Transcriptional regulation of host-pathogen interactions from the point of view of *Staphylococcus aureus*

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Summary

Compared to other human pathogens, *S. aureus* outstands with a remarkably broad spectrum of deseases: from minor skin infections over endocarditis, pneumoniae, and osteomyelitis, to septic shock. The prerequisite is an arsenal of adaptation strategies, encoded in the core and variable genome. It includes the coordinated expression of adhesins and toxins, evasion of the immune system, response to stress and starvation, adaptation of the metabolism, formation of biofilms and capsules, antibiotic resistance, and persistence on the skin, in nasal epithelial cells, and even in the inner of macrophages after phagocytosis. All these adaptation strategies enable *S. aureus* to colonize a diversity of niches within the human host.

The inevitable requirement is the ability to activate the appropriate adaptation strategy at the right time and at the right place. *S. aureus* overcomes this challenge with a sophisticated regulatory network. This PhD thesis covers a broad spectrum of transcriptional regulators, involved in *S. aureus* pathogenesis: (1) the quorum sensing system Agr (regulation of early- and late stage virulence factors), (2) the Sar family (regulation of early- and late stage virulence factors), (3) SaeRS (regulation of accessory exotoxins and adhesins), (4) CodY (response to amino acid starvation, including extracellular proteases), (5) Sigma B (general stress response, including virulence factors), (6) Rex (anaerobic energy metabolism), (7) CtsR and HrcA (protein quality control), (8) PerR and Fur (oxidative stress response), and (9) antibiotic resistance.

Traditionally, Proteomics constitute the long-lasting reputation of the Institute. In fact, the majority of investigations presented in this PhD thesis was initialized by proteomic analyses as the ultimate starting point. From the first day, a major goal of this PhD thesis was to add regulator-promoter interaction studies to the methodical spectrum. In particular, to complement transcriptomic and proteomic results by answering the logical follow-up question: Which regulator is responsible for the observed changes in gene expression and protein synthesis after application of a specific stimulus?

The first chapter provides specific analyses for three major regulators: Rex, CodY, and SarA. Publications were achieved for Rex (Hecker *et al.*, 2009; Pagels *et al.*, 2010). Results were mainly achieved by establishing regulator-promoter interaction methods (in particular EMSA and *"footprinting"*). Additionally, this chapter describes method development of a novel easy-to-apply method, named REPA (**r**estriction **e**ndonuclease **p**rotection **a**ssay).

The second chapter presents method development for the genome-wide identification of regulator-promoter interactions, named *"global footprinting"*. This approach combines two already well-established methods: (A) Purification of a recombinant *Strep*-tagged regulator via *Strep*-tag affinity chromatography. The modification in *"global footprinting"* is to incubate the regulator with fragmented genomic *S. aureus* DNA, resulting in co-purification and enrichment of DNA streches with specific regulator binding sites. (B) Identification and quantification of these DNA streches via *"next generation sequencing"* (NGS). Using this combined approach, this PhD thesis was able to localize the most affine promoter binding site for the regulator Rex precisely down to one single base pair across the whole *S. aureus* genome.

The third chapter describes the assembly of a data library, collecting the majority of DNA microarray data and regulator-promoter interaction studies from the worldwide literature. This data library summarizes more than 50,000 regulatory events and more than 2,000 regulator binding sites. As published in the perspectives in Fuchs *et al.* (2018), this data library can be incorporated into the free-accessible online data base "*Aureowiki*" (provided and maintained by the Department of Functional Genomics, University of Greifswald). The major effort is the consolidation of these "*big data*" via *in silico* cluster analysis, comparing 282 different experimental conditions at once. The major finding of this analysis is the identification of seven functional and regulatory gene clusters in *S. aureus* pathogenesis that are conserved across *S. aureus* strain diversity. These findings allowed the creation of a prediction tool, to provide novel experimental starting points for the worldwide *S. aureus* research community. This prediction tool was successfully applied on several topics, and partially published: functional and regulatory prediction for a set of 20 selected lipoproteins as potential virulence factors (Graf *et al.*, 2018), and prediciton of protein complexes (Liang *et al.*, 2016).

Alltogether, this PhD thesis provides new insights into the molecular mechanisms of three pathogenesis-relevant regulators: Rex, CodY, and SarA. It describes the development of three novel experimental methods for wet and dry lab applications that can be used on research topics beyond *S. aureus*: REPA, *"global footprinting"*, and cluster analysis. Finally, cluster analysis identifies seven conserved fuctional and regulatory gene clusters, involved in *S. aureus* pathogenesis. This cluster anaysis is used as a prediction tool to provide novel experimental starting points, and to predict the physiological mode of action of newly discovered anti-staphylococcal agents.

Zusammenfassung

Im Vergleich zu anderen Krankeitserregern sticht *S. aureus* mit einem bemerkenswert breiten Spektrum an Krankeitsbildern im Menschen heraus: von Hautinfektionen über Endocarditis, Pneumonie und Osteomyelitis bis hin zum septischen Schock. Die Grundvoraussetzung ist ein Arsenal an Adaptationsstrategien, die im Kern- und variablen Genom kodiert sind. Es beinhaltet die koordinierte Expression von Adhäsinen und Toxinen, die Umgehung des Immunsystems, die Antwort auf Stress und Hunger, die Anpassung des Stoffwechsels, die Bildung von Biofilmen und Kapseln, Resistenz gegenüber Antibiotika und die Persistenz auf der Haut, in nasalen Epithelzellen und sogar im Inntern von Makrophagen nach Phagozytose. All diese Adaptationsstrategien ermöglichen *S. aureus* die Besiedelung einer Vielzahl von Nischen im menschlichen Wirt.

Die notwendige Bedingung ist die Fähgigkeit, die passende Adaptationsstrategie zur richtigen Zeit am richtigen Ort zu aktivieren. *S. aureus* meistert diese Herausforderung mit einem ausgefeilten regulatorischen Netzwerk. Diese Promotionsarbeit deckt ein breites Spektrum von Regulatoren der Transkription ab, welche relevant für die Pathogenese von *S. aureus* sind: (1) Das Quorum-Sensing-System Agr (Regulation von Virulenzfaktoren der frühen und späten Phase), (2) die Sar-Familie (Regulation von Virulenzfaktoren der frühen und späten Phase), (3) SaeRS (Regulation von zusätzlichen Exotoxinen und Adhäsinen), (4) CodY (Antwort auf Aminosäuremangel, inklusive extrazelluläre Proteasen), (5) Sigma B (generelle Stressantwort, inklusive Virulenzfaktoren), (6) Rex (anaerober Energiestoffwechsel), (7) CtsR und HrcA (Proteinqualitätskontrolle), (8) PerR und Fur (oxidative Stressantwort), und (9) Resistenz gegenüber Antibiotika.

Die traditionelle Reputation des Instituts basiert auf Proteomics. Tatsächlich wurde der Großteil der Untersuchungen dieser Promotionsarbeit durch Proteomanalysen als ultimativer Startpunkt initialisiert. Vom ersten Tag dieser Promotionsarbeit an war es eines der Hauptziele, Regulator-Promoter-Interaktionsstudien dem methodischen Spektrum hinzuzufügen. Insbesondere um die transkriptomischen und proteomischen Ergebnisse zu ergänzen und die logische Folgefrage zu beantworten: Welcher Regulator ist verantwortlich für die beobachteten Änderungen in Genexpression und Proteinsynthese nach Anwendung eines bestimmten Stimulus?

Das erste Kapitel bietet spezifische Analysen für drei Hauptregulatoren: Rex, CodY und SarA. Publikationen wurden für Rex erreicht (Hecker *et al.*, 2009; Pagels *et al.*, 2010). Die Ergebnisse wurden hauptsächlich durch die Etablierung von Regulator-Promoter-Interaktionsstudien ermöglicht (insbesondere EMSA und *"footprinting"*). Zusätzlich beschreibt dieses Kapitel die Entwicklung einer neuen, leicht anwendbaren Methode, genannt REPA (**r**estriction **e**ndonuclease **p**rotection **a**ssay).

Das zweite Kapitel präsentiert die Entwicklung einer neuen Methode zur Genom-weiten Identifizierung von Regulator-Promoter-Interaktionsstudien, genannt *"global footprinting"*. Dieser Ansatz kombiniert zwei bereits gut etablierte Methoden: (A) Reinigung eines rekombinanten *Strep*-tag-Regulators mittels *Strep*-tag-Affinitätschromatografie. Als Modifikation in *"global footprinting"* wird der Regulator mit fragmentierter genomischer *S. aureus* DNA inkubiert, was zur Co-Purifikation und Anreicherung von DNA-Abschnitten führt, welche spezifische Regulator-Bindestellen tragen. (B) Die Identifizierung und Quantifizierung dieser DNA-Abschnitte mittels *"next generation sequencing"* (NGS). Mit diesem Ansatz ist es dieser Promotionsarbeit gelungen, die affinste Promoter-Bindestelle für den Regulator Rex bis auf das Basenpaar genau im gesamten *S. aureus* Genom zu lokalisieren.

Das dritte Kapitel beschreibt die Zusammenstellung einer Daten-Bibliothek, bestehend aus der Gesamtheit aller DNA-Microarray-Daten und Regulator-Promoter-Interaktionsstudien aus der weltweiten Literatur. Sie umfasst mehr als 50.000 regulatorische Ereignisse und mehr als 2.000 Regulator-Bindestellen. Wie in den *„perspectives"* in Fuchs *et al.* (2018) publiziert kann diese Daten-Bibliothek in die frei zugängliche Online-Datenbank "*Aureowiki*" integriert werden (betrieben vom Institut für funktionelle Genomforschung, Universität Greifswald).

Die Hauptleistung ist die Zusammenfassung der "*big data*" dieser Daten-Bibliothek mittels *in silico* Cluster-Analyse, welche 282 verschiedene experimentelle Bedingung auf einmal vergleicht. Das Ergebnis dieser Analyse ist die Identifizierung von sieben funktionellen und regulatorischen Gen-Clustern, die pathogenese-relavant und konserviert über die Stammvielfalt von *S. aureus* sind. Mit diesen Ergebnisse wird ein Vorhersagewerkzeug erschaffen, um der weltweiten *S. aureus* Forschungsgemeinschaft neue experimentelle Startpunkte bereitzustellen. Es wurde bereits in verschiedenen Forschungsthemen erfolgreich angewendet und teilweise veröffentlicht: funktionelle und regulatorische Vorhersage für 20 ausgewählte Lipoproteine als potentielle Virulenzfaktoren (Graf *et al.*, 2018) und die Vorhersage von Protein-Komplexen (Liang *et al.*, 2016).

Zusammengefasst bietet diese Promotionsarbeit neue Einblicke in die molekularen Mechanismen von drei Pathogenese-relevanten Regulatoren: Rex, CodY und SarA. Sie beschreibt die Entwicklung von drei neuen experimentellen Methoden die auf Forschungsthemen über diese Promotionsarbeit hinaus angewendet werden können: REPA, *"global footprinting"* und Cluster-Analyse. Abschließend identifiziert die Cluster-Analyse sieben konservierte funktionelle und regulatorische Gen-Cluster die in der Pathogenese von *S. aureus* involviert sind. Diese Cluster-Analyse wird als Vorhersagewerkzeug für neue experimentelle Startpunkte benutzt und trifft Vorhersagen für den Wirkmechanismus von neu entdeckten anti-staphylokokkalen Substanzen.

List of publications

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Int J Med Microbiol. 2018 Aug;308(6):569-581.

Manuscript is being written for submission:
Identification of seven conserved functional and regulatory gene clusters in *Staphylococcus aureus* pathogenesis.
Pagels M, Pané-Farré J, Engelmann S, Mäder U, Dandekar T, Riedel K, Fuchs S.

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List of abbrevations

A	adenosine
ATP	adenosine tri-phosphate
B. subtilis	*Bacillus subtilis*
CAP	cationic antimicrobial peptides
C	cytosine
EMSA	electro mobility shift assay
G	guanine
GF	global footprinting
GTP	guanosine tri-phosphate
Ig	immune globuline
MGE	mobile genetic elements
MSCRAMM	microbial surface component recognizing adhesive matrix molecules
N	a variable DNA consensus position: A, G, C or T
NAD or NADH	nicotinamide adenine dinucleotide
OD	optical densitiy
PDH compex	pyruvate dehydrogenase complex
PMN	polymorphonuclear neutrophils
REPA	restriction endonuclease protection assay
ROS	reactive oxygen species
S. aureus	*Staphylococcus aureus*
SAB	*Staphylococcus aureus* bacteremia
SSSS	staphylococcal scalded skin syndrome
T	thymidine
W	adenosine or thymidine
2KS	two-component system

1. Introduction

1.1 *Staphylococcus aureus*

The Gram-positive Firmicute *Staphylococcus aureus* (translated from greek language as "golden grape cluster berry") earns its name from its appearance under the microscope, and from the colour of its colonies when grown on agar plates. It is characterized as a facultative anaerobe that is incapable of forming spores.

Today, the genus *Staphylococcus* is separated into 58 distinct species (https://lpsn.dsmz.de/genus/staphylococcus). 15 of these species are capable to colonize mammal body surfaces as commensals. Of these species, 4 are well-known human pathogens: *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*. But only *S. aureus* is able to colonize a multitude of niches within the human host, and consequently, causes a broad spectrum of diseases. The species *S. aureus* splits into a multitude of strains that are summarized in 3 clades (Holden *et al.*, 2004; Lindsay and Holden, 2004). *S. aureus* shows a remarkably high prevalence among the human population, with 20% as continuous and another 30% as intermittent carriers (J. R. Fitzgerald, 2014). Colonization sites are the skin and the Nasopharynx, from where *S. aureus* can enter the inner of the human body via open wounds as an opportunistic pathogen. *S. aureus* nasal carriage manifests as intracellular long-term persistence in nasal epithelial cells (Clement *et al.*, 2005, Sakr *et al.*, 2018). Furthermore, *S. aureus* is also able to persist in endothelial cells, fibroblasts, osteoblasts, and professional phagocygtes of the immune response (Ogawa *et al.*, 1985; Sinha *et al.*, 1999; Jevon *et al.*, 1999; Dziewanowska *et al.*, 1999; Hoefnagels-Schuermans *et al.*, 1999; Lacoma *et al.*, 2017; Guerra *et al.*, 2017).

Pathological appearance of *S. aureus* was initially described by Sir Alexander Ogston, as a cause of pyogenic clinical pictures in humans (Ogston, 1882). With today's knowledge, the deseases caused by *S. aureus* range from minor skin infections over endocarditis, osteomyelitis, and pneumonia, to septic shock (Easmon and Adlam, 1983; Foster and Höök, 1998; Dinges *et al.*, 2000). This is in contrast to other staphylococcal pathogenic species that appear to be restricted to specific host sites. There are two major prerequisites that make *S. aureus* such a highly adaptive pathogen, able to exploit a variety of ecological niches within the human host: (1) a broad repertoire of virulence factors and adaptation strategies to deal with stress and nutrient starvation, encoded in the core genome, and (2) its genetic variability. (1) Virulence factors that are encoded in the *S. aureus* core genome include adhesins (e. g. fibrinogen, fibronectin, and collagen), exotoxins (e. g. hemolysins, leucocidins, proteases, lipases, and nucleases), and factors that manipulate or enable *S. aureus* to evade the

immune response of the human host (e. g. superantigens that activate non-staphylococcal specific T-lymphocyte proliferation, Protein A that binds and scavenges the Fc domains of host immunglobulin G, and factors that enable biofilm and capsule formation, and intracellular persistence). (2) Genetic variability is caused by horizontal and vertical gene transfer. On the one hand, horizontal gene transfer is caused by mobile genetic elements (MGEs), e. g. bacteriophages, transposons, plasmids, and pathogenicity islands. These MGEs cover 15% of *S. aureus* genomes (Alibayov *et al.*, 2014). On the other hand, vertical gene transfer is caused by spontaneous point mutations that might be selectively enriched within a *S. aureus* sub-population, given that these mutations are beneficial for bacterial survival (Lindsay, 2010). The most prominent virulence factors and MGEs are further discussed in 1.2 and 1.3.

On the basis of this broad spectrum of adaptability, *S. aureus* has become a worldwide concern in healthcare. In the industrialized world, the prevalence of *S. aureus* bacteremia (SAB) constantly increased over the last 60 years. SAB occurrence increased from 3 per 100,000 person-years in 1957 to 20 per 100,000 person-years in 1990 (Frimodt-Moller *et al.*, 1997). The term "person-years" describes the number of people in that study, multiplied with the number of years they spend in that study. In a nutshell, the survey data for 1 person over 10 years equals the survey data for 10 persons over 1 year. In recent studies, the occurrence of SAB was determied to be relatively stable at 10 to 30 per 100,000 person-years (Laupland *et al.*, 2013). Data from non-industrialized regions can differ significantly, from 2.5 per 100,000 person-years in north-east Thailand (Kanoksil *et al.*, 2013) to 48 per 100,000 person-years among children below the age of 15 in Manhica District, Mozambique (Sigauque *et al.*, 2009).

Despite these inhomogenous distributions, there are two common trends in the overall epidemiology of *S. aureus* infections during the last two decades: (1) a constant increase in health care-associated infections, and (2) a constant increase in community-associated skin and soft tissue infections, caused by β-lactam resistant strains (Aires de Sousa and de Lencastre, 2004; Tong *et al.*, 2015).

1.2 Expression of virulence factors

The simplified model for the course of *S. aureus* infection distinguishes between the early and the late stage. In the early stage, *S. aureus* colonizes host sites by attachment to host tissue, and by evading the immune response. In the late stage, the local *S. aureus* population grows in numbers, starts to exploit additional nutrient sources via exotoxins, and finally spreads to further infection sites. This simplified model can be reproduced *in vitro* in the shaking flask: *S. aureus* cultures with low cell

density produce adhesins during the exponential growth phase, whereas cultures with high cell density secrete exotoxins during the stationary growth phase. The major regulator of this sequential expression of virulence factors is the quorum sensing system Agr (**a**ccessory **g**ene **r**egulator). At low cell densities, Agr exhibits only basal activity, wherease at high cell densities it starts to autoinduce its own expression, and consequently, represses early stage virulence factors and activates the expression of late stage virulence factors. This regulation occurs on post-transcriptional level via the regulatory mRNA, called RNAIII, that binds to the mRNAs of several virulence factors and regulators, altering its translation efficiency and mRNA stability. This mode of action also includes the regulation of secondary virulence regulators (SarS, SarT, and SarU) that belong to the Sar family (**s**taphylococcal **a**ccessory **r**egulators). Consequently, these regulators bind to the promoters of virulence genes and regulate their expression in an Agr-dependent manner.

1.2.1 Early stage virulence factors

The most prominent member of the early stage virulence factors is Protein A (encoded by *spa*, SACOL0095). Protein A is anchored to the cell wall and belongs to the family of MSCRAMMs (**m**icrobial **s**urface **c**omponent **r**ecognizing **a**dhesive **m**atrix **m**olecules). It contains 5 repeats of an immunoglobulin-binding domain that binds the Fc domain of IgGs with high affinity, and to a lesser degree the Fab region of the VH3 family, giving Protein A a lesser binding capability to IgA, D, E and M as well (Cowan *et al.*, 1979). Consequently, Protein A is capable of scavenging *Staphylococcus*-specific Ig's, and thus prevents the opsonization of the bacterial surface. As a result, the recruitment of professional phagocytes and the activation of the complement system are impeded.

The regulation of Protein A expression is multifactorial, including the quorum sensing system Agr and a set of regulators being counted to the Sar family: SarA, SarR, SarS, SarT, SarU, Rot, TcaR, and MgrA. At present, this regulatory network is not fully understood, and most of the stimuli for these regulators are unknown. Furthermore, indirect regulations and strain-specific differences (due to spontaneous mutations) are highly prevalent and complicate the breakdown of this regulatory network. Nonetheless, there is one clear message from the literature: the quorum sensing system Agr acts as the major regulator of Protein A expression. This regulation occurs indirectly via the regulation of members of the Sar family (Tegmark *et al.*, 2000; Cheung *et al.*, 2001; Schmidt *et al.*, 2003; Gao and Steward, 2004; Oscarsson *et al.*, 2005), and directly via RNAIII, the effector of the Agr system, that binds the *spa* mRNA and inhibits the formation of the translation initiation complex (Huntzinger *et al.*, 2005).

Besides Protein A, the MSCRAMM-family also includes other adhesins as well, e. g. fibronectin-binding proteins (FnbpA, FnbpB), clumping factor proteins (ClfA, ClfB), and a collagen-binding protein (Coa) (Lowy, 1998). The expression of these adhesins is as well regulated by the Agr system and members of the Sar family. Additionally, the SaeRS two-component system also plays an important role (Rogasch *et al.*, 2006; Liu *et al.*, 2016).

1.2.2 Late stage virulence factors

The most prominent member of the late stage virulence factors is the α-hemolysin (SACOL1173, Hla). Five of these proteins assemble as a heptamer in the cellular membrane of host cells and form a β-barrel pore, leading to apoptosis of the target cell (Bhakdi and Tranum-Jensen, 1991; Bantel *et al.*, 2001). The specificity of Hla upon human cells is dependent on Hla-concentration and ranges from erythrocytes over endothelial cells to lymphocytes of the immune system (for a review please note Berube and Bubeck Wardenburg, 2013). Hla expression is regulated by the same set of regulators as for Protein A, however, with the opposite effect. For example, RNAIII, the effector of the Agr system, binds to the mRNA of Rot (**r**epressor **o**f **t**oxins; a Sar homolog) with an inhibitory effect on Rot translation, leading to de-repression of *hla* transcription. Additionally, the 5′ end of RNAIII binds to the translation site of the *hla* mRNA itself, causing a conformational change of the secondary structure that opens the ribosomal binding site (Novick *et al.*, 1993; Morfeldt *et al.*, 1995; Huntzinger *et al.*, 2005).

The members of late stage virulence factors can be divided into four groups of exotoxins. (1) Next to the α-hemolysin, Hla, other exotoxins are capable of host cell lysis as well: β-hemolysin, γ-hemolysin, and several leukocidins (e. g. PVL, LukF, LukM, LukS) (Kaneko *et al.*, 2004; Keitaro *et al.*, 2011). (2) The members of the second group are described as pyrogenic superantigens that are capable to stimulate non-*Staphylococcus* specific T-lymphocyte proliferation. This group includes the toxic shock syndrome toxin-1 (TSST-1), and a set of enterotoxins (e. g. SEA, SEB, SEI) (Lina *et al.*, 2004). (3) The third group includes enzymes that exploit alternative nutrient sources by degrading host components: proteases (SspABC, SplABC), lipase (Lip), nuclease (Nuc), phosphodiesterase (Plc), and coagulase (Coa). (4) The fourth group includes the exfoliative toxins ETA and ETB that degrade superficial skin layers, causing SSSS (**s**taphylococcal **s**calded **s**kin **s**yndrome) (Bukowski *et al.*, 2010; Melishand *et al.*, 2010). Regulation of these virulence factors depends on multiple intra- and extracellular signals and conditions. These regulatory systems include the major regulators Agr, the Sar family, and SaeRS, but other two-component systems and regulators as well (for reviews

please note Arvidson and Tegmark (2001), Novick (2003), Cheung *et al.* (2004), Bien *et al.* (2011), Jenul and Horswill (2018), and Gimza *et al.* (2019)).

1.2.3 The quorum sensing system Agr

The expression of the majority of virulence factors in *S. aureus* is dependent on quorum sensing by the Agr system. This regulatory system is composed of two operons that are divergently transcribed from a mutual promoter region (Kornblum *et al.*, 1990). One operon, AgrBDCA, is transcribed into the RNAII. This mRNA includes a so-called pheromone (AgrD, SACOL2024), the processor of the pheromone (AgrB, SACOL2023), a sensor kinase (AgrC, SACOL2025) that senses the concentration of the secreted pheromone, and consequently, phosphorylates and activates the response regulator (AgrA, SACOL2026). Phosphorylation of AgrA leads to dimerization and binding to the *agr* promoter region, causing up-regulation of RNAII and the second operon that encodes the RNAIII. RNAIII not only encodes the δ-hemolysin (Hld, SACOL2022), but more importantly constitutes the regulatory effector molecule of the quorum sensing system itself. RNAIII is able to bind to the mRNAs of several virulence factors, including *spa* and *hla*, and regulates regulators of the Sar family on post-transcriptional level as well (Novick *et al.*, 1993; Morfeldt *et al.*, 1995; Huntzinger *et al.*, 2005; Shu *et al.*, 2008; Chabelskaya *et al.*, 2014). For example, RNAIII stabilizes the mRNA of MgrA (Gupta *et al.*, 2015), but binds the mRNA of Rot with an inhibitory effect on transcription (Boisset *et al.*, 2007). Consequently, the Agr system causes a regulatory cascade effect and thus indirectly activates and represses a broad set of early- and late stage virulence factors, as it was demonstrated by several DNA-Microarray analyses (Dunman *et al.*, 2001; Cassat *et al.*, 2006; Queck *et al.*, 2008; Majerczyk *et al.*, 2010).

It should be noted that an increase in the activity of the quorum sensing system Agr might not only be caused by a growing cell count in a shaking flask with a constant volume. It could also be caused *vice versa*, at a constant cell count and a shrinking volume. Consequently, the Agr system might play a role in the escape from endosomes and phagosomes or even the host cell itself (Gresham *et al.*, 2000). Additionally, it might also be involved in local destruction of self-made *S. aureus* biofilms to facilitate spreading to new colonization sites (Yarwood *et al.*, 2004).

Additionally to quorum sensing as the major activatory stimulus of Agr transcription, other intra- and extracellular factors have been identified as well. On one hand, acidic pH has an inhibitory effect on the Agr system, from both, intracellular origin (due to glucose catabolism and lactate production) and extracellular orign. On the other hand, basic pH also inhibits Agr expression, giving an optimum of Agr activity at neutral pH (Regassa *et al.*, 1992; Weinrick *et al.*, 2004). The activity

of Agr can also be directly modulated by ROS (reactive oxygen species) that inactivate the pheromone via oxidation of the C-terminal methionine residue (Rothfork *et al.*, 2004).

Agr deletion mutants show reduced virulence in a rabbit endocarditis model (Cheung *et al.*, 1994), chronic osteomyelitis (Suligoy *et al.*, 2018), and in invasive infection of the lung (Heyer *et al.*, 2002). The Agr system also appears to be prone to accumulate spontaeous mutations *in vitro* and *in vivo*. Particularly, the 2KS-encoding *agrCA* appear as a hot spot for Agr mutations in strain NCTC8325 and its derivatives (Somerville *et al.*, 2002; Traber *et al.*, 2008; Shopsin *et al.*, 2010; James *et al.*, 2013; Shambat *et al.*, 2016; Sloan *et al.*, 2019). Naturally, these mutations can influence clinical pictures and epidemiology, and it was suggested that these mutations can be beneficial for *S. aureus* survival in human host infections, but are unfavorable for transmission among the human population (Shopsin *et al.*, 2010).

1.2.4 The Sar family

The major regulator of the Sar family is SarA (SACOL0672). Transcription occurs from 3 promoters in an Agr-dependent manner, with one promoter driven by a Sigma B consensus sequence (Cheung and Projan, 1994; Bayer *et al.*, 1996; Heinrichs *et al.*, 1996). SarA was first described as a regulator of *spa* and *hla* transcription. Similar to the Agr system, SarA exerts direct and indirect positive and negative regulation upon a broad set of early- and late stage virulence factors (Dunman *et al.*, 2001; Cassat *et al.*, 2006). However, some of these regulatory effects differ in a strain- and growth phase-dependent manner. The explanation for this observation are strain-specific mutations, e. g. in *rsbU* (needed for Sigma B activity; defective in NCTC8325), *agr*C (spontaneous point mutations in diverse clinical isolates and strains), and *tcaR* (truncated in strain NCTC8325). Sigma B and TcaR are activators of SarS (the major activator of *spa*), while the Agr system is a modulator of SarT (an activator of SarS). Given that SarA regulates *spa* transcription not only directly, but also via repression of SarS and SarT, it is not surprising that strain-specific mutations in Agr, Sigma B and / or TcaR lead to contradictory regulatory effects in SarA mutants via SarS and SarT on *spa* transcription (Chien *et al.*, 1999; Schmidt *et al.*, 2003; McCallum *et al.*, 2004).

To date, the modulation of SarA activity is unclear. Three potentially functional phosphorylation sites have been identified (Didier *et al.*, 2010; Ohlsen and Donat, 2010; Bäsell *et al.*, 2014). Additionally, the formation of N-terminal disulfide bridges might be functionally responsive to oxidative stress (Ballal and Manna, 2009 and 2010). These modifications might influence the DNA-binding activity of SarA and probably lead to a change of the three-dimensional structure of target promoter regions by SarA. SarA binding sites do not offer a clear DNA-binding consensus

sequence, but instead a remarkably high A/T-content. A/T base pairs are linked with only two hydrogen bonds and thus give the DNA a more flexible structure, compared to G/C base pairs that contain three hydrogen bonds. Together with the observation that several promoter regions contain up to three SarA Dimer binding sites, it is speculated that SarA is able to bend whole promoter regions (Schumacher *et al.*, 2001; Liu *et al.*, 2006).

SarA deletion mutants show reduced virulence in rabbit endocarditis models (Cheung *et al.*, 1994), in murine biofilm formation due to increased protease production (Zielinska *et al.*, 2012), and in murine septic arthritis and osteomyelitis (Blevins *et al.*, 2003).

Next to SarA, the Sar family also includes SarR, SarS, SarT, SarU, SarV, SarX, SarY, SarZ, MgrA, Rot, and TcaR. These regulators share a common winged-helix domain that enables DNA-binding. Nonetheless, the overall homology in the amino acid sequence of the Sar homologs is weak. Most Sar homologs bind to their target genes as dimers, except SarS, SarU and SarY. These three homologs are already encoded as two dimer-like domains. The literature provides a plethora of regulatory interactions on the transcriptional level between these regulators among each other, and with their target virulence genes as well, leaving the overall structure of this branched regulatory network unclear (for a review please note Cheung *et al.*, 2008). Two Sar homologs, SarZ and MgrA, show high homology to OhrR in *Bacillus subtills*. In fact, the activity of these regulators is modulated via the break-up of N-terminal disulfide bridges due to oxidative stress via an OhrR-like mechanism (Chen *et al.*, 2009; Poor *et al.*, 2009).

1.2.5 The two-component system SaeRS

The SaeRS 2KS-system is composed of the sensor kinase SaeS (SACOL0765), the response regulator SaeR (SACOL0766), and two accessory components, SaeP (SACOL0768) and SaeQ (SACOL0767). Transcription is induced by hydrogen peroxide and human α-defensin, indicating a role of SaeRS during internalization in PMNs (polymorphonuclear neutrophils) and the escape from professional phagocytosis (Geiger *et al.*, 2008). Additionally, *sae* transcription is modulated by other virulence regulators, but not *vice versa* (for a review please note Liu *et al.*, 2016).

