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Novel therapeutic targeting of the quorum sensing transcriptional regulation in multi-antibiotic resistant bacterium *Staphylococcus aureus*

Terapeutické cílení na transkripční regulaci systému quorum sensing v multirezistentní bakterii *Staphylococcus aureus*

Bachelor´s thesis

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Statement of authorship

I declare that I am the sole author of this bachelor's thesis and I have stated all the used sources in the bibliography. Neither this work nor a substantial part of it has been submitted to gain a different or the same degree.

In Prague, 29.4.2024

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Abstract

The threat of antibiotic resistance is deemed as a ticking time-bomb, yet in spite of this, the number of newly discovered antibiotics continues to steeply decline. Each year, infections caused by multi-drug resistant bacteria kill more than 4.95 million people, which demonstrates that in order to tackle this crisis, a novel approach is desperately required. The solution could be found in targeting bacterial transcriptional regulators that are responsible for the control of various virulence factors and can be highly species-specific. This also applies to the most widespread multi-drug resistant bacterium *Staphylococcus aureus*, whose virulence is intricately linked to the transcriptional regulation of the quorum sensing system. The aim of this bachelor thesis is to summarize current knowledge about clinically relevant transcriptional regulators that are associated with this system (AgrA, SarA, SarR, MgrA, Rot), with focus on their 3D structures, mechanisms of action, and inhibition.

Key words: antibiotic resistance, transcriptional regulation, quorum sensing, *Staphylococcus aureus*

Abstrakt

Hrozba antimikrobiální rezistence vůči antibiotikům je považována za tikající bombu, avšak navzdory tomu počet nově vynalezených antibiotik strmě klesá. Každoročně si infekce způsobené multi-rezistentními bakteriemi vyžádají více než 4.95 milionů lidských životů, což dokazuje, že tato krize naléhavě vyžaduje nový přístup v léčbě. Řešení by mohlo spočívat v cílení na bakteriální transkripční regulátory, které jsou často zodpovědné za produkci virulentních faktorů a zároveň mohou být i velice druhově specifické. Tak je tomu i v případě nejrozšířenější multi-rezistentní bakterie *Staphylococcus aureus*, jejíž virulence je úzce spjata s transkripční regulací systému „quorum sensing“. Cílem této bakalářské práce je shrnout současné poznatky o klinicky relevantních transkripčních regulátorech spojených s tímto systémem (AgrA, SarA, SarR, MgrA, Rot), se zaměřením na jejich 3D struktury, mechanismus působení a inhibici.

Klíčová slova: bakteriální rezistence k antibiotikům, transkripční regulátory, quorum sensing, *Staphylococcus aureus*

List of Abbreviations:

3D	three-dimensional
Å	Ångström
A site	aminoacyl site
A549	adenocarcinomic human alveolar basal epithelial cell line
agr	accessory gene regulator
AIP	autoinducing peptide
AMR	anti-microbial resistance
ATB	antibiotic
ATP	adenosine triphosphate
aur	aureolysin metalloprotease gene
BCG	Bacillus Calmette-Guérin vaccine
bp	base pair
BsaA	glutathione peroxidase homolog BsaA
CAP	catabolite activator protein
CFU	colony forming unit
cna	collagen-binding adhesin gene
CPM	cytoplasmic membrane
CTD	C-terminal domain
DAP	daptomycin
DBD	DNA-binding domain
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EBD	effector-binding domain
EMSA	electrophoretic mobility shift assay
ESAT-6	early secreted antigenic target 6 kDa
Fc	fragment crystallizable region
FnbA	fibronectin-binding protein A
FnbB	fibronectin-binding protein B
FRET	Förster resonance energy transfer
GlcNAc	N-acetylglucosamine
HepG-2	human epithelial type 2 cell line
HIV	human immunodeficiency virus

