	MSSA476
φSa2	*lukSF*-PV	-
φSa3	*sea, sak, seg2, sek2*	*sea, sak, seg2, sek2*
φSa4	-	(hypothetical) phage proteins
νsa-α	*ssl* (11), *lpl* (5)	*ssl* (11), *lpl* (5)
νsa-β	*spl* (4), *lukDE*, *bsa*	*spl* (4), *lukDE*, *bsa*
SaPI3	*ear*, *sel2* and *sec4*	-
SCC*mec* type IV	*mecA*	-
SCC_{476}	-	*far1* homologue

Blood donor

Venous blood was obtained from a single healthy human volunteer and immediately mixed with 50 μg/ml lepuridin. Lepuridin was used as anticoagulant, because it does not activate components of the innate immune system, which was considered relevant in this study (Dr. K.P.M. van Kessel, personal communication).

Growth conditions

Cultures were incubated at 37°C and 180 rpm, unless stated otherwise. The two strains were grown overnight in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA, USA). These overnight cultures were diluted (1:10) in fresh prewarmed IMDM and grown to mid-log phase culture (A_{660} ~0.5) prior to the growth experiment. The mid-log phase culture was diluted to an A_{660} of 0.3 with prewarmed IMDM, directly transferred to fresh prewarmed IMDM to obtain an A_{660} of 0.03 and grown to A_{660} ~0.5.

Cultures (40 ml) were spun for 8 min at 4500 rpm and 37°C. Pellets were resuspended in 40 ml blood or IMDM, both maintained at 37°C and 5% CO_2. For t=0 min, RNA protect buffer (Qiagen, Valencia, CA, USA) was added immediately after resuspension. Cultures were further incubated at 37°C in a CO_2 incubator without shaking. Samples were taken at 30, 60, 90 and 180 min post inoculation. Immediately after sampling, RNA protect buffer was added and incubated for 5 min at room temperature before harvesting cells by centrifugation. Cells were snap-frozen in liquid nitrogen and stored at -80°C until further use.

RNA extraction

RNA extraction was performed at room temperature unless stated otherwise. RNA was purified using the NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol with some adjustments. In brief, pellets were thawed slowly and resuspended in 5 volumes Buffer EL, incubated for 20 min on ice and spun at 4500 rpm at 4°C for 6 min. Cells were rinsed with 2 volumes Buffer EL. For RNA isolation, the pellet was resuspended in 350 µl RA1 buffer supplemented with 3.5 µl β-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.8 µg bacteriophage MS2 carrier RNA and 250 µg glycogen and vortexed vigorously. Resuspended bacteria were added to 0.5 ml 0.1 mm silica beads (Merlin, Breda, The Netherlands) and disrupted using a mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 30 sec at 5000 rpm. Total RNA was eluted in 60 µl RNase-free MilliQ water. RNA yield was measured using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and quality was measured using a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Both the RNA integrity number (RIN) and the presence of degradation products were checked.

Labeling, hybridization and scanning

Total RNA was labeled with fluorescent dyes by direct labeling. A total of 5 µg RNA was randomly primed with Superscript II reverse transcriptase (Invitrogen), random octamers (100 ng/ µl) and actinomycine D, in a total volume of 10 µl, for 2 h at 42°C with the incorporation of Cy5- or Cy3-dUTP (GE Healthcare, Diegem, Belgium) with a ratio dUTP/dTTP of 3/1, yielding approximately 2.8 µg labeled cDNA. RNA template was removed by hydrolysis with 1.5 µl 2.5 M NaOH (Sigma-Aldrich) for 15 min at 70°C. Hydrolysis was stopped by neutralization with 7.5 µl 2 M MOPS (Sigma-Aldrich) and put on ice. Labeled cDNA was purified using Qia-quick PCR purification kit (Qiagen, Valencia, CA, USA). Incorporation of Cy3 or Cy5 was determined using a NanoDrop ND-1000 (Nanodrop Technologies,

Wilmington, DE, USA).
The common reference was created by pooling Cy5-labeled RNA samples. Labeled cDNA was hybridized according to manufacturer's protocol (Roche NimbleGen, Basel, Switzerland). A total of 1.1 µg Cy3-labeled cDNA and 1.1 µg Cy5-labeled common reference was mixed in 7.2 µl of NimbleGen hybridization cocktail. The mixture was heated to 65°C for 5 min, and a total of 6 µl was loaded onto a custom made multi-strain *S. aureus* array (MicroArray Department, University of Amsterdam) and hybridized for 18 hours at 42°C in a dedicated hybridization chamber (Roche NimbleGen).
After the hybridization the arrays were dismantled at 42 °C and washed in buffer 1 for 2 min at room temperature, then 1 min in wash buffer 2 at room temperature and finally 15 sec in wash buffer 3 at room temperature (Roche NimbleGen). Slides were spun for 30 sec at 300 rpm to dry and scanned immediately in an Agilent DNA MicroArray Scanner (Agilent Technologies, Palo Alto, CA, USA). Data was extracted and processed using NimbleScan™ software (version 2.6, Roche NimbleGen).

Data analysis

Processing of the data was performed using R (version 2.13.0). All slides were subjected to a set of quality control checks, i.e. visual inspection of the scans, pseudocolor plots, tests against criteria for signal to noise ratios, testing for consistent performance across samples and visual inspection of pre- and post-normalized data with box and ratio-intensity and principal component plots.
The microarray data was quantile normalized and strain specific probes were selected as follows. All probes were aligned to MW2 (accession number: NC_003923), MSSA476 (accession number: NC_002953) and MSSA476 plasmid pSAS (accession number: NC_005951). All probes with an alignment within the sequences belonging to the strain with a bitscore over 100 and no other alignment with a bitscore over 40 were selected. Using the GenBank annotation of the given accessions genes were assigned to the probes if the region and strand where the probe matches the chromosome is included in the gene.
The samples taken at 30, 60, 90 and 180 min post inoculation were compared with the sample taken at t=0 min, for each strain cultured in blood or IMDM separately. The data was screened for multiple probes, designed against subsequent genomic targets, showing a relatively high Log2 fold-change in mRNA. Such probe sets were assumed to indicate differential gene expression or differential intergenic expression. We

used methodologies from a CGH analysis (31). Basically, an Adaptive Weights Smoothing (AWS) procedure was applied to estimate a Log2 fold-change in mRNA expression for genomic regions. Statistical analysis on the AWS regions (31) established that a Log fold-change of ~0.8 was sufficient for differential expression ($p < 0.05$). We decided to work in a more conservative manner and interpret only the regions with AWS estimations of 1.5 and higher or -1.5 and lower.

Taqman real-time PCR

A subset of genes was validated using Taqman real-time RT-PCR. Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used in combination with random hexamers to reverse transcribe RNA used in the microarray experiments. cDNA synthesis was performed with 0.5 µg total RNA according to manufacturer's protocol for random hexamers. The cDNA products were subsequently used in RT-PCR with Taqman probes for *fnbA*, *scn*, *efb*, *ssl6*, *ssl8* and *ssl9* and SybR-green for RNAIII/*hld* and *isdE* according to manufacturer's protocol (Primers and probes are listed in Table 2). Data obtained are expressed as mean fold-change of transcripts for t=180 min relative to t=0 min. Samples were analyzed in duplicate.

Table 2. Primers used in study

Name	Sequence	Conc (nM)
SSL6.seq-126R	TGATTTATAATTGTGTTGACCTTGAGTTG	900
SSL6.seq-37F	TTGGGATTGTTAGCTACTGGTGTAAT	900
SSL6-Probe	FAM-ACAGAAAGTCAAACAGTAAAAGCGGCA-TAMRA	200
SSL8.seq-141F	TCAATACTATTCAGGACCTAGTTATGAGTTAAC	300
SSL8.seq-250R	CTTGGAACTTTTGATTTTGTTGGTT	900
SSL8-Probe	FAM-TAGTGGCCAAAGTCAAGGTTATTATGACTCTAACG TTT-TAMRA	200
SSL9.seq-353F	GCGTTGGCGGTATAACAAAGA	300
SSL9.seq-449R	AAGCCATGTTTAGGATCGATTTTT	900
SSL9-Probe	FAM-AAATGTGAGATCAGTGTTTGGATTTGTAAGTAATC CAA-TAMRA	200
Fnba-F	GCTTGTACTGCTTTTGGTGCTTCT	300
Fnba-R	ATACAGCGCAACAGCAACAGA	300
Fnba-probe	FAM-CTTGTGTTGCGTTTGACGGTT-TAMRA	200
Scn-F	TTTTAGTGCTTCGTCAATTTCATTAT	900
Scn-R	AGCAATGTATGCTCTTAAGTCAAAAGAC	900
Scn-probe	FAM-TTTGAAGTTGATATTTTGCTTCTGAC-TAMRA	200
Efb_F	CTACAATTGCGTCAACAGCAGAT	900
Efb_R	ACCATCATTGTACTCTACGATATTGTGA	900

Efb_probe	FAM-CGAGCGAAGGATACGGTCCAAGAGAAA-TAMRA	200
RNAIII-F	GCCATCCCAACTTAATAACCA	900
RNAIII-R	CATAGCACTGAGTCCAAGGAAA	900
IsdE-F	AATGGAGCCGAATGTTGAAG	900
IsdE-R	TCGACTTTTGCATCCCTTTT	900

Results

Analysis of survival in human blood vs IMDM and quality of the data

To extend the knowledge on the pathophysiology of *S. aureus*, we analyzed the gene expression levels of MW2 and MSSA476 during culture in human blood. Survival of the bacteria in human blood was determined by CFU count on sheep blood agar plates. We confirmed that there was no significant difference in the number of inoculated bacteria (~5*10^8 CFU/ml) between the two strains. After 180 minutes culturing in human blood we noticed a decrease in CFUs of ~1 log for both strains (data not shown), probably due to aggregation of bacteria or phagocytosis and killing of bacteria (7).

Mid-exponential phase *S. aureus* cells grown in IMDM without CO_2 were resuspended in fresh human blood or IMDM both maintained at 5% CO_2 and incubated for 0, 30, 60, 90 and 180 minutes. At these time points RNA was isolated and reverse transcribed cDNA was used for hybridization to microarrays. Evaluation of pre- and post-normalized data with box and ratio-intensity plots indicated high quality of the data (results not shown). The resultant Principal Component Analysis (PCA) plot discriminates between culture medium, time and strain (Fig. 1A). The t=0 min time points in the two conditions differ from each other, but this might be explained by the time between resuspension and addition of RNA protect buffer. A venn diagram illustrates the differences and similarities between the number of differentially expressed genes in MSSAA476 and MW2 in blood and IMDM at the four time points of sampling as compared to t=0 min (Fig.1B).

Gene profiling upon changing environments

RNA transcript levels were determined for each strain and time point. The samples of the different time points of the blood and IMDM cultures were compared with the start of the culture (t=0 min). Even though statistical analysis on the AWS regions showed that a Log fold-change of ~0.8 was

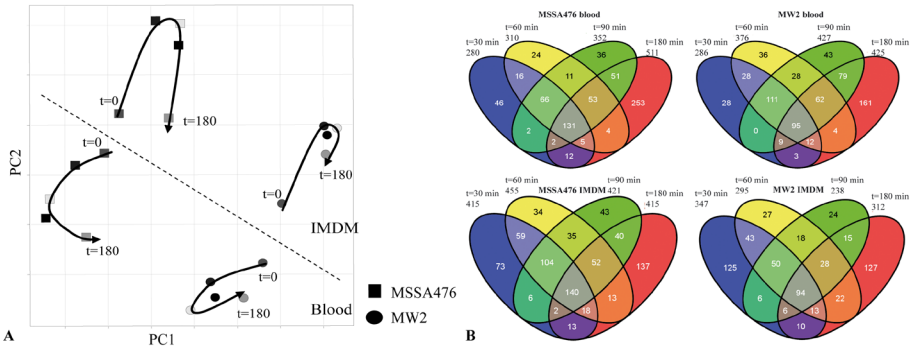

Figure 1. PCA analysis and venn diagrams.
(A) Principal Component Analysis (PCA) of microarray data showing all time points of the growth curves of MSSA476 and MW2 in blood or IMDM maintained at 5% CO_2. Treatment, time and strain are separated on the PC1 and PC2 axis. (B) Venn diagrams showing number of regulated genes per strain, condition and time point.

sufficient for differential expression ($p < 0.05$), we decided to examine only transcripts with log fold-changes of >=1.5 and <=-1.5.
Remodeling of the expression profiles after exposure to human blood occurred in both strains. Within 30 minutes 136 genes were up-regulated in MW2 and 180 genes in MSSA476, while 150 genes were down-regulated in MW2 and 100 genes in MSSA476. Over time, 699 genes (26.5% of the genome) were differentially regulated in MW2. In MSSA476, 712 genes (27.4% of the genome) were differentially regulated. Exposure to IMDM with 5% CO_2 showed within 30 minutes up-regulation of 212 genes in MW2 and 249 genes in MSSA476, while 135 genes were down-regulated in MW2 and 166 genes in MSSA476. Over time, 608 genes (23.1% of the genome) were differentially regulated in MW2. In MSSA476, 769 genes (29.6% of the genome) were differentially regulated. The number of regulated genes in the two conditions varied over time compared to t=0 min and between the two strains (Fig. 1B).
Subsequently, gene expression profiles were visualized using a heatmap in which differentially expressed genes are shown (Fig. 2 and Supplementary Fig. S1). In total, 897 genes present in both genomes were differentially expressed at one or more time points in at least one of the strains or conditions. Large differences in gene expression were identified between the strains, but also between the conditions. Both strains showed differentially expressed clusters of genes that were not significantly regulated in the other strain. A large cluster of genes encoding phage proteins, also present in MW2, is up-regulated in MSSA476 in both conditions. Another cluster consisting of genes involved in pyrimidine synthesis was down-regulated in blood in MSSA476. In MW2, the operon containing genes involved in iron and heme binding and/or uptake (*isdABCDEFG*) was highly up-regulated after exposure to blood. A cluster of

genes mainly encoding hypothetical proteins involved in transport and binding was down-regulated in both conditions in MW2. No delay in expression could be observed between the strains. Genes encoding for energy metabolism were up-regulated in both strains and both conditions. Additionally, both strains showed up-regulation of genes encoding cellular processes, mainly toxin and resistance proteins as well as virulence factors, after exposure to blood. When exposed to IMDM with 5% CO_2 a more diverse expression pattern was observed for these genes.
The genes located on the different mobile genetic elements were up-regulated in both conditions. In MSSA476 genes encoding proteins involved in mobile and extrachromosomal element functions, located on bacteriophage φSa4, were up-regulated at all time points. In MW2 genes located on bacteriophage φSa2 (*lukSF*-PV) and SaPI3 (*ear*, *sel2* and *sec4*) were up-regulated in both conditions at all time points (data not shown).
More than 30% of the genes in the staphylococcal genome code for hypothetical proteins or have an unknown or unclassified function. On exposure to human blood or IMDM with 5% CO_2, 25% of these genes is significantly regulated. This percentage of differentially expressed genes is comparable to that for genes with a known function.