SaeRS has been described as the major activator of a set of more than 20 virulence factors. Most of them are secreted exotoxins (including hemolysins, leukocidins, proteases, lipases, nucleases), and immune-response manipulating factors (e. g. the extracellular fibrinogen-binding protein Efb, the extracellular adhesion protein EAP, and superantigen-like exoproteins) (Rogasch *et al.*, 2006; Kusch *et al.*, 2011). The respective promoters do not offer a clear SaeR consensus sequence,

but since a regulatory influence on other regulators is missing, it is likely that regulation by SaeR occurs directly upon its target genes.

A point mutation in the sensor kinase SaeS was identified as a cause of high expression levels of *coa*, *fnbA*, *eap*, and *efb* in strain Newman (Mainiero *et al.*, 2010). In animal models, *sae* mutants showed reduced virulence in murine survival and skin infections (Voyich *et al.*, 2010), endocarditis (Xiong *et al.*, 2006), pneumonia (Montgomery *et al.*, 2010), and sepsis (Beenken *et al.*, 2014), underlining the pathogenic relevance of SaeRS in the regulation of virulence factors.

1.2.6 CodY: response to amino acid starvation

In many Firmicutes, the response to amino acid starvation has been well characterized as part of the so-called "*stringent response*". Amino acid-free tRNA blocks the ribosomal protein synthesis and acts as a signal for ppGpp synthesis (also referred to as ppGpp(p)) via the ribosome-associated RelA , with GTP and ATP as substrates (Natori *et al.*, 2009; Wolz *et al.*, 2010). The alarmone ppGpp affects several vital cellular processes, including transcription, translation, and DNA replication (Artsimovitch *et al.*, 2004). Furthermore, the consequent decrease in the intracellular GTP concentration leads to inactivation of the repressor CodY and consequently to a de-repression of CodY target genes that encode for amino acid biosynthesis and transport in *Bacillus subtilis* (Molle *et al.*, 2003; Mäder *et al.*, 2004; Tojo *et al.*, 2005; Natori *et al.*, 2009). This is because CodY repressor activity is dependent on the binding of GTP and branched-chain amino acids (Shivers *et al.*, 2004). Same for *S. aureus*, CodY (SACOL1272) represses a broad set of amino acid synthesis operons and transporters, but additionally, virulence factors as well (most notably extracellular proteases; Majerczyk *et al.*, 2008 and 2010). Many of these genes show a conserved DNA-binding consensus sequence in their promoter regions: the inverted repeat AATTTTC W GAAAATT. This is in accordance with the common CodY binding motif that was initially identified in *Lactococcus lactis* (den Hengst *et al.*, 2005; Guedon *et al.*, 2005). Taken these observations together, *S. aureus* stringent response to amino acid starvation does not only lead to up-regulation of amino acid biosynthesis and uptake, but also to the secretion of invasive proteases that generate new amino acid sources via destruction of host components.

A *codY* mutant was more susceptible during neutrophil phagocytosis (Geiger *et al.*, 2012). This is in accordance with the observations that the global expression pattern during neutrophil phagocytosis shows high similarity with that of amino acid starvation and mupirocin treatment (an inducer of stringent response) (Voyich *et al.*, 2005; Anderson *et al.*, 2006; Geiger *et al.*, 2010; Reiss *et al.*, 2012). Additionally, CodY defective strains produced more robust biofilms due to an elevated

expression of the *icaADBC* operon (Majerczyk *et al.*, 2008). Altogether, these findings indicate a significant role of CodY in *S. aureus* pathogenesis.

1.2.7 Sigma B: general stress response

In bacteria, the majority of the transcription of DNA into mRNA is carried out by a single RNA polymerase that is capable of elongation and termination, but fails to initiate transcription. Consequently, this core RNA polymerase is dependent on an additional factor for transcription initiation. In *S. aureus*, the major factor for transcription initiation is called the *"housekeeping Sigma factor"*, Sigma A, that recruits the core RNA polymerase to its respective promoters. Additionally, the transcription of minor sets of genes is initiated by the alternative Sigma factors B, H, and S (Morikawa *et al.*, 2003, Pané-Farré *et al.*, 2006, Miller *et al.*, 2012). Sigma B (SigB, SACOL2054) is the major regulator of general stress response in *S. aureus* and is activated by diverse environmental stress stimuli: heat shock, $MnCl_2$, NaCl, and alkaline shock (Pané-Farré *et al.*, 2006). The SigB system includes a set of modulatory proteins. Notably, the number of modulators is significantly reduced in *S. aureus*, compared to e. g. *Bacillus subtilis*. In non-stressed cells, SigB is unable to initiate transcription due to its binding to the anti-sigma factor RsbW. During stress, the anti-anti sigma factor RsbV is dephosphorylated by RsbU and RsbP. Dephosphorylated RsbV competes with SigB for the RsbW binding site and thus releases SigB. After stress, RsbV is phosphorylated again by RsbW, causing a restoration of the initial RsbW-SigB complex. This mechanism is called *"partner switching"* (Benson *et al.*, 1993; Dufour *et al.*, 1994; Pané-Farré *et al.*, 2009)

SigB binds to a conserved DNA-binding consensus sequence at its respective promoters: GTTTWW N_{12-15} GGGWAW (Petersohn *et al.*, 1999). This sequence can be found in the regulatory regions of 126 operons. Genes that are down-regulated in SigB mutants usually contain this sequence, whereas genes that are up-regulated do not (Gertz *et al.*, 2000; Bischoff *et al.*, 2004; Pané-Farré *et al.*, 2006). Genes under activatory control of SigB encode for stress response, cell envelope biosynthesis and turnover, metabolism, signaling pathways, and virulence factors. Genes encoding for adhesins, immune evasion, and biofilm formation are under activatory control of SigB, whereas genes encoding for toxins and exoproteins are under negative control. Consequently, it was supposed to understand SigB as an antagonist of the Agr system. Regulatory effects of SigB on its target genes can also be of indirect nature via SarA and SarS, whose transcription is driven by SigA and SigB promoters.

Despite the explicit involvement of SigB in the regulation of virulence factors, several attempts to demonstrate an impact on *S. aureus* pathogenesis in animal models failed (Chan *et al.*,

1998; Nicholas *et al.*, 1999; Horsburgh *et al.*, 2002). At least, in a more recent study, SigB mutants failed to form small colony variants (SCV) and were cleared by the host in chronic lung infections (Tuchscherr *et al.*, 2015).

1.3 Other pathogenesis-relevant adaptation mechanisms

1.3.2 Rex: anaerobic energy metabolism

The adaptation to an inoperative respiratory chain is important to cope with environmental oxygen starvation, but also plays a role in *S. aureus* pathogenesis. (1) Spontaneous mutations in *hemB* and *menD* cause deficiencies in cytochrome, and respectively menaquinone biosynthesis, as components of the respiratory chain. Due to their inoperative respiratory chain, these mutants exhibit a speudo-anaerobic gene expression profile. Growth of these mutants is impaired, causing small colonies on agar plates. These SCVs (small colony variants) play an important role in chronic and persistent infections, show resistance to antibiotic treatment due to their impaired growth rate, and are more resistant to the oxidative burst of professional phagocytes (Baumert *et al.*, 2002; Kohler *et al.*, 2003 and 2008; Bates *et al.*, 2003; Brouillette *et al.*, 2004; von Eiff *et al.*, 2006; Painter *et al.*, 2017). (2) The oxidative burst during phagocytosis into professional phagocytes is the major mechanism of *S. aureus* clearance by the immune system. Reactive oxygen species (ROS) lead to damage of intracellular components, including iron sulfur cluster-containing proteins of the TCA cycle and the respiratory chain. As a results, respiration is decreased and anaerobic metabolic pathways are up-regulated. This adaptation enables *S. aureus* to generate ATP in an alternative way, and simultaneously protects itself from addidional ROS production by its own respiratory chain (Chang *et al.*, 2006; Hochgräfe *et al.*, 2008; Painter *et al.*, 2015 and 2017; Rowe *et al.*, 2017). (3) Oxygen limitation can occur in deeper tissues within the human host and in *S. aureus* biofilms and abscesses (Coleman *et al.*, 1983; Park *et al.*, 1992; Cendra *et al.*, 2019).

Under aerobic conditions, *S. aureus* utilizes glucose by glycolysis, followed by the pyruvate dehydrogenase complex, and the TCA cylce. These pathways generate NADH (also referred to as $NADH+H^+$) that is reverted to NAD (also referred to as NAD^+) by the respiratory chain. This constant NAD / NADH cycle is the basis for ATP production (adenosine triphosphate). ATP can be understood as the universal energy currency for vital cell processes, e. g. biosynthesis of proteins, DNA, and RNA, and cell division. In the aerobic metabolic circuit, ATP is primarily produced by the respiratory chain due to the establishment of an electron transport chain across the membrane, generating a proton gradient that drives ATP synthases. Additionally, a minor amount of ATP is directly generated by glycolysis. Under anaerobic conditions (or in general: conditions where the respiratory chain is

impaired), NADH cannot be coverted back to NAD by the respiratory chain, and consequently, the NAD / NADH cycle is interrupted. As a response, the respiratory chain is replaced by anaerobic metabolic pathways, primarily anaerobic lactate fermentation, ethanol fermentation, and anaerobic nitrate and nitrite respiration (Fuchs *et al.*, 2007). These pathways restore the intracellular NAD / NADH cycle, with glycolysis now as the major producer of ATP.

In *Bacillus subtilis*, anaerbobic lactate production and secretion are repressed under aerobic conditions by the redox-sensing regulator Rex (YdiH) (Larrson *et al.*, 2005). This regulator contains a Rossman fold-like domain that is usually present in enzymes, e. g. dehydrogenases. Remarkably, in Rex this domain doesn't carry out enzymatic function, but instead, the binding of NAD / NADH acts as a sensory stimulus that activates (NAD) or inactivates (NADH) this repressor. Rex homologs are wide-spread among Gram-positives, but are also present in some Gram-negatives as well. Interestingly, some Rex homologs can also be found in obligate aerobes (e. g. *Streptomycetes*) and obligate anaerobes (e. g. *Chlortridiae*), usually not experiencing aerbic to anaerobic shifts (Fig. 1).

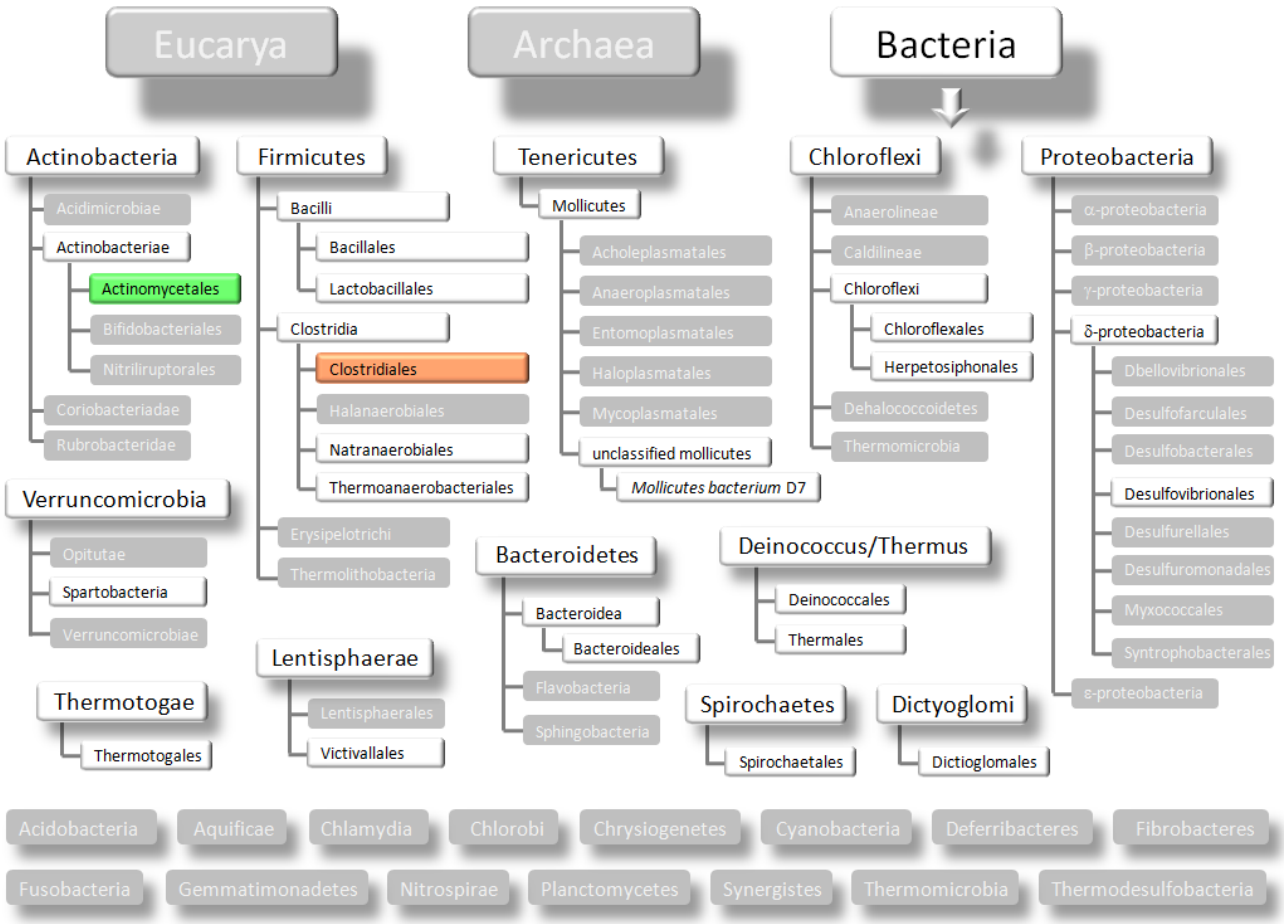


Fig.1. Distribution of Rex homologs among bacteria. White boxes indicate the presence of Rex homologs in the respective bacterial genera and phyla. Grey boxes indicate the absence. Two examples for obligate aerobes and obligate anaerobes are coloured (green: *Actinomycetales* = obligate aerobes; red: *Clostridiales* = obligate anaerobes).

These Rex homologs maintain the intracellular NAD / NADH homeostasis via the regulation of cytochrome expression, as part of the respiratory chain. Finally, a Rex homolog (SACOL2035) was identified in *S. aureus* as well, together with the localisation of a putative Rex DNA-binding consensus sequence in the regulatory regions of a set of anaerobically induced genes (Fuchs *et al.*, 2007). In the course of this PhD thesis, Rex is characterized as the major repressor of anaerobic metabolic pathways in *S. aureus* (Pagels *et al.*, 2010).

1.3.3 CtsR and HrcA: protein quality control

The regulatory system of protein quality control has been linked to pathogenesis in the comtext of protein repair, e. g. during the oxidative burst of professional phagocytes, and the modulation of virulence gene expression, probably due to degradation of Rot / RNAIII complexes (Clements and Foster, 1999; Frees *et al.*, 2003, 2005 and 2014; Chatterjee *et al.*, 2005; Michel *et al.*, 2006; Singh *et al.*, 2012; Gunaratnam *et al.*, 2019). In *S. aureus*, CtsR (**c**lass III **s**tress gene **r**epressor) appears as the major regulator of chaperone and Clp protease expression that refold, respectively degrade damaged proteins within the cell. CtsR binds to a direct repeat, A/GGTCAAANANA/GGTCAAA, in the promoter regions of *clpB*, *clpP*, the *clpC* operon (including *mcsA* and *B*), the *groELS* operon, and the *hrcA-dnaK* operon (Qoronfleh *et al.*, 1998; Kuroda *et al.*, 1999; Chastanet *et al.*, 2003). HrcA itself also represses its own *hrcA-dnaK* operon, and the *groELS* operon as well. Thus, in contrast to other bacteria, the HrcA regulon in *S. aureus* is embedded within the CtsR regulon, resulting in an overlap of regulation for class I (chaperones; usually repressed by HrcA) and class III (Clp proteases; usually repressed by CtsR) heat shock genes. Both operons are repressed under non-stress condition.

In *Bacillus subtilis*, CtsR is stabilized by McsA under non-stress conditions, but is destabilized by McsB under heat shock. As a conseqeunce of heat stress, CtsR is degraded by ClpP, and the expression of CtsR target genes is elevated. The resulting heat shock response includes protein refolding and degradation (Hecker *et al.*, 1996; Derré *et al.*, 2000, Krüger *et al.*, 2001; Elsholz *et al.*, 2010 and 2011). Folding of HrcA requires the chaperone GroE. If GroE is titrated due to an excess of misfolded proteins under stress conditions, HrcA becomes inactive and its target genes are de-repressed (Mogk *et al.*, 1997). Similar modes of action can be proposed for *S. aureus* as well.

1.3.4 PerR and Fur: oxidative stress response

In the course of infection, *S. aureus* faces oxidative burst during phagocytosis by professional phagocytes. Additionally, cell wall active antibiotics can induce oxidative stress as well (Kohanski *et al.*, 2007). As a consequence, DNA, proteins, and lipids are damaged by reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), superoxide ($^{o}O_2^-$), and hydroxyl radicals (OH^o). Surviving this part of the immune response can result in intracellular persistence of *S. aureus*. Nonetheless, oxidative stress response is not just about countering oxidative burst, but also has to adress ROS production as an unwanted by-product of the respiratory chain, and the uptake of iron that together with H_2O_2 causes DNA damage due to Fenton reaction (Repine *et al.*, 1981; Winterbourn *et al.*, 1995). Iron is a ligand of several detoxifying enzymes and a limiting factor for *S. aureus* in the human host. Consequently, *S. aureus* has to balance, and if needed, reduce its respiratory chain activity and iron uptake according to the current state of the intracellular oxidative stress level. *S. aureus* responds to oxidative stress with three different mechanisms: (1) Detoxification of ROS via superoxide dismutases (SodA, SodM), catalases (KatA), and alkyl hydroperoxide reductases (AhpC, AhpF), (2) repair of oxidized proteins via thioredoxin reductases (TrxA, TrxB) and a flavohemoprotein (Hmp), and (3) scavenging of ROS and oxidized amino acid residues via the small molecules Staphyloxanthin and Bacillithiol (Uziel *et al.*, 2004; Wolf *et al.*, 2008; Pöther *et al.*, 2013; Gray *et al.*, 2013; Posada *et al.*, 2014; for a review please note Beavers and Skaar, 2016). For some of these adaptation factors, a contribution to *S. aureus* pathogenesis has been demonstrated: KatA and AhpC are required for survival, persistence, and nasal colonization in mouse models (Cosgrove *et al.*, 2007). The presence of KatA also increased bacterial survival upon phagocytosis by macrophages (Das *et al.*; 2008 and 2009). Furthermore, *S. aureus* mutants of SodA and SodM were impaired during colonization in a mouse skin abscess model (Karavolos *et al.*, 2003), and particularly SodM was linked to *S. aureus* infections in cystic fibrosis (Treffon *et al.*, 2018).

It is noteworthy that this adaptation response is sequential and fine-tuned, activating only a set of components dependent on the kinds of oxidative stress and the level of current cell damage. The two major regulators in this scenario are PerR (SACOL1919) and Fur (SACOL1541). PerR was characterized as a repressor of *katA*, *trxB*, *ahpCF*, and iron storage. Under oxygen stress, especially in the presence of H_2O_2, PerR is inactivated due to histidine oxidation. This oxidation is more pronounced when iron is bound to PerR (Horsburgh *et al.*, 2001; Ji *et al.*, 2015). PerR also represses its own expression and that of Fur. Fur is a repressor of three different iron uptake systems: *sstABCD*, *sirABC*, and *fhuD2* (Horsburgh *et al.*, 2001). Fur binds to a conserved inverted repeat, GATAATGATAATCATTATC, when the regulator protein is associated with iron. There is evidence

that the expression of these transport systems is sequentially turned on an off, dependent on the extracellular and intracellular iron availability and the intracellular oxidative stress status (Morrissey *et al.*, 2002 and 2004).

1.3.5 Antibiotic resistance

Antibiotics are classified according to their chemical nature and the resulting mode of action: possible targets are the cell wall (penicillins), the cell membrane (polymyxins), protein biosynthesis (tetracyclins), vital enzymes (rifamycins), DNA synthesis (quinolones), and RNA synthesis (rifampicins). Since this PhD thesis presents results for cell wall active and cell wall stress-inducing antibiotics, the introduction will focus on that specific class.

Penicillin resistance in *Staphylococcus* was first observed in 1928 by Alexander Fleming as an inhibition of growth on agar plates due to a contamination with the fungus *Penicillium notatum*. Shorty after, Fleming predicted resistance development and demanded reasonable usage of this antimicrobial compound. In 1943, penicillin went into mass production during the Second World War, and at the end of the 1950s, further development from penicillin was made in terms of ampicillin, oxacillin, and the β-lactamase resistant methicillin. For all these antibiotics, resistant Staphylococci occured already several years after the starting of therapeutical treatments. The cellular target of β-lactam antibiotics is the transglycolylase-transpeptidase PBP2 (Giesbrecht *et al.*, 1998). It enables peptidoglycan envelope formation and cross-linking. Resistance to penicillin and ampicillin is caused by the β-lactamase blaZ that is located at transposon Tn552, either episomal on plasmid pI524 or chromosomal in the *S. aureus* chromosome (Massova *et al.*, 1998; Jensen and Lyon, 2009). Furthermore, PBP2a, a variant of the original PBP2, can cause resistance to methicillin and oxacillin as well. PBP2a is encoded as *mecA* on the staphylococcal chromosome cassette *mec*.

Over the last decades a large number of β-lactam antibiotics have been developed, e. g. cephalosporins, monobactams, carbapenems, and their semi-synthetic derivatives. However, only some of them were approved to treat *S. aureus* infections (Saravolatz *et al.*, 2011; Walsh and Wencewicz 2016). Additionally, glycopeptide (vancomycin) and semi-synthetic lipoglycopeptides (telavancin) were approved for MRSA (**m**ethicillin **r**esistant ***S***. ***a****ureus*), or rather VISA treatment (**v**ancomycin **i**ntermediate ***S***. ***a****ureus*) (Zeng *et al.*, 2016). The term „intermediate“ refers to the multi-step development of vancomycin resistance that requires the accumulation of several mutations for full resistance (for a review please note Foster, 2017).

At subinhibitory concentrations, cell wall active and cell wall stress-inducing antibiotics are able to cause a regulatory adaptation response in *S. aureus*. The major regulators that are involved in

this response are the two-component systems VraRS, WalKR (formerly known as VicRK or YycFG), and GraRS. These regulatory systems have been characterized to facilitate cell wall biosynthesis and / or turnover (Kuroda et al, 2000; Dubrac *et al.*, 2007; Meehl *et al.*, 2007; Dengler *et al.*, 2011). Furthermore, mutations in these regulatory systems have been observed in numerous VISA isolates and have been directly linked to that phenotype (Cui *et al.*, 2009; Galbusera *et al.*, 2010; Howden *et al.*, 2008 and 2010; Hafer *et al.*, 2012; Koch *et al.*, 2014). Among these regulatory systems, WalKR is already well-described to positively control genes, encoding for autolysis and cell wall degradation via a direct repeat: TGTAAT N_6 TGTAAT. Lowered levels of WalKR increase the length of peptidoglycan chains and its cross-linking. Cell wall biosynthesis was significantly reduced, giving an explanation for the findings of WalKR mutations in clinical VISA (Dubrac *et al.*, 2007).

2. Materials and methods

2.1 Strains, locus tags, and annotations

Most experiments and genomic analyses in this PhD thesis were carried out with strain COL (Shafer *et al.*, 1997). Gene constructs for the expression of recombinant regulator proteins in *E. coli* were derived from strain N135 genomic sequence (Kuroda *et al.*, 2001). For Rex transcriptional analyses, SH1000 was used (*Horsburgh et al.*, 2002). Functional annotations were carried out with the help of the online data base AureoWiki (https://aureowiki.med.uni-greifswald.de/Main_Page; Fuchs *et al.*, 2018). Locus tags for strain COL were used as standard reference in this PhD thesis because of two reasons: First, this PhD thesis analyzes DNA microarray data from more than 200 publications from the worldwide literature that predominantly used strain COL annotation. Second, most experiments in this PhD thesis were carried out in strain COL. Recently, NCBI / RefSeq provided a re-annotation for *S. aureus* genomes, resulting in new locus tags (Tatusova *et al.*, 2015, http://www.ncbi.nlm.nih.gov/refseq/about/prokaryotes/reannotation/). Both, the previous and the corresponding updated locus tags are presented in Table 1. These tags can also be found in the online database *AureoWiki*.

Table 1. *S. aureus* COL locus tags

symbol	previous tag	new tag	symbol	previous tag	new tag
	SACOL0007	SACOL_RS00055	*lukM*	SACOL2006	SACOL_RS10480
walR	SACOL0019	SACOL_RS00120	*hld*	SACOL2022	SACOL_RS10560
spa	SACOL0095	SACOL_RS00470	*agrB*	SACOL2023	SACOL_RS10565
adhE	SACOL0135	SACOL_RS00685	*agrD*	SACOL2024	SACOL_RS10570
narT	SACOL0166	SACOL_RS00845	*agrC*	SACOL2025	SACOL_RS10575
	SACOL0166	SACOL_RS00845	*agrA*	SACOL2026	SACOL_RS10580
pflB	SACOL0204	SACOL_RS01040	*rex*	SACOL2035	SACOL_RS10620
ldh	SACOL0222	SACOL_RS01135	*ilvA*	SACOL1772	SACOL_RS09065
rbsD	SACOL0252	SACOL_RS01280	*sigB*	SACOL2054	SACOL_RS10750
nirC	SACOL0301	SACOL_RS01520	*mtlF*	SACOL2146	SACOL_RS11235
sarA	SACOL0672	SACOL_RS03475		SACOL2333	SACOL_RS12265
sarX	SACOL0726	SACOL_RS03730	*lctP2*	SACOL2363	SACOL_RS12410
pgi	SACOL0966	SACOL_RS04950	*narK*	SACOL2386	SACOL_RS12520
	SACOL1045	SACOL_RS05335	*narG*	SACOL2395	SACOL_RS12565
hla	SACOL1173	SACOL_RS06010	*nirR*	SACOL2399	SACOL_RS12585
codY	SACOL1272	SACOL_RS06495		SACOL2491	SACOL_RS13055
ald1	SACOL1478	SACOL_RS07530		SACOL2517	SACOL_RS13180
adh1	SACOL1487	SACOL_RS10385	*ddh*	SACOL2535	SACOL_RS13280

Table 1. (continued) *S. aureus* COL locus tags

symbol	previous tag	new tag	symbol	previous tag	new tag
srrA	SACOL1535	SACOL_RS07825	*clpL*	SACOL2563	SACOL_RS13425
fur	SACOL1541	SACOL_RS07855	*arcA*	SACOL2657	SACOL_RS13915
hemE	SACOL1889	SACOL_RS09730		SACOL2706	SACOL_RS14155
perR	SACOL1919	SACOL_RS10030			

2.2 Primers

Primers that were used in this PhD thesis for regulator-promoter interaction studies, transcriptional analyses, and mutant construction are presented in Table 2.

Table 2. Primers that were used for regulator-promoter interaction studies

name	sequence (5‘-3‘)
SACOL0019_for	ATAAGACGGAAAATGCGCAC
SACOL0019_rev	CTTCCATACCATCACGACCA
SACOL0301_for	ACTTAATAAATGCTCACTGCC
SACOL0301_rev	CCTCACAATGACTCCTCGC
SACOL0660_for	GACACATTTTTTTGATCATAGC
SACOL0660_rev	GCTCTCATAATAATGTCCTCC
SACOL1778_for	ATACAACTATAAATCAAATGGAG
SACOL1778_rev	AACTAACATTTGCAACACTCC
SACOL1535_for	AAATGTTGTCGGTTTGAATGC
SACOL1535_rev	TTCGACATACAGGTCATACC
SACOL2006_for	AAGATGCAGGATATTATTTAGC
SACOL2006_rev	GCACATGATAATGATGACGC
SACOL2035_for	CATTCGATCTTCACCTTTCG
SACOL2035_rev	GTCACTCATTCGCTATTTCC
SACOL2363_for	GTACAATTCATTTTGATGAACAG
SACOL2363_rev	AAACGTATTTACTAACATAGGC
SACOL1889_for	GAAAATTGGAATAGTTGATGGG
SACOL1889_rev	GTTTTTATTATGCACCATAAAGG
SACOL2399_for	ATATACACTACAAGCGACCG
SACOL2399_rev	CCATTCACATTTACCAACCC
SACOL2535_for	TATTGCTCATTGAACATAGCC
SACOL2535_rev	TTGTCATTATTAAAAACCTCGC
SACOL2563_for	ATATTGAAAATGCAATGGATCC
SACOL2563_rev	CCGTTATTCATATAACATCACC
SACOL2657_for	TATACAACGTGTTTTTGTGGG
SACOL2657_rev	CGCTATTTACTTTAATTGGACC
SACOL_Sa5SA_for	AAAATTGTATAGAATGTGTATGG
SACOL_Sa5SA _rev	ATAGCCACCAGACAAAATAAC
Sa_ldh1_for	TAAAATGTGAAATAAATCACAATTTAAT

Table 2. (continued) Primers that were used for regulator-promoter interaction studies

name	sequence (5‘-3‘)
Sa_ldh2_for	TTATATGTGAAATAAATCACAAACTTAA
Sa_adhE_for	ATATGAAACACTTAATAAAGTGTTTGTA
Sa_nirC_for	AATTTTGTGAAATTATTCACAAATAAGA
Sa_adh1_for	TATAATGTGAATTAATTCACAAAGTATA
SACOL0135_for2	CATTTTTGAATCATCTAGCAGG
SACOL0135_rev2	ATATGTTGATCAACAGCTGC
SACOL0135_for	GCGATATAACAAGCTTTTTAGG
SACOL0204_for	GTGTAACAGAATGCAATTAGC
SACOL0204_rev	TCTTCCATTTTTAAATCCTTGCC
SACOL0222_for	ATCGTATACAAATTAAAAAGGTG
SACOL0222_rev	TTTGTTCATTACAAAAACTCCC
hla for1	CCCGACGAAATTCCAAACAT
hla rev1	ACTGAGCTGACTATACGTGT
SA1544_for1G60	CCATCCTCACATTGAAATTCC
SA1544_rev1G62	GGCCAGGTGTTAATAACAACGG

2.3 Purification of recombinant regulator proteins

S. aureus N315 whole genomic DNA was extracted by a comercially available kit (Promega). An overnight culture was grown in LB medium and centrifuged the next morning in a 1.5 ml Eppendorf tube. The cell pellet was resuspended in 480 µl TE buffer (10 mM Tris, 50 mM EDTA, pH 7.5). Cells were disrupted with the addition of 5 µl lysostaphin (10 µg / ml) at a temperature of 37°C for 30 minutes to 2 hours until the cell suspension had reached a viscous consistency. Cell debris were centrifuged for 5 minutes at 13,000g. The supernatant was removed and the remaining cell pellet was resuspended in 600 µl nucleic lysis buffer. Cell lysis was completed for 10 minutes at 80°C. After adding 3µl RNAse (4 mg / ml), incubation was carried out for 60 minutes at 37°C. The mixture was cooled to room temperature and 200 µl protein precipitation solution was added. After vortexing for 20 seconds, the mixture was cooled on ice for 5 minutes and centrifuged for 3 minutes at 13,000 G. The supernatant was transferred to a 1.5 ml tube and mixed with 600 µl 100 % isopropanol. After several turns, precipitated DNA threads became visible. The mixture was centrifuged for 3 minutes at 13,000G. After decanting the supernatant, the pellet was washed with 70 % ethanol and centrifuged for 3 minutes at 13,000G. The supernatant was removed and the pellet was air-dried for 15 minutes. Depending on the size of the pellet, 50-100 µl aqua bidest was added and the pellet was dissolved for 1 hour at 65°C or alternatively overnight at room temperature. The isolated chromosomal DNA was separated on a 1% TBE agarose gel at 150 V for 1 hour and checked for quality and quantity. The isolated DNA was stored at 4 ° C.