HK	histidine kinase
hla	α-hemolysin
hlb	β-hemolysin
hld	δ-hemolysin
HTH	helix-turn-helix
IOCB	Institute of Organic Chemistry and Biochemistry
K_D	dissociation constant
lukED	leukocidin ED gene
MTB	*Mycobacterium tuberculosis*
MTBVAC	*Mycobacterium tuberculosis* vaccine
MDSA	5,5′-methylenedisalicylic acid
MHC-II	major histocompatibility complex class II
MIC	minimal inhibitory concentration
Mo-A	methylophiopogonanone
mRNA	messenger RNA
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-susceptible *S. aureus*
MurNAc	N-acetylmuramic acid
NTD	N-terminal domain
PABA	*p*-aminobenzoic acid
PBP	penicillin-binding protein
PDB	Protein Data Bank
PG	phosphatidylglycerol
PSM	phenol-soluble modulin
PVL	Panton-Valentine leukocidin
QS	quorum sensing
RMSD	root mean square deviation
RNA	ribonucleic acid
RNAP	DNA dependent RNA polymerase
RR	response regulator
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
sar	staphylococcal accessory regulator
SarABI	4-[(2,4-diflurobenzyl)amino] cyclohexanol
Sbi	second immunoglobulin-binding protein
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Seb	enterotoxin B
Spa	staphylococcal protein A
spp.	species
ssl7	staphylococcal superantigen-like protein 7 gene
sspA	V8 serine protease gene
Stk1	serine-threonine kinase 1
Stp1	serine-threonine phosphatase 1
TCS	two-component system
THF	tetrahydrofolate
TOPO IV	DNA topoisomerase IV
TR	transcriptional regulator
Tst	toxic shock syndrome-toxin 1
VF	virulence factor
VRSA	vancomycin-resistant *S. aureus*
W	loop region of the wing of the wHTH motif
WHO	World Health Organization
wHTH	winged-helix-turn-helix

Contents

1. Introduction

Antibiotic resistance has been under scientific scrutiny for decades, and while various antibiotics (ATBs) have reached the market over the past 90 years, bacteria somehow always find a way to get one step ahead. In 2019, antimicrobial resistance (AMR) was reported to cause death of approximately 1.27 million people and to be associated with 4.95 million deaths in total. Infections caused by multi-drug resistant bacteria therefore annually kill more people than HIV or malaria, making AMR one of the leading causes of death worldwide (Murray *et al.,* 2022). Without action, predictions estimate that by the year 2050, ATB resistance could be responsible for over ten million deaths per year and might thus even surpass the annual death toll of cancer (O'Neill, 2014).

The urgency of this crisis was stressed in 2017 by the World Health Organization (WHO) by publishing a list of the twelve most threatening multi-drug resistant bacteria. These strains were further separated into three groups (critical, high, and medium) based on their severity of drug resistance and incidence, with the hope of spurring ATB development against these priority pathogens. Methicillin- and vancomycin-resistant strains of *Staphylococcus aureus* (*S. aureus*) have been listed among the pathogens of the high priority (WHO.int, 2017). However, the latest studies imply that out of all bacterial pathogens, *S. aureus* is the leading cause of infections as well as deaths and hence belongs on top of the list of the most critical priority pathogens (Asokan *et al.,* 2019; Murray *et al.,* 2022).

A promising strategy that could alleviate the AMR crisis has been found in the development of anti-virulence compounds (Dickey *et al.,* 2017). Various studies have revealed that pathogenic bacteria control the expression of virulence factors and biofilm formation via transcriptional regulation of the quorum sensing (QS) system (Chien & Cheung, 1998; Whiteley *et al.,* 1999). Transcriptional regulators (TRs) associated with this system are oftentimes species-specific, which makes them potential targets of next generation of ATBs (Arya & Princy, 2013b). In *S. aureus*, the *agr* and *sar* QS systems have been identified as central virulence regulators and sparked the interest in the development of QS-interfering anti-virulence compounds (Cheung *et al.,* 1992; Sully *et al.,* 2014). Nonetheless, further research of this transcriptional regulatory network has to be carried out as many participating TRs are still not fully functionally and structurally annotated.