Virulence gene profiling upon changing environments
Pathogenicity related expression of the two strains was studied in more detail by examining the differential expression of virulence genes and regulators. A hierarchical clustering was created showing only the virulence genes of interest and important global regulators, differentially expressed after exposure to blood and/or IMDM (Fig. 3).
From the heatmap, it becomes apparent that after exposure to blood, up-regulation of genes encoding staphylococcal superantigen-like proteins 12-14 (*ssl12-14*), cytolytic toxins and the gamma-hemolysin subunits (*hlgA*, *hlgB* and *hlgC*) was observed for both strains. Different gene regulation was detected for genes encoding serine proteases (*splA*, *splB* and *splC*) as well as leukocidin D and E (*lukD* and *lukE*) as they were more up-regulated in MW2. Beta phenol soluble modulins (PSMβ1 and PSMβ2) were down-regulated in MW2, while no differential expression was observed in MSSA476. On the other hand, genes encoding for factors involved in immune evasion, especially staphylococcal superantigen-like proteins 1-10 (*ssl1-10*) and fibronectin binding protein A (*fnbA*) were up-regulated more and earlier in MSSA476. An apparent down-regulation of *ssl11* was observed in MW2, while no differential expression occurred in MSSA476. The gene encoding fibronectin binding protein B (*fnbB*) was less down-regulated in MSSA476 compared to MW2. Normalized expression levels at t=0 min of all these genes were comparable

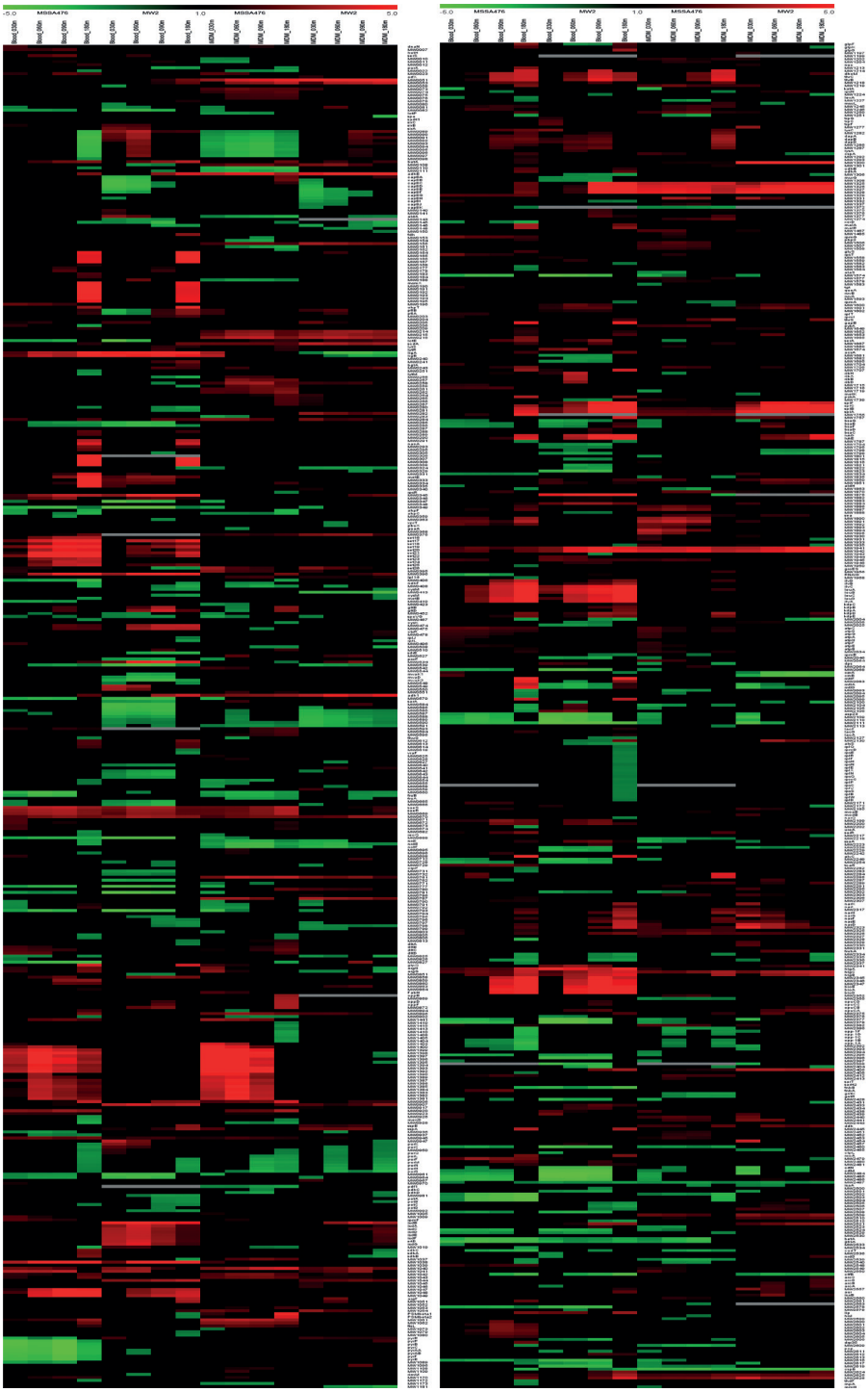
-5.0
MSSA476
MW2
1.0
MSSA476
MW2
5.0

Figure 2. Heatmap of MSSA476 and MW2 in blood and IMDM both at 5% CO_2.
Gene expression profiles of all differentially expressed genes in MSSA476 and MW2 in blood and IMDM both at 5% CO_2. Genes are listed according to occurrence in MW2. Significantly regulated genes are indicated by green (down-regulated) or red (up-regulated). Results are presented as log2 fold-changes compared to time point t=0.

for both strains (results not shown).

Differential expression of global regulators and/or regulatory systems was visualized to study the influence on virulence gene regulation. As observed previously (7), the *saeRS* genes were up-regulated in both strains after exposure to human blood. Down-regulation was observed for *sarA* in MW2, whereas a slight up-regulation was present in MSSA476. Another notable difference in significantly regulated genes was the up-regulation of *rot* and the down-regulation of RNAIII in MSSA476 from t=30 min, while *rot* was not differentially expressed in MW2. Normalized expression levels of the regulators were comparable at t=0 min

Transcripts involved in iron and heme uptake and/or binding (*isdABCDEFG*, *sbnACDEFGHI*, *srtB* and *sirABC*) showed high up-regulation in MW2, while in MSSA476 no significant regulation was observed. The *sbnACDEFGHI* genes were down-regulated in MSSA476 from t=180 min and up-regulated in MW2 at 60 min. Normalized expression levels of the iron and heme uptake and/or binding genes in MSSA476 at t=0 min were already as high as the up-regulated expression levels at t=90 min for MW2, which probably explains the differences found in regulation between the strains.

Comparing differential expression of virulence genes after exposure to IMDM at 5% CO_2 with expression in human blood, showed some clear differences. One of the most notable differences was the lack of differential expression of *ssl1-10* and *ssl12-14* and the up-regulation of *ssl11* in IMDM at 5% CO_2. Also the genes encoding enterotoxin H (*seh*), PSMβ1 and PSMβ2 were up-regulated in IMDM with 5% CO_2. No differential expression in IMDM was observed for *fnbB* and the genes encoding iron and heme uptake and/or binding (*sirABC*, *isdABCDEFG* and *srtB*). The genes encoding siderophore synthesis proteins (*sbnACDEFGHI*) were more down-regulated in MSSA476 compared to blood. Normalized expression levels at t=0 min were comparable between the two conditions. For the global regulators no major differences in expression were observed between the two

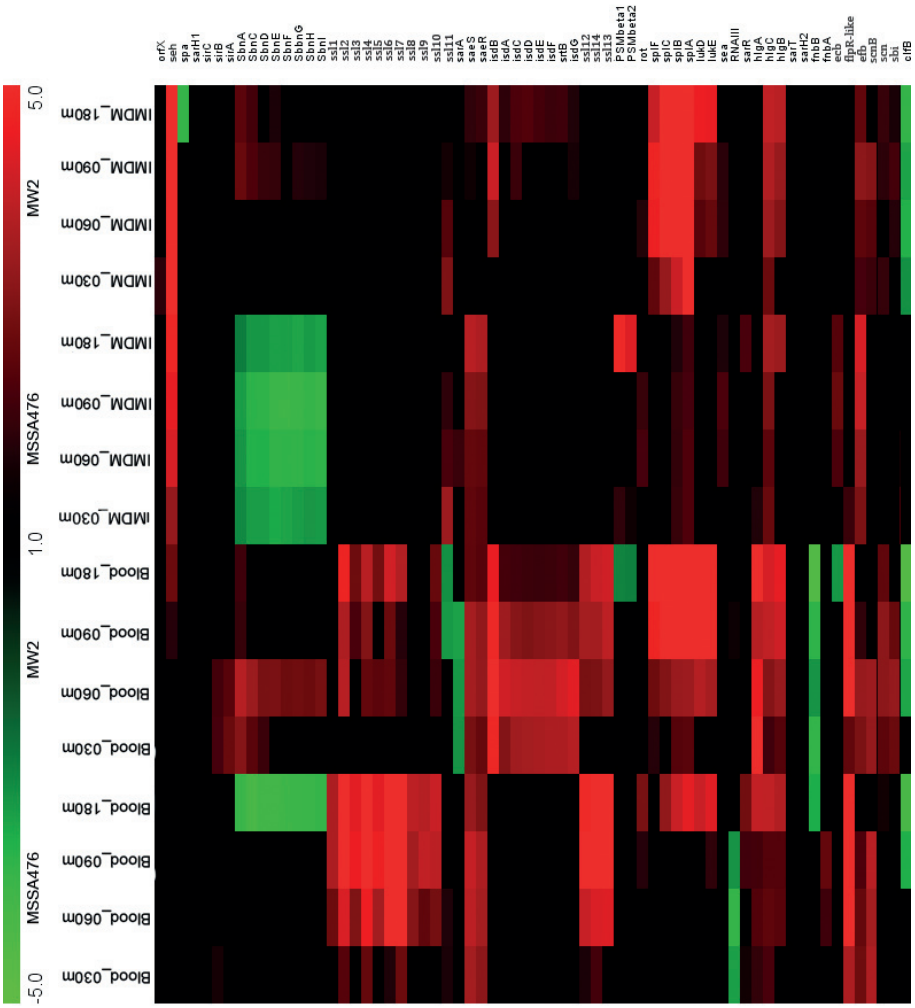

Figure 3. Heatmap visualizing genes involved in virulence, regulation and iron uptake and/or binding.

Blow-up of complete heatmap for virulence and regulator genes significantly regulated in human blood and/or IMDM are indicated by green (down-regulated) or red (up-regulated). Results are presented as log2 fold-changes compared to time point t=0.

conditions, except RNAIII which was not differentially expressed in MSSA476. Taken together, these data indicate a large difference in gene regulation after exposure to infection related conditions for two genetically similar strains.

Microarray results of eight genes showing differential regulation were verified by Taqman real-time quantitave (RT)-PCR (Fig. 4). Transcript levels of the chosen genes identified by microarray analysis and Taqman RT-PCR after 180

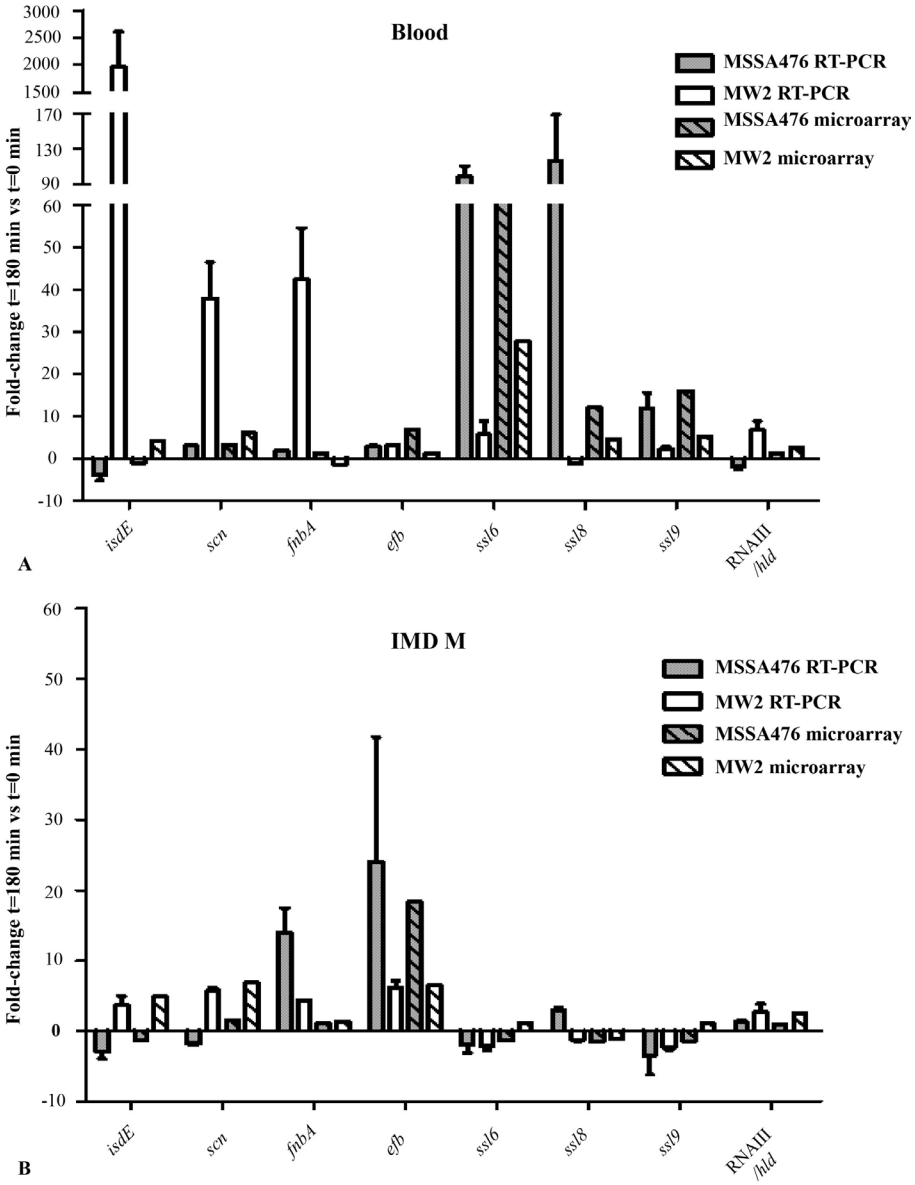

Figure 4. Changes in expression of nine transcripts in human blood or IMDM both at 5% CO_2.

Validation of microarray data by Taqman RT-PCR. Results are expressed as the average fold-change in transcripts in blood at t=180 min compared to t=0 min. (A) Verified transcripts in human blood. (B) Verified transcripts in IMDM.

min were compared to t=0 min. For *ssl6*, *ssl8*, *ssl9* and *isdE*, cDNA levels were undetermined at t=0, as was anticipated from the microarray data. A strong positive correlation between the microarray and RT-PCR data was observed.

Small RNA transcript profiling

Small RNAs (sRNAs) have been postulated as powerful regulators of many genes, including virulence genes. Numerous sRNAs are encoded in the intergenic regions. For this reason, differential expression (Log fold-changes of >=1,5 or <=-1,5) of transcript levels in intergenic regions was determined upon exposure to blood and IMDM both at 5% CO_2 (Fig. 5 and Supplementary Table 2). When exposed to blood, 59 sRNA candidates were identified for MW2 and 48 sRNA candidates for MSSA476, 12 of these sRNA candidates were identified in both strains. We observed Log2 fold-changes ranging from -4 to 5 for the putative sRNAs. Several differentially expressed sRNA candidates were previously identified (32,33), but have not yet been functionally characterized. The hierarchical clustering of these putative sRNAs showed more RNA transcripts down-regulated for MW2 compared to MSSA476. This could hint at more regulation of metabolic pathways in MW2 compared to MSSA476 after exposure to blood. Moreover, changes in expression levels of the putative sRNAs in MW2 were more pronounced than in MSSA476.