PCR products for the regulators of interest were amplified and cloned into pPR-IBA1 (IBA, Göttingen, Germany), following the manufacturer's instructions. Plasmid amplification was carried out in *E. coli* strain DH5α (Invitrogen). The insert was confirmed by sequencing (4base lab GmbH, Reutlingen, Germany). The recombinant *Strep*-tagII tagged regulators were expressed in *E. coli* that contained the plasmids BL21 and pLysS (Invitrogen) and purified by a Streptag purification column (IBA) according to the manufacturer's protocol.

It was reported for Rex in *Thermus thermophilus* that stoichiometric levels of NADH co-purified with the protein (Sickmier et al., 2005). To ensure that the Rex protein from *S. aureus* is free from NADH, the absorbance at 340 nm of the purified Rex protein was measured with nanodrop DN-1000 (Agilent, Böblingen, Germany) and ultrospec 3100 pro (Amersham biosciences, Freiburg, Germany). No absorbance peak at 340 nm (which would have been characteristic for NADH), was detected.

2.4 Regulator-promoter interaction studies

The upstream regions of several genes were amplified by PCR with synthetic oligonucleotides (Table 2). Purified PCR products (4 nM) were incubated with different amounts of purified regulator and 5 µg sheared salmon testis DNA as an unspecific competitor. Reaction buffers were set with 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 3% (w/v) Ficoll 400, 0.5 mM DTT, and 150 mM NaCl. Dependent on the regulator of interest, ligands were added: NAD / NADH for Rex, Ile and GTP for CodY, and DDT or H_2O_2 for SarA. Since NAD / NADH interfered with DNA separation, Tris buffer was replaced with HEPES buffer in the case of Rex. The regulator and the corresponding PCR product of the target gene were incubated for 15 min at room temperature. To confirm specific regulator-promoter interactions, replicates were carried out with 5 mg sheared salmon testis DNA. Each determination was repeated at least one time with PCR products that were obtained from an independent PCR.

For EMSA, non-denaturing 5.4% acrylamide / bisacrylamide gels and a mini gel apparatures with 1.5 mm spacers were used. The gel was polymerized for 10 minutes, run for 2 hours at 60 V, and stained with ethidium bromide (10µl / 50 ml aqua dest) for 2 minutes shaking. Afterwards, the gel was washed several times with aqua dest and PCR products were detected on a UV table.

For footprint analyses, regulator-promoter interaction samples were set as described above. Sequencing lanes and sample separation were carried out on an agarose sequencing gel and were detected by phospho-imager as described for primer extension analyses in 2.8 below.

For *"global footprinting"* (GF), salmon testis DNA was replaced by sonicated *S. aureus* COL whole genomic DNA as competitor DNA. Incubation of the regulator of interest with DNA was

performed as above, except that PCR products were replaced by sonicated *S. aureus* COL whole genomic DNA. The purification procedure was performed with a *Strep*-tag column as described for the purification of recombinant Rex protein. Consequently, regulator and DNA were separated by a PCR purification kit (QiaGen). Labeling, detection, and quantification of the enriched DNA fragments (*"next generation sequencing"*) was carried out by LGC Genomics (Berlin, Germany).

2.5 Site-directed mutagenesis

The regulatory region of *adhE* was amplified by PCR with synthetic oligonucleotides (Table 2) and cloned into the plasmid pRSETA (Invitrogen) with XhoI and PvuI digest. *E. coli* DH5α (Invitrogen) was used for plasmid amplification. Site-directed mutagenesis was performed with the GeneTailor site-directed mutagenesis kit (Invitrogen) according to the manufacturer's protocol. The mutated inserts were confirmed by sequencing (4base lab GmbH) and analysed in EMSA.

2.6 Construction of chromosomal *rex* mutants

The construction of *S. aureus* Δ*rex* in strain SH1000 that was used in primer extension analyses was carried out by Proctor *et al.* (for details please see Pagels *et al.*, 2010). Chromosomal deletion mutants and their complementants that were used in all other experiments were constructed in the course of this PhD thesis, following the same protocol that was described by Kraemer and Iandolo (1990). The wild type *rex* gene was derived from *S. aureus* N315 genomic DNA as described for the expression of Rex protein. The sequence of wild type *rex* and its regulatory region upstream are identical for all strains that were used in this study. PCR was used to amplify the flanking regions with the following primers:

F1 (GCGAATTCCAGAATCATAGTTTTCTACACCAGC)
R1 (CATTCACTCACTCACCATGGTCGCTATTTCCTCCTTCG)
F2 (CGACCATGGTGAGTGAGTGAATGTGATGTTAGCTTTGAATGATAA)
R2 (CGGAATTCCGTCAAAATCAATAATCCTATACTTTG)

PCR products were digested with NcoI, ligated, cloned, and amplified with PCR. This procedure resulted in a Δ*rex* locus with no antibiotic cassette. The deletion locus was digested with EcoR1 and cloned into the vector pMAD (Arnaud *et al.*, 2004). The amplified plasmid was electroporated into *S.*

aureus strain RN4220 and subsequently transferred into the respective target strains via phage transduction. The vector pMAD carries an erythromycin resistance, an X-Gal system, and *in vivo* replication of this vector is thermosensitive. Consequently, growth at 42°C in the presence of erythromycin forced the integration of the vector into the chromosome via homologous replication at the *rex* locus. The following excision of the vector was carried out at 30°C and verified by picking white colonies on X-Gal agar plates. Clones that still carry the vector appear blue, whereas clones that lost the vector appear white. The *rex* loci of the resulting white clones were checked for deletion of *rex* via PCR and sequencing.

Electroporation: *S. aureus* RN4220 was used as a passage strain to transfer plasmid DNA into staphylococci. 50 µl of the electro-competent cells were incubated with 300 ng plasmid on ice for 30 min. The cells were placed into an electroporation cuvette and were suscepted to electric shock (1.5 kV; 100 Ω; 25 µF). Cells were immediately incubated with pre-warmed (37°C) 900 µl SOC medium (10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, and SOB medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0). The mix was incubated for 90 min at 37° and plated on erythromycin-containing agar plates.

Phage lysates: Overnight cultures (LB medium, 37°C) of *S. aureus* RN4220 that contained the plasmid were incubated with $CaCl_2$ to a final concentration of 5 mM. Cultures were grown for another 30 minutes. 0.3 ml of each culture was incubated at 52°C for 2 minutes. 0.1 ml of a dilution series of phage lysate (phage 80, taken from *S. aureus* NCTC8325-4) was added. After 15 minutes of incubation at room temperature, 4 ml of pre-warmed liquid soft agar (52°C) was added, containing 5 mM $CaCl_2$. The mixtures were poored on blood agar plates and incubated at 30°C over night. The following day, plates with confluent lysis were harvested. For the harvesting of the phages, 3 ml of phage buffer (LB, containing 5 mM $CaCl_2$) were poored on the plates. After mingling the buffer with the soft agar layer, the liquid was transferred to an Eppendorf tube and centrifuged at 7,900 g for 10 minutes. The supernatant was sterile-filtered with a pore size of 0.45 µm. The phage lysates were stored at 4 ° C.

Phage transduction: To transduce the plasmid into the target strains, overnight cultures were supplemented with $CaCl_2$ to a final concentration of 5 mM and grown for an additional 30 minutes. After incubation of 0.3 ml of the culture for 2 min at 52°C, phage lysates were added in a dilution series. After 15 minutes of incubation at room temperature, the suspension was mixed with 3 ml pre-warmed LB soft agar (52°C), supplemented with 20 mM Na_3-citrate, and poored on LB agar plates, supplemented with X-Gal. The subsequent incubation was performed at 42°C for integration or 30°C for excision respectively. After several days, white colonies were picked from the plates, chromosomal DNA was isolated, and the *rex* locus was amlified with PCR and sequenced. Verified rex deletions were subsequently used in follow-up experiments.

This method was also used to complement the chromosomal *rex* deletion mutants with *rex93* and its controls in *cis* and *trans*. Additionally, for the construction of the mutated *rex93* locus, site-directed mutagenesis was used as described above for the regulatory region of *adhE*.

2.7 Growth conditions

For cultivation experiments, *S. aureus* COL was grown under aerobic conditions in 100 ml liquid broth (LB, Invitrogen, Karlsruhe, Germany) in 500 ml Erlenmeyer flasks under vigorous agitation at 37°C to an optical density of 1.0. A part of the culture was shifted to anaerobic growth by transferring 50 ml of the culture to Falcon tubes which were completely filled with bacterial cell culture and incubated under vigorous agitation at 37°C. The remaining part of the culture was cultivated under aerobic conditions. Anaerobic conditions were verified in parallel cultures by using 0.001% resazurin as a redox indicator that changes its colour from violett over pink to colourless during oxygen depletion (Fuchs *et al.*, 2007).

2.8 Transcription analyses

For Northern blot analyses, total RNA was isolated with a modified acid phenol method (Fuchs et al., 2007). Northern blot analyses were performed according to the protocol that was published by (Levi *et al.*, 1992). Digoxygenin-labelled RNA probes were prepared with PCR and synthetic oligonucleotides:

rex_for	(TGAGTGACCAAGTTAAAATTCC)
rex_rev1	(CTAATACGACTCACTATAGGGAGATAATAATGACTGTAATTCTATACC)
ldh1_for	(CATGCCACACCATATTCTCC)
ldh1_rev1	(CTAATACGACTCACTATAGGGAGAGCTGATACAGTCAATACGGC)
ddh_for	(GCAGAATGTGCTTTTGCAGG)
ddh_rev1	(CTAATACGACTCACTATAGGGAGAGCAGAATGTGCTTTTGCAGG)
adhE_for	(ATGCTCTAGCTGACAAAGGG)
adhE_ rev1	(CTAATACGACTCACTATAGGGATGTGCACTTGGATGGAATGC)

T7 RNA polymerase was used for *in vitro* transcription of the respective PCR products with a NTP labelling mix (Roche Diagnostics, Mannheim, Germany). The mixture was centrifuged and incubated

for 2 hours at 37°C. The reaction was stopped by adding 2 µl EDTA (200 mM). Afterwards, the labeled RNA was precipitated by adding 2.5 µl LiCl (4 M) and 75 µl cooled pure ethanol for 30 minutes at -80°C. After centrifugation for 10 minutes at 12,000 G, the supernatant was removed and the RNA pellet was washed with 75 µl 70% ethanol. After centrifugation for 10 minutes at 12,000G, the supernatant was removed and the pellet was dried under the hood. The pellet was disolved in 100 µl sterlie aqua dest for 30 minutes at 37°C and stored at -80°C. For quality control, 1 µl of a dilution series of the probe (1:10 to 1:100,000) was applied to a nylon membrane, cross-linked with UV light, and detected. Depending on the quality, 5-20µl probe was mixed with 5 ml pre-hybridization solution and stored in a 15ml Falcon tube at -80°C.

The denaturating gel was prepared with 3 g agarose, 20 ml buffer (200 mM MOPS, 5 mM natrium acetate pH 5.2, 1 mM EDTA, pH adjusted to 7.0, autoclaved) and filled to 200 ml aqua dest. The mixture was placed in an autoclaved glass bottle and dissolved in the microwave with occasional mixing. The gel was tempered at 65°C for one hour. After adding 34 ml of formaldehyde (35%) and mixing, the gel was poured and polymerized for 30 minutes. 15 µg total RNA was used for northern blot analyzes. The samples were filled to a common total volume with sterilized aqua dest and supplemented with sample buffer. After 10 minutes of incubation at 65°C, the samples were centrifuged and applied to the gel. The gel was run at 150 V for 1.5 hours. Afterwards, the formamide gel was irradiated on the UV table for 2 minutes. The blot apparatus was assembled and the nylon membrane was washed with aqua bidest. The gel was placed on the membrane and a constant pressure of 55-60 mbar was applied. The gel was overlaid with denaturing solution (50 mM NaOH, 10 mM NaCl, autoclaved). After 5 minutes, the solution was removed and the gel was overlaid with neutralizing solution (0.1M Tris-HCl pH 7.4, autoclaved). After five minutes, the solution was removed and the gel was overlaid with transfer buffer. The buffer was occasionally replenished during the 6 hour blotting. After the blot, the RNA and the membrane were cross-linked for 1 hour by heat at 140°C. For quality and quantity control, the membrane was stained with methylene blue for 1 minute, washed twice with aqua bidest, and was scanned.

For detection, a detection tube and a turning oven were used. The tube was equipped with the membrane and 30 ml of pre-heated (65°C) pre-hybridization solution. The incubation was carried out with constant turning for 2 hours at 65°C. The pre-hybridization solution was then poured off and replaced by the probe-carrying hybridization solution. Hybridization was done overnight and at 65°C. Unbound probes were washed off the membrane with 50 ml of transfer buffer for 5 minutes at room temperature. The solution was decanted and the washing step was repeated. Afterwards, the membrane was washed with 50 ml transfer buffer for 15 minutes at 65°C. The solution was decanted and the step was repeated. The washing solution was decanted and the membrane was incubated with 50 ml of pre-detection buffer for 1 minute at room temperature. At this point, quality control

membranes with probe dilutions were added. From here, all steps were carried out at room temperature. The buffer was decanted and replaced with 100ml washing buffer. The incubation was carried out for 30 minutes at room temperature. The buffer was decanted and replaced with freshly prepared diluted antibody conjugate. After incubation for 30 minutes, the buffer was decated and replaced with 100 ml of washing buffer. The washing step was carried out over 15 minutes and was repeated. The buffer was decanted and replaced with 20ml detection buffer with an incubation for 4 minutes. The membrane was removed from the tube, incubated with substrate solution (light-sensitive "CDP star" stock solution diluted 1:200 in detection buffer), and covered with foil. The incubation was carried out for 4 minutes in a closed detection cassette. Afterwards, excess of the solution was carefully wiped with a cellulose paper. Chemiluminescent signals were detected with a Lumi-Imager and visualized with LumiAnalyst (RocheDiagnostics).

For primer extension analyses, synthetic oligonucleotides (Table S2) were labeled with [P^{32}]-ATP. For the sequencing lanes, upstream regions were amplified with synthetic oligonucleotides (Table 2) by PCR and used as templates. Sequencing was performed according to the method of Sanger *et al.* (1977). Five micrograms of total RNA were used for reverse transcription with SuperScriptII reverse transcriptase (Invitrogen). Sequencing and reverse transcription reactions were analysed on polyacrylamide sequencing gels and visualized with a phosphor storage screen and a typhoon scanner (Amersham Biosciences). All experiments were carried out in duplicate.

2.9 Cluster analysis

In the course of cluster analysis, the expression profiles of genes were clustered with the online tool Multi Experiment Viewer MeV (http://mev.tm4.org/). This tool is commonly used for the quality control of DNA microarray data. Cluster files were vizualised with the online tools iTOL (interactive tree of life, https://itol.embl.de/) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Cluster trees for homology analyses of Rex and SarA are based on amino acid seuqence alignments generated with the online tool *"ClustalW"* (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

3.1 Part one: Regulator-promoter interactions for three selected regulators

3.1.1 Rex: anaerobic energy metabolism

3.1.1.1 Rex homologs and their functional properties

A total of 246 Rex homologs is wide-spread across Gram-positive bacteria, but can also be found in some Gram-negatives as well (down to 35% identity and 55% similarity in amino acid sequence to *S. aureus* Rex). Rex homologs have been described to regulate the expression of the respiratory chain in *Streptomyces coelicolor* and *Thermus thermophilus*, and the respiratory chain and lactate fermentation in *Bacillus subtilis* (Brekasis and Paget, 2003; Sickmier *et al.*, 2005; Larsson *et al.*, 2005; Gyan *et al.*, 2006; Nakamura *et al.*, 2007; Wang *et al.*, 2008).

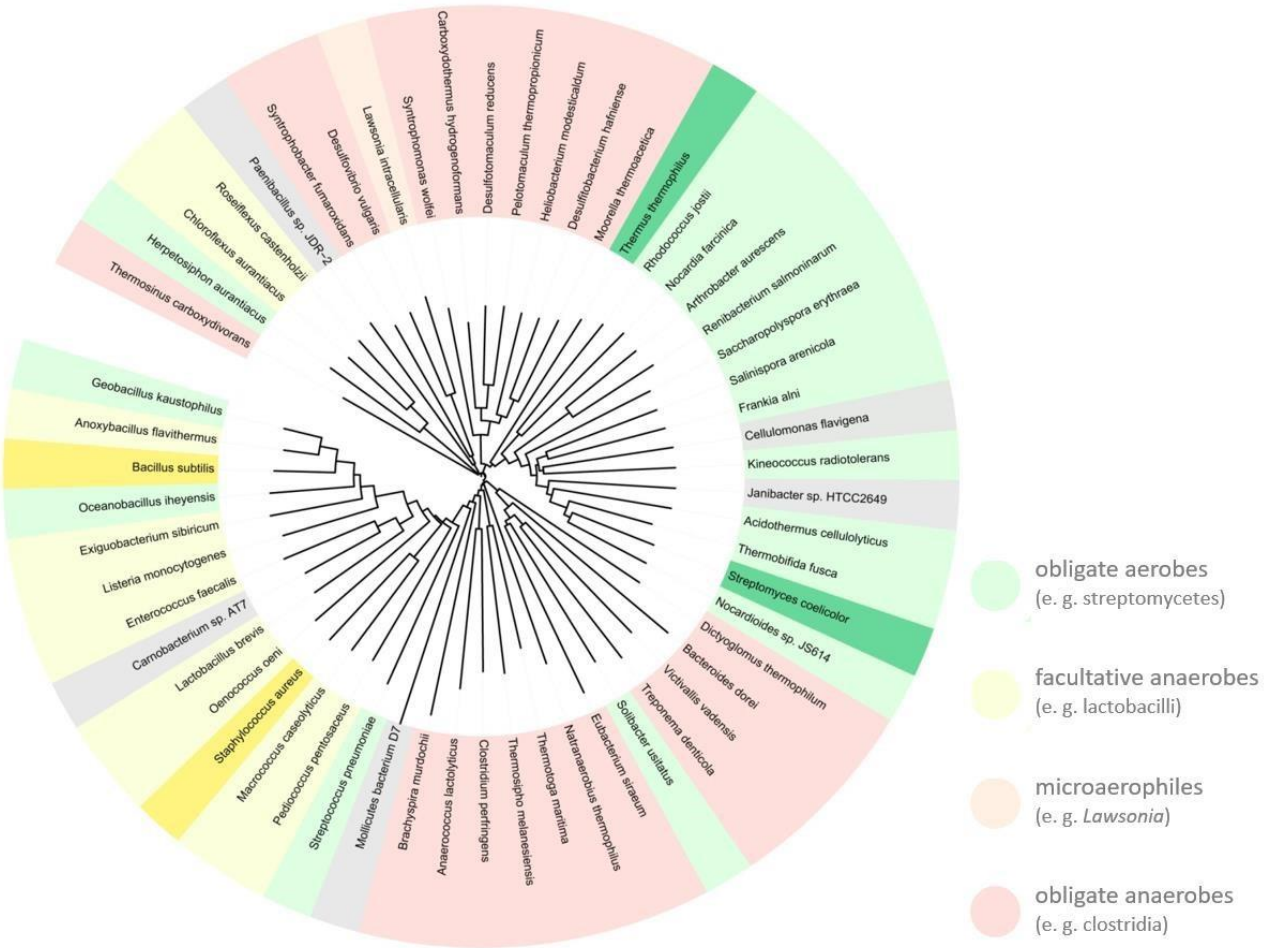


Fig. 2. Homology tree of Rex homologs among bacteria. The colours describe the behaviour of the respective bacteria towards oxygen availability. Bacteria whose Rex homologs are functionally described in the literature are in bold colours.

Astonishingly, some Rex homologs are present in obligate aerobes and obligate anaerobes that usually do not deal with aerobic to anaerobic shifts. This finding indicates that oxygen is unlikely to be the signal for Rex activity and function. A detailed overview of Rex homologs and their corresponding bacteria's behaviour towards oxygen availability is presented in Fig. 2 as a homology tree.

The Rex protein does not contain any oxygen-sensing domains, e. g. iron sulfur clusters. Instead, a Rossmann fold-like domain is present in all Rex homologs and contains highly conserved amino acid residues that can be found in enzymatic enzymes that are involved in NAD / NADH metabolism, e. g. dehydrogenases. Fig. 3 presents amino acid sequence alignment of 65 homologs, representing 65 bacterial genera from 12 bacterial phyla. Despite this taxonomic diversity, some Rossmann fold-like residues were 100% conserved among all Rex homologs: a glycine repeat, GXGXXG, and a flanking asparitc acid residue. A second domain shows similarity to common DNA-binding motives (helix-turn-helix) and also contains residues with 100% conservation among all Rex homologs. A third domain can be functionally linked to dimerization, though conservation in that domain was weaker.

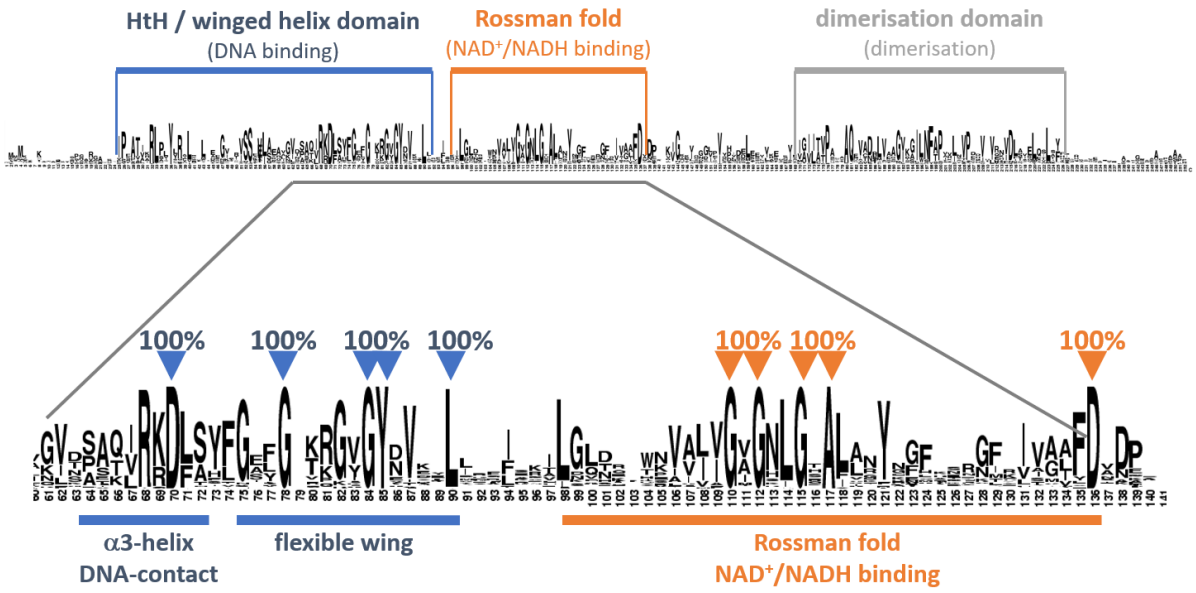


Fig. 3. Amino acid sequence alignment of 65 Rex homologs, representing 65 bacterial genera in 12 bacterial phyla. The *Seq2Logo* web tool was used to depict conservations: the bigger the letter, the higher the conservation. Positions with low conservation might contain two or more dominant residues. The DNA-binding domain is coloured in blue. The Rossmann fold-like domain is coloured in orange. The dimerization domain is greyed out. Residues that are perfectly conserved among all Rex homologs are labeled with "100%".

3.1.1.2 Identification of Rex target genes

Fuchs *et al.* (2007) identified a multitude of anaerobically induced genes in *S. aureus* via combining Proteomics, DNA microarray analysis, and determination of metabolites. A conserved DNA binding motif, TGTGA N_6 TCACA, was discovered in the regulatory regions of a set of these genes. This motif was already known as a regulatory motif of several regulators in other bacteria, particularly Rex in *Bacillus subtilis* (Wachenfeldt *et al.*, 2005). Consequently, this motif was proposed to be a putative Rex binding motif in *S. aureus*.

This PhD thesis verifies that Rex (SACOL2035) in *S. aureus* is able to bind to this motif. Recombinant *Strep*-tagged Rex protein was expressed in *E. coli*, purified, and incubated with a PCR product that was derived from the regulatory region of the anaerobically induced gene *adhE* (SACOL0135; ethanol dehydrogenase). The PCR product was digested with XmnI, a restriction endonuclease, cutting at GAANN / NNTTC, that overlaps with the putative Rex binding site. With increasing concentrations, Rex was able to protect its proposed binding site from enzymatic digest. DNA fragmentation was made visible by using agarose gel electrophoresis and Ethidium bromide staining (Fig. 4).

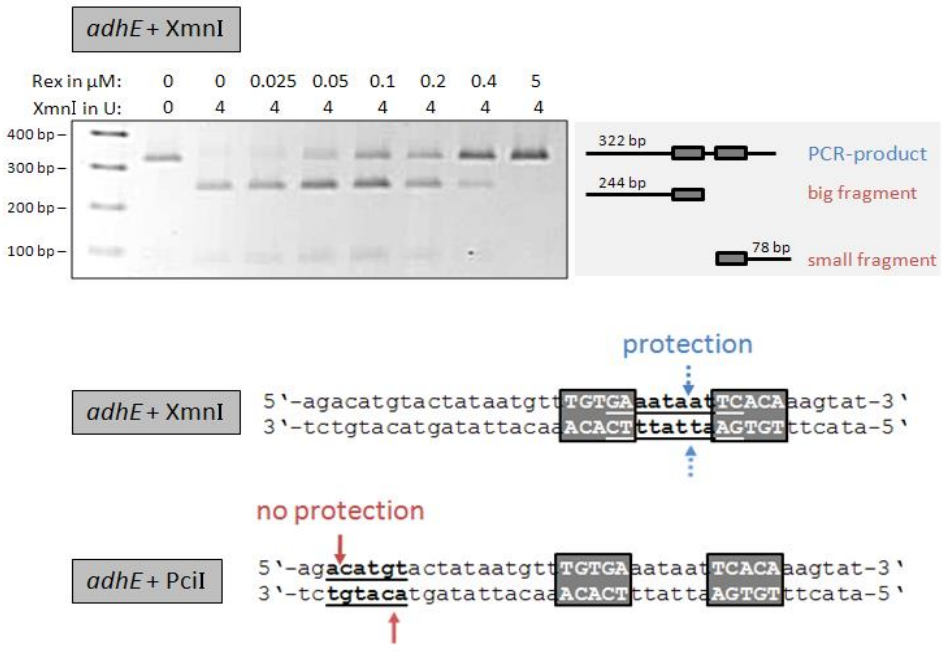


Fig. 4. REPA (*restriction endonuclease protection assay*) for the regulatory region of *adhE* (top left). Depiction of the fragmentation pattern (top right). Depiction of the DNA sequence, including protected XmnI-digest (middle). Depiction of unprotected PciI-digest (control; bottom). Restriction endonuclease cutting sequences are in bold and underlined. The putative Rex binding site is shown in grey boxes and white capital letters.

As a control, digest by PciI, another restriction endonuclease, cutting 12 base pairs upstream of the putative Rex binding site, was not protected by Rex (Fig. 4, bottom). This inability of protecting was specific for this regulatory region, since Rex was in fact able to protect from PciI digest in another regulatory region (SACOL2657; *arcA*) where the Rex motif and the PciI cutting sequence are overlapping (data not shown). These data confirm that Rex binds to the motif TGTGA N_6 TCACA in *S. aureus*.

After verification that Rex truly binds the proposed motif, all regulatory regions of anaerobic genes that contain this motif were subjected to regulator-promoter interaction studies. For this purpose, EMSA (***e****lectro* ***m****obility* ***s****hift* ***a****ssay*) was used. Recombinant Rex was incubated with the PCR product of the promoter region of each gene. Due to binding of Rex, the PCR product was attenuated during the run through a native polyacrylamid gel and subsecquently detected by Ethidium bromid staining. In these experiments Rex bound to the promoter regions of a high number of anaerobic genes (in contrast to e. g. *Bacillus subitlis*): *ddh* (SACOL2535, encoding a D-lactate dehydrogenase), *ldh1* (SACOL0222; L-lactate dehydrogenase), *lctP2* (SACOL2363; lactate transporter), *pflB* (SACOL0204; leading gene of the *pflBA*-operon; pyruvate formiate lyase), *adhE* (SACOL0135; ethanol dehydrogenase), *adh1* (SACOL1487; ethanol dehydrogenase), *narG* (SACOL2395; leading gene of the *nar*-operon; nitrate respiration), *nirR* (SACOL2399; regulator and leading gene of the *nir*-operon; nitrite respiration), *nirC* (SACOL0301; nitrite transporter), *arcA* (SACOL2657; leading gene of the *arc*-operon, encoding the arginine deiminase pathway and the regulator ArcR), *ald1* (SACOL1478; alanine dehydrogenase), *srrA* (SACOL1535; leading gene of the *srrAB* operon, encoding a regulatory two-component system), and *lukM* (SACOL2006; encoding a leukocidin) (Fig. 5).