The aim of this thesis is to summarize current knowledge about the molecular structure and function of key QS- and virulence-associated TRs in *S. aureus* and to discuss the potential of using these TRs as novel targets to develop anti-microbial compounds. Conventional ATBs against *S. aureus* and its mechanisms of resistance to them will be briefly introduced, followed by a general description of TRs that serves as an opening for the transcriptional regulation in MRSA. Attention will be devoted to TRs that have already been studied for their promising clinical impact (AgrA, SarA, SarR, MgrA, Rot), with emphasis on their three-dimensional (3D) structures, interactions with DNA, native effectors, or inhibitory compounds. The herein presented research work thus provides a crucial stepping stone for further experimental research in the mentoring laboratory.

2. The threat of ATB resistance and multi-drug resistant *S. aureus*

The harrowing death toll of AMR indicates that ATB resistance is undoubtedly one of the greatest challenges of the 21st century (Murray *et al.,* 2022). Due to their inherent rapid mutability and the ability of horizontal gene transfer, bacteria are evolving into almost non-druggable strains (McInnes *et al.,* 2020; Wheatley *et al.,* 2021). Even though ATB resistance is deemed as an inevitable process, it has been significantly aggravated by inappropriate use and overprescription of ATBs (Goossens *et al.,* 2005). While this crisis keeps escalating, the approval of novel ATBs appears to be stalled. Since 1962, only two new classes of ATBs have been administered (Coates *et al.,* 2011). To confront this pandemic, WHO issued a list of the most threatening multi-drug resistant bacteria that urgently require novel treatment (Figure 1) (WHO.int, 2017). Although *S. aureus* has been placed in the second group of priority pathogens, recent studies indicate that it actually takes a leading place among the listed bacteria with a staggering annual death toll of over 100,000. In high-income countries, *S. aureus* accounts for roughly 26% of the total AMR burden (Murray *et al.,* 2022).

Priority:	Bacterium/Species:	Alarming resistance to:
1. Critical	*Acinetobacter baumannii*	carbapenems
	Pseudomonas aeruginosa	carbapenems
	Enterobacteriaceae	carbapenems
2. High	*Enterococcus faecium*	vancomycin
	Staphylococcus aureus	methicillin, vancomycin
	Helicobacter pylori	clarithromycin
	Campylobacter spp.	fluoroquinolones
	Salmonellae	fluoroquinolones
	Neisseria gonorrhoeae	fluoroquinolones, cephalosporins
3. Medium	*Streptococcus pneumoniae*	penicillin
	Haemophilus influenzae	ampicillin
	Shigella spp.	fluoroquinolones

Figure 1: WHO list of priority pathogens published in 2017. Inspired by WHO.int, 2017.

S. aureus is a gram-positive bacterium, which is naturally found in the human microbiome. However, it is also an opportunistic pathogen capable of causing an exceptionally wide range of infections targeting the skin, soft tissue, pleuropulmonary, cardiac and osteoarticular systems or evolving into systemic life-threatening bacteremia, sepsis, and endocarditis (Tong *et al.,* 2015). This incredible flexibility arises from the high plasticity of its relatively small genome (ca 2.8 Mbp in size) and intricate transcriptional regulation (Holden *et al.,* 2004). Thanks to this, *S. aureus* has been bestowed with the ability to gain resistance to virtually every ATB that has been administered so far, making the bacterium a true master of AMR (Vestergaard *et al.,* 2019).