After exposure to IMDM with 5% CO_2, 51 putative sRNAs could be identified in MSSA476 and 66 in MW2 of which 15 were identified in both strains. In MSSA476, more sRNA candidates were down-regulated than in MW2. Compared to blood, less pronounced Log2 fold-changes were observed for both strains. Only two of the putative sRNAs identified in MSSA476 were differentially regulated in blood and CO_2. For MW2, seven putative sRNAs were differentially regulated in both conditions. In all, the appearance of differentially expressed probes in IGRs, may imply the relevance of non-translated RNAs. Moreover, the differences between the strains and the conditions suggest that different regulation strategies can be used. The highly differentially expressed sRNA candidates and the candidates identified in both strains are of special interest for further characterization of their function in *in vitro* and *in vivo* studies.

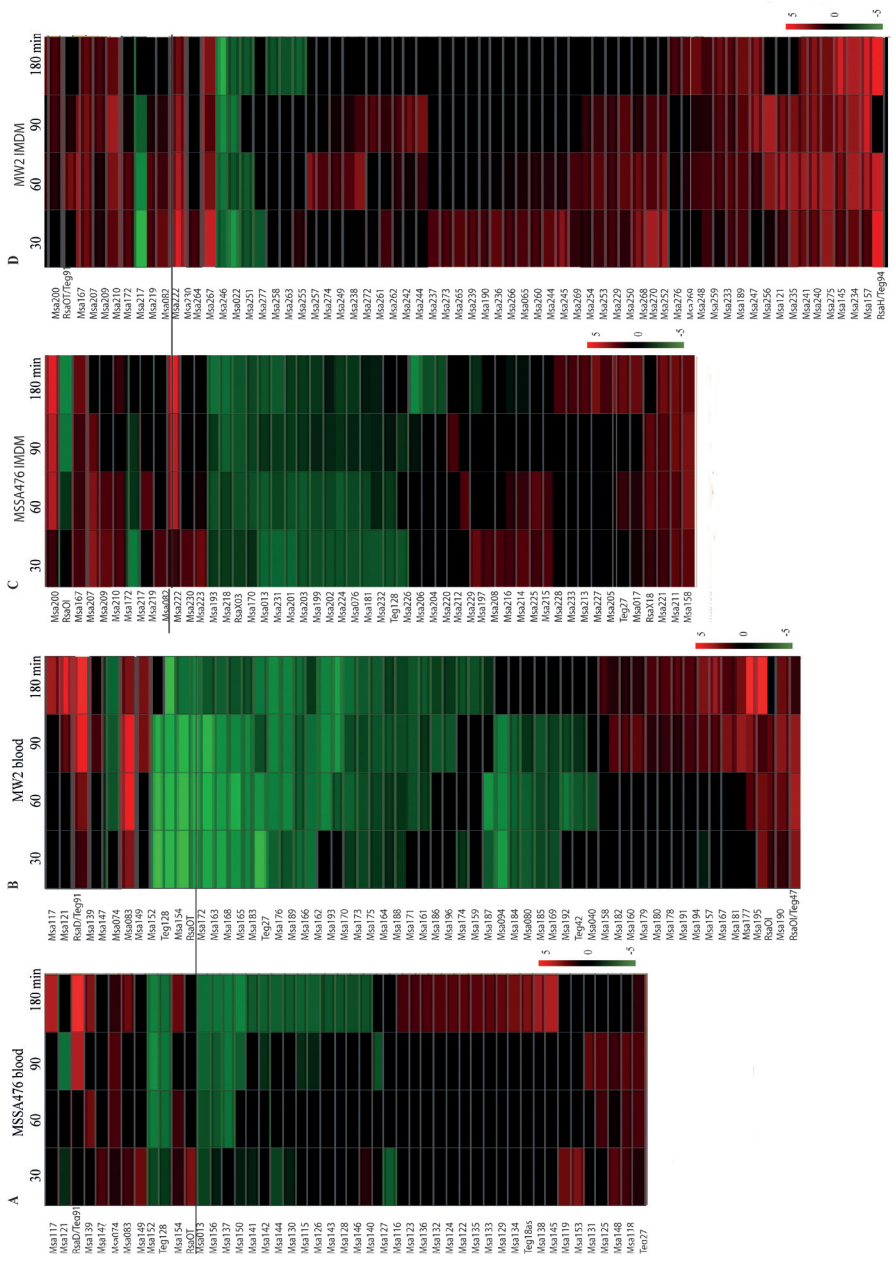

Figure 5. Heatmap of sRNA candidates in intergenic regions.
Differentially expressed sRNA candidates in intergenic regions over time on the y-axis upon exposure to human blood for (A) MSSA476 and (B) MW2 or when exposed to IMDM with 5% CO_2 (C) MSSA476 and (D) MW2, indicated by green (down-regulated) or red (up-regulated). Results are presented as log2 fold-changes compared to time point zero (t=0 min).

Discussion

S. aureus is the leading cause of various hospital-acquired infections and antibiotic resistance is a serious problem (1). In addition to hospital-acquired infections it seems that *S. aureus* infections in healthy individuals with no apparent risk factor are also increasing (14). Although the molecular epidemiology of *S. aureus* is extensively studied, no apparent link between genetic background, gene content and the different infections it can cause has yet been identified. In fact, genetically similar strains can cause different diseases (5).

For this reason, we have compared the adaptive gene expression upon interaction with human blood and IMDM both at 5% CO_2 of two genetically similar *S. aureus* strains, MSSA476 and MW2, to generate a more comprehensive view. Any differences in gene expression between the strains might give an indication for the ability to cause various types of infection.

The experiments presented in this pilot study mimic the infectious environment directly after entering the bloodstream. The most remarkable observation was the high up-regulation of *ssl1-10* and *ssl12-14* after exposure to human blood compared to the lack of regulation after exposure to IMDM at 5% CO_2. Up-regulation of *ssl* transcripts was previously shown in USA300 after exposure to human blood (7) and upon contact with azurophilic granule proteins (27). Influence of human serum (7) and H_2O_2 (27) on the gene expression of *ssl* transcripts on the other hand was only mild. Also exposure to purified neutrophils compared to human blood showed a less pronounced up-regulation in multiple MRSA strains (MW2, LAC (USA300), MnCop, MRSA252 and COL) (26). Only *ssl12-14* showed up-regulation in MW2, LAC and MnCop. These results indicate that *ssl* transcripts are up-regulated once *S. aureus* is in contact with human blood. A probable explanation for the fact that exposure to purified neutrophils resulted only in minor up-regulation compared to blood might be that bacterial uptake by neutrophils is required. This is dependent on multiple factors present in blood, like antibodies and complement factors, that were not present in the assay with purified neutrophils in combination with serum. The production of SSLs by opsonized bacteria may in turn be a trigger to evade destruction by PMNs. However, no intracellular functions of SSLs have been identified yet.

Another relevant marker for *S. aureus* during infections is most probably iron limitation (34). Iron-regulated surface determinant A (IsdA) is able to bind transferrin and hemin and is most likely important in the iron uptake across the membrane (34,35). IsdA also functions as an adhesin and is able to bind fibrinogen and fibronectin (34). An isogenic *isdAB*-negative strain of MW2 showed increased susceptibility to human neutrophils and thereby demonstrated the importance of iron-regulated surface determinant proteins in evading the innate immune defense system (27). Upon exposure to blood, genes involved in iron and heme uptake and/or binding are up-regulated in MW2. Gene profiling of USA300 in human blood previously showed highly up-regulated genes involved in iron and heme uptake and/or binding (7). For MSSA476, high expression levels were already detected after growth in IMDM (9). This might imply a different trigger for up-regulation of genes involved in iron and heme uptake and/or binding in *S. aureus* strains. Some strains might be activated for up-regulation by the presence of bound iron, like heme, while other strains might up-regulate the genes already when low levels of free iron are present, as is the case in the synthetic medium IMDM. Another condition triggering strong up-regulation of iron associated genes in MW2 was H_2O_2 (27), an important neutrophil microbicide created in activated PMNs. However, exposure to azurophilic granule proteins, released by activated PMN, stimulated down-regulation of these genes in MW2 (27). This indicates that different molecules present in blood might be able to activate genes involved in iron and heme uptake and/or binding and highlights the ability of *S. aureus* to quickly adapt gene expressions to be able to survive harsh environments.

Different gene expression patterns between the strains were characterized by a more pronounced up-regulation of genes encoding immune evasion proteins, like *ssls* in MSSA476, whereas MW2 showed higher up-regulation of genes encoding proteases and toxins, like *hlgABC*, *lukDE* and *splABCD*. It has been described previously for MW2 that *hlgABC* and *lukDE* were up-regulated after phagocytosis (26,27). Moreover, a deletion mutant of gamma-hemolysin subunits (*hlgABC*) was shown to reduce neutrophil lysis (7). For the two strains described here, the higher up-regulation of *ssl* transcripts in MSSA476 as well as the higher up-regulation of *hlgABC*, *splABCD* and *lukDE* in MW2 might indicate a

difference in survival strategy after phagocytosis. A hypothesis could be that MW2 is better able to lyse the neutrophils and thereby prevent killing, whereas MSSA476 could be able to evade the antimicrobial compounds produced by the phagosomes and thus able to survive within the cell.

Regulation of virulence genes occurs mostly via global regulators, two-component gene regulatory systems and the regulatory sRNAs. An important sRNA in *S. aureus*, RNAIII, coordinately represses the expression of virulence genes, but also at least one transcription factor, Rot, is repressed by RNAIII (22,36). Recently, Rot has been reported to positively regulate genes encoding SSLs (17). The simultaneous down-regulation of RNAIII and up-regulation of *rot* as observed after exposure to blood in MSSA476 might be a trigger for up-regulation of *ssl* transcripts. Up-regulation of *rot* was shown, but no down-regulation of RNAIII was observed after exposure to IMDM with 5% CO_2. As a consequence, the *ssl* transcripts might not be regulated which was also observed for both strains.

Large differences in gene expressions were observed between the strains, despite the high genetic similarity. Approximately 600 single nucleotide polymorphisms (SNPs) have been identified in MW2 compared to MSSA476 in IGRs and coding regions, mostly nonsynonymous (30). The location of the SNPs was not up- or downstream of the genes or within the genes that showed large variation in expression levels. The presence of SNPs is probably not the reason for the observed differences between the strains. Since there is no delay in gene expression between the strains and SNPs likely do not explain the dissimilarities, it seems that a different strategy of altering gene expression is used to survive the same environments by two genetically similar strains.

In summary, we used a microarray based approach to study the differences in gene regulation between two genetically similar *S. aureus* strains upon exposure to human blood and IMDM both at 5% CO_2. Major differences in gene expression were observed between the conditions but also between the strains. Gene regulation in blood compared to IMDM both at 5% CO_2 showed the major difference in regulations of genes encoding *ssls1*-10 and *ssl12-14*. The main differences between the strains consisted of regulation of genes encoding proteases and toxins in MW2, whereas in MSSA476 genes encoding immune evasion proteins were more pronounced regulated. In all, this suggests a major role for SSLs upon contact with human blood as well

as different survival strategies for the two strains upon exposure to infection related conditions.

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References

1. Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N.Engl.J.Med.*, **339**, 520-532.
2. Sibbald, M.J.J.B., Ziebandt, A.K., Engelmann, S., Hecker, M., de, J.A., Harmsen, H.J.M., Raangs, G.C., Stokroos, I., Arends, J.P., Dubois, J.Y.F. *et al.* (2006) Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol.Biol.Rev.*, **70**, 755-788.
3. Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T. *et al.* (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, **359**, 1819-1827.
4. Lindsay, J.A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler, R.A., Husain, S.E., Butcher, P.D. and Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J.Bacteriol.*, **188**, 669-676.
5. Gillet, Y., Issartel, B., Vanhems, P., Fournet, J.C., Lina, G., Bes, M., Vandenesch, F., Piemont, Y., Brousse, N., Floret, D. *et al.* (2002) Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet*, **359**, 753-759.
6. Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., Renzoni, A., Lucchini, S., Lew, D.P., Vaudaux, P. *et al.* (2007) A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC.Genomics*, **8**, 171-185.
7. Malachowa, N., Whitney, A.R., Kobayashi, S.D., Sturdevant, D.E., Kennedy, A.D., Braughton, K.R., Shabb, D.W., Diep, B.A., Chambers, H.F., Otto, M. *et al.* (2011) Global changes in *Staphylococcus aureus* gene expression in human blood. *PLoS One*, **6**, e18617.
8. Ray, B., Ballal, A. and Manna, A.C. (2009) Transcriptional variation of regulatory and virulence genes due to different media in *Staphylococcus aureus*. *Microb Pathog*, **47**, 94-100.
9. ten Broeke-Smits, N.J.P., Pronk, T.E., Jongerius, I., Bruning, O., Wittink, F.R., Breit, T.M., van Strijp, J.A.G., Fluit, A.C. and Boel, C.H.E. (2010) Operon structure of *Staphylococcus aureus*. *Nucleic Acids Res*, **38**, 3263-3274.
10. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.