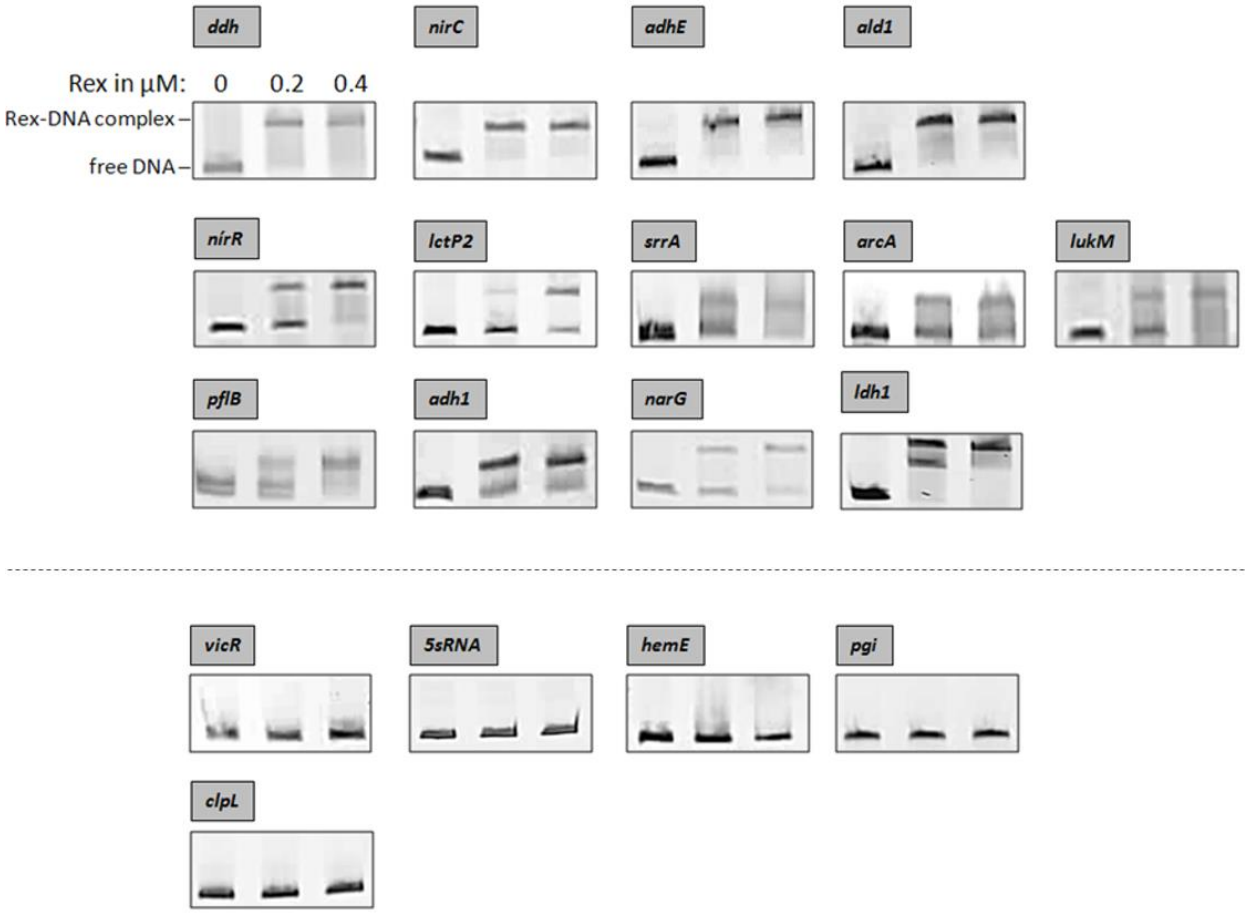


Fig. 5. EMSA with regulatory regions of anaerobic genes that contain a Rex motif (top), and controls (bottom). The four genes in the upper row showed highest affinity in Rex-binding.

Rex did not bind to the following five genes: (1) *vicR* (SACOL0019; also known as *yycF* or *walR*), a response regulator that facilitates cell wall turnover and thus could play a role in slow-growing anaerobic cells, (2) the ribosomal *5sRNA* (SACOLSa5SA), (3) *hemE* (SACOL1889), which contributes to the synthesis of components of the respiratory chain (known to be under Rex control in other bacteria), (4) *pgi* (SACOL0966), as part of the glycolysis, and (5) *clpL* (SACOL2563; Clp-protease). *5sRNA* contains a putative Rex motif, but is not anaerobically induced. *VicR* and *pgi* contain lesser conserved Rex motives, only the latter gene is anaerobically induced. *HemE* and *clpL* do not contain Rex motives, and only the latter gene is anaerobically induced. Rex did not bind to any of these regulatory regions.

Please note that all EMSA were performed with PCR products, using chromosomal DNA from strain COL, except for *narG*. The Rex binding site in this strain has a base pair deletion in its spacer. Consequently, chromosomal DNA from strain N315 was used in the case of *narG* to verify Rex binding.

3.1.1.3 Characterization of the Rex consensus sequence

The finding that Rex is unable to bind to the regulatory region of *5sRNA* was expected from the physiological point of view, since this gene is not up-regulated under anaerobic conditions. Nonetheless, it was very surprising from the molecular point of view, since the promoter region of *5sRNA* contains a perfect putative Rex binding site: TGTGA N_6 TCACA. As a conclusion, there must be additional base pairs and / or yet unknown features that prevent the binding of Rex to this motif. Consequently, the verified Rex binding sites were investigated with a detailed *in vitro* sequence alignment. This analysis resulted in the identification of an extended Rex motif: TTGTGAA W_4 TTCACAA, from this point on referred to as the Rex consensus sequence.

Hereafter, EMSA were used to quantify the exact dissociation constats (K_d) of four selected Rex binding sites. The dissociation constant is the concentration of regulator protein that is needed to achieve a shift of 50% of the PCR product. According to the predicted Rex consensus sequence, the binding site with a perfect consensus sequence provided the highest affinity (*adhE*) (Fig. 6). The presence of one mismatch led to a two-fold decrease in Rex affinity (*adh1*). The presence of three mismatches led to a 4.5-fold decrease in affinity (*nirR*). The regulatory region of *5sRNA* (no affinity) also contains three mismatches, and additionally, two of these mismatches showed a shift from A/T to G/C. Since Rex has no affinity to the *5sRNA* promoter region, it was consequently proposed that the more structural flexibility of A/T base pairs, resulting from two hydrogen bonds, instead of three (as it is for G/C base pairs), is crucial for Rex binding. This presumption explains why Rex does not bind to the *5sRNA* promoter region.

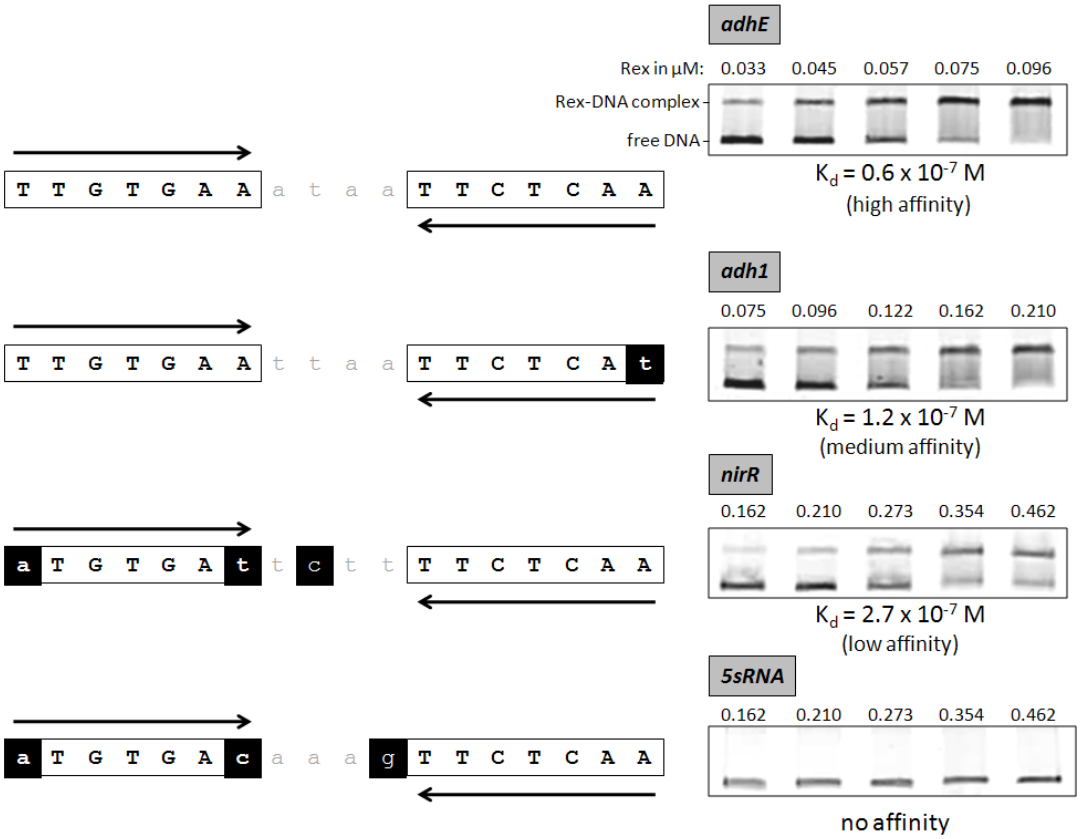


Fig. 6. Characterization of mismatches in Rex binding sites in the regulatory regions of selected genes (left). The inverted repeat is marked with arrows and white boxes. Matches to the Rex consensus sequence are shown in capital letters. Mismatches are shown in lower letters, marked with black boxes. EMSA were used to determine the respective dissociation constant (K_d) for Rex (right).

Subsequently, the proposed Rex consensus sequence, TTGTGAA W_4 TTCACAA, was additionally verified experimentally with site-directed mutagenesis for the Rex binding site of *adhE* (Fig. 7). This regulatory region was chosen because it contains a perfect Rex consensus sequence and showed highest affinity. Several single to triple mutations were introduced into the inverted repeat, as well as a deletion, an insertion, and A/T to G/C shifts in the spacer region. Mutations in the centre of the inverted repeat led to pronounced decreases in Rex affinity (mutants 1 to 3). Deletions and insertions in the spacer region led to a complete loss of Rex affinity (mutant 4 and 5). These findings suggest that the sequence of the inverted repeat and the spacer size are crucial. A mediate decrease was observed for a double mutant in the spacer region that caused A/T to G/C shifts (mutant 5). This decrease was abscent in a mutant at the same positions that preserved the A/T content (mutant 4). This finding finally proves that solely the structural flexibility of the A/T spacer region is important, but not the exact sequence.

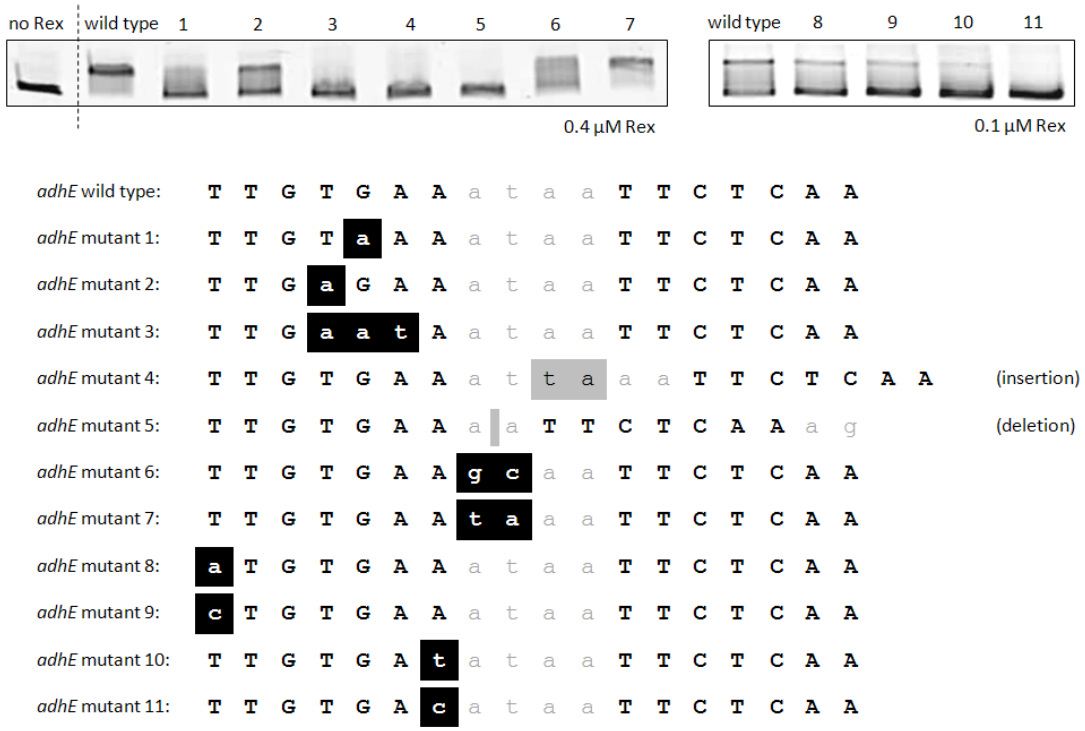


Fig. 7. Rex affinity, determined by EMSA (top), to several consensus sequence mutants (bottom). The consensus sequence is in bold and capital letters. Mutations are indicated by boxes.

To investigate sequence properties that have a minor impact on Rex binding, Rex concentration was lowered to 1/4 (Fig. 7, top right). This is because single mutations in less important positions were not sufficient to generate a visible effect under high Rex concentrations, since the affinity of Rex to the *adhE* binding site is high. A comparison between mutant 8 and 9 indicates that the sequence of the outermost base pair of the inverted repeat has only a minor effect on Rex affinity, and that the structural flexibility is not important at this position. In contrast, an A/T to G/C shift in the innermost base pair of the inverted repeat truly led to a significant decrease in Rex affinity (mutant 10 and 11). This finding indicates that the importance of structural flexibility even exceeds the spacer region for one base pair, reaching into the inverted repeat itself.

Altogether, these data verify the Rex consensus sequence, TTGTGAA W_4 TTCACAA, and the importance of a structurally flexible A/T rich spacer (W_4). Logically, it was of interest to use this fully characterized sequence in a genome-wide search in the *S. aureus* genome, to identify additinional Rex target genes that have not been detected in the first run. As a result, additional 13 motives with one or two mismatches were found, including the anaerobically induced genes *mtlF* (SACOL2146; leading gene of the *mtl*-operon; manitol-speficic PTS system), SACOL0166 (conserved hypothetical protein), and SACOL2491 (hypothetical protein), indicating the existence of additional Rex target genes.

3.1.1.4 The effect of Rex-binding

After the comprehensive identification of Rex binding sites in the promoter regions of target genes, the logical follow-up study was to identify the regulatory mode of action of Rex-binding on the transcription of these genes. Consequently, transcription start point mapping („*primer extension*“) was performed for the genes *ldh1* (lactate fermentation), *pflB* (pyruvate formiate lyase complex), and *adhE* (ethanol fermentation). To that date, the promoters of these genes have not been identified. All respective Rex binding sites appeared as typical repressor sites that overlap the -35 region or locate in close poximity of 5 or less base pairs to the -35 region or the -10 TATA box of Sigma A promoters (Fig. 8).

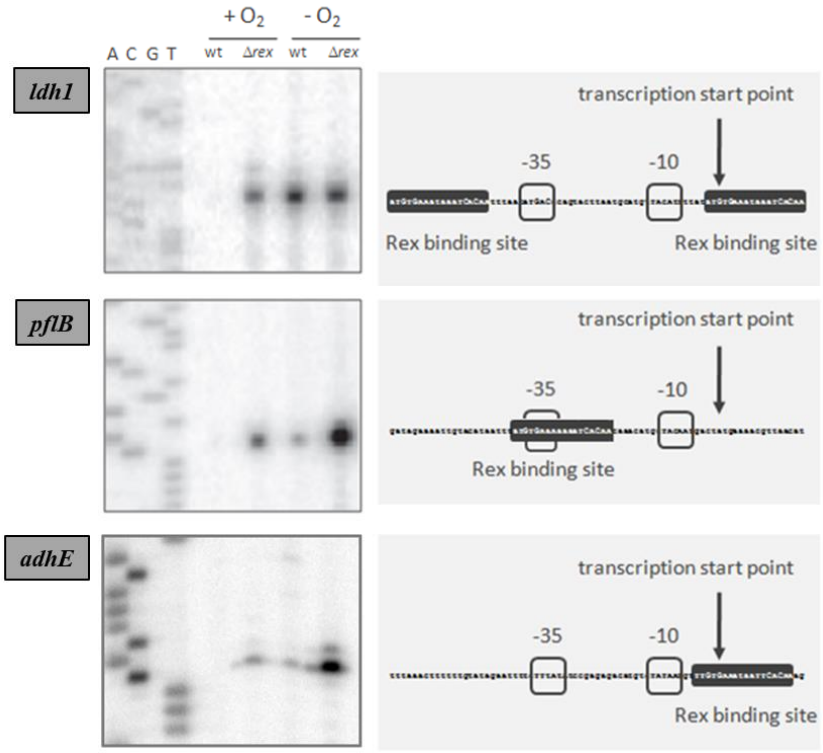


Fig. 8. Transcription start point mapping (left) and co-localization of transcription start points with verified Rex binding sites (right). Sequencing lanes are labled with A, C, G, and T. Whole mRNA samples were taken from strain SH1000 wild type (wt) and its isogenic *rex* deletion mutant (Δ*rex*) under aerobic (+O_2) and anaerobic conditions (-O_2). Verified Rex binding sites and promoters are shown in black and white boxes, respectively.

As expected, under aerobic conditions, the expression of *ldh*, *pflB*, and *adhE* was absent in the wild type, but enhanced in the isogenic *rex* deletion mutant. Under anaerobic conditions, wild type and mutant both showed elevated expression. This verifies that Rex acts as a repressor under aerobic conditions and is inactivated under anaerobic conditions. The *rex* deletion mutant was

provided by Richard A. Proctor and co-workers (Pagels *et al.*, 2010).

To verify if repressor motives are common among Rex-regulated genes, additional regulatory regions were screened *in silico*, resulting in the identification of putative Sigma A promoters for five additional genes and their co-localization with verified Rex binding sites in close proximity (Fig. 9).

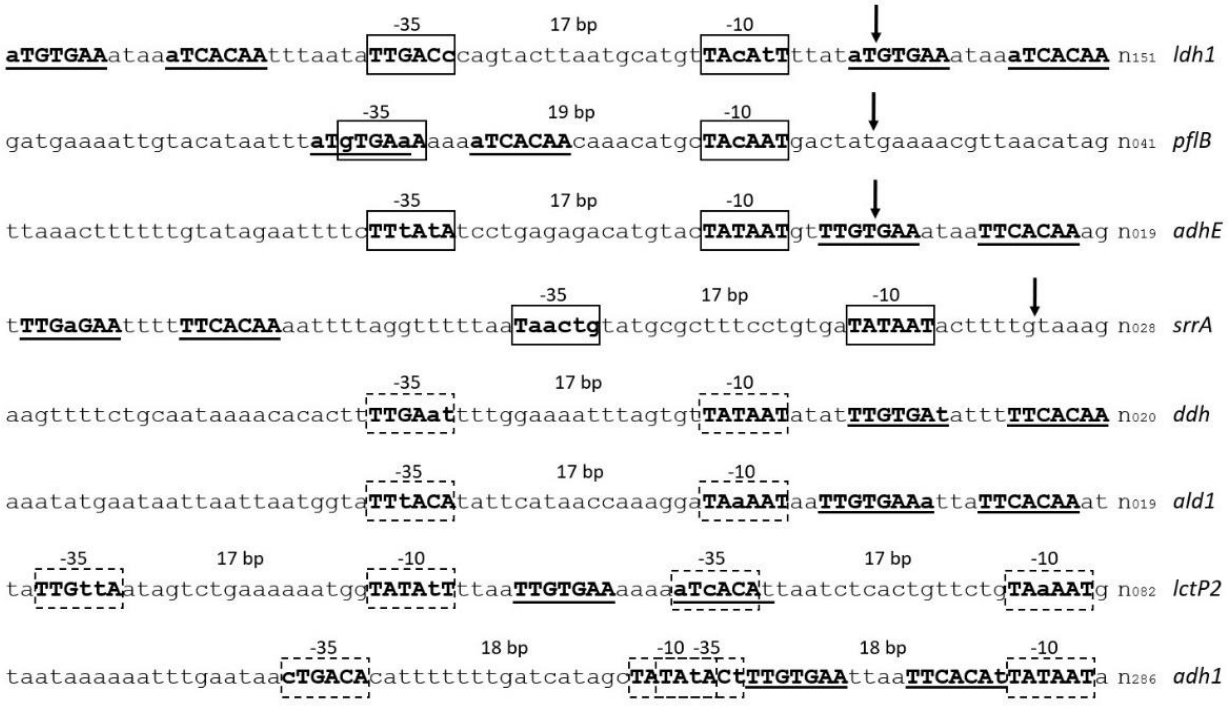


Fig. 9. Co-localization of verified Rex binding sites (underlined) with verified (boxes) and predicted (dotted boxes) Sigma A promoters. -35 and -10 boxes are marked and their spacer sizes are indicated. The respective consensus sequences are in bold letters. Matching bases are in capital letters. Verified transcription start points are indicated by arrows. The transcription start point of *srrA* was published by Pragman *et al.* (J. Bacteriol, 2004).

3.1.1.5 Influence of NAD / NADH on Rex activity

After the verification that Rex acts as a repressor, the focus was placed on the characterization of the conditions under which Rex occupies its repressor sites. The Rex protein does not contain an oxygen-sensing domain, but a Rossmann fold. This protein domain is responsible for NAD / NADH binding in metabolic enzymes, e. g. dehydrogenases. Under aerobic conditions, NADH is generated from NAD by the primary metabolic energy pathways (glycolysis, the PDH complex, and the citric acid cycle) and is reverted to NAD by the respiratory chain, providing a constant NAD / NADH cycle. Under anaerobic conditions, this cycle is interrupted due to an inoperative respiratory chain, leading to NADH accumulation. Consequently, NADH can be suggested to act as an inactivatory ligand of

Rex. To verify this, recombinant Rex protein was expressed, purified, and verified to be free of NAD as well as NADH by spectroscopy (data not shown). The Rex protein was again used in EMSA with the promoter region of *adhE* (high affinity) at several concentrations of NAD and NADH (Fig. 10). With increasing concentrations, NAD was able to significantly increase Rex binding activity, while NADH was able to fully inactivate Rex. In a competition, a ten-fold concentration of NAD was needed to fully prevent the inhibitory effect of NADH. This observation can be explained by the fact that Rex binds as a dimer, and / or that NADH might have a higher affinity. Consequently, two NADH-free monomers of Rex would be required for efficient binding.

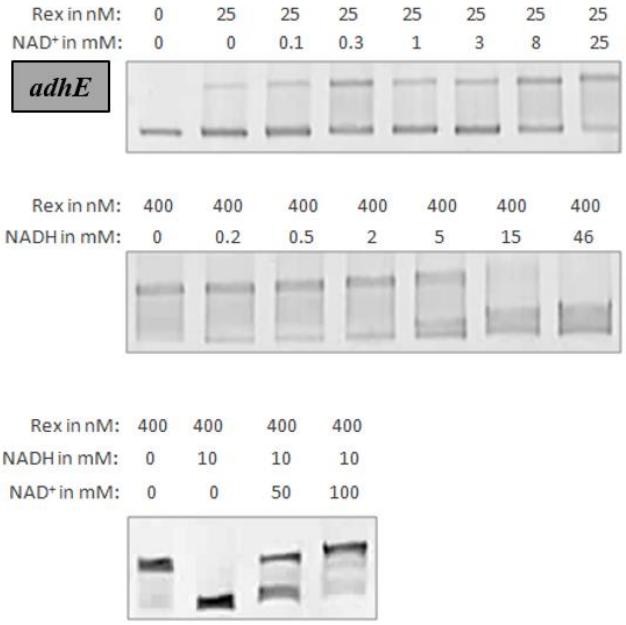


Fig. 10. EMSA of the regulatory region of *adhE* with Rex and its ligands NAD and NADH.

The enhanced Rex-binding activity in the presence of NAD may be caused by a conformational change in the three-dimensional structure of Rex. To adress this, X-ray crystallography was attempted, but the production of appropriate crystals failed for recombinant *Strep*-tagged Rex as well as for *Intein*-tagged Rex. Subsequently, DNAseI protection assay („*footprinting*“) was used as the method of choice. As shown in Fig. 11, NAD-free Rex was only able to protect from DNAseI digest 5 base pairs up- and downstream of its consensus sequence. In contrast, addition of NAD led to a significant increase in the protection area up to 45 base pairs distant from its consensus sequence. This increases the overall protected area from a 42 base pair „*toe"* print to a 108 base pair footprint. This is in accordance with the results from 3.1.1.2, where Rex was not able to protect a PciI-cutting site that was located only 12 base pairs upstream of the Rex binding site in the same regulatory region. Since the high A/T content in the centre of the Rex consensus sequence

was demonstrated to be important for Rex binding, it can be assumed that, upon binding to NAD**,** Rex undergoes a conformational change that enhances DNA-binding and causes an elevated protection efficiency, most likely by bending of the DNA region.

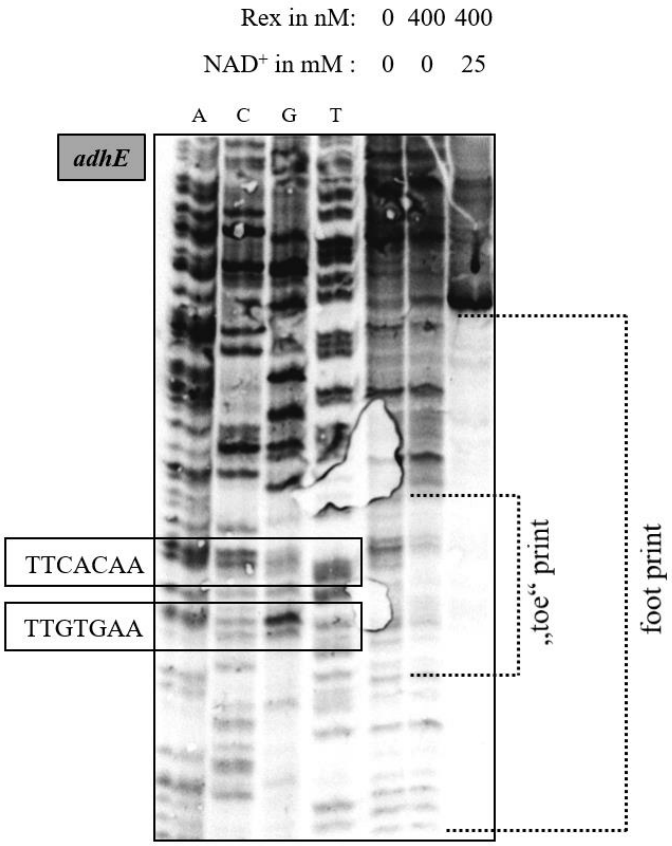


Fig. 11. DNAseI protection assay („*footprinting*") for the regulatory region of *adhE*, using NAD-free and NAD-bound Rex. Sequencing lanes are labled with A, C, G, and T. The Rex consensus sequence is marked with boxes. Protected areas are marked with „*toe*" print and footprint, respectively.

Alltogether, these findings demonstrate that Rex activity responds to the intracellular NAD / NADH ratio. Both ligands compete for the Rossmann fold domain. NADH exhibits an inactivating effect, while NAD is able to displace NADH from the Rossmann fold in competition and consequently keeps Rex active. Additionally, the binding of NAD has also a direct positive effect on Rex activity, most likely by causing a conformational change that leads to a bending of the target DNA.

3.1.1.6 Physiological importance of Rex inactivation

Transcription start point mappings showed that Rex target genes are de-repressed in a *rex* deletion mutant, even in the presence of oxygen (3.1.1.4). Nonetheless, our group also demonstrated that *S. aureus* growth is not affected by this mutation (reasons are discussed in the Discussion and in Pagels *et al.*, 2010). Since the constitutively absence of Rex doesn't affect *S. aureus* growth, a reverse approach was made where the effect of a constitutively active Rex on *S. aureus* growth was adressed. For this purpose, the goal was to create a Rossmann fold mutation that is incapable of NADH-binding, keeping Rex constitutively active and its target genes constitutively repressed, even under anaerobic conditions. Consequently, *S. aureus* should not be able to generate an alternative NAD / NADH cycle by replacing the respiratory chain with anaerobic metabolic pathways, resulting in a significant lack in ATP synthesis.

It should be considered that the wild type Rossmann fold is also important for NAD-binding, which significantly enhances Rex binding activity. Consequently, the desired mutation should not just prevent NADH-binding, but would ideally still allow NAD-binding as well. For this purpose, several single- to tripple-point mutations were introduced into the conserved amino acid sequence of the Rossmann fold, including the highly conserved GXGXXG motif (residues G91, G93, and G96), the conserved N-terminal lysine residue (L79), and the conserved C-terminal aspartic acid residue (D117). Please note that the respective numbers of position differ from the sequence alignment of multiple Rex homologs presented in Fig. 3, because some Rex homologs are longer than others. Instead, the numbers of position presented above refer to the positions in the amino acid sequence of Rex in *S. aureus*.

Mutated residues were replaced by alanine, which is a common strategy since the simple structure of this amino acid usually avoids side effects. The mutated *rex* genes, including the wild type promoter region 500 base pairs upstream, were cloned into the single-copy plasmid pRB473. These plasmids were used to trans-chromosomally complement a chromosomal *rex* deletion mutant in strain COL. As a result, one single mutant, G93->A, from this point on referred to as "*rex93"*, showed a significant deficiency in anaerobic growth (Fig. 12). After reaching full anaerobiosis at OD 1.3, the *rex* deletion mutant, complemented in *trans* with empty plasmid or wild type *rex* as controls, continued growth to a final OD of 2.27 after 3.5 hours. In contrast, the *rex* deletion mutant, complemented with *rex93*, remained at the initial OD of 1.3, confirming a significant retardation of *S. aureus* growth due to the constitutional repression of anaerobic metabolic pathways by the constitutively active Rex93.

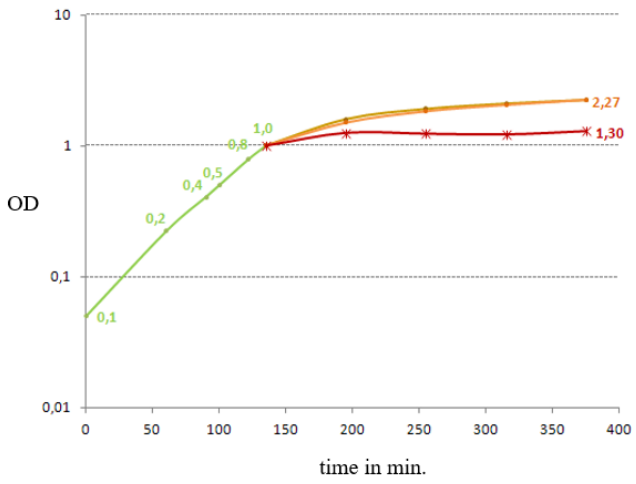


Fig. 10. Aerobic (green, all strains) and anaerobic growth of *rex* deletion mutants in strain COL, complemented with pRB473 in *trans*. Anaerobic growth of strains with empty plasmid (brown), with the plasmid that contains wild type *rex* (orange), and with the plasmid that contains *rex*G93->A (red) were compared.

Northern blot analyses verified that Rex93 was capable to fully repress its target genes *ldh* (L-lactate dehydrogenase), *ddh* (D-lactate dehydrogenase), and *adhE* (alcohol dehydrogenase), even under anaerobic conditions (highlighted with red lines in Fig. 13). As controls, the first four lanes confirm that the *rex* deletion mutant, complemented with wild type *rex* in *trans*, exhibits a similar expression profile for *ldh*, *ddh*, and *adhE* compared to the wild type strain, as expected. The following four lanes compare the effects of constitutively absent Rex (*rex* deletion mutant) and constitutively active Rex (*rex* deletion mutant, complemented with *rex93* in *trans*). The former strain shows constitutive de-repression of the respective target genes, even under aerobic conditions. The latter strain shows constitutive repression, even under anaerobic conditions. This verifies that Rex93, containing the mutated Rossman fold, cannot be inactivated by NADH.