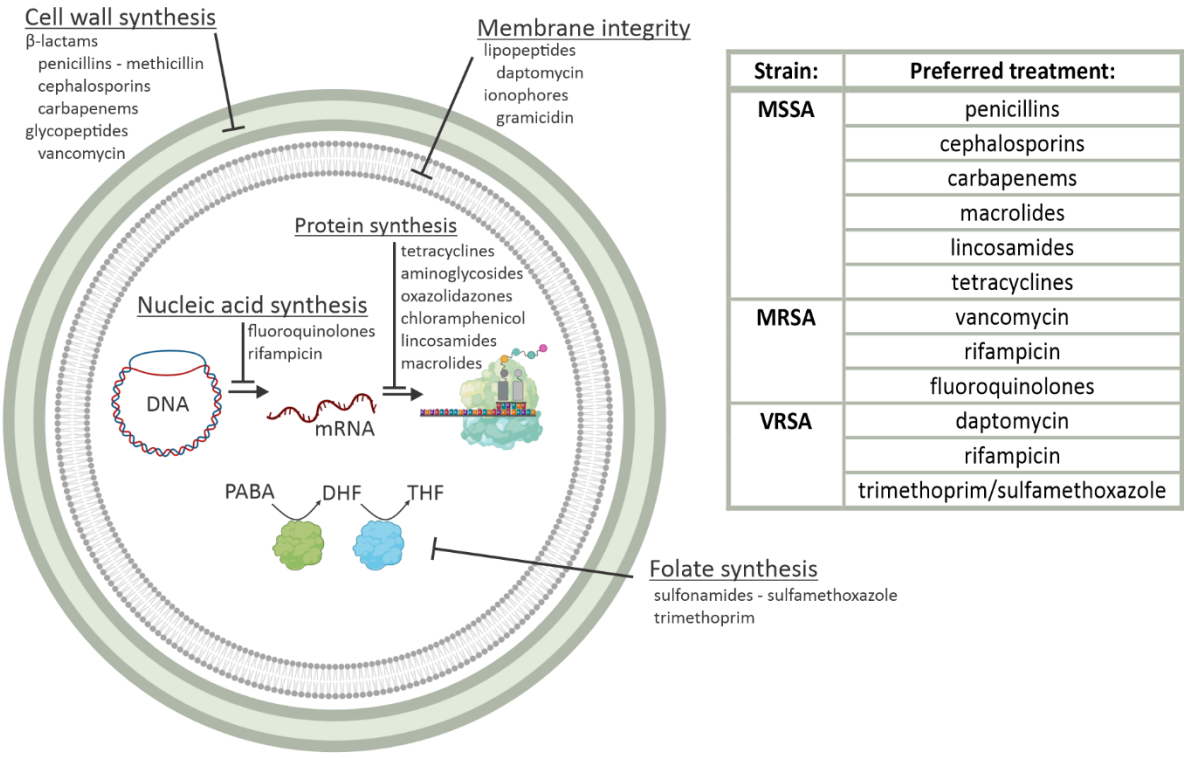
Based on the magnitude of ATB resistance, three main categories of *S. aureus* strains are recognized: MSSA (methicillin-susceptible), MRSA (methicillin-resistant) and VRSA (vancomycin-resistant). The surge of these strains poses a global threat to public health, and the particularly rapidly spreading MRSA is considered to be the most alarming one (Tarai *et al.,* 2013). For severe MRSA infections, vancomycin oftentimes represents a last-resort ATB, yet this treatment is associated with

a high rate of failure (up to 50% (Gentry *et al.,* 1997)) and nephrotoxicity (with incidence ranging from 10 to 40% depending on the dosage (Elyasi *et al.,* 2012; Lodise *et al.,* 2020; Murray *et al.,* 2013)).

In addition, MRSA has been accompanied by the emergence of VRSA strains, suggesting that this crisis calls for a completely novel approach (Cong *et al.,* 2020; Hiramatsu *et al.,* 1997). Addressing this emergency, the mentoring Laboratory of Structural Biology at IOCB Prague decided to join the investigation of alternative treatments based on the interference with QS- and virulence-associated TRs. However, prior to introducing the prospective unconventional TR-based therapy, the following chapters will briefly recapitulate the mechanisms of available ATBs against *S. aureus* and its mechanisms of resistance to them.

3. Conventional ATB targets against *S. aureus* and its mechanisms of resistance

Although hundreds of ATBs against *S. aureus* (and other related bacteria) have been developed, the vast majority of them aim at these five fundamental cellular processes: cell wall synthesis, membrane integrity, nucleic acid, protein, and folate synthesis (Figure 2) (Lade & Kim, 2021). This narrow spectrum of targets stems from the lack of funding for innovative research as well as the difficulty of finding targets that have no homologs in eukaryotes (Coates *et al.,* 2011).