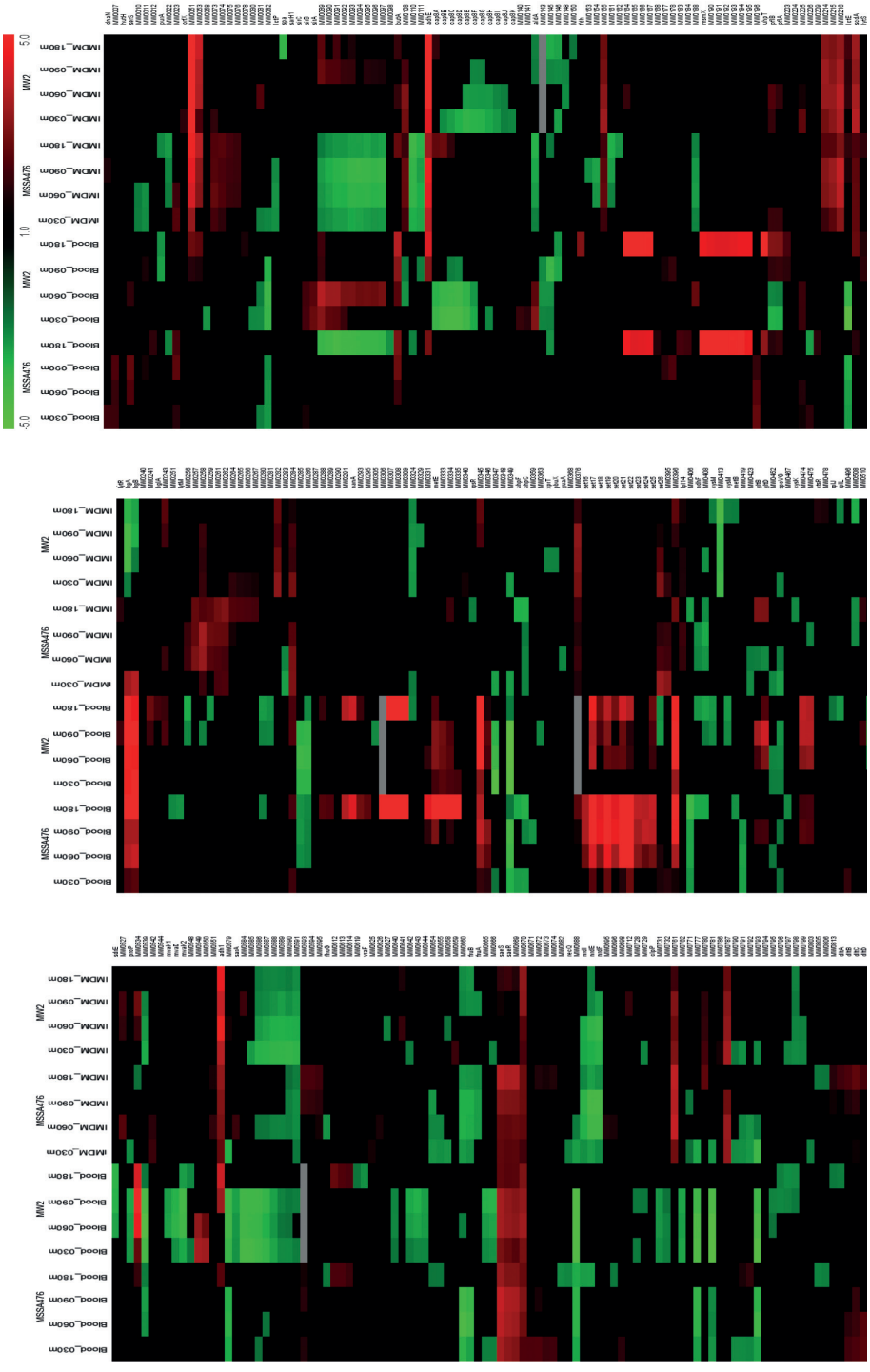
11. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
12. Chan, P.F. and Foster, S.J. (1998) The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. *Microbiology*, **144 (Pt 9)**, 2469-2479.
13. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol.Med.Microbiol.*, **40**, 1-9.
14. Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E., McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J. and Kreiswirth, B.N. (2003) Global regulation of *Staphylococcus aureus* genes by Rot. *J.Bacteriol.*, **185**, 610-619.
15. Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J. and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *Rna*, **6**, 668-679.
16. Benito, Y., Lina, G., Greenland, T., Etienne, J. and Vandenesch, F. (1998) trans-complementation of a *Staphylococcus aureus agr* mutant by *Staphylococcus lugdunensis agr* RNAIII. *J Bacteriol*, **180**, 5780-5783.
17. Benson, M.A., Lilo, S., Wasserman, G.A., Thoendel, M., Smith, A., Horswill, A.R., Fraser, J., Novick, R.P., Shopsin, B. and Torres, V.J. (2011) *Staphylococcus aureus* regulates the expression and production of the staphylococcal superantigen-like secreted proteins in a Rot-dependent manner. *Mol Microbiol*, **81**, 659-675.
18. Manna, A. and Cheung, A.L. (2001) Characterization of *sarR*, a modulator of sar expression in *Staphylococcus aureus*. *Infect Immun*, **69**, 885-896.
19. Giraudo, A.T., Cheung, A.L. and Nagel, R. (1997) The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level. *Arch.Microbiol.*, **168**, 53.
20. Novick, R.P. and Jiang, D. (2003) The staphylococcal *saeRS* system coordinates environmental signals with *agr* quorum sensing. *Microbiology*, **149**, 2709.
21. Papenfort, K. and Vogel, J. (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe*, **8**, 116-127.
22. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**, 1353-1366.
23. Chabelskaya, S., Gaillot, O. and Felden, B. (2010) A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog*, **6**, e1000927.
24. Wiltshire, M.D. and Foster, S.J. (2001) Identification and analysis of *Staphylococcus aureus* components expressed by a model system of growth in serum. *Infect.Immun.*, **69**, 5198.
25. Yarwood, J.M., McCormick, J.K., Paustian, M.L., Kapur, V. and Schlievert, P.M. (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. *J.Bacteriol.*, **184**, 1095-1101.
26. Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Said-Salim, B., Porcella, S.F., Long, R.D., Dorward, D.W., Gardner, D.J., Kreiswirth, B.N. *et al.* (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J.Immunol.*, **175**, 3907-3919.
27. Palazzolo-Ballance, A.M., Reniere, M.L., Braughton, K.R., Sturdevant, D.E., Otto, M., Kreiswirth, B.N., Skaar, E.P. and DeLeo, F.R. (2008) Neutrophil microbicides

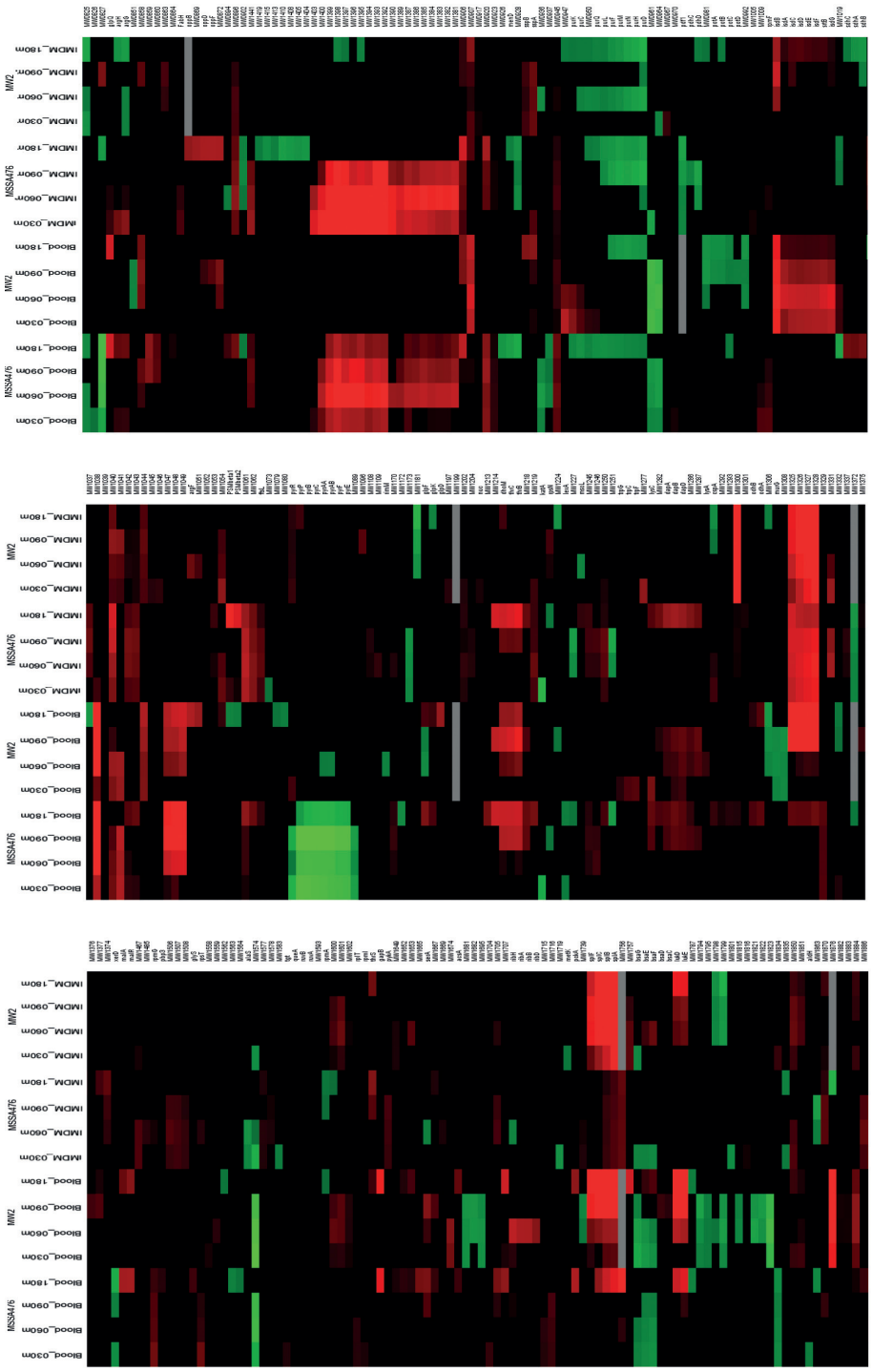
induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J.Immunol.*, **180**, 500-509.
28. Loughman, J.A., Fritz, S.A., Storch, G.A. and Hunstad, D.A. (2009) Virulence gene expression in human community-acquired *Staphylococcus aureus* infection. *J.Infect. Dis.*, **199**, 294-301.
29. Joost, I., Blass, D., Burian, M., Goerke, C., Wolz, C., von Muller, L., Becker, K., Preissner, K., Herrmann, M. and Bischoff, M. (2009) Transcription analysis of the extracellular adherence protein from *Staphylococcus aureus* in authentic human infection and *in vitro*. *J Infect Dis*, **199**, 1471-1478.
30. Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R. *et al.* (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc.Natl.Acad.Sci.U.S.A*, **101**, 9786-9791.
31. Hupe, P., Stransky, N., Thiery, J.P., Radvanyi, F. and Barillot, E. (2004) Analysis of array CGH data: from signal ratio to gain and loss of DNA regions. *Bioinformatics*, **20**, 3413-3422.
32. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J. and Francois, P. (2010) Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One*, **5**, e10725.
33. Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C. *et al.* (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res*, **37**, 7239-7257.
34. Clarke, S.R., Wiltshire, M.D. and Foster, S.J. (2004) IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Mol.Microbiol.*, **51**, 1509-1519.
35. Mazmanian, S.K., Skaar, E.P., Gaspar, A.H., Humayun, M., Gornicki, P., Jelenska, J., Joachmiak, A., Missiakas, D.M. and Schneewind, O. (2003) Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science*, **299**, 906-909.
36. Novick, R.P. and Geisinger, E. (2008) Quorum sensing in staphylococci. *Annu Rev Genet*, **42**, 541-564.

Supplementary figure and table

Supplementary figure S1. Heatmap of complete genomes.

Gene expression profiles of all genes in MSSA476 and MW2 in blood and IMDM. Genes are listed according to occurrence in MW2. Significantly regulated genes are indicated by green (down-regulated) or red (up-regulated). Results are presented as log2 fold-changes compared to time point t=0.


5.0
1.0
-5.0
MW2
MSSA476
IMDM_180m
IMDM_090m
IMDM_060m
IMDM_030m
Blood_180m
Blood_090m
Blood_060m
Blood_030m


MW2
MSSA476
IMDM_180m
IMDM_090m
IMDM_060m
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Blood_180m
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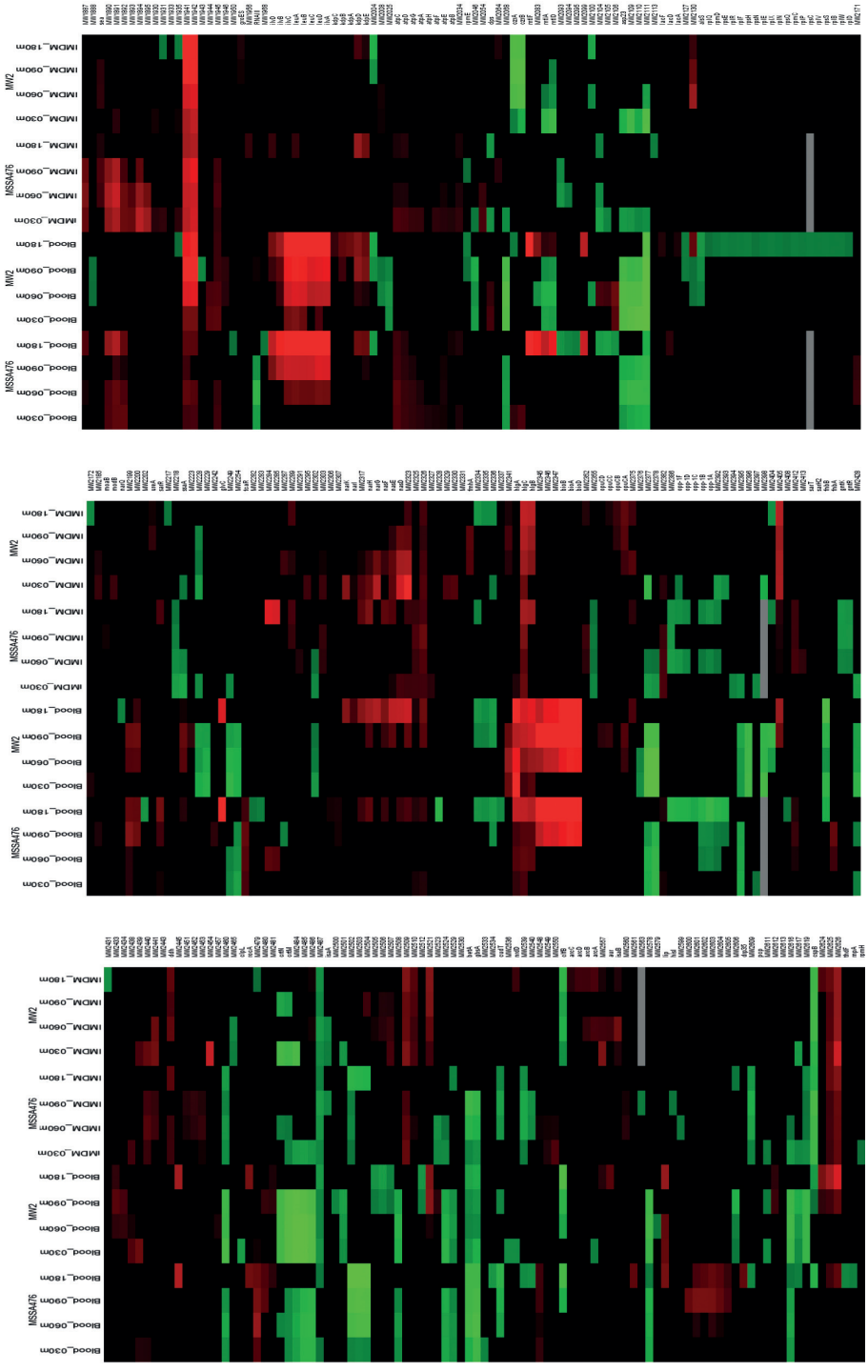
MW2
MSSA476
MW2
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IMDM_180m
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IMDM_060m
IMDM_030m
Blood_180m
Blood_090m
Blood_060m
Blood_030m
Blood_180m
Blood_090m
Blood_060m
Blood_030m

Supplementary table S1 Differentially regulated sRNA candidates

Name	start_MSSA476	end_MSSA476	start_MW2	end_MW2	Strand
Msa115	44299	45826	-	49373	+
Msa116	87705	88105	88999	89399	-
Msa117	199051	199370	200352	200671	+
Msa118	252375	252455	253675	253755	+
Msa119	293725	294445	295080	295800	-
Msa120	378538	378618	379893	379973	-
Msa121	405616	405838	406971	407193	-
Msa122	428687	428752	430026	430091	-
Msa123	429503	429663	430842	431002	-
Msa124	434417	434497	435756	435836	-
Msa125	436574	436734	437913	438073	+
Msa126	485819	485893	487157	487231	+
Msa127	759933	760013	761505	761585	+
Msa128	803046	803285	804618	804857	+
Msa129	845731	845815	861738	861822	-
Msa130	886988	887308	903019	903339	-
Msa131	932645	933205	948676	949236	-
Msa132	992502	994241	-	1562254	-
Msa133	1000980	1007781	1555872	1549079	-
Msa134	1008181	1012901	1548679	1543959	-
Msa135	1013302	1016022	1543558	1540838	-
Msa136	1016902	1024421	1539958	1532439	-
Msa137	1163938	1164295	1135152	1135509	+
Msa138	1170165	1170284	1141379	1141498	-
Msa139	1171299	1171379	1142626	1142706	-
Msa140	1180286	1180606	1151613	1151933	-
Msa141	1371207	1371367	1342711	1342871	+
Msa142	1379523	1379754	1350969	1351200	+
Msa143	1384630	1384790	1356076	1356236	+
Msa144	1571272	1571352	1591514	1591594	+
Msa145	1802507	1802587	1822854	1822934	-
Msa146	1860245	1861525	1880910	1882190	-
Msa147	1903170	1903914	1923836	1924580	-
Msa148	1926474	1926602	1947140	1947268	+