Transcription of *rex* in *trans* was highly elevated, both for the wild type *rex* and *rex93*, due to unknown reasons. The plasmid pRB473 is a single-copy plasmid, and the promoter region in front of the gene constitutes the genomic wild type region 500 base pairs upstream of *rex* and is not autoregulated. Consequently, an explanation for this elevated *rex* transcription in *trans* is missing. This elevated transcription might also be an explanaiton of the anaerobic repression of *ldh*, *ddh*, and *adhE* in the first place. But this assumption seems unlikely, since the elevated expression of *rex* in *trans* was also elevated in the *rex* deletion mutant, complemented with wild type *rex*, and still led to a similar de-repression of target genes under anaerobic conditions, compared to the wild type strain. Nonetheless, the results from this experiment should be confirmed with a chromosomal *rex93* mutant to avoid overexpression from *rex* in *trans* in a future study.

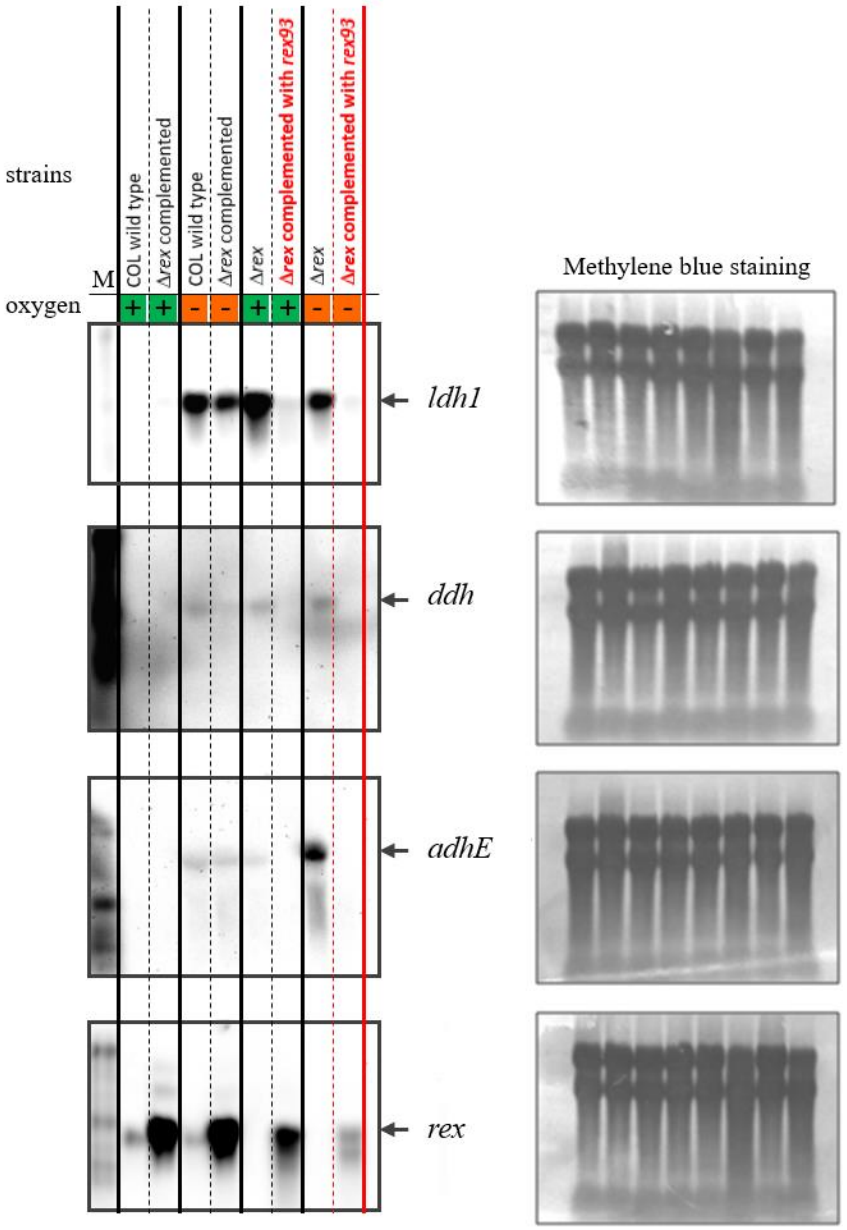


Fig. 13. Northern blots of *ldh*, *ddh*, *adhE*, and *rex* under aerobic (green +) and anaerobic (red -) conditions in respective strains (left). Anaerobic repression in *rex93* is highlighted with red lines. M: marker. Methylene blue staining of whole mRNA (right).

To characterize the growth-impairing effect of Rex93 *in vivo*, a macrophage internalization assay was set as a trial run before going into animal models. The oxidative burst of professional phagocytes can impair the respiratory chain, creating a suitable experimental condition to check for Rex93 effects (Richardson *et al.*, 2006; Rowe *et al.*, 2017). Surprisingly, survival after phagocytosis into macrophages was not diminished for the tested strains: Δ*rex* Newman and Δ*rex* RN1HG that were both complemented with *rex93* in *trans*. To achieve an explanation for these negative results, intracellular *S. aureus* was recovered from the macrophages, and it was checked, if the plasmids were

still present. Strains that were complemented with wild type *rex* only showed a partial loss of the respective plasmid, whereas the plasmids that contain *rex93* were almost completely depleted from the intracellular *S. aureus* population. This clearly indicates a selective pressure upon *rex93* in *trans* after internalization, that is missing for wild type *rex*. As a consequence, *rex93* was introduced into the chromosome instead of a plasmid, to generate stable *rex93* mutants. These mutants can be used in macrophage internalization assays and follow-up animal models in future studies.

Alltogether, these results suggest that inactivation of Rex and the consequent de-repression of its target genes play an important role for *S. aureus* viability during anaerobic adaptation and probably during pathogenesis as well. Nonetheless, the respective experiments have to be repeated with stable chromosomal *rex93* mutants in the future to obtain unambiguous results.

3.1.2 CodY: response to amino acid starvation

The role of CodY in stringent response to amino acid starvation has already been studied in the past. CodY was characterized as a repressor of amino acid biosynthesis, corresponding amino acid transporters, and the expression of extracellular proteases that exploit alternative amino acid sources within the human host (Bennett *et al.*, 2007, Pohl *et al.*, 2009). As already shown for other bacteria, CodY activity is positively regulated by its ligands GTP and branched-chain amino acids. During amino acid starvation, GTP is converted into the alarmone ppGpp (also referred to as ppGpp(p)). The drop in intracellular amino acid and GTP levels leads to inactivation of CodY and a consequent de-repression of CodY target genes. Regulator-promoter interaction studies identified an inverted repeat, AATTTTC N GAAAATT, as the CodY consensus sequence and demonstrated binding of CodY to a multitute of target genes that are de-repressed in a *codY* deletion mutant (Majerczyk *et al.*, 2010). Consequently, this chapter does not focus on experimental results, but on the development of a new method for regulator-promoter interaction studies that was applied.

Wet lab approaches to identify regulator binding motives include DNAseI protection assays (as presented in 3.1.1.5) and site-directed mutagenesis (as presented in 3.1.1.3). Since these methods are time-consuming, this Ph. D. thesis aimed at the development of a quick and easy-to-use method to identify and verify regulatory DNA motives. A small-scale application of this method was performed beforehand for Rex (presented in 3.1.1.2), as a restriction endonuclease protection assay, from this point on referred to as REPA. For this method, the researcher needs purified regulator as well, but renounces hard-to-handle radioactive sequencing gels and time-consuming site-directed

mutagenesis in exchange for an easy-to-use agarose gel electrophoresis with Ethidium bromid staining. Basically, this method is a simple digest of a PCR product with comercially available restriction endonucleases, a daily standard procedure in every molecularbiological lab. The only difference: the PCR product is pre-incubated with the regulator of interest to protect its binding site. The only bottleneck is the availability of an appropriate restriction endonuclease, whose cuting side overlaps with the putative binding site of the regulator of interest, and a buffer that allows activity for both proteins simultaneously. Nonetheless, a plethora of several hundred restriction endonucleases, most of them functioning in a set of different buffers, are comercially available to date and provide the researcher with a set of choices.

For CodY, the experimental setup included the regulatory region of *ilvA* (SACOL1772; threonine dehydratase), several concentrations of the regulator CodY, several concentrations of the ligands GTP and isoleucine (Ile), and the restriction endonuclease Hpy188I, cutting at four distinct positions within the PCR product at TCN/GA. Three of these cutting sites were not affected by CodY, whereas the fourth, overlapping the putative CodY binding site, was completely protected from enzymatic digest (Fig. 14). This REPA allowed the verification of CodY binding to its pruposed DNA

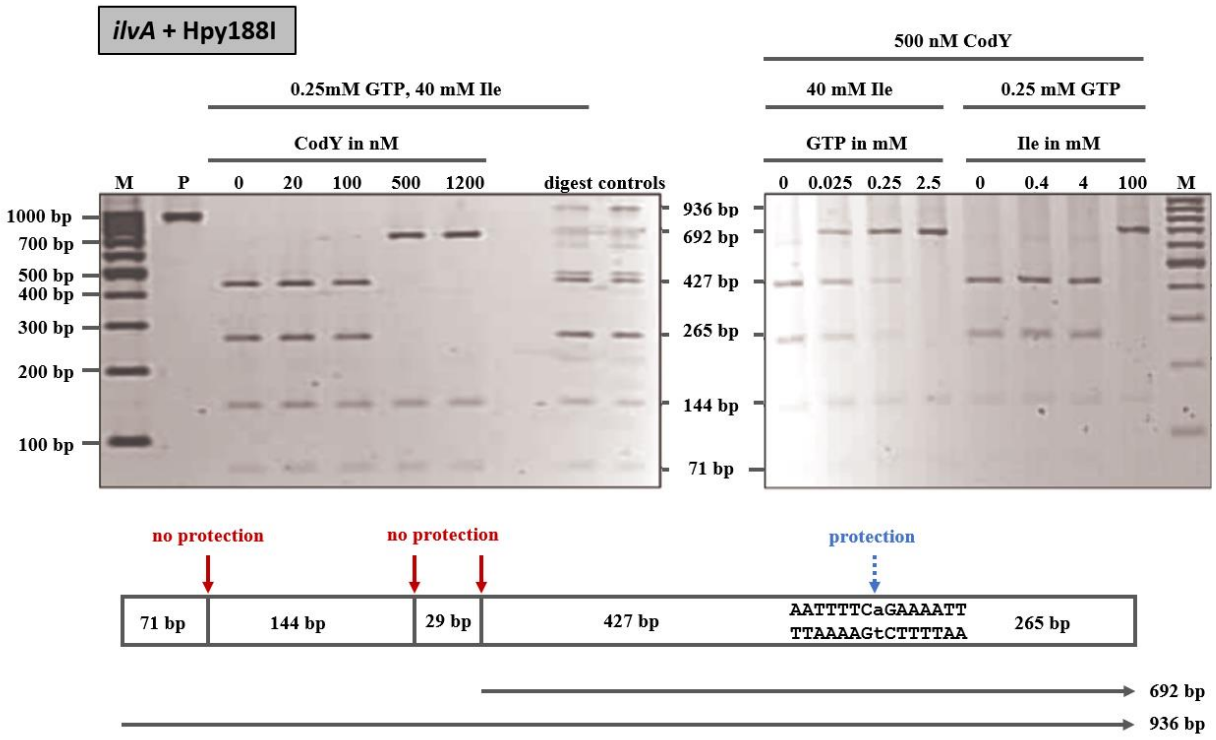


Fig. 14. REPA for the regulatory region of *ilvA*, using CodY and its activatory ligands isoleucine (Ile) and GTP (top). Hpy188I was used as the restriction endonuclease, cutting at the putative CodY binding site only in the absence of CodY, and at three additional distinct sites even in the presence of CodY. M: marker. P: undigested PCR product (all other sample lanes contain Hpy188I). The theoretical fragmentation pattern is depicted, including the CodY binding motif (bottom). Hpy188I-cutting sites are marked with arrows.

motif. It also quantified the inhibitory effects of GTP and / or Ile on CodY activity, independent from and combined with each other (discussed later, and in the diploma thesis of Nina Köhler, who acted as a co-worker at the Institute during the experimental procedure; unpublished data).

Alltogether, REPA proves to be a quick and easy-to-use method for the identification and verification of regulator-promoter binding sites and subsequent applications as well. With this method, a high number of experimental setups can be performed in short time. Consequently, this method can also be used to identify regulator ligands, to characterize their effects on the regulator of interest (inhibitory or activatory), and to quantify this effect.

3.1.3 SarA: early- and late stage virulence factors

3.1.3.1 Functional grouping of the Sar family

Among the Sar family of virulence regulators, SarA (SACOL0672) is the most intensively studied regulator to date. The Sar family constitutes a highly-branched regulatory network that fine-adjusts the expression of early- and late stage virulence genes and regulators as well (for reviews please see Cheung *et al.*, 2008; Jenul and Horswill, 2018). Interestingly, current literature does not provide an appropriate grouping of these Sar homologs by functional and evolutionary aspects. This PhD thesis provides a homolgy tree of Sar homologs, based on amino acid sequence alignment. It comprises all Sar homologs from *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*, and their closest relatives in other bacteria (Fig. 15).

Please note that some Sar homologs are not present in all of these staphylococcal species. Additionally, Sar homologs function as dimers, but some of them (SarS, SarU, and SarY) are encoded as one single protein, already containing both subunits that are linked by a linker domain. These Sar homologs are referred to as two-domain Sar homologs in the literature.

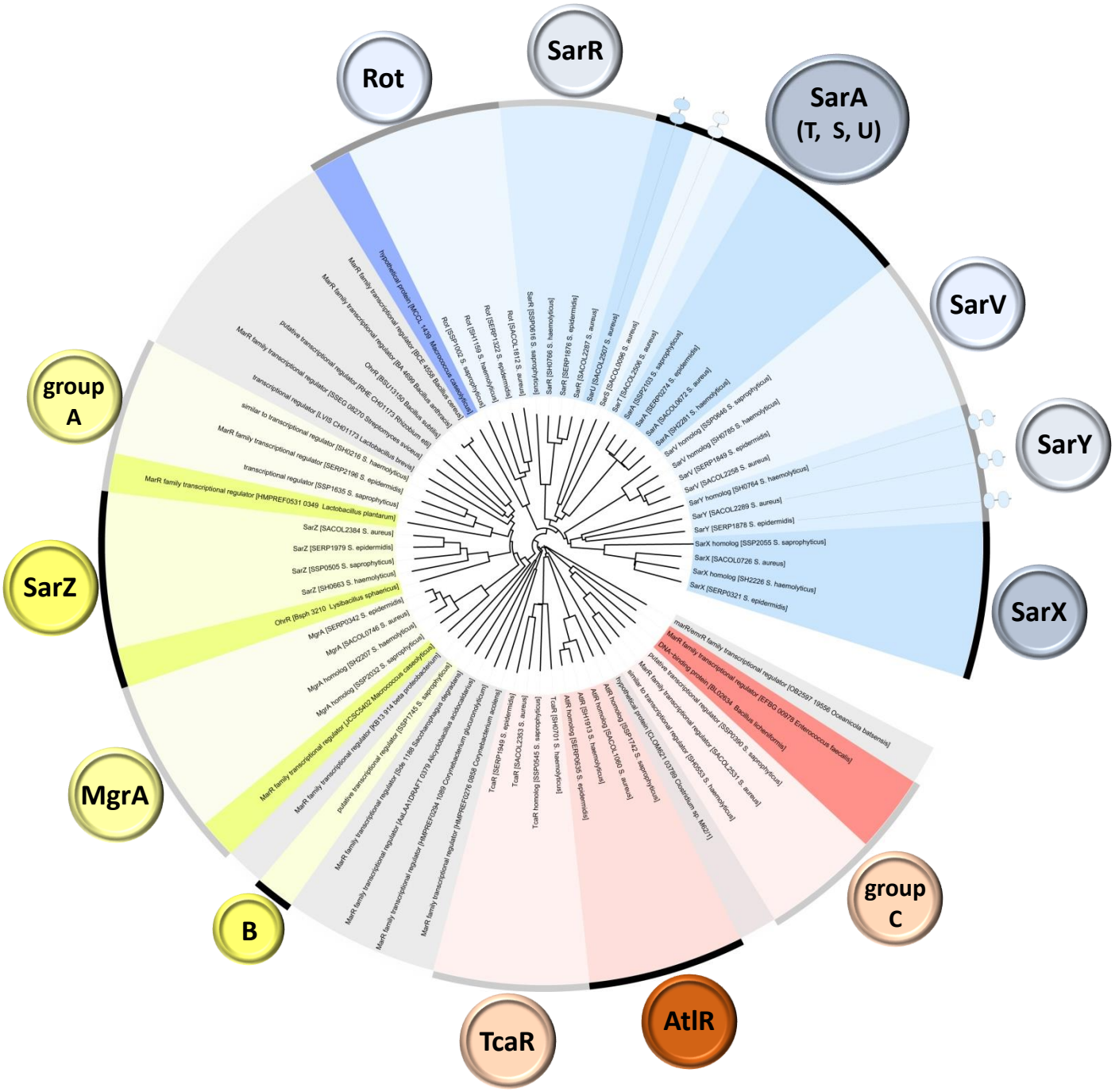


Fig. 15. Homology tree of the Sar family from *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*, based on amino acid sequence alignment. Homologs are grouped in three coloured branches, based on similarity: (1) blue, (2) yellow, and (3) red. Homologs from other bacteria that show high similarity to staphylococcal Sar homologs are highlighted in strong colours. Homologs from other bacteria with lower similarity are greyed out. Homologs that have not been named to date are referred to as „*group A*“, „*B*“, and „*group C*“. Two-domain Sar homologs are marked by two connected blue circles.

The major finding of this analysis is the splitting of the Sar homologs into three separate branches. Each branch contains one or more homologs from other bacteria, indicating that members of the staphylococcal Sar family may have evolved from different unrelated progenitors, instead of one single Sar progenitor in *Staphylococcus spec*.

(1) The blue branch contains the major regulator SarA. Notably, SarT, SarS, and SarU are only present in *S. aureus* and show highest similarity to SarA. Consequently, one might speculate that SarA was the progenitor for these Sar homologs in the species *S. aureus*. SarT, SarS, and SarU can be described as fine-adjusting secondary regulators, downstream in the regulatory cascade. Intriguingly, they are also directly and / or indirectly regulated by SarA itself. The blue branch also contains a Rot homolog (***r****epressor* ***o****f* ***t****oxins*) from *Maccrococcus caseolyticus* (the genera *Macrococcus* and *Staphylococcus* belong to the same family: *Staphylococcaceae*). This regulator shares 36% identity and 66% similarity to its closest relative in *Staphylococcus*. It is noteworthy, that this Rot homolog appears as the closest relative (outside of *Staphylococcus*) for every single Sar homolog from the blue branch. This strongly suggests that all Sar homologs from the blue branch evolved from one single progenitor that is closely related to the Rot homolog in *Macrococcus*.

(2) The yellow branch includes homologs from other bacteria that are characterized as OhrR-like regulators and are involved in oxidative stress response. An N-terminal cysteine residue can generate an intermolecular disulfide bond that leads to dimerization and consequently binding to a conserved DNA motif. Intriguingly, dimerization is impeded by oxidative agents, e. g. Diamide and H_2O_2, that oxidize the cysteine residue and thus prevent dimerization (Fuangthong and Helmann, 2002). The respective N-terminal amino acid sequence, including the reactive cysteine residue, is conserved as well in staphylococcal Sar homologs of the yellow branch. Two members of the yellow branch, SarZ and MgrA, were already described to act via an OhrR-like mechanism in *S. aureus* (Chen *et al.*, 2006 and 2009). Notably, there are no cysteine residues in any of the Sar homologs from the other two branches. The only exception appears to be SarA, which also contains a cysteine residue in the N-terminal region, but does not contain a conserved OhrR-like amino acid sequence. Furthermore, in contrast to SarZ and MgrA, the cysteine residue of SarA is not part of the dimerization domain (Schumacher *et al.*, 2001). In contrast to the blue branch, each of the three subgroups of the yellow branch shows higher similarity to a homolog from other bacteria than to the other two staphylococcal subgroups in their own branch.

(3) The red branch contains the Sar homolog TcaR (an activator of SarS transcription) and additional regulators that are not counted to the Sar family to date. This branch appears to be ambiguous and is not linked to any so far known regulatory mechanisms.

Alltogether, these findings suggest that Sar homologs from the blue branch can truely be assigned to one family, whereas other homologs, e. g. MgrA, SarZ, and TcaR, segregate into seperate groups with distictive functional and evolutionary properties, and their similarities are higher to homologs from other bacteria than to SarA in *S. aureus*.

3.1.3.2 Influence of H_2O_2 and diamide on SarA activity

Previously, the author of this work verified binding of SarA to the regulatory regions of a set of virulence factors (diploma thesis at the Institute; unpublished data). For three of these genes, transcriptional analyses were carried out and showed an increased transcription in a *sarA* deletion mutant, indicating SarA to function as a repressor. For most genes, multiple SarA binding sites were verified. These binding sites do not show a clear SarA consensus sequence, but instead a remarkably high A/T content, remarkably high even when considering that *S. aureus* is an A/T rich organism in general. Please note that A/T-rich regions are susceptible to DNA bending by regulatory proteins due to only two hydrogen bonds that provide more structural flexibility than C/G pairs (three hydrogen bonds).

The cysteine residue in the N-terminal region (described in 3.1.3.1) led to the hypothesis of the formation of disulfide bonds under oxidative stress conditions. These bonds could lead to the covalent linking of several SarA dimers, and consequently, to a tighter packing of the regulatory region and a more pronounced repression. In contrast to SarZ and MgrA, the cysteine residue of SarA is not located in the dimerization domain (Schumacher *et al.*, 2001). In this PhD thesis, SarA was subjected to disulfide-bond inducing agents (H_2O_2 and Diamide) and analyzed in denaturating polyacrylamide gel electrophoresis. Both agents were able to covalently link SarA monomers, as verified by SDS PAGE (data not shown). This effect was revertable by Dithiothreitol (DTT), a reducing agent, capable of cleaving disulfide bonds. Subsequently, covalently linked SarA dimers were tested under native conditions in non-denaturating EMSA, using the regulatory region of *hla* (SACOL1173; α-hemolysin). Since this region contains three SarA binding sites, binding of multiple SarA dimers results in a ladder pattern during the run through a native gel. The addition of the respective oxidating agents after incubation of the PCR product with SarA caused a more pronounced retardation of the PCR product (Fig. 16). However, this effect was marginal and not observed at low SarA concentrations (data not shown).

Alltogether, these results do not rule out disulfide bond formation as part of the mode of action for SarA, but this possibility appears to be doubtful. Instead, an alternative mechanism for SarA

activity, depending on three phosphorylation sites, was suggested in the literature and will be reviewed in the Discussion.

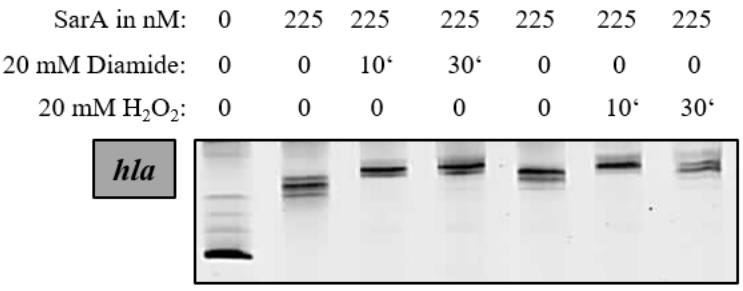


Fig. 16. EMSA of the regulatory region of *hla*, incubated with SarA in the presence of disulfide-bond inducing agents for 10 and 30 minutes.

3.2 Part two: *"global footprinting"*

3.2.1 Method development

In the course of this PhD thesis, a novel method for the genome-wide identification of regulator binding sites was developed. This global approach represents an alternative to ChIP-on-chip (**ch**romatin **i**mmune **p**recipitation, detected **on** a DNA microarray **chip**) and can be understood as a DNAseI protection assay (*footprinting*) that is applied across the whole genome. Therefore, this method is from this point on referred to as *"global footprinting"*. In the first place, ChIP-on-chip seems to be the method of choice, since it allows a valuable *in vivo* snapshot from the inner of the living bacterium. Nonetheless, ChIP-on-chip can only identify target genes, if the regulator is active and occupies its binding sites when the snapshot is made. Additionally, the use of formaldehyde does not only generate covalent cross-linkings between the regulator and its DNA motifs, but can also lead to a denaturation of epitopes that are essential to immune-precipitate the regulator-DNA complexes, making the application of this method challenging. To date, a plethora of protocols are online available that differ significantly from one research group to the other, indicating that this method is not easy-to-apply. This assumption is bolstered by the fact that only two examples for ChIP-on-chip have been published for *S. aureus* so far, characterizing the regulators CodY and Hfq (Majerczyk *et al.*, 2010, Liu *et al.*, 2010).

As part of this PhD thesis, the potential of Rex to be cross-linked with DNA via formaldehyde

was examined. This approach failed, since formaldehyde treatment disturbed the separation of DNA in native gel electrophoresis in EMSA. Consequently, the development of an alternative to ChIP-on-chip was focussed. This new method is carried out *in vitro* and follows an easy-to-apply protocol: (1) Chromosomal DNA is extracted, purified, and fragmented into fragments of a size of approximately 500 base pairs. In this step, sonification appeared to be more suitable than enzymatic digest. (2) Fragmented DNA is incubated with a recombinant *Strep*-tagged regulator of interest that was expressed in *E. coli*. (3) Regulator-DNA complexes are separated from unbound DNA due to a run through a *Strep*-tag column, retaining the tagged regulator and the DNA stretches that the regulator specifically bound to. (4) The enriched regulator-DNA complexes are purified, using a comercial available DNA purification kit, and are subsequently used in *next generation sequencing* (NGS). In NGS, quantification is usually part of the quality control during the sequencing of unknown genomes. Obviously, in the method presented in this PhD thesis, NGS is not used to sequence the DNA (the *S. aureus* genome is already known), but to quantify the enrichment of DNA fragments that contain regulator binding sites, as the major result.

Enrichment potential and appropriate conditions were tested for Rex, CodY, and SarA in a first trial run (that does not need NGS). PCR products were added in high concentrations to the fragmented genome. Some PCR products contained already known regulator binding sites, some did not. After purification with a *Strep*-tag column, samples were separated on an agarose gel and stained with Ethidium bromid. For Rex, significant enrichment of PCR products that contain Rex binding sites was obtained (data not shown). For SarA, enrichment was only observed under specific SarA concentrations. CodY did not yield any enrichment, even with the addition of the activatory ligands GTP and isoleucine, and was thus ceased from further testings. In a second trial run, Rex and SarA were used to enrich genomic DNA without the addition of PCR products. Enrichment was validated with semi-quantitative PCR. Only for Rex, a significant enrichment was obtained. These first two trial runs were also performed to optimize the method, e. g. the washing and purification procedure. Subsequently, Rex samples were prepared, and finally analyzed with NGS. This final approach yielded the majority of Rex binding sites across the whole genome, that were already verified by EMSA in this PhD thesis (3.1.1.2).

3.2.2 The „*global footprint*“ of Rex

The final run, described in 3.2.1, led to the verification of Rex binding sites and to the identification of new ones via NGS. The cut-off for enrichment peaks was set at four-fold compared to the background, resulting in 0.2 % of the whole genome to be regarded as significantly enriched. The

enriched base pairs constitute a total of 18 enrichment sites. Of these regions, 9 sites have already been identified by EMSA in this study (Fig. 17A). For the Rex binding site upstream of *adhE*, which was demonstrated to provide highest Rex affinity in EMSA, the enrichment peak was located precisely to one single base pair across the whole genome. As false negative results among the remaining Rex binding sites (that were already identifed by EMSA), *srrA*, *ald1*, and *lukM* also showed enrichment peaks but fell below the cut-off. Additionally, *narG* didn't show an enrichment peak at all, since it contains a mutation in its Rex binding site in the genome of strain COL that was used in this experiment.

Another 2 enrichment sites were located in the regulatory regions of anaerobically induced genes that have not been linked to Rex regulation to date. One of these genes is of yet unknown function (SACOL0166), encoding an integral membrane protein. SACOL0166 contains a motif with 1 mismatches to the Rex consensus sequence in its regulatory region (TTGTGAtTATTTTCACAA), making direct regulation by Rex likely. The other gene, *narT* (also referred to as *narK*, SACOL2386), encodes a nitrite transporter (Fig. 17B). SACOL2386 (*narT*) contains a motif with 4 mismatches to the Rex consensus sequence in its regulatory region (aTGTtcAcTTTTTCACAA), making direct regulation by Rex doubtful. The remaining 7 enrichment sites did not contain appropriate matches to the Rex consensus sequence and can be regarded as false positives.

Alltogether, these results suggest that this newly developed method, from this point on referred to as „*global footprinting*" (GF), proves to be valuable for global-scale analyses of regulator-promoter interactions across the whole genome. Nonetheless, the trial runs from this PhD thesis also demonstrate that this method is not yet applicable for every regulator and thus demands for additional optimization in the future.

Fig. 17. „Global footprint“ for Rex in *S. aureus* strain COL. Target genes are coloured in red. Rex motives are marked in green. Y-axes show the coverage by reads. Gene organisations of the respective region are depicted below each enrichment diagram. **A:** binding sites that have already been identified by EMSA in this study. **B:** additional enrichment peaks upstream of anaerobically induced genes.

3.3 Part three: Cluster analysis

3.3.1 Creation of a prediction model

Besides specific regulator-promoter interaction studies, „Omic"-technologies as well contribute to the investigation of the regulatory network on a global scale, primarily via global profiling of regulator knockout strains. To date, the literature provides more than two hundred DNA microarray analyses for *S. aureus*. Consequently, a major concern of this work was to collect these „*big data*", to standardize them, and to subject them to a comprehensive analysis to get the whole picture. The major goal was to extract and compare the expression profiles for each single gene within the *S. aureus* genome. When the expression profiles of two genes share high similarity, these genes can be suspected to function in the same physiological context and might share the same regulation. If the function and / or regulation of one of these two genes is already characterized, the other gene, whose function and regulation is unknown, might be assigned to the same functional and regulatory context. Consequently, the application of such a prediction model is of high value for the creation of novel experimental starting points.