Strain:	Preferred treatment:
MSSA	penicillins
	cephalosporins
	carbapenems
	macrolides
	lincosamides
	tetracyclines
MRSA	vancomycin
	rifampicin
	fluoroquinolones
VRSA	daptomycin
	rifampicin
	trimethoprim/sulfamethoxazole

Figure 2: **The five common targets of ATB treatment against *S. aureus* with examples of ATBs and their classes.** The abbreviations stand for: PABA - *p*-aminobenzoic acid, DHF - dihydrofolic acid, THF - tetrahydrofolic acid. Inspired by Lade & Kim, 2021 and created with BioRender.com. The predominantly used ATBs in clinical practice against MSSA, MRSA and VRSA are listed in the table which is inspired by Liu *et al.,* 2011 and Rayner & Munckhof, 2005.

3.1 Cell wall synthesis

For benign MSSA infections, β-lactam ATBs (specifically penicillins and cephalosporins), remain the preferred treatment, owning to their low cytotoxicity (Rayner & Munckhof, 2005). β-lactams inhibit the transglycosylation and transpeptidation of bacterial cell-wall peptidoglycans by binding to transpeptidases (Tipper *et al.,* 1965). Hence transpeptidases susceptible to these ATBs have been designated penicillin-binding proteins (PBPs) (Suzuki *et al.,* 1980). In MRSA, spontaneous point mutations gave rise to a transpeptidase PBP2a with a diminished affinity to β-lactams (Hartman & Tomasz, 1984; Lim & Strynadka, 2002).

Additionally, resistance to β-lactams can be mediated by β-lactamases – enzymes specialized for the hydrolysis of the strained amide bond found within the β-lactam ring of β-lactams. Several attempts have been made to develop β-lactamase inhibitors, nonetheless, the efficiency of these compounds is limited as β-lactamases have evolved into more than two thousand species (Bush, 2018). For this reason, vancomycin became the first-line ATB for the treatment of MRSA infections. This glycopeptide mimics the terminal D-Ala moieties of peptidoglycan oligopeptides and prevents their subsequent cross-linking (Cooper *et al.,* 2000). Nonetheless, in late 1980s, VRSA strains have emerged and endangered the treatment of malignant *S. aureus* infections (Figure 3) (Hiramatsu *et al.,* 1997; Schwalbe *et al.,* 1987). Even though the development of β-lactam and glycopeptide analogs is still ongoing, these compounds eventually become either too difficult or too expensive to synthesize (Coates *et al.,* 2011).

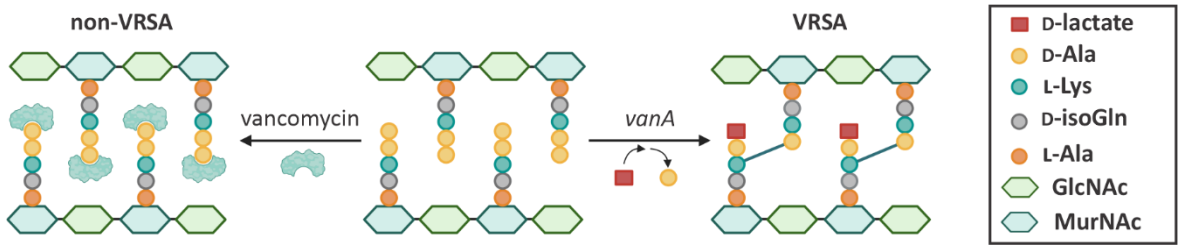


Figure 3: A schematic representation of vancomycin resistance. Vancomycin resistance is mediated by the *vanA* gene cluster encoding enzymes that exchange the terminal D-Ala of peptidoglycan oligopeptides for D-lactate. The exchanged D-lactate cannot be bound by vancomycin and thus restores peptidoglycan cross-linking (Arthur & Courvalin, 1993; Hiramatsu *et al.,* 1997). The abbreviations stand for: GlcNAc - N-acetylglucosamine, MurNAc - N-acetylmuramic acid. Inspired by Li *et al.,* 2022 and created with BioRender.com.