Msa149	2121755	2121995	2142630	2142870	-
Msa150	2122315	2122395	2143190	2143270	-
Msa151	2405006	2405326	2425901	2426221	-
Msa152	2235134	2235253	2256029	2256148	-
Msa153	2471530	2471729	2492362	2492561	+
Msa154	2471769	2472013	2492601	2492845	+
Msa155	2686369	2686529	2707023	2707183	-
Msa156	62639	63679	63932	64972	+
Msa157	86345	88105	87639	89399	-
Msa158	147522	147606	148822	148906	-
Msa159	289046	289165	290401	290520	+
Msa160	365478	365958	366833	367313	+
Msa161	452191	452591	453530	453930	-
Msa162	452751	453869	454090	455208	-
Msa163	489579	489817	490917	491155	+
Msa164	510604	510846	511942	512184	+
Msa165	627244	627404	628553	628713	+
Msa166	651819	651899	653127	653207	+
Msa167	825079	825159	826596	826676	+
Msa168	843852	843932	859859	859939	+
Msa169	1034715	1034845	1005992	1006122	-
Msa170	1058739	1058899	1029998	1030158	+
Msa171	1087003	1087083	1058217	1058297	+
Msa172	1164098	1164178	1135312	1135392	+
Msa173	1359165	1359243	1330669	1330747	-
Msa174	1383433	1384790	1354879	1356236	+
Msa175	1680826	1682107	1701174	1702455	-
Msa176	1896778	1897258	1917444	1917924	-
Msa177	1900469	1900947	1921135	1921613	-
Msa178	2024907	2025227	2045661	2045981	-
Msa179	2026427	2026507	2047181	2047261	-
Msa180	2068982	2069072	2089740	2089830	-
Msa181	2097872	2097952	2118747	2118827	-
Msa182	2201497	2201657	2222392	2222552	-
Msa183	2352435	2352515	2373330	2373410	-
Msa184	2379907	2380690	2400802	2401585	-

Msa185	2405406	2405646	2426301	2426541	-
Msa186	2406487	2406567	2427382	2427462	+
Msa187	2428085	2428405	2448980	2449300	-
Msa188	2605227	2605268	2625869	2625910	+
Msa189	2612226	2613824	2632868	2634466	+
Msa190	2620782	2621022	2641424	2641664	+
Msa191	2743720	2743880	2764380	2764540	-
Msa192	2746893	2747293	2767553	2767953	+
Msa193	2785490	2787479	2806150	2808139	-
Msa194	-	-	848571	849451	-
Msa195	-	-	849531	850331	-
Msa196	-	-	850291	850721	+
Msa197	30847	30925	30847	30925	+
Msa198	44285	45826	-	49373	+
Msa199	49647	49807	-	-	+
Msa200	63599	63679	64892	64972	+
Msa201	71188	71988	72481	73281	+
Msa202	86665	88105	87959	89399	-
Msa203	100963	101165	102257	102459	-
Msa204	485616	485696	486954	487034	-
Msa205	735865	736505	737437	738077	+
Msa206	800246	800368	801818	801940	+
Msa207	822315	823259	823887	824831	+
Msa208	893871	894747	909902	910778	-
Msa209	924504	924705	940535	940736	+
Msa210	951825	952545	967856	968576	-
Msa211	1269557	1269717	1241062	1241222	+
Msa212	1297156	1297316	1268661	1268821	+
Msa213	1353885	1354445	1325389	1325949	-
Msa214	1360325	1360450	1331829	1331954	-
Msa215	1364646	1364806	1336150	1336310	+
Msa216	1544255	1544815	1518607	1519167	-
Msa217	1716440	1716520	1736788	1736868	+
Msa218	1853803	1853883	1874468	1874548	-
Msa219	2022624	2022876	2043377	2043629	+
Msa220	2089477	2089597	2110352	2110472	-

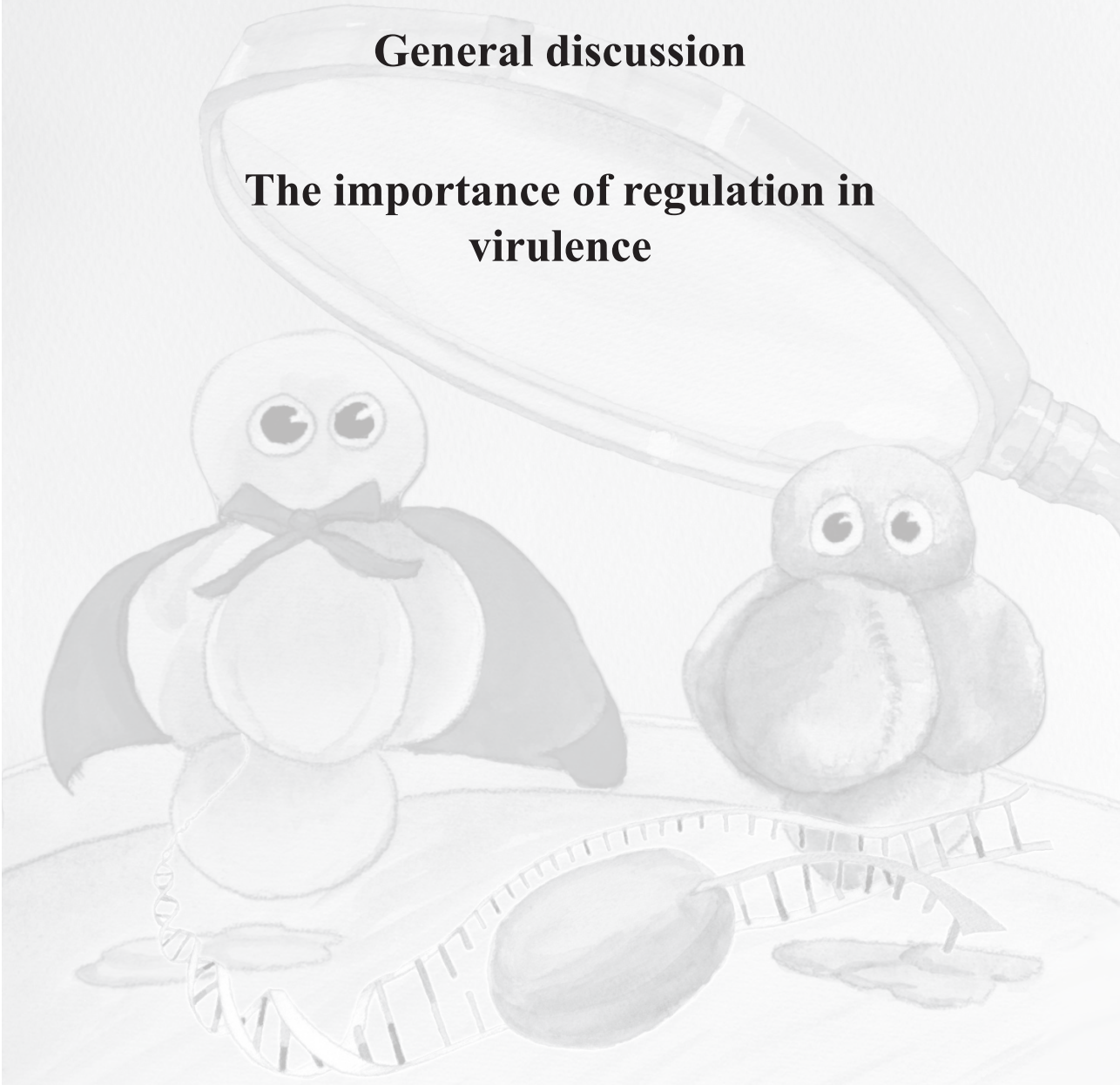
Msa221	2089637	2089877	2110512	2110752	-
Msa222	2141430	2141689	2162304	2162563	-
Msa223	2305950	2306068	2326845	2326963	+
Msa224	2342429	2342749	2363324	2363644	-
Msa225	2365308	2365550	2386203	2386445	+
Msa226	2410049	2410688	2430944	2431583	-
Msa227	2410889	2411289	2431784	2432184	+
Msa228	2411369	2411770	2432264	2432665	+
Msa229	2426525	2426925	2447420	2447820	+
Msa230	2465737	2465817	2486569	2486649	-
Msa231	2484142	2484222	2504974	2505054	-
Msa232	2508870	2508950	2529702	2529782	+
Msa233	2538113	2538260	2558821	2558968	+
Msa234	63199	63519	64492	64812	+
Msa235	144008	144173	145302	145467	-
Msa236	163465	164425	164765	165725	-
Msa237	319396	319556	320751	320911	-
Msa238	328638	328878	329993	330233	-
Msa239	354354	354834	355709	356189	+
Msa240	378738	379058	380093	380413	+
Msa241	405998	406159	407353	407514	-
Msa242	421176	421336	422531	422691	-
Msa243	547784	548264	549153	549633	-
Msa244	554463	554704	555832	556073	+
Msa245	618512	618912	619821	620221	-
Msa246	626483	626684	627792	627993	+
Msa247	762333	762413	763905	763985	+
Msa248	823539	823739	825111	825311	+
Msa249	824619	824719	826191	826291	+
Msa250	924504	924705	940535	940736	+
Msa251	941347	941670	957378	957701	+
Msa252	951825	952545	967856	968576	-
Msa253	1034744	1034845	1006021	1006122	-
Msa254	1064834	1064923	1036111	1036200	+
Msa255	1115821	1116221	1087035	1087435	+
Msa256	1225240	1225560	1196567	1196887	-

Msa257	1319058	1319218	1290563	1290723	-
Msa258	1393710	1393751	1365156	1365197	+
Msa259	1549900	1550060	1524252	1524412	+
Msa260	1903884	1903914	1924550	1924580	-
Msa261	1908769	1908890	1929435	1929556	+
Msa262	1922636	1922716	1943302	1943382	-
Msa263	2022624	2022876	2043377	2043629	+
Msa264	2025947	2026267	2046701	2047021	-
Msa265	2029099	2029179	2049853	2049933	+
Msa266	2077800	2078600	2098558	2099358	+
Msa267	2121915	2121995	2142790	2142870	-
Msa268	2253326	2253966	2274221	2274861	-
Msa269	2254046	2254606	2274941	2275501	-
Msa270	2342469	2342749	2363364	2363644	-
Msa271	2365228	2368028	2386123	2388923	+
Msa272	2368108	2368188	2389003	2389083	+
Msa273	2368268	2368588	2389163	2389483	+
Msa274	2368748	2369470	2389643	2390365	+
Msa275	2515013	2515093	2535845	2535925	+
Msa276	2785731	2787479	2806391	2808139	-
Msa277	2790432	2790592	2811092	2811252	-
Msa278	-	-	849611	850411	-
Msa279	-	-	58004	58164	+
Msa280	-	-	850641	850721	+

Chapter 6

General discussion

The importance of regulation in virulence



A successful microorganism is characterized by the ability to quickly adapt to different (host) environments, which requires a tight regulation of genes. Regulation of bacterial transcription has been an interesting topic over the last decades. The classic regulation mechanism consists of many protein based regulatory elements. The RNA polymerase (RNAP) holoenzyme complex accomplishes bacterial transcription by binding to various σ-factors (1). These in turn recognize different promoters and as such control particular clusters of genes. An additional regulatory function is provided by a multitude of transcription factors that can repress or activate transcription. The presence of the Shine-Dalgarno sequence is required for initiation of protein translation (2). Transcription is terminated via Rho-dependent or Rho-independent termination (3).

This basic regulatory mechanism is supplemented with additional global regulators and regulatory elements which have long been considered as 'peculiarities': small RNAs (sRNAs), riboswitches and operons with internal promoters that affect transcription and translation; protein translation is affected by leaderless mRNA and untranslated regions (UTRs); and extra transcription terminators that regulate termination (4). Recent transcriptome studies in *Staphylococcus aureus* have suggested the presence of these 'peculiarities' as well (5,6).

Regulation in *S. aureus*

S. aureus causes a wide variety of diseases in humans ranging from impetigo to invasive infections, like endocarditis and osteomyelitis (7). *S. aureus* is also a harmless commensal, frequent colonizing multiple human body sites, like the nose, perineum and skin (8). The nose has been suggested as the main seeding place for *S. aureus* from where it is able to establish infections (9) and therefore knowledge of the niche is essential. We determined the exact niche in the nose and hypothesize a potential difference in persistent and intermittent carriers (**Chapter 2**). During different stages of infection, from colonization via immune evasion to bacterial spread, *S. aureus* needs to coordinately express a diverse array of virulence factors.

Expression of these virulence factors is dependent on energy availability, cell density and environmental signals and tight regulation is crucial to survive in changing and harsh environments (10).

A complex network of global and accessory regulators has been identified to control virulence. The best described regulators are the staphylococcal accessory regulator (*sarA*) and the accessory gene regulator (*agr*). SarA enhances post-exponential expression of α, β and δ toxins both directly and indirectly via up-regulation of *agr* (11). The *agr* locus acts as molecular switch and up-regulates toxin genes while down-regulating synthesis of cell wall proteins during post-exponential phase in culture medium (10). Furthermore, it is an autoinducing system. The *agr* locus is a quorum-sensing system that consists of two transcription units (12). The P2 transcript encodes AgrC (the signal receptor) and AgrA (the response regulator), together forming a two-component signaling (TCS) module. The AgrB and AgrD proteins produce and secrete the activating ligand for AgrC, thiolactone-containing autoinducing peptide (AIP). AgrC in turn activates AgrA, which up-regulates its own P2 promoter as well as the RNAIII promoter, P3. RNAIII is a 514 nt regulatory RNA that directly or indirectly controls a variety of virulence genes. Indirect regulation of virulence genes occurs via post-transcriptional repression of a transcription factor, repressor of toxins (Rot). Rot is a *sarA* homolog that was initially shown to down-regulate toxin synthesis and up-regulate genes encoding cell wall proteins (13). Recently, it was described that *rot* also regulates the expression of genes encoding immunomodulatory proteins known as staphylococcal superantigen-like proteins (SSLs) (14).

In addition to the protein based regulation of virulence genes, RNA regulators, the so-called small RNAs (sRNAs), have been identified to play important roles in virulence in *S. aureus* (15-18). RNAIII is the best characterized and of major importance in virulence regulation (15,17,18). RNAIII is a dual function sRNA (19) that has multiple regulatory functions, but also encodes delta toxin (*hld*). Its RNA structure is characterized by 14 stem-loops and two long helices at the 5'-and 3'-end (20). The 5'-end is involved in activation of the expression of hemolysin A *(hla*) (21), whereas the 3'-end is necessary for repression of the expression of staphylococcal protein A (*spa*), extracellular fibrinogen binding protein (*efb*) and coagulase (*coa*) (15,18). In addition, the virulence regulator *rot* is repressed by RNAIII (15). Another virulence gene regulating sRNA is SprD which was identified to regulate *S. aureus* binder of IgG (16).

The regulatory role of sRNAs

Bacterial sRNAs have been proposed as powerful regulators of gene expression. Transcriptome studies during the last years, have revealed many potential virulence regulating sRNA candidates in various bacteria (22). Two classes of sRNAs, *trans*-acting and *cis*-acting, are characterized by different binding and regulation mechanisms. *Cis*-acting sRNAs are encoded opposite of the target mRNA and have an extensive base-pairing interaction (23), while *trans*-acting sRNAs are encoded elsewhere on the genome and are able to regulate multiple mRNA targets in differing degrees and outcomes (19). The regulatory role of sRNAs includes translation inhibition or activation, translation elongation and mRNA stability, but also indirect transcription regulation of adjacent genes by changing DNA supercoiling can be induced by sRNAs (24). Even though sRNAs seem to play an important role in regulation, the functional characterization of sRNAs only progresses slowly, especially in Gram-positive bacteria. Most techniques, like *in silico* prediction programs and overexpression systems, are based on Gram-negative bacteria. In addition, *in vivo* interaction of sRNAs from Gram-negative bacteria can be tested in *Escherichia coli* with the use of a high copy plasmid (encoding the sRNA) and a low copy plasmid (encoding the target mRNA coupled to green fluorescent protein (GFP)) (25). Such system is not available for Gram-positive bacteria. As a consequence, sRNA research in Gram-positive bacteria is progressing more slowly. Nonetheless, virulence regulating sRNAs have been recognized in *Listeria monocytogenes* (26) and *S. aureus* (12,16,27).