For the creation of a data library, all available DNA microarray data and regulator-promoter interaction studies were collected from the literature (including publications until October 2019). During this literature search, 800 publications were reviewed. As a result, two data sets were assembled. The first set comprises DNA microarray data under 282 different conditions, including 53 regulator knockout analyses and more than 50,000 regulatory events. A total of 59 different strains were used in the respective publications. The most dominant strains comprise Newman (13% of the experiments were carried out in this strain), MW2 and UAMS-1 (10% each), and COL, NCTC8325, SA113, and SH1000 (6-7% each). Strains are distributed across all three clades of *S. aureus*, underlining the heterogeneity of this data set (for an overview of *S. aureus* clades please note Liang *et al.*, 2016). This strain diversity is not to be regarded as an obstacle, but instead perfectly fits the second goal of this analysis: The identification of expression patterns that are conserved in *S. aureus* in general, and not specific just for one single strain due to regulator mutations.

Strain differences have evolved as a major concern in the characterization of the regulatory network of *S. aureus*. Spontaneous mutations in the quorum sensing system Agr and a non-functional *rsbU*-gene as part of the Sigma B-dependent general stress response are the most prominent examples to mention. Consequently, for the characterization of the regulatory network of *S. aureus*, two approaches appear to be viable in concert: (1) The selection of one specific strain that is subjected to a variety of stimuli (Mäder *et al.*, 2016), and (2) The selection of one specific stimulus that is applied to a variety of strains. This PhD thesis has chosen the latter approach, with the aim to distinguish

conserved expression profiles in *S. aureus* from specific expression profiles in specific strains.

The second set of the data library comprises more than 2,000 regulator-promoter interactions. It is used to confirm regulations that were observed in DNA microarray analyses, and to distinguish between direct and indirect regulations.

3.3.2 Identification of functional and reguatory gene clusters

After collection and standardization of DNA microarray data from the literature, a comprehensive cluster analysis was carried out. The basic concept: when a gene with unknown function shares a similar expression profile with a gene with known function (under various stimuli), the gene with unknown function can be predicted to act in the same physiological and regulatory context as the gene with known function. For this analysis, the free-available software *"Multi Experiment Viewer"* (MeV) was used. This tool is commonly used for quality control of DNA microarray experiments and for other „*big data*" applications as well (Howe *et al.*, 2011). Genes that are up- or down-regulated after the application of a specific stimulus can be quantified and grouped. MeV also generates a small-scale cluster tree for quality assurance: experimental parallels upon stress locate at the one end, whereas parallels of the untreated control locate on the other end. This PhD thesis exploits the software MeV for a large-scale cluster analysis, comparing data from DNA microarrays under 282 different experimental conditions at once (provided by the world-wide literature). The major finding of this analysis is the identification of seven functional and regulatory gene clusters in *S. aureus* pathogenesis that are conserved across *S. aureus* strain diversity (Fig. 18). Other unidentified clusters that do not meet the topic of this PhD thesis might be present as well.

The following sub-sections describe the identified clusters in detail. Since experiments were usually carried out by different research groups under different experimental conditions, gene regulation does not appear to be universally consistent. For example, even if two different research groups investigated an *agr* deletion mutant in the same strain, different results can be expected due to the use of e. g. different growth phases and medium. Considering this issue, genes are marked as up- or down-regulated if the respective effect was present in at least 40% of the DNA-microarrays for a specific condition. For the generation of the cluster tree, the original induction ratios for each gene under the respective conditions were used. Nonetheless, for the sake of clarity, regulatory events in the final tables are summarized and marked with black boxes instead of the original induction ratios, since regulatory events occurred in a set of different DNA-microarray analyses.

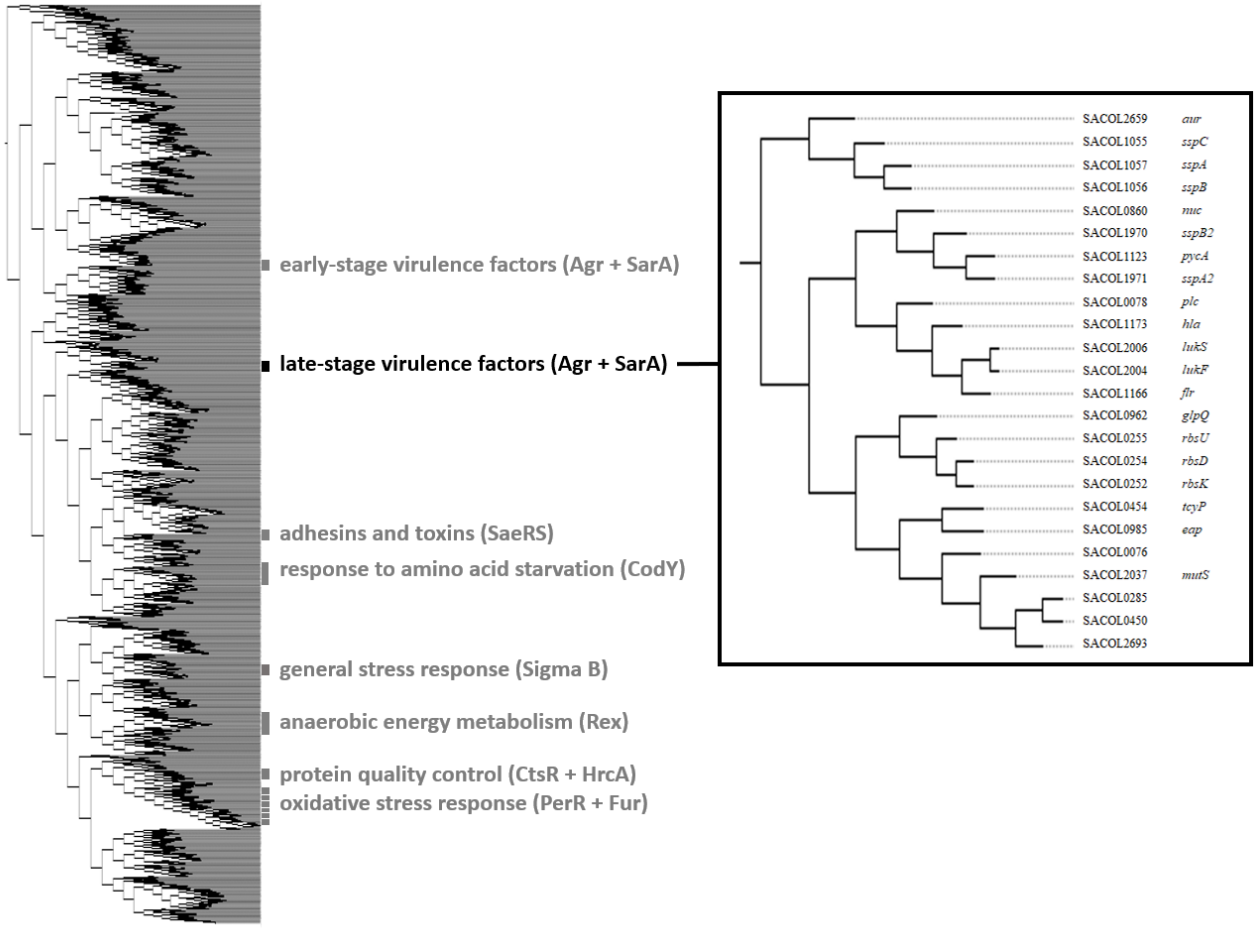


Fig. 18. Cluster tree of more than 2,000 genes, based on similarities in their expression profiles under 282 different experimental conditions (left). Functional and regulatory clusters were manually identified and are labeled. As an example, the functional cluster of late stage virulence factors is zoomed in (right).

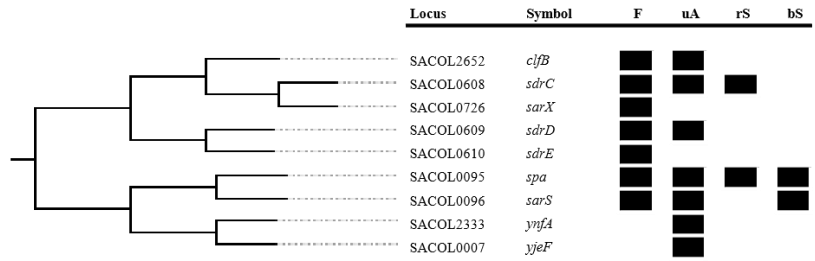
(1) <u>Early stage virulence factors</u> (Table 3, top): This cluster contains genes that encode for surface-bound virulence factors, known to facilitate the establishment of *S. aureus* infection in the human host. These host-pathogen interactions include adhesion, mediated by surface-bound adhesins, e. g. clumping factor B (*clfB*), and immune evasion, e. g. Protein A (*spa*), a surface-bound protein that is able to bind the Fc domain of human Immunglobulin G (IgG) and thus scavenges human antibodies. Notably, the regulator SarS, the major activator of *spa* transcription (Cheung *et al.*, 2001), was located directly adjacent to *spa*, confirming that SarS plays a crucial role in *spa* activation.

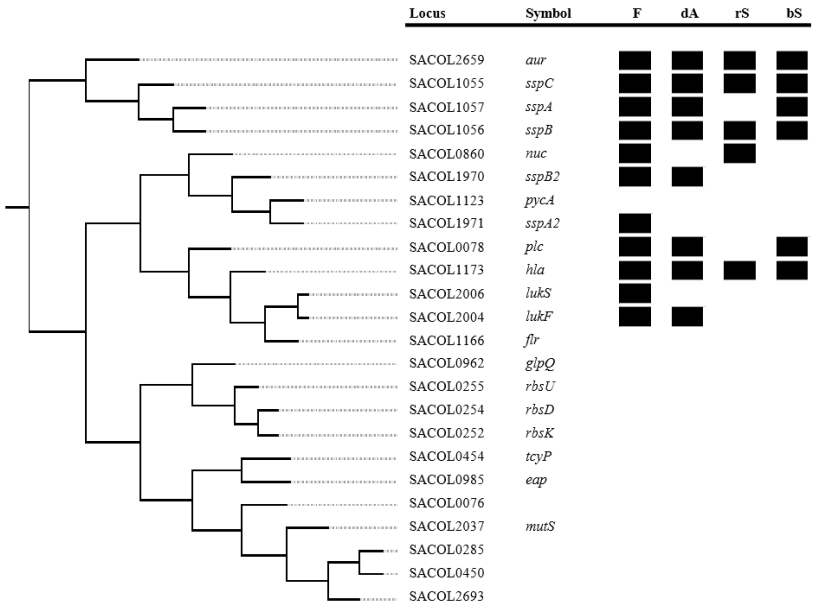
The majority of the genes within this cluster is negatively regulated by the quorum sensing system Agr, leading to up-regulation in the early growth phase in the shaking flask *in vitro* when cell density is low. Some of these genes are directly and / or indirectly regulated by SarA. The influence of SarA on the expression of these genes is not consistent but highly dependent on the strain, growth phase, growth medium, and other experimental conditions.

Interestingly, two genes that are also present in this cluster have not been linked to pathogenesis to date: SACOL2333 (membrane protein) and SACOL0007. Additionally, the regulator SarX (SACOL0726) is also present in this cluster, indicating an activatory role in the regulation of this cluster. SarX is classified as a member of the Sar family, but is so far uncharacterized in terms of regulation of virulence factors. These 3 genes present compelling targets for follow-up studies.

(2) <u>Late stage virulence factors</u> (Table 3, bottom): This cluster is dominated by genes that encode for secreted virulence factors, representing the invasive phase of pathogenesis. Destruction of host cells and components are mediated by toxins (*Hla*), proteases (Sspa), lipases (Plc), and nucleases (Nuc). The according genes are positively regulated by the quorum sensing system Agr, causing activation in the late growth phase in the shaking flask *in vitro* when cell density is high. A set of these genes is directly repressed by SarA. The lower part of this cluster has not been described to be regulated by Agr to date. It contains genes for ribose metabolism (*rbsD* and SACOL0252) and corresponding transport (*rbsU*), a glycerophosphoryl diester phosphodiesterase (*glpq*), a cystine transporter (*tcyP*), and a surface protein (*eap*). Whether these genes are involved in uptake and processing of host compounds that are released by secreted toxins is open for investigation.

Table 3. Early- and late stage virulence factors (Agr + SarA)





F: genes whose fuction is already described to be linked to this physiological context

uA: genes whose expression is up-regulated in *agr* deletion mutants

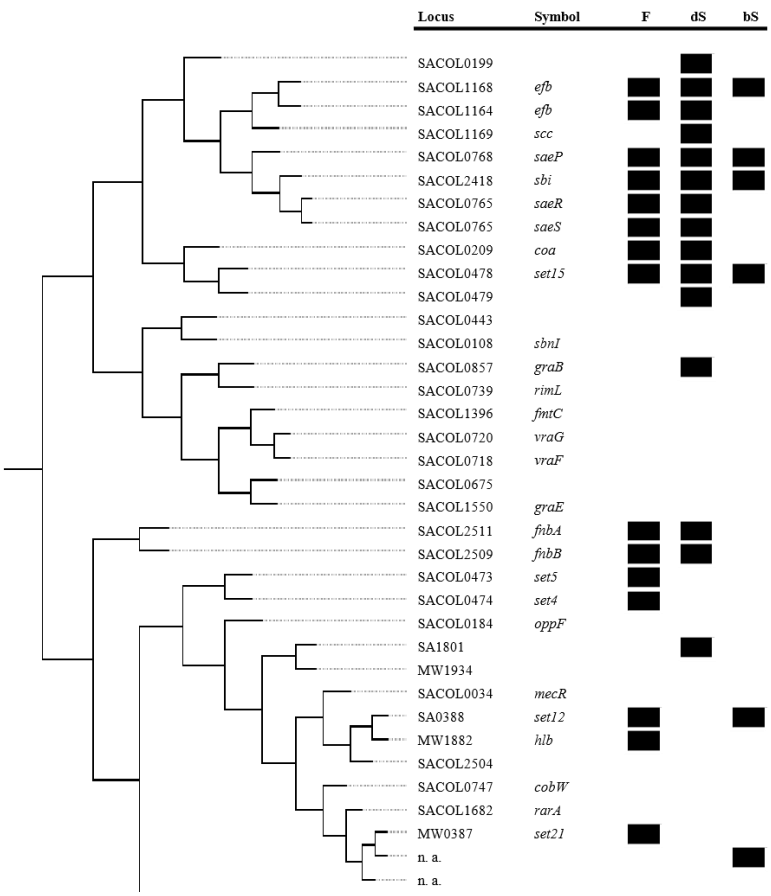
dA: genes whose expression is down-regulated in *agr* deletion mutants

rS: genes whose expression is changed in a *sarA* deletion mutant

bS: genes that contain verified SarA binding sites in their regulatory regions

(3) <u>Accessory adhesins and toxins</u> (Table 4): This cluster contains additional virulence factors that are positively regulated by the two-component system SaeRS. The exact stimulating signal is yet unknown. Virulence factors in this cluster mediate the binding of host factors (*coa*, *efb*, and *fnbAB*), and comprise a toxin (*hlb*) and superantigens (*set4*, *set5*, *set12*, *set15*, and *set21*). Another set of genes in this cluster encodes for transporters and proteins of unknown function, and has not been linked to virulence or SarRS regulation to date.

Table 4. Accessory adhesins and toxins (SaeRS)



F: genes whose fuction is already described to be linked to this physiological context

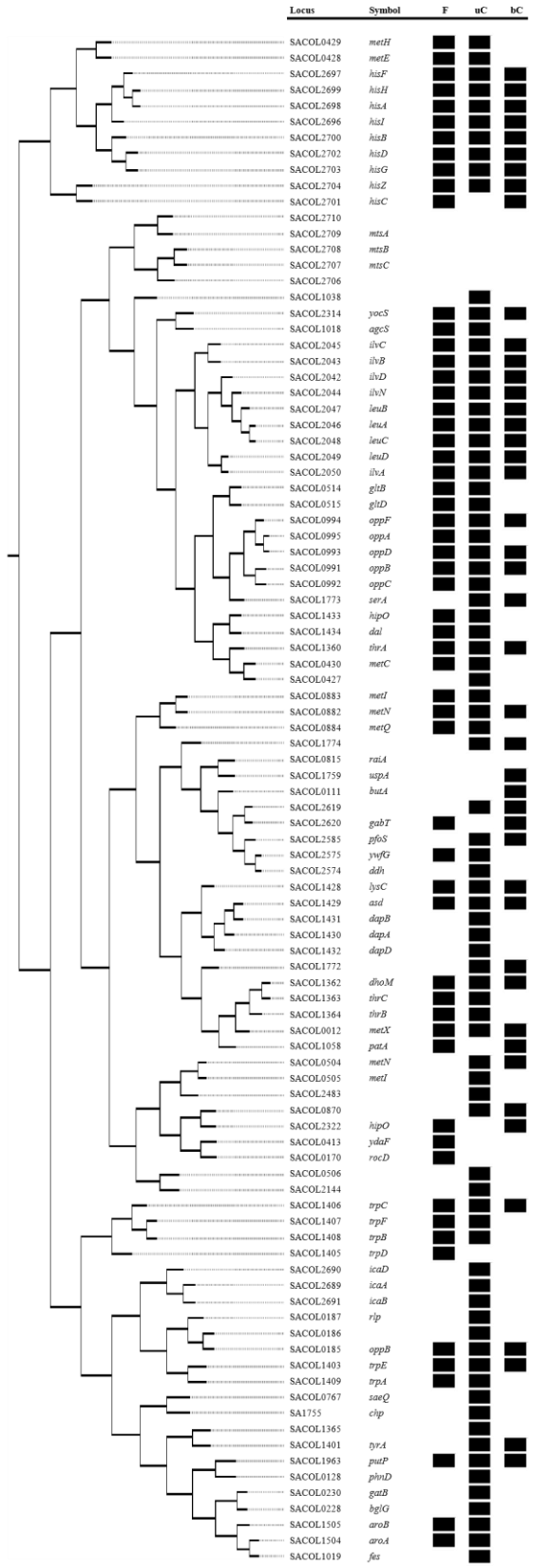
dS: genes whose expression is down-regulated in *saeRS* mutants

bS: genes that contain a verified SaeR binding site in their regulatory regions

n. a.: these genes are no longer annotated

(4) <u>Response to amino acid starvation</u> (Table 5): This cluster contains a total of 95 genes, whose function is assigned to the biosynthesis and transport of amino acids. The majority of these genes is up-regulated in a *codY* deletion mutant and partially contain verified CodY binding sites in their promoter regions. Interestingly, the first three genes of the *ica*-operon (*icaA*, *B*, and *D*), important for biofilm formation, are also present. This finding provides an explanation for the observation of increased PIA-dependent biofilm formation in a *codY* deletion mutant (Majerczyk *et al.*, 2008). Additionally, an operon that comprises two ABC-transporters and genes with unknown function (SACOL2706 to SACOL2710), were also located within this cluster, though they have failed to show up-regulation in a *codY* deletion mutant so far. Nonetheless, the similarity of the overall expression profiles of these genes with CodY-repressed genes hinds to a functional involvement in this physiological context.

Table 5. Response to amino acid starvation (CodY)



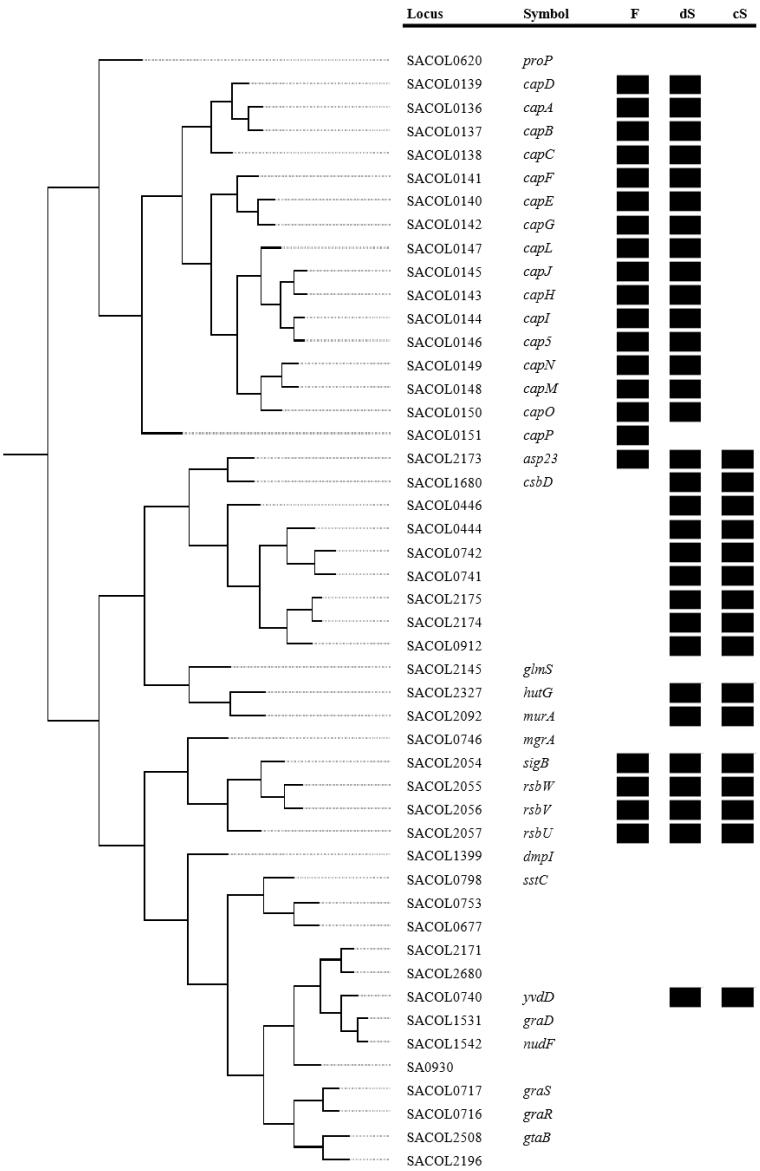
F: genes whose function is already described to be linked to this physiological context

uC: genes whose expression is up-regulated in *codY* deletion mutants

bC: genes that contain a verified CodY binding site in their regulatory regions

(5) General stress response (Table 6): Sigma B is known to directy and indirectly regulate a large set of genes that are inducible by heat, salt, and other environmental stress stimuli. A total of 126 Sigma B consensus sequences was identified in *S. aureus* so far. Most of the corresponding genes are scattered across the cluster tree, but their locations are increasingly focused in and around this cluster. It contains *sigB*, its own positively autoregulated *rsbVW-sigB* operon, and *rsbU*. These components regulate Sigma B activity under environmental stress stimuli.

Table 6. General stress response (Sigma B)



F: genes whose fuction is already described to be linked to this physiological context

dS: genes whose expression is down-regulated in *sigB* deletion mutants

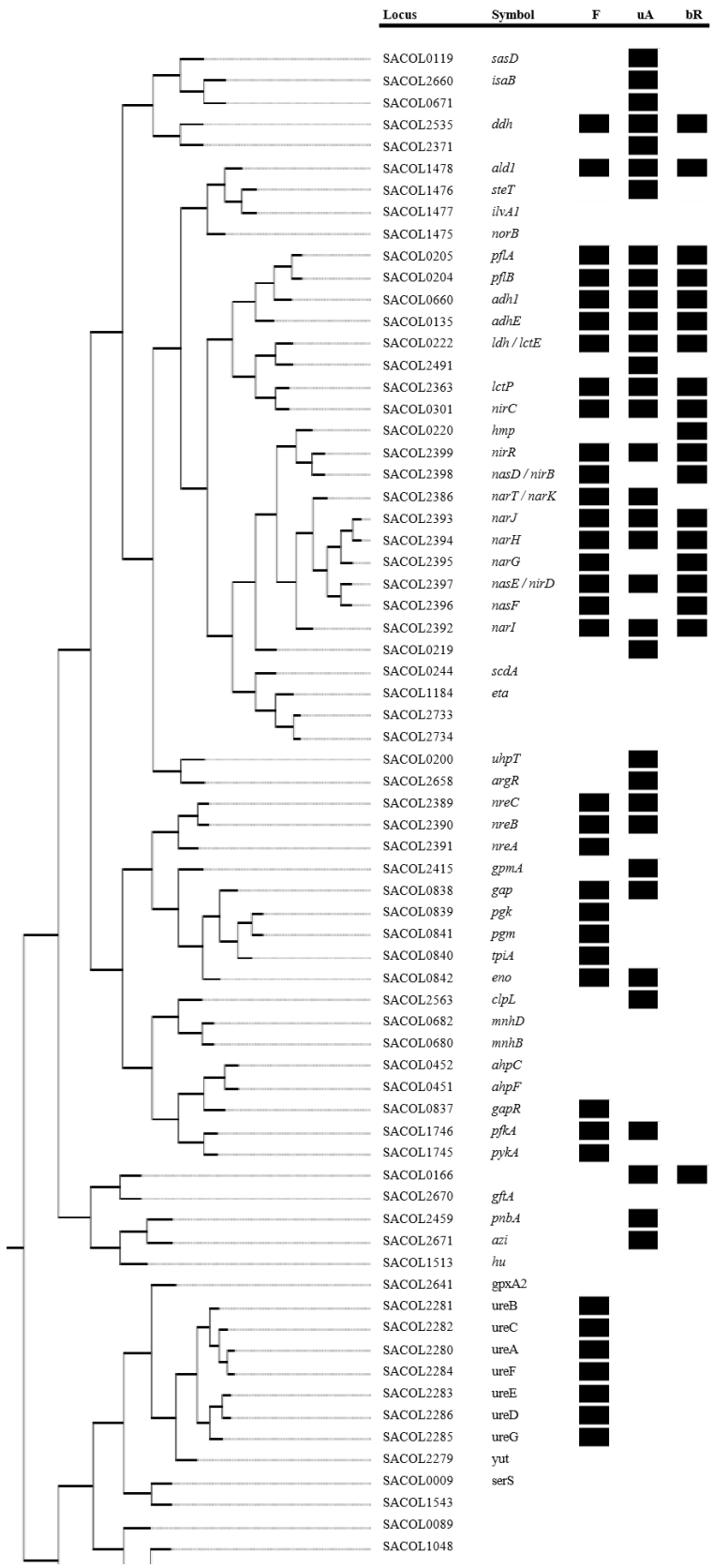
cS: genes that contain a Sigma B consensus sequence in their regulatory regions

Another gene, *asp23*, is well known to be under direct positive regulation by Sigma B (Müller *et al.*, 2014). Asp23 highly accumulates under alkaline shock, is linked to a membrane-anchored adaptor protein at the inner cell membrane, and is supposed to function in cell morphology and / or division. Additionally, the Sigma B cluster contains the *cap*-operon that facilitates capsule formation in *S. aureus*, and MgrA (formerly known as Rat or NorR), a positive regulator of capsule formation (Luong *et al.*, 2003 and 2006). In fact, Sigma B itself has already been connected to capsule formation as well (Meier *et al.*, 2007). The Sigma B cluster also contains a set of genes with unknown function that also contain Sigma B promoters in their regulatory regions.

(6) <u>Anaerobic energy metabolism</u> (Table 7): During pathogenesis, *S. aureus* can be confronted by an inoperative respiratory chain. This can occur due to low oxygen levels, e. g. in deeper or infected tissue, and in deeper layers of self-produced biofilms. Additionally, the oxidative burst of professional phagocytes can impair the respiratory chain. Furthermore, spontaneous mutantions in *hemB* and *menD* (encoding synthesis pathways for components of the resiratory chain), are involved in clinical small colony variants and lead to pseudo-anaerobic expression patterns (Kohler *et al.*, 2003 and 2008). As a response, up-regulation of glycolysis compensates for the drop in ATP production. NADH, generated from NAD by glycolysis, is no longer reverted to NAD by the respiratory chain, but instead by anaerobic metabolic pathways, including fermentation and anaerobic respiration. This results in the upkeep of an alternate constant NAD / NADH cycle between glycolysis and the respective anaerobic metabolic pathways.

The majority of these pathways appears to be repressed by the regulator Rex (this study; Pagels *et al.*, 2010), and the corresponding genes are found in close proximity to each other in the cluster presented here. These pathways include lactate dehydrogenases (encoded by *ldh1* and *ddh*), pyruvat-formiate lysase (*pflBA*), alcohol dehydrogenases (*adhE* and *adh1*), nitrate and nitrite respiration (*narG*- and *nirR*-operon respectively), and corresponding transporters (*lctp2* and *nirC*). Genes that encode for acetate and butandiol production are not regulated by Rex and thus are not located within this cluster.

Table 7. Anaerobic energy metabolism (Rex)



F: genes whose fuction is already described to be linked to this physiological context

uA: genes whose expression is up-regulated under oxygen limitation

bR: genes containing a verified Rex binding site in their regulatory regions (this study)

Additionally, serveral genes with unknown function are located within this cluster as well. The most interesting of these genes appears to be SACOL2491. This gene contains a Rex binding motif with only two mismatches in its regulatory region and the expression of this gene is anaerobically induced. Consequently, SACOL2491 can be suspected to be directly repressed by Rex and to play a yet unknown role in anaerobic adaptation. Homologs with unknown function are present in other bacteria as well, including the best match in Listeria monocytogenes with a truncation of the C-terminal 10 amino acids but a 100% identity for the remaining sequence.

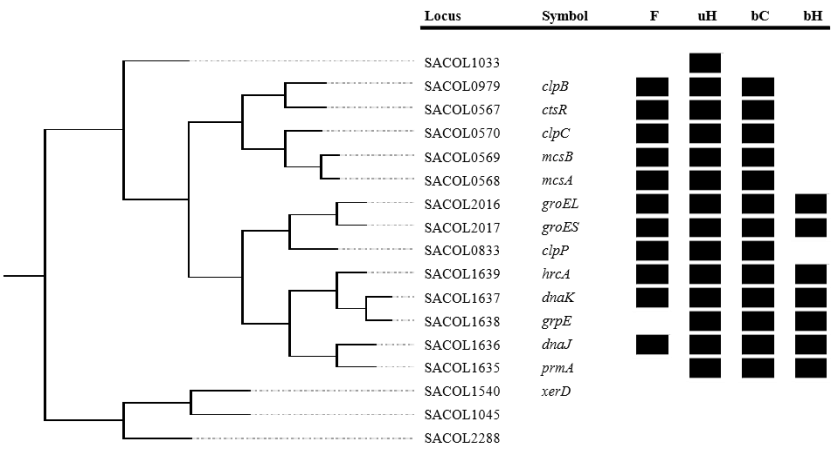
The anaerobic cluster contains two major sub-clusters. The upper one contains genes that are repressed by Rex. The lower one contains the majority of glycolysis-encoding genes that are not regulated by Rex (*gap*, *pgk*, *pgm*, *tpiA*, *eno*, and *pfkA*). Three glycolytic genes, encoding for the initial three enzymatic steps in glycolysis, are not located within this cluster. Nonetheless, two of them can be found in close proximity (*pgi* and *pfkA*). The gene *glk*, encoding for the first enzymatic step in glycolysis, is not located in close proximity to this cluster. The lower part of this cluster tree also contains an urease-encoding operon that is involved in buffering the elevated intracellular lactate level, caused by anaerobic lactate production, with the production of NH_4^+. Another member of the lower sub-cluster is the *nre*-operon, whose regulatory components are activators of nitrate and nitrite respiration (Sc*hla*g *et al.*, 2008). These genes are also not regulated by Rex.

Some anaerobically induced genes that are directly repressed by Rex are not located in this cluster: *srrAB*, the *arc*-operon, and *lukM*. This is in accordance with the finding that Rex affinity to the respective binding sites is weak, and that transcription in a *rex* deletion mutant was only marginally elevated or even absent.