3.2 Membrane integrity

Contrary to the cell wall, bacterial cytoplasmic membrane (CPM) has a eukaryotic counterpart, which complicates ATB development. Daptomycin (DAP), predominantly prescribed against VRSA strains, circumvents this obstacle by interacting with phosphatidylglycerol (PG) (Figure 4) (Straus & Hancock, 2006). This ensures high selectivity as PG is much more abundant in the bacterial CPM (Hiraoka *et al.,* 1993; Uran *et al.,* 2001). Astonishingly, while penicillin-resistant MRSA started appearing just two years after its approval for clinical use (Rammelkamp & Maxon, 1942), resistance to DAP emerged at

a much slower rate and remains sporadic to this day (Lee *et al.,* 2010). In *S. aureus*, occasional resistance to DAP can be conferred by decreased membrane fluidity or increased net charge, for instance by linking D-Ala to negatively charged teichoic acids of the cell wall (Bertsche *et al.,* 2013). Unfortunately, DAP use is associated with myotoxicity (5-10% incidence) and an elevated risk of eosinophilic pneumonia (2% incidence) (Garreau *et al.,* 2023).

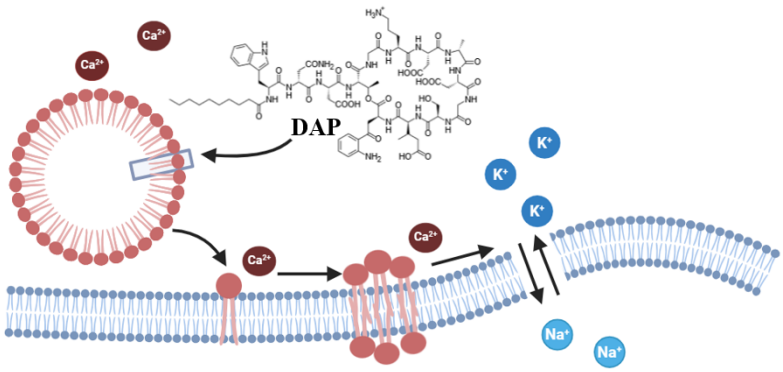


Figure 4: DAP structure and mechanism of action. DAP is a cyclic tridecapeptide with three non-canonical amino acids and an N-terminal decanoyl residue (Debono *et al.,* 1987). In a solution, DAP forms micelles whose hydrophilic moieties interact with Ca^{2+} ions. When this complex gets near the negatively charged PG of bacterial CPM, DAP inserts itself in the outer leaflet of the membrane. Upon the insertion, DAP starts to oligomerize and gets partially translocated into the inner part of CPM (Jung *et al.,* 2004). This leads to a curvature of the membrane and formation of pores that completely disrupt the transport of ions and protons, inexorably resulting in cell death (Straus & Hancock, 2006). Inspired by Straus & Hancock, 2006 and created with BioRender.com.

3.3 Nucleic acid synthesis

Only two main classes (rifampicins and fluoroquinolones) of ATBs targeting nucleic acid synthesis have been discovered so far. Rifampicin, broadly used against MRSA and VRSA, selectively binds to the bacterial RNA polymerase (RNAP). Structural studies have revealed that it binds within the cleft in between the β and β′ subunits of the RNAP and thus inhibits formation of oligonucleotides longer than three bases (Campbell *et al.,* 2001). The resistance to rifampicin arises from point mutations in the *rpoB* gene encoding the β subunit of RNAP (Gao *et al.,* 2013), which mitigate the affinity of RNAP to the drug (O'Neill *et al.,* 2006).

Fluoroquinolones, such as norfloxacin, simultaneously target the bacterial topoisomerase IV and DNA gyrase (Figure 5). Resistance to fluoroquinolones can be evoked even in the course of the treatment by mutations in the drug-binding regions or decreased uptake/increased efflux of the ATBs (Horcajada, 2002; Ng *et al.,* 1996; Schmitz *et al.,* 1998). In MRSA, fluoroquinolone resistance has been directly linked to an increased expression of the NorA efflux pump (Ng *et al.,* 1994) and the identification of its inhibitors is currently in the scientific spotlight (Tintino *et al.,* 2023).