Functional characterization was established via different techniques. For *S. aureus*, SprD was characterized in mouse models with a knock-out strain where the sRNA was replaced with an erythromycin cassette (16). However, introducing antibiotic cassettes has major drawbacks, since regulation could be affected by the presence of the cassette and not the result of the knock-out. This was recently identified for the introduction of a chloramphenicol cassette in *Enterococcus feacium* (unpublished results and personal communication Dr. J. Top). RNAIII has been characterized using a strain lacking RNAIII (27). An RNAIII knock-out has not been constructed yet, probably due to the major importance of RNAIII in the *agr* system (12). Overexpression constructs of sRNAs in S. aureus have not been described before. In *L. monocytogenes*, sRNAs

were identified in a wide range of infection related conditions and thus far, no sRNA knock-out strains have been tested (26).
In **Chapter 4**, we identified five *trans*-acting sRNA candidates, located on the chromosome, with the potential to regulate virulence genes based on *in silico* predictions and expression data from *S. aureus* growth curves described in **Chapter 3**. Additionally, we described functional tests of two sRNA knock-out strains in MSSA476. Comparison of the two independent knock-out strains to the wild-type MSSA476 showed trends of post-transcriptional regulation of the predicted targets, Efb and Hld. Though, *in vitro* analysis did not fully support our *in vivo* results and more research will be needed to understand the regulatory effect of these sRNAs. In this, the role of a potential chaperone protein, like Hfq, to establish sRNA-mRNA interactions has to be investigated. We also attempted the construction of sRNA overexpression strains, but large biological variations were obtained in levels of sRNA overexpression. The addition of chloramphenicol might have played a role in this. Also considering unintended consequences that have been suggested to appear in prokaryotes, like regulation of other proteins or mRNA (28), a more conclusive result will probably be found with the use of knock-out strains.
Quickly changing environments require fast regulation of genes and sRNAs are expected to be faster in their regulatory function compared to their protein counterparts (29,30). Therefore, we searched for sRNA candidates upon changing environments. Two infection related conditions, human blood and IMDM both maintained at 5% CO_2, and two genetically similar strains, MSSA476 and MW2, were used (**Chapter 5**). Around one hundred differentially expressed sRNA candidates were identified. Six sRNA candidates identified during growth in IMDM were also differentially regulated in blood and one in IMDM at 5% CO_2. In addition, eight putative sRNAs differentially expressed in either blood or IMDM at 5% CO_2, were identified in previous studies (5,31). This differential regulation in multiple environments suggests an important function for these sRNAs and further characterization might show the relevance. To date, two sRNAs in *S. aureus* have been identified and validated to regulate virulence genes (16,27). An additional two sRNAs have been proposed to be involved in virulence regulation and many candidates identified under different conditions with various technologies are of

interest to explore further.

The role of operons in regulation

The definition of an operon, functional genomic unit with multiple open reading frames under influence of one promoter, was linked to the regulation of genes essential in one (metabolic) pathway to ensure equal levels of expression (4). Transcriptome analysis over the past years however, showed different expression levels of genes organized in an operon. The presence of internal promoters and terminators has been shown in various bacteria. In *Bacillus subtilis* for example, many genes in polycistronic operons are transcribed from multiple promoters (4). In addition, read-through terminators exist as well, from where partial continuation of transcription can occur. This has also been shown in other bacteria, like *Helicobacter pylori* and *Mycoplasma pneumoniae*. Moreover, a more complex regulatory mechanism might exist for overlapping promoters in operons. These observations highlight the importance of gene regulation at an operon level, where multiple factors influence operon regulation which leads to higher regulatory flexibility and responsiveness.

For *S. aureus*, only *in silico* operon predictions were available at the start of this thesis (32,33), which were based on the length of intergenic regions (IGRs), conserved gene clusters and Rho-independent terminators. We wanted to extend the knowledge on gene regulation in *S. aureus* to better comprehend its pathogenicity. Therefore, experimental identification of the operon structure was an essential step. We have identified the operon structure of *S. aureus* using RNA isolated from five highly reproducible growth curves in standard medium (IMDM) (**Chapter 3**). Pearson correlations between adjacent probes in coding regions and IGRs were used to accurately compile the operon structure. Approximately 60% of the genes were shown to be regulated within operons. Remarkably, also genes separated by large IGRs were identified to be transcribed in one operon. We did not expect to detect virulence genes within an operon, since these are usually located on mobile genetic elements and exchanged regularly. Nevertheless, multiple virulence genes were identified and validated in various operons. Also the synergistic effect of two in an operon transcribed genes encoding innate immune evasion proteins, Efb and ScnB, was shown. We observed potential differential

regulation of multiple operons and additional experiments indeed showed differential regulation of the operon containing lantibiotic genes, while split operons were not predicted *in silico*.
Taken together, recent studies have shown different regulatory elements involved in the operon organization. Along with the identification of virulence genes organized in an operon, this shows the importance of experimental determined operon structures to study pathogenicity. To ensure the best possible regulation and organization of genes upon changing (infection related) environments, operons are probably differentially regulated. Of interest for *S. aureus* is to determine the operon structure under different conditions.

Gene regulation upon changing environments
Pathogens that are able to cause a wide variety of infections require fast regulation of genes upon changing and harsh environments for survival. However, the molecular basis to understand bacterial responses to host signals is still limited. Therefore, gene regulation was studied upon infection relevant conditions for various pathogens, like effects of human blood in Group A Streptococcus (34). This has provided crucial insight into bacterial strategies to evade host defenses and survive under harsh conditions.
S. aureus is able to live as a commensal and is simultaneously able to cause major infections. In addition, genetically similar *S. aureus* strains were identified to be able to cause different disease types (35). This implies a key role for gene regulation during infections. Also the knowledge that *S. aureus* gene expression during standard growth in different media is markedly different (6,36) supports the importance of regulation. Nonetheless, only limited studies have been conducted to determine gene expression upon changing environments. To date, most tudies on gene expression in *S. aureus* upon changing environments have been performed to study the differential expression of a limited set of virulence genes upon contact with human serum (37-39). Recent studies to identify global gene expressions in *S. aureus* upon contact with human blood (40), neutrophils (41) or neutrophil microbicides (42) indicated the importance of fast regulation of genes to be able to survive harsh conditions.
We have identified the global gene expression of two genetically similar

strains after exposure to two conditions, human blood and IMDM both at 5% CO_2 (**Chapter 5**). This indeed showed that genetically similar strains can regulate the gene expression in rather different ways upon exposure to the same condition, which indicates the ability of *S. aureus* strains to cause a wide variety of infections. A comparison between the conditions revealed the importance of staphylococcal superantigen-like genes (*ssls*) upon contact with human blood. Possibly SSLs are essential to survive within the cell after uptake by neutrophils. This hypothesis is supported by the regulation of *ssl* transcripts upon exposure to neutrophil microbicides (42).
Altogether, the regulation of *ssl* transcripts upon exposure to blood indicates a potential role in survival of the bacteria after phagocytosis. In addition, the differences in gene regulation of two genetically similar strains indicate the relevance of gaining more knowledge on global gene expression of different strains to be able to fully comprehend *S. aureus* pathogenicity.

Concluding remarks

Bacterial transcriptomics have shown the complexity of the transcriptome of pathogens. The abundance of regulatory RNAs, the differentially regulated operons, but also the presence of other regulatory elements, like long UTRs, riboswitches, RNA thermometers, leaderless mRNA and even changes in DNA supercoiling, suggest complex layers of regulation (4, **this thesis**). Extending the current knowledge on regulation will be essential to gain a more complete comprehension of pathogenicity. Future directions should include exact mapping of transcription start sites and terminators as well as to experimentally determine the complex RNA structure. With this, alternative transcripts of an operon and all different lengths of 3'- and 5'-UTRs will be identified. Experimentally mapped RNA structures will provide insight in the secondary and tertiary RNA structures important in sRNAs, riboswitches and RNA thermometers. The presence of multiple layers of regulation of bacterial transcription, indicate a crucial role for gene regulation during colonization, infection and survival. However, a complete comprehension of *S. aureus* pathogenicity is still a future prospect.

References

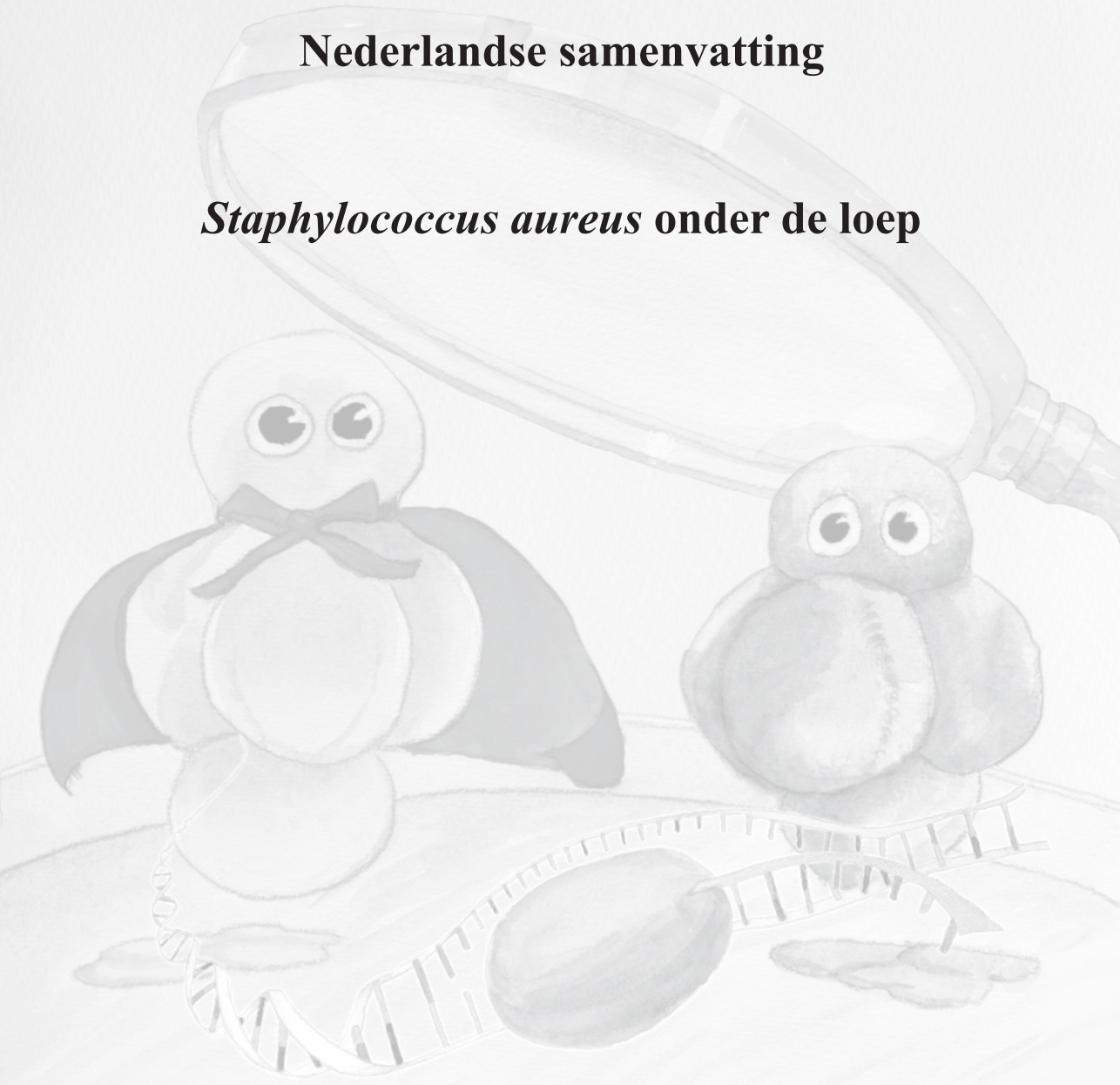
1. Wosten, M.M. (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev*, **22**, 127-150.
2. Shine, J. and Dalgarno, L. (1975) Determinant of cistron specificity in bacterial ribosomes. *Nature*, **254**, 34-38.
3. Santangelo, T.J. and Artsimovitch, I. (2011) Termination and antitermination: RNA polymerase runs a stop sign. *Nat Rev Microbiol*, **9**, 319-329.
4. Guell, M., Yus, E., Lluch-Senar, M. and Serrano, L. (2011) Bacterial transcriptomics: what is beyond the RNA horiz-ome? *Nat Rev Microbiol*, **9**, 658-669.
5. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J. and Francois, P. (2010) Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One*, **5**, e10725.
6. ten Broeke-Smits, N.J.P., Pronk, T.E., Jongerius, I., Bruning, O., Wittink, F.R., Breit, T.M., van Strijp, J.A.G., Fluit, A.C. and Boel, C.H.E. (2010) Operon structure of *Staphylococcus aureus*. *Nucleic Acids Res*, **38**, 3263-3274.
7. Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N.Engl.J.Med.*, **339**, 520-532.
8. Wertheim, H.F.L., Verveer, J., Boelens, H.A.M., van Belkum, A., Verbrugh, H.A. and Vos, M.C. (2005) Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. *Antimicrob Agents Chemother*, **49**, 1465-1467.
9. Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin.Microbiol.Rev.*, **10**, 505-520.
10. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
11. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol.Med.Microbiol.*, **40**, 1-9.
12. Novick, R.P. and Geisinger, E. (2008) Quorum sensing in staphylococci. *Annu Rev Genet*, **42**, 541-564.
13. Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E., McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J. and Kreiswirth, B.N. (2003) Global regulation of *Staphylococcus aureus* genes by Rot. *J.Bacteriol.*, **185**, 610-619.
14. Benson, M.A., Lilo, S., Wasserman, G.A., Thoendel, M., Smith, A., Horswill, A.R., Fraser, J., Novick, R.P., Shopsin, B. and Torres, V.J. (2011) *Staphylococcus aureus* regulates the expression and production of the staphylococcal superantigen-like secreted proteins in a Rot-dependent manner. *Mol Microbiol*, **81**, 659-675.
15. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**, 1353-1366.
16. Chabelskaya, S., Gaillot, O. and Felden, B. (2010) A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog*, **6**, e1000927.
17. Geisinger, E., Adhikari, R.P., Jin, R., Ross, H.F. and Novick, R.P. (2006) Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol.Microbiol.*, **61**, 1038-1048.
18. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus*

RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.
19. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615-628.
20. Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J. and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *Rna*, **6**, 668-679.
21. Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B. and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.*, **12**, 3967-3975.
22. Papenfort, K. and Vogel, J. (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe*, **8**, 116-127.
23. Brantl, S. (2007) Regulatory mechanisms employed by cis-encoded antisense RNAs. *Curr Opin Microbiol*, **10**, 102-109.
24. Blot, N., Mavathur, R., Geertz, M., Travers, A. and Muskhelishvili, G. (2006) Homeostatic regulation of supercoiling sensitivity coordinates transcription of the bacterial genome. *EMBO Rep*, **7**, 710-715.
25. Urban, J.H. and Vogel, J. (2007) Translational control and target recognition by Escherichia coli small RNAs in vivo. *Nucleic Acids Res.*, **35**, 1018.
26. Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K. *et al.* (2009) The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature*, **459**, 950-956.
27. Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T., Vandenesch, F. and Romby, P. (2010) Staphylococcus aureus RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. *PLoS Pathog*, **6**, e1000809.
28. Storz, G., Vogel, J. and Wassarman, K.M. (2011) Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Mol Cell*, **43**, 880-891.
29. Gripenland, J., Netterling, S., Loh, E., Tiensuu, T., Toledo-Arana, A. and Johansson, J. (2010) RNAs: regulators of bacterial virulence. *Nat Rev Microbiol*, **8**, 857-866.
30. Romby, P., Vandenesch, F. and Wagner, E.G. (2006) The role of RNAs in the regulation of virulence-gene expression. *Curr Opin Microbiol*, **9**, 229-236.
31. Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C. *et al.* (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res*, **37**, 7239-7257.
32. Brouwer, R.W.W., Kuipers, O.P. and Hijum, S.A.F.T. (2008) The relative value of operon predictions. *Brief.Bioinform.*, **9**, 367-375 Epub.
33. Wang, L., Trawick, J.D., Yamamoto, R. and Zamudio, C. (2004) Genome-wide operon prediction in *Staphylococcus aureus*. *Nucleic Acids Res.*, **32**, 3689-3702.
34. Graham, M.R., Virtaneva, K., Porcella, S.F., Barry, W.T., Gowen, B.B., Johnson, C.R., Wright, F.A. and Musser, J.M. (2005) Group A Streptococcus transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *Am J Pathol*, **166**, 455-465.
35. Lindsay, J.A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler, R.A., Husain, S.E., Butcher, P.D. and Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J.Bacteriol.*, **188**, 669-676.
36. Ray, B., Ballal, A. and Manna, A.C. (2009) Transcriptional variation of regulatory and virulence genes due to different media in *Staphylococcus aureus*. *Microb Pathog*,

47, 94-100.
37. Oogai, Y., Matsuo, M., Hashimoto, M., Kato, F., Sugai, M. and Komatsuzawa, H. (2011) Expression of Virulence Factors by Staphylococcus aureus Grown in Serum. *Appl Environ Microbiol*, **77**, 8097-8105.
38. Wiltshire, M.D. and Foster, S.J. (2001) Identification and analysis of *Staphylococcus aureus* components expressed by a model system of growth in serum. *Infect.Immun.*, **69**, 5198.
39. Yarwood, J.M., McCormick, J.K., Paustian, M.L., Kapur, V. and Schlievert, P.M. (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. *J.Bacteriol.*, **184**, 1095-1101.
40. Malachowa, N., Whitney, A.R., Kobayashi, S.D., Sturdevant, D.E., Kennedy, A.D., Braughton, K.R., Shabb, D.W., Diep, B.A., Chambers, H.F., Otto, M. *et al.* (2011) Global changes in *Staphylococcus aureus* gene expression in human blood. *PLoS One*, **6**, e18617.
41. Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Said-Salim, B., Porcella, S.F., Long, R.D., Dorward, D.W., Gardner, D.J., Kreiswirth, B.N. *et al.* (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J.Immunol.*, **175**, 3907-3919.

Nederlandse samenvatting

Staphylococcus aureus onder de loep



Een succesvol micro-organisme wordt gekarakteriseerd door zijn vermogen zich snel aan de veranderende omgeving aan te kunnen passen. Hiervoor is het van belang genen strikt te reguleren. Regulatie van bacteriële transcriptie is al tientallen jaren van interesse in de wetenschappelijke wereld. Het klassieke regulatie mechanisme bestaat uit een veelvoud van eiwit gebaseerde regulatie elementen. Transcriptie vindt plaats door RNA polymerase (RNAP) complex holoenzym dat bindt aan verschillende σ-factoren (1). Deze herkennen promoters en controleren de specifieke genclusters. Een extra regulerende functie wordt gegeven door meerdere transcriptiefactoren, welke transcriptie kunnen activeren of onderdrukken. Daarnaast is de aanwezigheid van een Shine-Dalgarno sequentie noodzakelijk voor eiwit translatie (2). Terminatie vindt plaats via Rho-afhankelijke of Rho-onafhankelijke terminatie (3).
Deze basiselementen voor regulatie worden aangevuld met algemene regulatoren en elementen die lang bekend stonden als "vreemdheden": kleine RNAs (sRNAs), riboswitches en operonen met interne promoter beïnvloeden trancriptie en translatie; eiwit translatie wordt beïnvloed door niet-getransleerde regionen (UTRs); en meerdere terminaters voor terminatie van transcriptie (4). Recent transcriptoom onderzoek in *Staphylococcus areus* suggereert ook de aanwezigheid van deze "vreemdheden" (5,6).

Regulatie in *S. aureus*

S. aureus veroorzaakt vele verschillende humane ziektes variërend van krentenbaard tot invasieve infecties, zoals endocarditis en osteomyelitis (7). Daarnaast is *S. aureus* ook een onschadelijke commensaal en koloniseert het meerdere lichaamsdelen, zoals de neus, perineum en de huid (8). De neus is waarschijnlijk de belangrijkste plaats van waaruit *S. aureus* zich kan verspreiden (9) en daarom is kennis van deze niche belangrijk. Wij hebben de exacte niche in de neus beschreven en hypothetiseren dat er een mogelijk verschil tussen persistente en niet-persistente dragers is (**Hoofdstuk 2**). Tijdens de verschillende fases van infecties, van kolonisatie via ontwijking van het immuunsysteem tot bacteriële spreiding, moet *S. aureus* een verscheidenheid aan virulentie factoren gecoördineerd tot expressie brengen.
Expressie van deze virulentie factoren is afhankelijk van aanwezigheid van energie, cel-dichtheid en omgevingssignalen. Strakke regulatie is essentieel in snel veranderende en gevaarlijke omgevingen (10). Een complex netwerk van algemene regulatoren die virulentie regelen is bekend. De best beschreven regulatoren zijn staphylococcal accessory regulator (*sarA*) en accessory gene regulator (*agr*). SarA verhoogt de expressie van α, β en δ toxins in de na-exponentiële groei fase, zowel direct als indirect via activatie van het *agr* systeem (11). Het *agr* systeem werkt als moleculaire switch en activeert de toxine genen terwijl de synthese celwand eiwitten geremd wordt tijdens de

na-exponentiële groei fase in medium (10). Bovendien functioneert *agr* als een autoinductie systeem.
De agr locus is een systeem dat reageert op celdichtheid en bestaat uit twee transcriptie eenheden (12). Het P2 transcript codeert voor AgrC (signaal receptor) en AgrA (regulator van reactie), samen vormen ze het twee-componenten signaal (TCS) module. De AgrB en AgrD eiwitten produceren en scheiden AIP uit dat uiteindelijk AgrC weer activeert. AgrC activeert AgrA vervolgens wat de eigen P2 promoter aanzet maar ook de RNAIII promoter, P3. RNAIII is een 514 nt regulatie RNA dat zowel direct als indirect een verscheidenheid aan virulentie genen reguleert. Indirecte regulatie van virulentie genen vindt plaats via post-transcriptionele remming van een transcriptie factor, repressor of toxins (Rot). Rot is een *sarA* homoloog dat in eerste instantie de toxine regulatie remde en de regulatie van celwand eiwitten activeerde (13). Recent is beschreven dat *rot* ook de expressie van genen coderend voor immuun ontwijkende eiwitten, bekend als staphylococcal superantigen-like proteins (SSLs) (14) reguleert.
Naast de eiwit gebaseerde regulatie van virulentie genen zijn er RNA regulatoren, de sRNAs, ontdekt die een belangrijke rol spelen in regulatie van virulentie in *S. aureus* (15-18). RNAIII is de bekendste en meest beschreven en is van groot belang in de regulatie van virulentie (15,17,18). RNAIII is een sRNA met duale functie (19) met meerdere regulerende functies, maar het codeert ook voor delta toxine (*hld*). De RNA structuur bestaat uit 14 haarpinnen en twee lange helices aan de 5'-en 3'-uiteinden (20). Het 5'-uiteinde is betrokken bij activatie van de expressie van hemolysine A (*hla*), terwijl het 3'-uiteinde essentieel is voor remming van de expressie van staphylococcal protein A (*spa*), extracellular fibrinogen binding protein (*efb*) en coagulase (*coa*) (15,18). Daarnaast wordt ook de virulentie regulator *rot* geremd door RNAIII (15). Een ander beschreven sRNA is SprD dat *S. aureus* binder of IgG reguleert (16).

De regulerende rol van sRNAs
Bacteriële sRNAs zijn krachtige regulatoren van gen expressie. Transcriptoomstudies in de afgelopen jaren hebben vele potentiële virulentie regulerende sRNA kandidaten aangewezen in verschillende bacteriën (22). Twee klasses van sRNAs, *trans*-werkende en *cis*-werkende, kunnen onderscheiden worden op basis van binding en regulatie. *Cis*- werkende sRNAs zijn gecodeerd tegenover het mRNA dat gereguleerd wordt en kent een extensieve interactie (23), terwijl *trans*-werkende sRNAs elders op het genoom gecodeerd liggen en in staat zijn meerdere mRNAs te reguleren met verschillende gradaties en resultaten (19). De regulerende rol van sRNAs

omvat translatie inhibitie of activatie, translatie en mRNA stabiliteit, maar ook indirecte transcriptie regulatie van naburige genen door veranderingen in DNA vouwing kan veroorzaakt worden door sRNAs (24). Ook al lijken sRNAs een uiterst belangrijke rol te spelen in regulatie, het functioneel karakteriseren van sRNAs is een traag proces, vooral in Gram-positieve bacteriën. De meeste technieken, zoals in silico voorspellingsprogramma's maar ook overexpressie systemen, zijn gebaseerd op Gram-negatieve bacteriën. Daarnaast kunnen sRNAs van Gram-negatieve bacteriën getest worden in *Escherichia coli*, met behulp van een high copy plasmide (met daarop het sRNA) en een low copy plasmide (met daarop het doel mRNA gekoppeld aan het groen fluorescent eiwit (GFP)) (25). Zo een systeem is niet beschikbaar voor Gram-positieve bacteriën. Vandaar dat sRNA onderzoek in Gram-positieve bacteriën zelfs nog langzamer verloopt. Toch zijn er virulentie regulerende sRNAs gvonden in *Listeria monocytogenes* (26) en *S. aureus* (12,16,27).

Functionele karakterisatie kan via verschillende technieken. Voor *S. aureus* is SprD gekarakteriseerd in muizen modellen met een knock-uit stam waar het sRNA vervangen is door een erythromycine cassette (16). De introductie van een antibioticum cassette kan echter flinke nadelen hebben, regulatie kan namelijk beïnvloed worden door de aanwezigheid van de cassette en niet resulterend vanuit de knock-uit. Dit fenomeen was recent gevonden na de introductie van een chlooramphenicol cassette in *Enterecoccus faecium* (niet gepubliceerde data en persoonlijke communicatie Dr. J. Top). RNAIII was getest met een stam die van nature RNAIII mist (27). Een RNAIII knock-uit is nog niet gemaakt, waarschijnlijk vanwege de rol in het *agr* systeem (12). Overexpressie stammen in *S. aureus* zijn nog nooit eerder beschreven. In *L. monocytogenes* zijn virulentie regulerende sRNAs gevonden op basis van van experimenten met een grote variëteit aan infectie gerelateerde condities en tot nu toe zijn er nog geen knock-uit stammen getest (26).

In **Hoofdstuk 4** beschrijven we vijf *trans*-werkende sRNAs die mogelijk virulentie genen reguleren, gebaseerd op *in silico* voorspellingen en *in vitro* groeicurves uit **Hoofdstuk 3**. Aanvullende beschrijven we functionele testen voor twee sRNA knock-uit stammen in MSSA476. Vergelijk van de onafhankelijke knock-uit stammen met wild-type MSSA476 resulteerde in trends van post-transcriptionele regulatie van de voorspelde eiwitten, Efb en Hld. Aanvullende *in vitro* analyse

ondersteunde de *in vivo* data echter niet geheel en meer onderzoek zal nodig zijn om het regulerende effect van deze sRNAs te begrijpen. Hierin zou een chaperone eiwit, zoals Hfq, een belangrijke rol kunnen spelen. We hebben ook geprobeerd sRNA overexpressie stammen te maken, maar vonden een te grote biologische variatie van het sRNA. Het toevoegen van chlooramphenicol zou hierin een rol kunnen hebben gespeeld. Ook gezien de onbedoelde consequenties die al gesuggereerd werden in prokaryoten, zoals regulatie van andere eiwitten of mRNA (28), leidde tot de conclusie dat knock-uit stammen waarschijnlijk betere resultaten geven.

Snel veranderende omgevingen eisen snelle regulatie van genen en sRNAs worden geacht sneller te zijn dan eiwit regulatoren (29,30). Om deze reden zijn we sRNA op zoek gegaan naar sRNA kandidaten in veranderende omgevingen. Twee infectie gerelateerde condities, humaan bloed en medium beide met 5% CO_2 en twee genetisch gelijkende stammen, MSSA476 en MW2, werden gebruikt (**Hoofdstuk 5**). Ongeveer 100 differentieel tot expressie gebrachte sRNA kandidaten werden gevonden. Zes kandidaten die eerder gevonden waren tijdens normale groei in medium waren ook in deze twee condities gevonden. Daarnaast zijn acht van de gevonden sRNAs in **Hoofdstuk 5** in eerdere studies beschreven (5,31). De differentiële regulatie in verschillende omgevingen suggereert een belangrijke functie voor de sRNAs en verdere karakterisatie zal de relevantie aan moeten tonen.

Tot op heden zijn er twee virulentie regulerende sRNAs in *S. aureus* gevonden en gevalideerd (16,27). Daarnaast zijn er twee sRNAs met potentiële functies in virulentie regulatie en er zijn nog vele kandidaten geïdentificeerd onder verschillende condities en andere technieken die van interesse zijn.

De rol van operonen in regulatie

De definitie van een operon, functionele genomische eenheid met meerdere open reading frames (ORFs) onder invloed van een promoter, was gekoppeld aan regulatie van genen zodat deze gelijke expressie niveaus zouden hebben (4). Transcriptoom analyse in de laatste jaren liet echter verschillende expressie niveaus zien van genen in een operon. In *Bacillus subtilis* bijvoorbeeld kunnen genen in een operon van meerdere promoters afgeschreven worden (4). Ook bestaan terminaters die lekken waardoor gedeektelijke transcriptie plaats kan vinden. Dit was eerder gezien in bacteriën, zoals *Helicobacter*

pylori en *Mycoplasma pneumoniae*. Bovendien kan er een nog complexer regulatie mechanisme bestaan door overlappende promoters in operonen. Deze observaties laten het belang zien van gen regulatie op operon niveau, waar meerdere factoren invloed hebben op operon regulatie wat leidt tot grote complexiteit.
Voor S. aureus waren alleen *in silico* voorspellingen beschikbaar aan het begin van dit proefschrift (32,33) die gebaseerd waren op de lengte van intergene regionen (IGRs), geconserveerde gen clusters en Rho-onafhankelijke terminaters. Wij wilden de kennis van gen regulatie in *S. aureus* vergroten om uiteindelijk de hoge infectiviteit te kunnen begrijpen. Hiervoor hebben we eerst de operon structuur van *S. aureus* in kaart gebracht met behulp van RNA geïsoleerd uit vijf reproduceerbare groeicurves in standaard medium (**Hoofdstuk 3**). Pearson correlaties tussen naast elkaar liggende probes in coderende en niet-coderende regionen waren berekend en gebruikt om de operon structuur te bepalen. Ongeveer 60% van de genen werden gevonden coderend in een operon. Opvallend waren ook genen gescheiden door grote IGRs gelegen in een operon. We hadden niet verwacht virulentie-genen in een operon te vinden, omdat deze vaak op mobiele elementen liggen en vaak uitgewisseld worden tussen stammen. Toch hebben we een aantal virulentie genen gevonden en gevalideerd die in operonen afgeschreven worden. Ook tonen we een synergetisch effect aan van twee in een operon afgeschreven genen van belang in ontwijking van het immuunsysteem, Efb en ScnB. Differentiële regulatie van operonen was ook opgevallen en verdere experimeten lieten inderdaad differentiële regulatie zien van het operon met lantibiotic genen. Gesplitste operonen waren *in silico* niet voorspeld.
Samennemend hebben recente studies aangetoond dat er verschillende regulatore elementen gemoeid zijn met de operon organisatie. Samen met de virulentie-genen gevonden in een operon, laat dit zien dat experimenteel bepaalde operonen van belang zijn. Om de best mogelijke regulatie te kunnen hebben in snel veranderende condities, zijn veel operonen waarschijnlijk differentieel gereguleerd. Voor *S. aureus* is het dus interessant om de operon structuur onder verschillende condities te bepalen.