Altogether, a broad set of anaerobically induced genes clusters in close proximity, and this cluster is separated into a Rex-repressed sub-tree and a Rex-indepentend sub-tree. These new findings provide a well-understandable total picture of the anaerobic adaptation in *S. aureus* and the hierarchy of its regulatory systems. As a prerequisite for anaerobic adaptation, Rex acts as a global repressor and the major regulator of anaerobic response. This major mechanism is accompanied by several activators that provide fine-adjustment of smaller and more specific sets of genes, dependent on the exact conditions, e. g. nutrient availability.

(7) <u>Protein quality control</u> (Table 8): Proteins undergo natural denaturation and misfolding during their life cycle, and might need assistane in correct folding after translation. In pathogenesis, proteins are additionally subjected to misfolding and denaturation during the oxidative burst of professional phagocytes. Cell wall stress-inducing antibiotics are able to induce oxidative stress as well (Kohanski *et al.*, 2007). Finally, entry into the host causes an increase in the environmental temperature, especially during fever. The cluster presented here contains class I and III heat shock genes that encode chaperones, facilitating correct (re-)folding, and Clp proteases respectively, facilitating degradation of irreparably damaged proteins. The respective operons also encode the regulators CtsR and HrcA. These regulators are repressors of their own operons and are subject to a sophisticated stress-dependent control by gene products that are repressed by these regulators in the absence of stress (Chastanet *et al.*, 2003). In *Bacillus subtilis*, CtsR is degraded by ClpP under heat stress, leading to de-repression of the CtsR regulon and up-regulation of chaperone- and Clp protease-expression. This mode of action involves the modulators McsA and McsB that are encoded in the *clpC*-operon (Derré *et al.*, 2000, Krüger *et al.*, 2001; Elsholz *et al.*, 2010). Additionally, the regulator HrcA is folded by GroE into its functional shape. During heat shock, intracellular misfolded proteins titrate GroE, leading to de-repression of HrcA target genes whose products respond to the elevated level of misfolded proteins (Mogk *et al.*, 1997). All these genes form a distinct cluster in *S. aureus*.

Table 8. Protein quality control (CtsR + HrcA)



F: genes whose fuction is already described to be linked to this physiological context

uH: genes whose expression is up-regulated under heat stress (48°C)

bC: genes that contain a verified CtsR binding site in their regulatory regions

bH: genes that contain a verified HrcA binding site in their regulatory regions

Three additional genes are located at the lower end of this cluster of protein quality control. Despite their lack of induction during heat stress, at least one of them, SACOL1045, an iron uptake component, has already been linked to the regulatory function of ClpP (Michel *et al.*, 2006).

(8) Oxidative stress response: As mentioned above, *S. aureus* faces oxidative stress during its pathogenesis. Several genes that encode for detoxifying enzymes and iron-uptake components, and their regulators PerR and Fur were located in the same region of the cluster tree, but did not form a distinct cluster. This observation can be explained by sequential activations and inactivations that are dependent on the kind of oxidative stress and the status of the intracellular and extracellular iron availability. E. g. three different iron uptake systems are divergently activated or repressed by PerR and Fur, providing the right iron uptake system at the right time and avoiding an excess of intracellular iron that can lead to toxic Fenton reaction under oxidative stress. Additionally, it should be considered that these genes might already be expressed under control conditions in the respective research studies.

(9) Antibiotic resistance: A screening for genes that facilitate resistance to cell wall stress-inducing antibiotics also did not lead to the identification of a clear cluster. This observation is being dealt with by another approach in the following chapter.

3.3.3 Cluster of expression profiles for cell wall stress-inducing antimicrobial compounds

As elaborated in 3.3.2, the screening for a gene cluster that facilitates resistance to cell wall stress-inducing antibiotics led to a negative result. Consequently, a reciprocal approach was applied. Alternatively to the clustering of the more than 2,000 genes, the 282 different conditions were clustered instead. The resulting cluster tree of conditions unveiled a cluster of cell wall stress-inducing antimicrobial compounds, e. g. the antibiotics Vancomycin and Oxacillin (Fig. 19). The clustering of Vancomycin expression profiles generated by 4 different research groups underlines the robustness of this analysis. Interestingly, this cluster also contains antimicrobial compounds whose mode of action is not fully understood so far, e. g. the membrane-active agent CCCP, Magnolol, human β-defensin, and several cationic antimicrobial peptides. The clustering of these compounds suggests a common interference with *S. aureus* cell wall turnover. This cluster also includes mutants of two regulatory systems, whose products are involved in cell wall turnover: the two-component system GraRS, and MvaAKS.

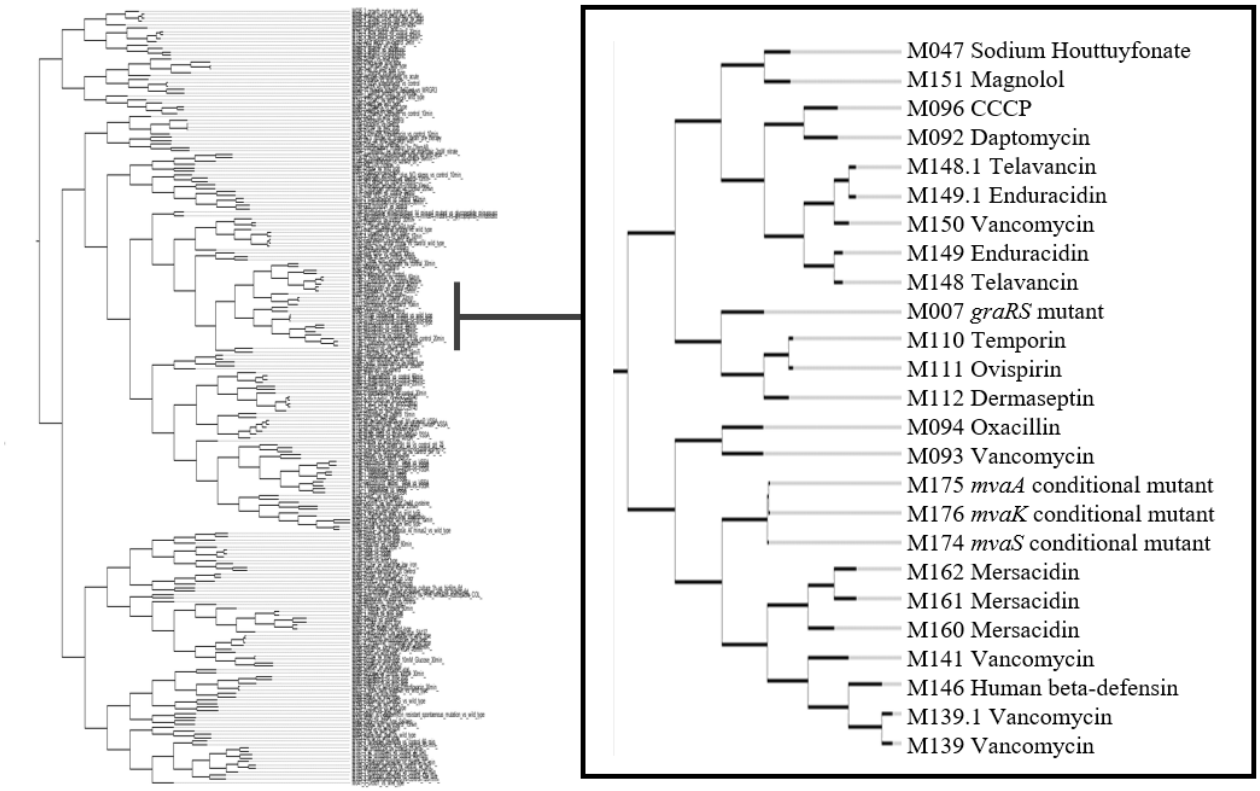


Fig. 19. Cluster tree of 282 different DNA microarray conditions (left). The identified cluster of cell wall stress-inducing antimicrobial compounds is zoomed in (right). Prefixes to the respective condition, e. g. M047, refer to the respective experiment in the data base.

Since there was no cluster for genes that are involved in resistance to cell wall stress-inducing antibiotics, the condition cluster, presented in Fig. 19, was used as a filter to identify genes whose expression was predominantly up-regulated under these conditions. The most dominant genes are listed in Table 9. Among the 10 most dominant genes, three regulatory systems were identified: (1) the two-component system *vraRS*, known as a major regulator of cell wall homeostasis (Galbusera *et al.* 2011, and Overton *et al.*, 2011), (2) the regulator-encoding *lytR*, known to be involved in autolysis (Brunskill and Bayles, 1996), and (3) the gene SACOL2517, encoding a MarR family regulator of unknown function. Additionally, this stimulon also includes several members of class I and III heat shock response, including the regulator CtsR, the modulator McsA, and the chaperone DnaK. All these genes are part of the CtsR regulon, and their up-regulation under the majority of microbial compounds in this condition cluster strongly indicates elevated protein damage and misfolding. In fact, cell wall stress-inducing antibiotics are known to induce oxidative stress (Kohanski *et al.*, 2007). The majority of the genes that are presented in Table 9 are of unknown function and thus might be suspected to be involved in antibiotic resistance to cell wall stress-inducing antibiotics and compounds, and / or cell-wall turnover in general.

Alltogether, the results presented in part 3 of this PhD thesis underline that cluster analysis is a highly valuable approach for the characterization of the regulatory network in *S. aureus*. It can be used as a prediction model for genes with unknown function and regulation, and for antimicrobial compounds with unknown mode of actions. Additionally, this cluster analysis contributes to the understanding of the setup and regulation of host-pathogen interactions and provides a plethora of valuable suggestions for novel experimental starting points. This cluster analysis was already used to predict funcions of lipoproteins with yet unknown function (Graf *et al.*, 2018), and for the identification of protein complexes (Liang *et al.*, 2016), as it will be discussed in the Discussion below.

Table 9. Genes that are up-regulated by cell wall stress-inducing compounds (black boxes)

Locus	Symbol	Va	Ox	Da	De	En	Te	Tm	Ov	Gr	Mv	So	Hu	Ma	CC
SACOL1944	*vraT*	■	■	■	■	■	■	■	■	■	■	■	■	■	
SACOL1897	*prsA*	■	■	■	■	■	■	■		■	■	■	■		
SACOL1943	*vraR*	■	■	■	■	■	■	■		■	■	■	■		
SACOL1943	*vraS*	■		■	■	■	■	■	■	■	■	■	■		
SACOL2517		■	■	■		■	■			■	■	■	■	■	
SACOL1457	*ptbA*	■	■	■		■	■	■	■	■	■	■			■
SACOL2302	*lytR*	■	■	■		■	■	■	■	■	■	■			■
SACOL1777	*htrA*	■	■	■		■	■	■		■	■	■	■		
SACOL0967		■	■	■		■	■	■	■	■	■	■	■		
SACOL2315		■			■	■	■	■	■	■	■	■	■		
SACOL0692		■	■	■	■	■			■	■	■		■	■	■
SACOL1438		■		■	■	■	■	■	■	■	■	■		■	■
SACOL2179			■	■	■	■	■	■	■	■	■	■			■
SACOL1440	*xpaC*	■	■	■		■	■		■		■	■	■		■
SACOL2571		■		■	■	■		■	■	■	■	■		■	
SACOL1956		■	■	■		■	■			■	■	■	■		
SACOL1945		■			■	■	■	■	■	■	■	■	■		
SACOL2550		■				■	■	■	■	■	■	■		■	■
SACOL1705		■			■	■	■	■	■	■	■	■		■	
SACOL1456		■	■	■		■	■	■	■	■	■				■
SACOL2518	*relP*	■				■		■	■	■	■	■			
SACOL2116	*murZ*	■	■	■		■	■			■	■				
SACOL2436		■	■	■		■	■			■	■	■	■		
SACOL0968	*spsA*	■	■	■		■	■	■	■		■		■		
SACOL0191		■			■		■	■	■	■	■	■	■		
SACOL1059		■	■	■	■	■		■	■	■	■		■		
SACOL1066	*fmtA*	■	■	■		■	■			■	■		■		
SACOL1439	*acyP*	■		■		■	■			■	■				■
SACOL1637	*dnaK*		■	■		■	■	■	■	■		■		■	■
SACOL2522			■	■	■	■	■	■	■	■		■			■
SACOL2713		■	■	■	■		■	■	■		■		■		
SACOL0163		■				■	■	■	■		■	■			■
SACOL1486		■	■	■		■	■			■	■				■
SACOL1831	*tal*	■		■		■	■	■	■		■	■			■
SACOL2352	*tcaA*	■	■	■		■	■	■	■		■				
SACOL2343				■		■	■		■		■	■			■
SACOL0625	*vraX*	■	■	■	■	■	■		■	■		■		■	
SACOL2435	*glxK*	■	■	■		■	■					■	■		■
SACOL0419					■	■	■	■	■		■		■		
SA1256	*msrB*	■				■	■		■	■	■	■			
SACOL1441	*telA*			■		■	■				■	■			■
SACOL0922	*fabK*			■		■	■	■	■		■				■
SACOL1003	*mecA*		■	■		■	■				■	■			■
SACOL0811			■	■		■	■				■	■		■	■
SACOL0567	*ctsR*			■		■	■	■	■	■		■			■
SACOL0783	*opuB*			■		■	■	■			■				
SACOL0161				■		■	■				■	■			■
SACOL0111	*butA*			■		■	■	■	■	■		■		■	■
SACOL0569				■		■	■	■	■	■		■		■	■
SACOL0568	*mcsA*			■		■	■	■		■		■		■	■

Va: Vancomycin (glycopeptide antibiotic)

Ox: Oxacillin (β-lactam antibiotic)

Da: Daptomycin (lipopeptide antibiotic)

De: Dermaseptin (cationic antimicrobial peptide)

En: Enduracidin (polypeptide antibiotic)

Te: Telavancin (cationic antimicrobial peptide)

Tm: Temporin (cationic antimicrobial peptide)

Ov: Ovispirin (cationic antimicrobial peptide)

Gr: *graRS* deletion mutant

Mv: *mvaASK* conditional mutants

So: Sodium Houttuyfonate

Hu: Human β-defensin 3

Ma: Magnolol

CC: CCCP

4. Discussion

This PhD thesis elaborates the transcriptional regulation of a variety of adaptation strategies that are involved in *S. aureus* pathogenesis. All these adaptation strategies would be completely useless if *S. aureus* would not be able to use the appropriate adaptation strategy at the right time and at the right place. *S. aureus* overcomes this challenge with a sophisticated regulatory network that senses a plethora of intra- and extracellular stimuli, and consequently fine-adjusts the expression of virulence factors and pathogenesis-relevant responses.

In the late 1980s to early 1990s, first steps in the characterization of the regulatory network of virulence factors in *S. aureus* were made by the research groups of Arvidson *et al.*, Cheung *et al.*, and Novick *et al.* Initial analyses identified the quorum sensing system Agr and the staphylococcal accessory regulator SarA as the two major regulators of α-heamolysin (Hla) and Protein A (Spa) expression. In the course of investigation, it quickly became clear that this regulatory network is much more sophisticated, and also includes several other regulators of the Sar family, the two-component system SaeRS, and regulators of metabolism (CodY) and stress response (Sigma B). With the inclusion of "Omic"-approaches, primarily carried out with regulator knockout mutants, research in this topic extended beyond the investigation of specific gene regulations to a global approach of expression profiling. Though, the contribution of "Omic"-approaches is of undoubtfully high value, several hurdles showed up. (1) Next to direct regulations, regulator knockout mutations also show indirect regulations via other regulators that are under the control of the regulator of interest. These indirect regulatory effects complicate the characterization of the sophisticated regulatory network. (2) Regulator knockout mutations can also cause regulatory side effects. E. g., a *rex* deletion mutant shows up-regulation of the urease operon under aerobic conditions (Pagels *et al.*, 2010). Since Rex does neither directly nor indirectly regulate this operon, it can be assumed that the artificial up-regulation of lactate production in the *rex* mutant under aerobic conditions causes an acidic shift of the intracellular pH. This change might be sensed by another regulator that is independent of Rex and, as a consequence, up-regulates the urease operon to buffer the intracellular pH via the production of ammonium ions. It can be suspected, that comparable physiolocigal side effects from regulator knock-outs could also influence the Agr system and CodY, since the expression and / or activity of these regulators depends on the metabolic state of the bacterium. (3) Spontaneous mutations have been described in a plethora of strains and clinical isolates, including the quorum sensing system Agr *in vitro* (Somerville *et al.*, 2002) and *in vivo* (Shopsin *et al.*, 2010), *rsbU* as an activator of Sigma B in strain NCTC8325 (Giachino *et al.*, 2001), the member of the Sar family TcaR in strain NCTC8325

and its derivatives (McCallum *et al.*, 2004), a point mutation in SaeS in strain Newman (Mainiero *et al.*, 2010), and several two-component systems that are often defective in vancomycin intermediate clinical isolates VISA (WalKR, VraRS, and GraRS) (Howden *et al.*, 2008 and 2010; Cui *et al.*, 2009; Galbusera *et al.*, 2010; Hafer *et al.*, 2012; Koch *et al.*, 2014). Since regulators of virulence gene expression are often interconnected with each other, the knockout of a regulator of interest might result in different expression profiles in different strains, dependent on the genomic background of these strains. (4) Different experimental conditions, e. g. growth phase, growth medium, and shaking velocity, used by different research groups lead to different or even contradictory results for the same regulator of interest. (5) The results of "Omic"-approaches usually come in big data sets, which make a clear depiction of the regulatory network challenging.

This PhD thesis deals with these hurdles via the application and development of regulator-promoter interaction studies (dealing with (1) and (2)), and via the use of a cluster analysis for "big data" from DNA microarray analyses from the worldwide literature (dealing with (3) and (4)). Finally, this cluster analysis can also be used as a tool for the depiction of the regulatory network as a whole (dealing with (5)).

Despite all contradictory results for the interwoven network of virulence gene expression in *S. aureus*, the literature agrees upon one point: The expression of virulence factors in *S. aureus* is dependent on multiple intra- and extracellular signals and conditions. Since the regulatory regions of genes are restricted to only several hundred DNA base pairs, a direct regulation via a dozen of regulators and sensory systems that bind to such a regulatory region of limited space is unfeasible. *S. aureus* overcomes this challenge with a highly sophisticated regulatory network including secondary regulators. Simplified, several sensory systems directly regulate specific secondary regulator, that finally directly regulate the respective target virulence gene. With this network, a multitude of intra- and extracellular signals can be incorporated into the regulation of one specific virulence gene.

4.1 Regulator-promoter interactions for three selected regulators

(A) Rex and the adaptation to anaerobic conditions: Since most of the regulatory systems that are presented in this PhD thesis were already described in the literature, this work focussed on the characterization of a novel Rex homolog in *S. aureus*. This study clearly demonstrates that Rex in *S. aureus* is not to be seen as an oxygen-dependent sensing system, but as the major repressor in the intracellular NAD / NADH homeostasis. In contrast to *Bacillus subtilis*, Rex in *S. aureus* does not regulate components of the respiratory chain. Instead, Rex represses anaerobic metabolic pathways under aerobic conditions in the presence of NAD (Fig. 20).

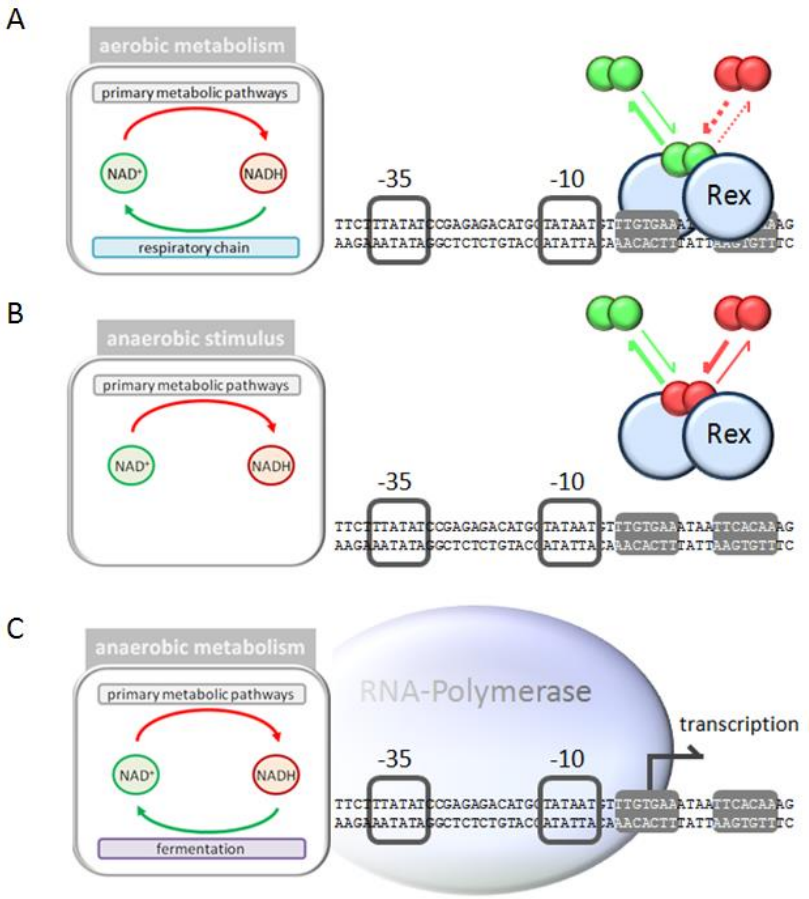


Fig. 20. Schematic depiction of the anaerobic adaptation in *S. aureus*. (A) Aerobic metabolism: Rex is bound by NAD and represses anaerobically induced genes. (B) Anaerobic stimulus: Rex is inactivated by NADH and leaves its repressor sites. (C) Anaerobic metabolism: Rex inactivation allows binding of the RNA polymerase and expression of anaerobic metabolic pathways that install an alternative intracellular NAD / NADH cycle. The DNA sequence and the corresponding promoter are presented for *adhE* (ethanol dehydrogenase).

In this scenario, NAD competes with NADH for a Rossman fold-like domain. Under conditions when the respiratory chain is inactive, NADH accumulates and consequently inactivates Rex, leaving genes for anaerobic adaptation de-repressed. As a result, an alternative intracellular NAD / NADH cycle is installed. Due to the instability of NAD and NADH, the exact measurement of the intracellular concentrations is difficult. Nonetheless, preliminary data from two independent research groups suggest that the intracellular NAD / NADH ratio can differ significantly between aerobic and anaerobic conditions, with a significant increase of the NADH concentration under the latter condition (Lalk *et al.* and Proctor *et al.*; unpublished data). The strategy of NAD / NADH-sensing appears to be particularly smart, since the presence or absence of oxygen alone does not tell much about the current environmental and intracellular situation in the first place. E. g., even in the presence

of oxygen, the respiratory chain may be disabled due to spontaneous mutations in *hemB* or *menD*, as it can be found in small colony variants (SCV), the oxidative burst of professional phagocytes, or needs to be tuned down in the presence of cell wall stress-inducing antibotics that induce ROS production. Instead, sensing and reacting directly to a drop in the intracellular NAD / NADH ensures the maintenance of NAD / NADH homeostasis universally when the respiratory chain is impaired.

Genes that are repressed by Rex under aerobic conditions cover a broad spectrum of anaerobic metabolic pathways, corresponding transporters, and specific activators. L-lactate dehydrogenase (Ldh), D-lactate dehydrogenase (Ddh), and the corresponding lactate extrusion component (LctP) appear to be solely under regulation of Rex. Transcription of these genes was already at its maximum in a *rex* mutant under aerobic conditions, indicating that no additional activation is required for full expression. This finding implies that the immediate response to NADH accumulation in *S. aureus* is lactate fermentation. In fact, metabolite measurements revealed an excess lactate production in the *rex* mutant even under aerobic conditions (Pagels *et al.*, 2010). This might also explain why the regulatory region of *ldh* contains two Rex binding sites, whereas all other Rex target genes contain a single one.

In contrast, all other operons are additionally induced under anaerobic conditions, compared to a *rex* deletion mutant under aerobic conditions. This indicates the existence of additional activators that are required for full expression. These operons encode for ethanol fermentation, nitrate and nitrite respiration, the arginine deiminase pathway, an alanine dehydrogenase pathway that generates ATP, corresponding transporters, and regulators (Fig. 21).

Most of these operons are activated by one out of four regulatory system. (1) One of these systems is the two-component system NreBC (Sc*hla*g *et al.*, 2008). This 2KS is an activator of components for nitrate and nitrite respiration, and the *arc*-operon that encodes the arginine deiminase pathway (further described below). Activity of NreBC is dependent on an oxygen-labile iron-sulfur cluster which is non-functional under aerobic conditions. The expression of this 2KS is not repressed by Rex, though most of its target genes are. (2) A further activator of anaerobic gene expression is NirR that activates its own operon, encoding for nitrite respiration, via an unknown mechanism. The transcription of this regulator is repressed by Rex and activated by NreBC. (3) Another activator of anerobic gene expression is the 2KS SrrAB (Yarwood *et al.*, 2001; Pragman *et al.*, 2004; Windham *et al.*, 2016). The activatory signal of SrrAB is unknown, though it is speculated that the redox state of components of the respiratory chain, particularly menaquinone, can influence the activity of the sensor kinase SrrB. SrrAB is homologous to ResDE in *Bacillus subtilis* (Larsson *et al.*, 2005; Nakano *et al.*, 2006). Under specific conditions, SrrAB activates, among other genes, the expression of components of the respiratory chain (*qoxABCD*, *cydAB*, and *hemABCX*; not repressed by Rex) and

of ethanol fermentation (*pflBA* and *adhE*; repressed by Rex). This 2KS is under weak repressory control of Rex. (4) Finally, the regulator ArcR activates the expression of its own operon that encodes the arginine deiminase pathway, generating ATP, and ammonium ions probably to buffer the pH during extensive lactate production. ArcR is active when bound to cyclic AMP (Makhlin *et al.*, 2007). This regulatory system is also under weak repression of Rex, and activation of NreBC as well.

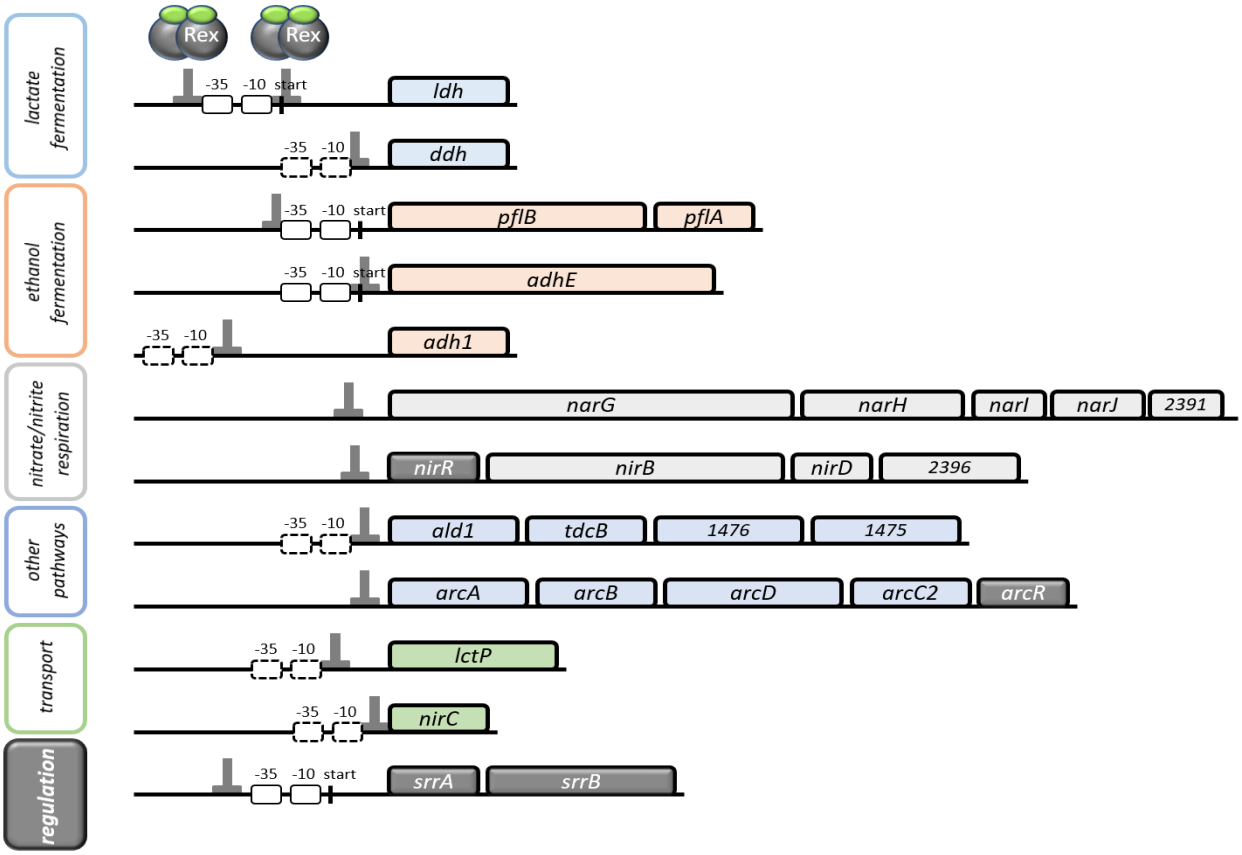


Fig. 21. Schematic depiction of the Rex regulon in *S. aureus*. When bound to NAD, Rex occupies typical repressor sites (marked with grey stamps). Verified promoters are shown in white boxes with continuous lines, whereas putative promoters are shown in white boxes with dashed lines. Verified transcription start points are marked with "start". Secondary activators are marked with black boxes. Gene numbers are taken from strain COL.

After identification of Rex repressor sites with EMSA, all verified Rex motifs were aligned and characterized in detail with the help of site directed mutagenesis. The resulting final Rex consensus sequence, TTGTGAA W_4 TTCACAA, provided explanation for the observed differences of Rex affinity to different binding sites. In fact, it also explains observations from *B. subtilis*. To date, 7 Rex binding sites were identified in *B. subtilis*, regulating the expression of cytochrome bd, lactate fermentation, a NADH dehydrogenase, and a putative transporter (Wang et al., 2008). The binding sites with the highest affinity, *ywcJ* and *ldh2*, also show the best matches to the Rex consensus sequence in *S. aureus*, with 1, respectively 2 mismatches. Additionally, these mismatches are located

at lesser important positions: in the spacer region and in the outermost flanking position of the inverted repeat. In contrast, all 3 binding sites in the regulatory region of the *cyd*-operon have 3 mismatches in the middle of one site of the inverted repeat. These findings strongly suggest, that the *S. aureus* Rex consensus sequence presented in this study is also applicable for *B. subtilis*.

To identify additional putative Rex binding sites, the final Rex consensus sequence was used in a search vs. the *S. aureus* genome. The resulting list was squared with the results from *"global footprinting"*, and with the anaerobic expression profile from Fuchs *et al.* (2007). This comprehensive search resulted in the identification of three additional Rex targets: SACOL0166 (integral membrane protein; unknown function), SACOL2491 (unknown function), and *narT* (also referred to as *narK*; SACOL2386; putative nitrate transporter). All three genes are anerobically induced.