Gen regulatie in veranderende omgevingen

Pathogenen zijn in staat om een grote variabiliteit aan infecties te veroorzaken door snelle regulatie van genen onder veranderende en gevaarlijke omstandigheden. Desondanks is de moleculaire basis om bacteriële reacties op host signalen te begrijpen gelimiteerd. Vandaar dat gen-regulatie in infectie gerelateerde condities bestudeerd is voor verschillende pathogenen, zoals de invloed van bloed op Groep A Streptococcus (34). Dit leverde cruciale inzichten in bacteriële strategieën om het host immuun systeem te omzeilen en te overleven in de host.
S. aureus is in staat om als commensaal te leven maar tegelijkertijd ook in staat levensbedreigende infecties te veroorzaken (35). Dit impliceert een belangrijke rol voor gen-regulatie tijdens infecties. Ook de wetenschap dat gen-expressie in *S. aureus* tijdens standaard groei in verschillende media anders is (6,36) ondersteunt de invloed van gen-regulatie. Desondanks zijn maar enkele studies gedaan om de gen-expressie onder invloed van verschillende omstandigheden te bepalen. Tot nu toe zijn de studies vooral gericht op gen-van een gelimiteerde set van genen na contact met humaan serum (37-39). Meer recentere studies hebben de algehele gen-expressie in *S. aureus* bepaald na contact met humaan bloed (40), neutrofielen (41) of neutrofiele microbiciden (42). Deze studies toonden aan dat snelle regulatie van genen van belang is om te overleven.
Wij hebben de algehele gen-expressie bekeken van twee genetisch gelijkende stammen na blootstelling aan twee condities, humaan bloed en medium beide met 5% CO_2 (**Hoofdstuk 5**). Dit bevestigde dat genetisch gelijkende stammen op verschillende manier kunnen reageren ondanks dezelfde omgeving. Dit geeft aan dat S. aureus in staat is om vele verschillende infecties te veroorzaken. Een vergelijking tussen de condities liet het belang van staphylococcal superantigen-like genes (*ssls*) zien na blootstelling aan bloed. SSLs zijn mogelijk essentieel voor overleving van de bacteriën in de cel na opname door neutrofielen. Deze hypothese wordt ondersteund door de regulatie van *ssl* transcripten na blootstelling aan neutrofiele microbiciden (42). Samengenomen impliceert de regulatie van *ssl* transcripten na blootstelling aan bloed dat deze een rol spelen in overleving van bacteriën na fagocytose. Daarnaast geeft het verschil tussen de twee stammen aan dat gen regulatie een belangrijke rol speelt tijdens infecties en dat meerdere stammen onder verschillende condities getest zullen moeten worden om de infectiviteit van *S. aureus* te begrijpen.

Concluderende opmerkingen

Bacteriële transcriptoom-studies hebben de complexiteit van het transcriptoom van pathogenen laten zien. De veelheid aan sRNAs, de differentieel

gereguleerde operonen, maar ook de aanwezigheid van andere regulatore elementen, zoals lange UTRs, riboswitches, RNA thermometers, en zelfs veranderingen in DNA vouwing, suggereren complexe lagen van regulatie (4, **dit proefschrift**). Uitbreiden van de huidige kennis over regulatie is essentieel om de complete virulentie van pathogenen te bepalen. Toekomstige experimenten zullen het bepalen van de exacte transcriptie start plaatsen en terminaters, maar ook de complexe RNA structuur moeten omvatten. Hiermee kunnen alternatieve start plaatsen in een operon en de verschillende lengtes van 5'- en 3'-UTRs bepaald worden. Experimenteel bepaalde RNA structuren zullen meer inzicht geven in de secundaire en tertiaire RNA structuren die van belang zijn in sRNAs, riboswitches en RNA thermometers.
De meerdere lagen van regulatie in bacteriële transcriptie geven de cruciale rol van gen-regulatie weer tijdens kolonisatie, infectie en overleving. Desondanks is het begrijpen van de virulentie van *S. aureus* nog steeds toekomst-perspectief.

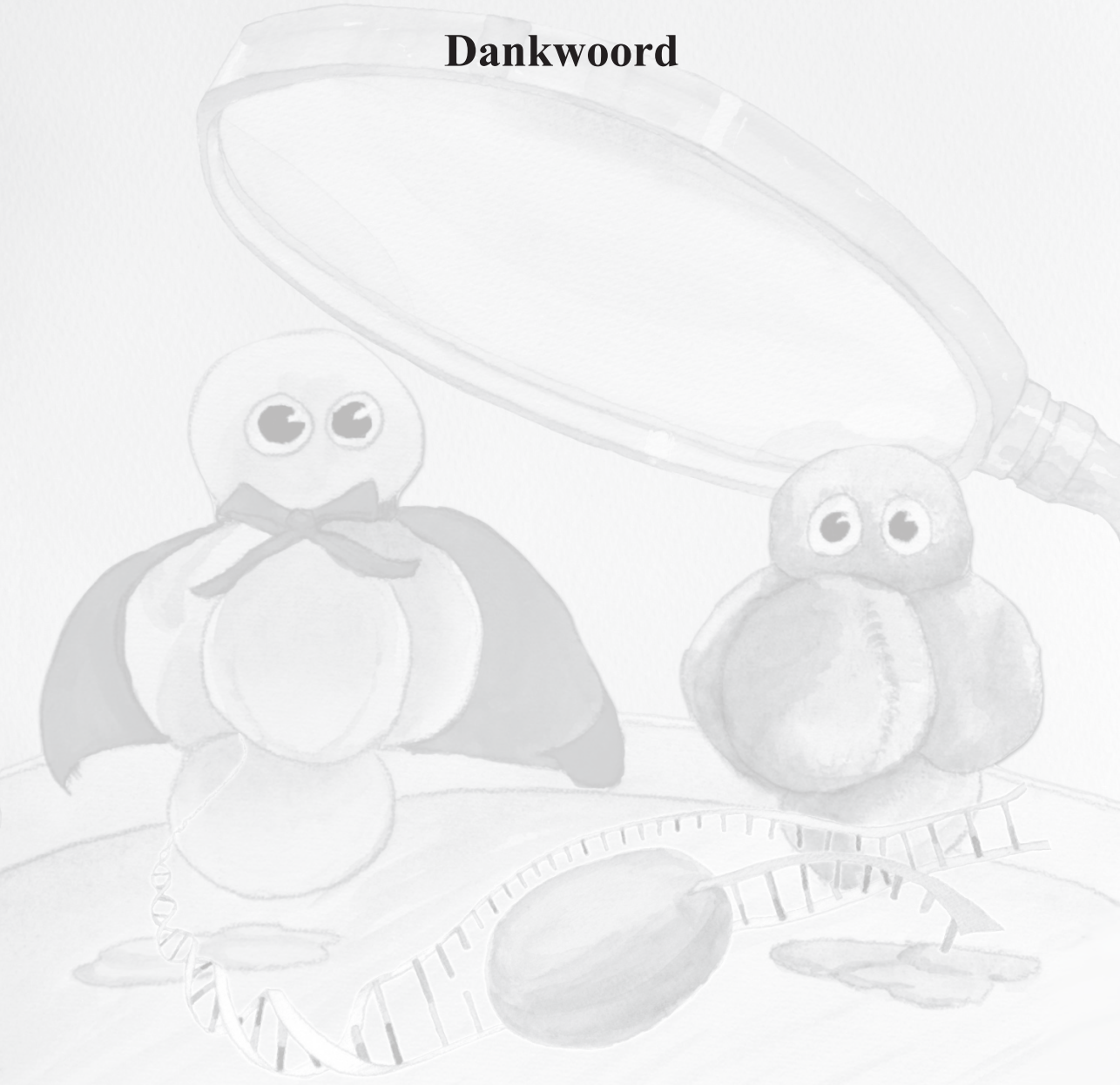
References

1. Wosten, M.M. (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev*, **22**, 127-150.
2. Shine, J. and Dalgarno, L. (1975) Determinant of cistron specificity in bacterial ribosomes. *Nature*, **254**, 34-38.
3. Santangelo, T.J. and Artsimovitch, I. (2011) Termination and antitermination: RNA polymerase runs a stop sign. *Nat Rev Microbiol*, **9**, 319-329.
4. Guell, M., Yus, E., Lluch-Senar, M. and Serrano, L. (2011) Bacterial transcriptomics: what is beyond the RNA horiz-ome? *Nat Rev Microbiol*, **9**, 658-669.
5. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J. and Francois, P. (2010) Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. *PLoS One*, **5**, e10725.
6. ten Broeke-Smits, N.J.P., Pronk, T.E., Jongerius, I., Bruning, O., Wittink, F.R., Breit, T.M., van Strijp, J.A.G., Fluit, A.C. and Boel, C.H.E. (2010) Operon structure of *Staphylococcus aureus*. *Nucleic Acids Res*, **38**, 3263-3274.
7. Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N.Engl.J.Med.*, **339**, 520-532.
8. Wertheim, H.F.L., Verveer, J., Boelens, H.A.M., van Belkum, A., Verbrugh, H.A. and Vos, M.C. (2005) Effect of mupirocin treatment on nasal, pharyngeal, and perineal carriage of *Staphylococcus aureus* in healthy adults. *Antimicrob Agents Chemother*, **49**, 1465-1467.
9. Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin.Microbiol.Rev.*, **10**, 505-520.
10. Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol.Microbiol.*, **48**, 1429-1449.
11. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004) Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol.Med.Microbiol.*, **40**, 1-9.
12. Novick, R.P. and Geisinger, E. (2008) Quorum sensing in staphylococci. *Annu Rev Genet*, **42**, 541-564.
13. Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E.,

McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J. and Kreiswirth, B.N. (2003) Global regulation of *Staphylococcus aureus* genes by Rot. *J.Bacteriol.*, **185**, 610-619.
14. Benson, M.A., Lilo, S., Wasserman, G.A., Thoendel, M., Smith, A., Horswill, A.R., Fraser, J., Novick, R.P., Shopsin, B. and Torres, V.J. (2011) *Staphylococcus aureus* regulates the expression and production of the staphylococcal superantigen-like secreted proteins in a Rot-dependent manner. *Mol Microbiol*, **81**, 659-675.
15. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**, 1353-1366.
16. Chabelskaya, S., Gaillot, O. and Felden, B. (2010) A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog*, **6**, e1000927.
17. Geisinger, E., Adhikari, R.P., Jin, R., Ross, H.F. and Novick, R.P. (2006) Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol.Microbiol.*, **61**, 1038-1048.
18. Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824-835.
19. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615-628.
20. Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J. and Vandenesch, F. (2000) Probing the structure of RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *Rna*, **6**, 668-679.
21. Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B. and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.*, **12**, 3967-3975.
22. Papenfort, K. and Vogel, J. (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe*, **8**, 116-127.
23. Brantl, S. (2007) Regulatory mechanisms employed by cis-encoded antisense RNAs. *Curr Opin Microbiol*, **10**, 102-109.
24. Blot, N., Mavathur, R., Geertz, M., Travers, A. and Muskhelishvili, G. (2006) Homeostatic regulation of supercoiling sensitivity coordinates transcription of the bacterial genome. *EMBO Rep*, **7**, 710-715.
25. Urban, J.H. and Vogel, J. (2007) Translational control and target recognition by Escherichia coli small RNAs in vivo. *Nucleic Acids Res.*, **35**, 1018.
26. Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K. *et al.* (2009) The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature*, **459**, 950-956.
27. Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T., Vandenesch, F. and Romby, P. (2010) Staphylococcus aureus RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. *PLoS Pathog*, **6**, e1000809.
28. Storz, G., Vogel, J. and Wassarman, K.M. (2011) Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Mol Cell*, **43**, 880-891.
29. Gripenland, J., Netterling, S., Loh, E., Tiensuu, T., Toledo-Arana, A. and Johansson, J. (2010) RNAs: regulators of bacterial virulence. *Nat Rev Microbiol*, **8**, 857-866.
30. Romby, P., Vandenesch, F. and Wagner, E.G. (2006) The role of RNAs in the regulation of virulence-gene expression. *Curr Opin Microbiol*, **9**, 229-236.

31. Geissmann, T., Chevalier, C., Cros, M.J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., Francois, P., Vandenesch, F., Gaspin, C. *et al.* (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res*, **37**, 7239-7257.
32. Brouwer, R.W.W., Kuipers, O.P. and Hijum, S.A.F.T. (2008) The relative value of operon predictions. *Brief.Bioinform.*, **9**, 367-375 Epub.
33. Wang, L., Trawick, J.D., Yamamoto, R. and Zamudio, C. (2004) Genome-wide operon prediction in *Staphylococcus aureus*. *Nucleic Acids Res.*, **32**, 3689-3702.
34. Graham, M.R., Virtaneva, K., Porcella, S.F., Barry, W.T., Gowen, B.B., Johnson, C.R., Wright, F.A. and Musser, J.M. (2005) Group A Streptococcus transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *Am J Pathol*, **166**, 455-465.
35. Lindsay, J.A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler, R.A., Husain, S.E., Butcher, P.D. and Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J.Bacteriol.*, **188**, 669-676.
36. Ray, B., Ballal, A. and Manna, A.C. (2009) Transcriptional variation of regulatory and virulence genes due to different media in *Staphylococcus aureus*. *Microb Pathog*, **47**, 94-100.
37. Oogai, Y., Matsuo, M., Hashimoto, M., Kato, F., Sugai, M. and Komatsuzawa, H. (2011) Expression of Virulence Factors by Staphylococcus aureus Grown in Serum. *Appl Environ Microbiol*, **77**, 8097-8105.
38. Wiltshire, M.D. and Foster, S.J. (2001) Identification and analysis of *Staphylococcus aureus* components expressed by a model system of growth in serum. *Infect.Immun.*, **69**, 5198.
39. Yarwood, J.M., McCormick, J.K., Paustian, M.L., Kapur, V. and Schlievert, P.M. (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. *J.Bacteriol.*, **184**, 1095-1101.
40. Malachowa, N., Whitney, A.R., Kobayashi, S.D., Sturdevant, D.E., Kennedy, A.D., Braughton, K.R., Shabb, D.W., Diep, B.A., Chambers, H.F., Otto, M. *et al.* (2011) Global changes in *Staphylococcus aureus* gene expression in human blood. *PLoS One*, **6**, e18617.
41. Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Said-Salim, B., Porcella, S.F., Long, R.D., Dorward, D.W., Gardner, D.J., Kreiswirth, B.N. *et al.* (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J.Immunol.*, **175**, 3907-3919.

Dankwoord



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