Altogether, the inactivation of Rex by NADH appears as the pre-requisite for effective anaerobic adaptation in *S. aureus*, with lactate fermentation as the immediate response. Other metabolic pathways require specific activation by additional regulatory systems, with some of them being under Rex repression as well. With this regulatory cascade, *S. aureus* is able to adjust its anaerobic response to multiple factors, including nitrate availability, the intracellular decrease of the pH due to an excess in lactate production, and the production of harmful ethanol as a by-product of ethanol fermentation. Nonetheless, *S. aureus* is also capable of butanediol and acetate fermentation as well (Fuchs *et al.*, 2007), whose regulation is independent of Rex via an unknown mechanism, leaving a reason for further investigations.

(B) CodY and the adaptation to amino acid starvation: The involvement of CodY in amino acid starvation has already been extensively characterized in other bacteria, and in *S. aureus* as well (Majerczyk *et al.*, 2008 and 2010). As part of the stringent response, GTP is converted to the alarmone ppGpp due to an attenuation in the ribosomal translation machinery. GTP and branched-chain amino acids act as activatory ligands of the repressor CodY. Consequently, a drop in the intracellular concentrations of these ligands causes inactivation of CodY and de-repression of its target genes. In contrast to other bacteria, CodY in *S. aureus* does not only repress a broad set of biosynthesis pathways for amino acids and according transporters, but also virulence factors, particularly extracellular proteases (Majerczyk *et al.*, 2010). Additionally, our group demonstrated significant changes in the extracellular proteome in *codY* mutants in strains Newman, RN1HG, and UAMS-1. In this scenario, CodY did not influence the expression of the major regulators of virulence factors, Agr, SarA, SaeRS, and Sigma B, indicating that the regulatory effects of CodY are direct (unpublished data from Nina Köhler). In the course of this PhD thesis, commonly used EMSA experiments with CodY led to ambiguous results due to methodical reasons. Consequently, a novel experimental setup

was developed and applied for the characterization of CodY (and beforehand for Rex as well). With this new method, from this point referred to as REPA (**r**estriction **e**ndonuclease **p**rotection **a**ssay), we were able to verify the binding of CodY to its consensus sequence, and quantify the effects of GTP and branched-chain amino acids as activatory ligands.

This novel easy-to-use method is a simple restriction endonuclease digest of a PCR product (encompassing the putative regulator binding site), followed by an agarose gel electrophoresis with Ethidium bromide staining, as it is carried out on a daily basis in every molecular biological laboratory these days. In addition, the researcher only needs his purified regulator of interest. Consequently, this method can be easily applied in the case that EMSA or other approaches fail, or as an alternative for time-consuming DNAseI protection assays that demand radioactive labeling and gel electrophoresis with hard-to-hande sequencing gels. It can also be used to easily test a multitude of buffer conditions to find the appropriate setup for the regulator of interest before starting more complex experimental methods. It can also be used to identify regulator ligands, and characterize and quantify their activatory or inhibitory effects on the regulator.

As a drawback, this method depends on the availability of an appropriate restriction endonuclease that overlaps with the binding site of the regulator of interest, generates an appropriate restriction pattern and shows activity under regulator-specific buffer conditions. Nonetheless, several hundreds of restriction endonuclease are commercially available to date, providing the researcher with a broad set of options. E. g. the current catalog of New England Biolabs (NEB) offers a collection of 285 different restriction endonucleases. Another drawback of this method is a usually lower resolution compared to DNAseI protection assays, not allowing the identification of the binding site to the exact base pair. Nonetheless, this method provides the researcher with the appealing option to use REPA for trial experiments before going into complicated DNAseI protection assay if a better resolution of the binding site is necessary. Altogether, REPA has proven its worth as a time-saving and easy-to-use method in this PhD thesis and can be used for various applications and research topics beyond this study.

(C) SarA and the members of the Sar-family: This PhD thesis provides a comprehensive picture of the relations between the members of the Sar-family, based on amino-acid sequence homology. The sequence alignment of Sar homologs resulted in the differentiation of three groups that are clearly separated from each other:

(1) The first group contains the majority of Sar homologs, including SarA and Rot, and probably evolved early on from one common ancestor that is homologous to Rot in *Maccrococcus caseolyticus*. This Rot homolog is the best match (based on amino acid sequence) outisde of *S. aureus* for all Sar homologs that cluster together in the first group. Consequently, this group can clearly be

described as a Sar family of regulators. Interestingly, some members of this group (SarT, SarS, and SarU) are absent in all other 57 *Staphylococcus* species and show highest similarity to SarA. Thus, it can be speculated that these homologs evolved from SarA in *S. aureus*. SarT, SarS, and SarU can be seen as secondary regulators of virulence gene expression, since they are directly and / or indirectly regulated by SarA itself, and other major regulators, e. g. the Agr system. These secondary regulators generate a branched network that is unique for the species *S. aureus*.

Among the Sar family members of the first group, only SarA contains a cysteine residue. This residue might be responsive to oxidative stress and could lead to the formation of disulfide bonds between several SarA dimers in the presence of oxidizing compounds. Nonetheless, this PhD thesis demonstrated only a slight positive effect of H_2O_2 and Diamide on SarA-binding activity to the promoter region of the α-hemolysin (Hla). This is even in contrast to the finding of Ballal and Manna (2010) who demonstrated a negative [sic] effect on SarA binding activity to the promoter region of *trxB* (thioredoxin reductase) under comparable conditions. This contradiction can be explained by differences in the proposed mode of action and consequent differences in the experimental procedure: The authors suggested an OhrR-like mechanism for SarA, as it was already demonstrated for SarZ and MgrA. Though, this assumption seems unlikely, since this PhD thesis shows that SarA only shares weak homology to SarZ, MgrA, and OhrR homologs in other bacteria, and in contrast to these OhrR-like regulators, the cysteine residue of SarA is not located in the dimerization domain of SarA (Schumacher *et al.*, 2001). However, both models of SarA activity are possible in theory, since H_2O_2 and Diamide are able to generate disulfide bonds in a first oxidation step, but can also lead to the destruction of disulfide bonds via higher oxidation to sulfenic acid (Luo *et al.*, 2005; Wolf *et al.*, 2008). In this PhD thesis, SarA was simultaneously incubated with PCR product and oxidizing agents to allow cross-linking between several SarA dimers that already occupy their binding sites. In contrast, Ballal and Manna performed pre-incubation of SarA with oxidizing agents to inactivate SarA and subsequently added the PCR product. Despite this propable explanation, these contrary results make the model of disulfide bonds between multiple SarA dimers doubtful.

Given that the amount of SarA protein appeared astonishingly constant in strain COL during several stress conditions and growth phases (data not shown), it is likely that SarA target genes might not be regulated by a change in the concentration of SarA protein, but by a change in its activity. In fact, several research groups have identified three phosphorylation sites in the SarA protein (Didier *et al.*, 2010; Ohlsen and Donat, 2010; Bäsell *et al.*, 2014). However, the impact of phosphorylation by the Ser/Thr kinase PknB (also named Stp1) on SarA activity still has to be evaluated.

(2) The second group of the members of the Sar family includes SarZ and MgrA. Amino acid homology between these regulators and OhrR in *Bacillus subtilis* was higher than the homology to

other Sar family members in *S. aureus*. These two regulators share 45%, respective 39% amino acid identity with OhrR, contain an N-terminal OhrR-like motif around the cysteine residue, and regulate their target genes via an OhrR-like mode of action: Repression of target genes requires the formation of disulfide bonds that lead to dimerization and binding to DNA in the absence of oxidative stress (Fuangthong and Helmann, 2002; Chen *et al.*, 2006 and 2009). Under oxidative stress, disulfide bonds are further oxidized, leading to a loss in dimerization and de-repression of respective target genes.

(3) The third group of the homolgy tree contains the Sar homolog TcaR. Since homologies between TcaR and the Sar family members of group 1 and 2 are weak, and instead higher to other regulators that are not members of the Sar family, it is somewhat questionable if members of this group should truly be recognized as members of the Sar family.

Alltogether, the results from this PhD thesis suggest the grouping of Sar homologs into three separate groups from both, the evolutionary and the functional point of view. These groups evolved from different evolutionary progenitors and have different functional properties: Group 1 consists of Sar homologs that are only present in the *Staphylococcaceae* family (including *Macrococcus*). Group 2 consists of OhrR homologs that regulate their target genes via an OhrR-like sensing of oxidative stress. Group 3 consist of three ambiguous regulators, including TcaR, that only show marginal homology to other Sar homologs. Based on their homology to regulators from other bacteria, only the Sar homologs from group 1, including SarA, truly form a distinct family of regulators.

4.2 Method development *"global footprinting"*

The first part of this work presents specific regulator-promoter interaction studies for three selected regulators of host-pathogen interactions. Global-scale approaches are already well established in the terms of Genomics, Transcriptomics, Proteomics, and Metabolomics, providing comprehensive data sets. However, a well-established global scale approach to regulator-promoter interactions is still missing in the arsenal of molecular-biological research tools. Initial approaches were made in the development of *ChIP-on-chip* (**c**hromatine **i**mmuno **p**recipitation, blotted on a DNA microarray chip) that delivers a snap shot of the regulatory status from the inner of the living cell (Blat and Kleckner, 1999; Buck and Lieb, 2004). This method opens with treatment of formaldehyde that crosslinks DNA-bound regulators with its binding sites within the living cell. Subsequently, cells are disrupted and the isolated chromosome is fragmented. Regulator-DNA complexes are immuno-precipitated by anti-regulator IgGs that retain the respective complexes during purification. Finally, DNA is dissolved from the regulator, translated into RNA, and quatified with a DNA microarray chip.

Although, this concept is highly appealing, many bottlenecks can occur during the process. (1) The highly critical fixation of regulator-DNA complexes. Formaldehyde crosslinks free amino groups that are in very close proximity of up to 1 Angström to each other. Since many bacterial regulators appear to have small sizes, e. g. 124 amino acids for SarA, the absence of such amino groups in the regulator protein and / or the absence of an adjacent group in the DNA can limit this method in its application. Low treatment with formaldehyde can lead to insufficient cross-linking, whereas high treatment can lead to partial denaturation of the regulator, eliminating precious epitopes that are required for the following immuno-precipitation. Since cell envelope and intracellular components quench formaldehyde, the appropriate concentration of formaldeyde is hard to estimate and depends on a multitude of factors. (2) The separation and purififaction of the regulator-DNA complexes from whole cell extract. A search in the literature resulted in the finding of a plethory of fundamentally different protocols from different research groups that tried to apply this method. (3) The regulator of interest might not occupy all of its binding sites due to inactivity or competition with other regulators for overlapping DNA binding sites. Regarding these obstackles, is it not surprising, that to date only two global-scale studies have been published for *S. aureus* (Majerczyk *et al.*, 2010, Liu *et al.*, 2010).

Since trial runs for a *ChIP-on-chip* setup for the regulator Rex failed in this PhD study (data not shown), an alternative *in vitro* approach was developed. In this method, whole genomic *S. aureus* DNA was fragmented, incubated with *Strep*-tagged Rex, and consequently, Rex binding sites were enriched via the run through a *Strep*-tag purification column. The enriched DNA fragments were identified and quantified by *next generation sequencing* (NGS). This method yielded 9 out of 13 Rex binding sites that were already identified in this study by EMSA. The missing of one of these binding sites was as expected, since this site contains a mutation in the Rex binding site in the genome of strain COL that was used in this experiment. Two of the other 3 missing binding sites show several mismatches in their Rex motives, were already verified to have only low affinity to Rex, and the influence of Rex on the transcription of these genes was marginal. The Rex binding site with the highest affinity in the promoter region of *adhE* was detected exactly to one base pair among the whole genomic sequence. This approach also yielded two novel Rex target genes, *narT* and SACOL0166 (a membrane protein with unknown function). These genes are induced under anaerobic conditions and contain a Rex motif in their regulatory regions.

The remaining 7 enriched sites that were also enriched can be regarded as false positive, since they did not contain a Rex consensus sequence and most of them are not induced under anaerobic conditions. An explanation for the finding of false positives can be found in the experimental procedure: Whole genomic fragmented DNA was labeled for NGS before the enrichment procedure with *Strep*-tagged regulator. NGS labeling results in a dramatic loss of sample DNA and thus the

regulator might bind to unspecific sites due to a lack of competition. Regulator-DNA interactions are a steady-state process with low dissociation constants for specific target sites and high dissociation constants for unspecific target sites. Consequently, the regulator of interest would preferably occupy specific sites. Nonetheless, if the regulator is incubated with a residual amount of DNA, unspecific sites would be occupied as well, resulting in false positives. As a consequence, the author of this PhD thesis recommends the testing of an alternative protocol where the labeling for NGS is carried out after the enrichment procedure, instead of beforehand.

Altogether, this work values *"global footprinting"* as a useful addition to the arsenal of methods for the characterization of regulator-promoter interaction studies. *ChIP-on-chip* still appears as the method of choice to start with, however, if this method fails, *"global footprinting"* offers a valuable and easy-to-apply alternative for the identification of regulator target genes across the whole genome. Nonetheless, the presence of false positives demands further optimization, and besides that, results inevitably have to be confirmed by follow-up studies, e. g. with a regulator knock-out mutant in proteomic and / or transcriptomic approaches. The combination of expression profiling "Omics" and the genome-wide identification of regulator target genes present the current state-of-the-art for the breakdown of sophisticated regulatory networks.

4.3 Cluster analysis

Besides "Omic"-approaches and regulator-promoter interaction studies, the investigation of a sophisticated regulatory networks also demands to deal with several hurdles. These are (1) strain-specific differences that lead to contradictory results, (2) differences in experimental results due to different research groups that used different methods and experimental conditions, (3) incomplete expression patterns and analytical windows, and (4) the confusing arrangement of a plethora of regulatory systems that are interwoven with each other. To date, two approaches appear to be viable. The first approach is to select one specific strain, whose genomic status is well-characterized, and subject it to an array of stress and starvation stimuli to get a clear picture of the whole regulatory network (at least for this specific strain). This approach was recently carried out by Mäder *et al.* (2016) in strain HG001, a derivate of NCTC-8325. In this strain, a *rsbU*-deficiency has been repaired, and it was cured from prophages (Caldelari *et al.*, 2017). The other approach is to select one specific stimulus and apply it to a set of different strains, to identify common response patterns and separate them from strain-specific deviations. This latter approach was chosen for this PhD thesis, because the current state of the literature allows the creation of a comprehensive model for reviewing and

prediction purposes. The bottleneck for the creation of such a prediction model is the extent of data that are available in the literature. On one hand, if too few data are available, the creation of a prediction model would be urgent, but there would be no basis for the creation of such a prediction model due to the lack of initial data. On the other hand, if everything is already known, the creation of a prediction model would be easy, but futile. After reviewing the current state of literature, the author of this PhD thesis evaluates *S. aureus* to be perfectly in between these two extremes. Consequently, the creation of a prediction model appears to be both, doable and useful.

A comprehensive literature research was carried out for DNA microarray data and regulator-promoter interaction studies, leading to a data library of more than 50,000 regulatory events and more than 2,000 regulator binding sites. With a cluster analysis of transcriptomic data, it was possible to not only compare the response patterns of several strains under one stimulus, but also to discover overlaps between the response patterns of several different stimuli. E. g. anaerobic gene expression patterns can occur under anaerobiosis, due to mutations in components of the respiratory chain (*hemB* and *menD*), and in *rex* deletion mutants under aerobic conditions. Additionally, several other conditions can be linked to at least a partly anaerobic response pattern: *arcR* mutants, *nreBC* mutants, *clpP* mutant, and hydrogen peroxide. Consequently, cluster analysis confirms stimulons and regulons from the literature in a more comprehensive way. It also elcidates, if the observed expression pattern is only applicable for the one specific strain that was used in that study, or if it is applicable for *S. aureus* in general. Furthermore, the discovery of 7 out of 9 pathogenesis-relevant functional and regulatory clusters (that were searched for in this study) validates the robustness of this data analysis and provides a quality control for the cluster analysis itself. Overall, this cluster analysis served several major goals. (1) The overall expression profiles of each single gene across the whole genome were compared. If two genes share a similar pattern, these genes can be suspected to function in the same physiological context. Consequently, this cluster analysis provides valuable functional predictions for genes with unknown function, and generates novel experimental starting points. (2) These two genes might also share the same regulation. Therefore, cluster analysis does not only contribute to the identification and complementation of stimulons, but regulons as well. (3) By comparing various expression patterns with patterns that are caused by antibiotic treatment, the mode of action of potential novel antimicrobial compounds can be elucidated.

(1) Stimulon complementation and functional prediction: This cluster analysis provides several functional predictions for genes with yet unknown function. Some of the most promising ones will be mentioned in the following: SACOL2333 and SACOL0007 were found directly adjacent to *spa* (Protein A) and *sarS* (the activator of *spa*) as part of the early stage virulence gene cluster. Since this cluster consists of cell envelope-anchored adhesins, it is intriguing that SACOL2333 is annotated as

a membrane protein. The cluster of late stage virulence factors contains two transporters, SACOL0454 (cysteine transport) and SACOL0255 (ribose transport). Since this cluster also contains genes encoding for extracellular proteases and nucleases, these transporters might be directly involved in the invasive phase of *S. aureus* pathogenesis, facilitating uptake of degradation products and consequently, the expoitation of new nutrient sources. Another interesting gene can be found in the midst of the anaerobic Rex cluster: SACOL2491 contains a Rex motif in its promoter region, is anaerobically induced, and clusters directly adjacent to *ldh* and *lctP*. Consequently, a role in lactate fermentation or at least anaerobiosis can be postulated. Additionally, the anaerobic cluster contains the cell wall-anchored adhesins SACOL0119 (*sasD*) and the antibody response-inducing membrane-anchored SACOL2660 (*isaB*; immunodominant antigen B). So far, these two genes have only been vaguely associated with *S. aureus* pathogenesis (Lorenz *et al.*, 2000; Ythier *et al.*, 2012; Liu *et al.*, 2015). The clear link to anaerobic adaptation, presented in this cluster analysis, might facilitate further investigations.

(2) Regulon complementation and regulatory prediction: The cluster of early stage virulence factors consists of cell envelope-anchored adhesins. The Protein A-encoding *spa* clusters directly adjacent to its own activator *sarS*, confirming the observations from several publications. Interestingly, another member of the Sar family, SarX, also adds itself to this cluster. So far, SarX has been described as a negative regulator of late stage virulence factors (SspA, Hla, and Hlb) via the Agr system and a positive regulator of biofilm production via the *ica*-operon (Manna and Cheung, 2006; Rowe *et al.*, 2011; Cue *et al.*, 2013). The results from the cluster analysis in this study suggest that SarX also has a strong positive effect on early stage virulence factors. This would be in accordance with the speculations from Manna and Cheung, that SarX is an antagonist of the Agr system.

The Sigma B cluster contains the *cap*-operon (capsule formation) due to an indirect Sigma B-dependent regulation. Previously, this regulation has been linked to the two-component system ArlRS, a positive regualtor of the *ica*-operon (Meier *et al.*, 2007). However, *arlRS* also lacks a Sigma B promoter. Interestingy, another positive regulator of *ica*-expression, MgrA (Luong *et al.*, 2003 and 2006), interacts with ArlRS regulation in this regulatory circuit (Luong and Lee, 2006, Crosby *et al.*, 2016 and 2019; Kwiecinski *et al.*, 2019), and the *mgrA* gene clusters directly adjacent to *rsbVW-sigB* in this cluster analysis. However, *mgrA* also lacks a Sigma B promoter as well, but still might be regulated on a posttranscriptional level via the directly Sigma B-dependent non-coding RNA RsaA (Geissmann *et al.*, 2009, and Romilly *et al.* 2014).

Cluster analysis showed that SarA binding sites (verified by the author of this PhD thesis in unpublished data, and by publications of other research groups) are located in front of both, early- and late stage virulence factors. Considering all available data, there is no clear pattern for SarA-

dependent regulation of early stage virulence genes. In *sarA* deletion mutants, some of these genes are up-regulated, some are down-regulated, and some are up- or down-regulated, dependent on the experimental conditions. Since SarA regulates several secondary regulators, e. g. SarS and SarT, the overall influence of SarA on the transcription of a specific virulence gene appears as a concert of direct and indirect regulations and thus can vary dependent on the experimental conditions and point mutations in other regulatory systems (Chien *et al.*, 1999; Schmidt *et al.*, 2003; McCallum *et al.*, 2004). In contrast to early stage virulence genes, a clearer picture can be drawn for SarA-dependent regulation of virulence genes of the late stage. These genes usually show the opposite expression pattern to SarA, assigning SarA the role of a repressor. When SarA expression is up-regulated, the expression of e. g. extracellular proteases is usually down-regulated. This was verified by a modified stand-alone run of the cluster analysis: The values of the expression pattern of SarA were manually inverted, e. g. a 3-fold increase in *sarA* transcription under a specific condition was artificially manipulated, by turning it into a 3-fold decrease. As a result, the artificially inverted *sarA* clustered within the cluster of late stage virulence factors (data not shown). This result strongly indicates that SarA is a repressor of late stage toxins and extracellular proteases in the first place.

(3) Characterization of the mode of action of antibiotic compounds: The first part of the cluster analysis compared the expression profiles of each single gene across the whole genome. The other way around, this part of the cluster analysis clusters and compares the more than 200 conditions, instead of all genes. There are several applications for this approach, e. g. the confirmation that mutations in *hemB* and *menD* (synthesis of components of the respiratory chain) cause anaerobic-like expression profiles, and the confirmation that the regulons of MgrA and ArlRS (an activator of MgrA) are strongly overlapping.

The most interesting application deals with antimicrobial compounds. As presented in Fig. 19, cell wall stress-inducing antibiotics (Vancomycin and Oxacillin) and antimicrobial compounds cause similar expression profiles in *S. aureus*. The 14 conditions of this cluster are astonishingly heterologous regarding the chemical origins of the anti-staphylococcal substances that were applied. They include β-lactam and glycopeptide antibiotics that inhibit peptidoglycan formation and cross-linking (Oxacillin, Vancomycin, and Enduracidin), antibiotics that act upon the membrane instead of the cell wall (Daptomycin), and cationic antimicrobial peptides (CAPs) that act upon the membrane as well (Dermaseptin, Temporin, and Ovispirin; Pietiäinen *et al.*, 2009). Additionally, this cluster of stimuli contains several anti-staphylococcal compounds whose modes of action are not fully characterized to date. (1) Magnolol, a chinese herb that is obtained from the stem bark of *Magnolia obovata* and *Magnolia officinalis*. Magnolol has been used in therapeutic treatments in China, Japan, and Korea (Huang, 1993) and inhibits *S. aureus* autolysis via an unknown mechanism (Wang *et al.*,

2011). (2) Sodium Houttuyfonate also inhibits *S. aureus* autolysis (Liu *et al.*, 2011). (3) CCCP (carbonyl cyanide m-chlorophenylhydrazone) may act in a Daptomycin-like mode of action (Muthaiyan *et al.*, 2008). (4) Human β-defensin 3 is a short antimicrobial peptide that is part of the innate immune response (Sass *et al.*, 2008). Finally, mutations in *graRS* and *mvaASK*, which are involved in cell wall turnover, also led to a cell wall stress expression pattern. GraRS has already been linked to the response to cationic antimicrobial peptides (Yang *et al.*, 2012).

Interestingly, some regulators and chaperones of the class-III heat shock response stimulon are up-regulated under most condition (*ctsR*, *mcsA*, and *dnaK*; Table 8 and 9). Since this stimulon facilitates the re-folding and degradation of damaged proteins under oxidative stress, this observation confirms that antibiotics (particularly cell wall stress-inducing ones) are able to induce oxidative stress in *S. aureus* (Kohanski *et al.*, 2007). This induction is usually mediated via an increased TCA cycle activity that may lead to an increase of respiratory chain activity as a result. Consequently, *S. aureus* itself would produce ROS (reactive oxygen species) via its own respiratory chain. The list of commonly up-regulated genes under cell wall stress-inducing compounds also includes the regulatory systems VraRS and LytR, known to play a role in cell wall turnover, and in the case of VraRS a role in clinical VISA (vancomycin intermediate *S. aureus*) has been verified (Brunskill and Bayles, 1996; Howden *et al.*, 2008 and 2010; Dengler *et al.*, 20011; Galbusera *et al.* 2011; Overton *et al.*, 2011).

Alltogether, these data suggest, that Magnolol, Sodium Houttuyfonate, CCCP, and Human β-defensin 3 act in a comparable physiological mechanism as Vancomycin and Oxacillin. Interestingly, a regulator with yet unknown function, SACOL2517, was up-regulated under 10 out of 14 cell wall stress-inducing conditions in this cluster, presenting a tempting target for further investigations.

4.4 Perspectives

The results from cluster analysis provide a comprehensive overview of pathogenesis-relevant stimulons and regulons in *S. aureus*. It also characterizes genes with unknown function by assigning these genes to specific functional and regulatory clusters. Consequently, new experimental starting points are suggested. The cluster analysis also facilitates the understanding of the adaptation to cell wall stress-inducing antimicrobial compounds in *S. aureus*. The comprehensive comparison of a multitude of stimuli in one cluster analysis depicts common expression profiles and adds to the characterization of modes of action of potential novel antibiotics in the future.

This prediction model has already been used in two publications so far. Recently, our group started to investigate the roles of 20 selected lipoproteins with unknown function in *S. aureus* pathogenesis (Graf *et al.*, 2018). With the aid of cluster analysis, predictions were already made for some of them. This prediction allowed the selection of appropriate experimental conditions for the testing of knock-out mutants of the respective lipoprotein-encoding genes. As a result, one lipoprotein was found in the Sigma B cluster. In fact, this gene is up-regulated in *sigB* mutants in several strains and contains a Sigma B consensus sequence in its regulatory region. Consequently, Sigma B-inducing stress conditions will be used to verify a potential importance of this lipoprotein in the adaptation process. Another pair of lipoproteins did cluster in close proximity to each other, indicating a shared functional and regulatory background. Both genes are up-regulated under Mupirocin, indicating a role in stringent response. Consequently, single or even double mutants will be subjected to Mupirocin in the future. Another lipoprotein clusters in close proximity to early stage virulence factors. Since these virulence factors are anchored to the cell envelope, a similar functional context was proposed for the membrane-anchored lipoprotein of interest. Finally, another lipoprotein clustered in moderate proximity to the cell wall stress response 2KS VraRS. In fact, the expression of this lipoprotein is up-regulated under Vancomycin, Enduracidin, Mersacidin, Telavancin, Daptomycin, and CCCP, indicating a role of this lipoprotein in cell wall stress response, and providing a set of appropriate experimental conditions under which the respective deletion mutant will be tested in the future.

Finally, the data library, containing more than 50,000 regulatory events and more than 2,000 regulator binding sites, will be made available for the public in the online data base *AureoWiki* in the future (Fuchs *et al.*, 2018). The corresponding cluster tree will be made available in an upcoming publication, whose manuscript is currently being written.

This PhD thesis covers experimental data for 33% of all known regulators in *S. aureus*, either due to the incorporation of DNA microarray data from the worldwide literature into cluster analyses or specific analyses of regulators of interest that are linked to *S. aureus* pathogenesis (Tab. 9). Another 28% of regulators have also been described in the literature so far, but were not included in this PhD thesis due to the lack of DNA microarray data and / or because they are part of a physiological context that is not linked to pathogenesis. The remaining 39% of all regulators have not yet been investigated at all. The cluster analysis, presented in this PhD thesis might facilitate the future course of investigations. It should be considered that additional regulators might be encoded in the *S. aureus* genome as well, partly annotated as putative regulators, and that regulation does not only include regulators of gene transcription, but also a plethora of other regulatory mechanisms, e. g. post-transcriptional regulation via regulatory RNAs. Altogether, this PhD thesis provides a comprehensive overall picture for the pathogenesis-relevant regulatory network, but still, the understanding of the whole regulatory network of *S. aureus* is not fully understood and demands further investigations.

Table 9. A list of *S. aureus* regulators and their current status of investigation.

A reglators that were incorporated into this PhD thesis

name	gene regulation			DNA binding	
	positiv	negativ	total	binding	motif
AirRS	209	214	423	11	0
MgrA	172	166	338	15	0
Fur	136	142	278	7	22
SigB	207	58	265	0	126
CodY	20	150	170	30	30
Agr	107	45	152	3	2
Rot	76	67	143	6	0
SarA	83	51	134	14	10
CymR	47	83	130	9	9
NorG	69	56	125	4	0
ArlRS	36	77	113	0	0
SarZ	36	54	90	5	0
SaeRS	65	14	79	0	0
NreBC	26	43	69	0	0
Rbf	21	31	52	1	0
VraSR	44	0	44	0	0
XdrA	20	21	41	0	0
SrrAB	24	14	38	0	0
Rex	11	21	32	14	14
MsrR	17	15	32	0	0
SarV	21	3	24	0	0
SarX	7	10	17	10	0
CtsR	0	13	13	13	13
PerR	0	12	12	13	13
SarR	5	3	8	12	0
HrcA	0	7	7	7	7
MntR	4	3	7	3	4
TcaR	2	5	7	5	0
SarT	2	3	5	3	0
NirR	5	0	5	0	0
SarS	1	3	4	6	0
IcaR	0	4	4	4	0
ArcR	4	0	4	0	0
Zur	0	3	3	0	0
SarU	1	1	2	0	0

B published

BlaI	30
SmtB/ArsR	25
LytRS	23
LrgAB	22
TetR	20
LexA	19
CzrA	17
CcpA	15
WalKR	14
SpxA	13
GraR	13
LuxS	13
MepR	10
CidR	7
LysR	7
BlaR1	6
CspA	6
PurR	4
SigS	4
Rsp	4
GapR	3
PhoR	3
TRAP	3
CspC	3
HssR	3
DeoR	2
LacR	2
KdpE	2
FapR	1
RpiRA	1
SarY	1

C unknown

GntR
MalR
MtlR
NrdR
PyrR
RbsR
TreR
AcrR
AhrC
ArgC2
ArgR2
BglG
BglG2
BirA
CspD
DinB2
GltC
GraE
GtlC
Hex
HisR
Hit
HU
LysR2
LytR2
MarR
NsaR
NusG
PadR
PfoR2
RbsR
RinB
RpiRB
RpiRC
ScrR
SlyA
SpoIIIE
SpoVG
TenA
YvnA

A: A list of regulators that were incorporated into this PhD study. The numbers of positive and negative regulations of target genes that have been described so far are listed under “gene regulation”. The amount of genes with a verified binding site and / or motif in their regulatory regions is listed under “DNA binding”.

B: A list of regulators that were not part of this PhD thesis, and the number of publications that refer to these regulators.

C: A list of regulators that have not been described so far.

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"To travel hopefully is a better thing than to arrive, and the true success is to labour."

quoted from an 1878 essay entitled *"El Dorado"*
by Robert Louis Stevenson

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