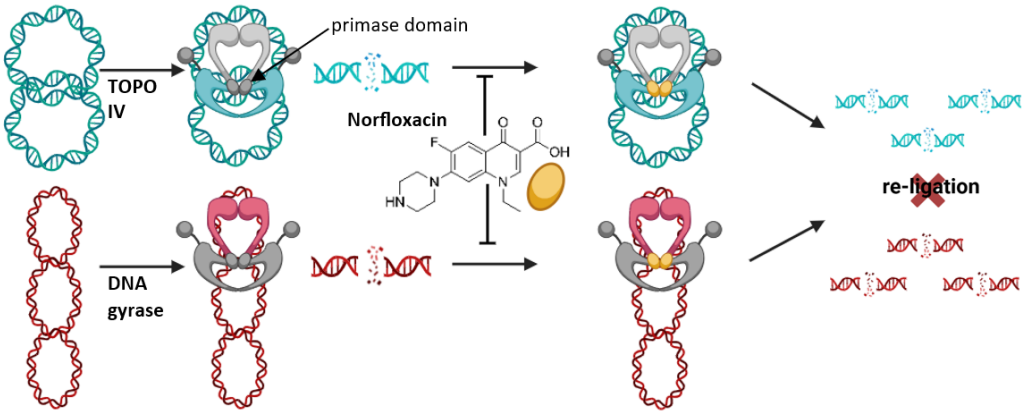


Figure 5: Norfloxacin mechanism of action. DNA gyrase is an enzyme specialized for maintaining the negative supercoiling of the chromosomal DNA, whereas its homolog topoisomerase IV (TOPO IV) is crucial for decatenation of inter-linked DNA molecules. Both enzymes have the ability to introduce transient double-stranded breaks into DNA, which is misused by fluoroquinolones. These ATBs bind to the DNA-topoisomerase and DNA-gyrase complex and inhibit the re-ligation of generated breaks by sterically blocking the primase domains. This promotes DNA fragmentation and inevitably leads to cell death (Drlica & Zhao, 1997). Inspired by Rusu *et al.,* 2021, created with BioRender.com.

3.4 Folic acid synthesis

The folate pathway provides the cofactor tetrahydrofolate (THF), which is necessary for the biosynthesis of nucleic acids, amino acids, and transfer of single-carbon molecules. Enzymes catalyzing this conversion thus represent broad spectrum targets for ATB treatment (Bermingham & Derrick, 2002).

The most renowned ATBs from this class are sulfonamides and trimethoprim, targeting dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively (Figure 6). DHPS has no mammalian counterparts, which allows selective inhibition. Albeit bacterial DHFR has a homologous mammalian enzyme, they only share a 35% sequence identity and have many structural differences that enable specific inhibition (Lee *et al.,* 2010). However, resistance to both agents spread rapidly (Sköld, 2001). In most cases, the resistance stems from the production of mutated isoforms of the targeted enzymes that decrease the susceptibility to the treatment (Gleckman *et al.,* 1981). For this reason, a combined therapy of sulfonamides and trimethoprim became the golden standard. Startlingly, simultaneous resistance to both ATBs is becoming prevalent in MRSA strains, making the targeting of the folic acid pathway essentially non-applicable (Ham *et al.,* 2023).

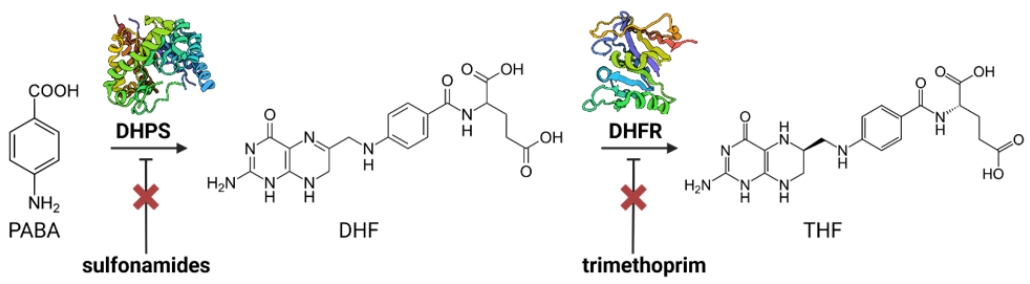


Figure 6: **A scheme of the resistance of MRSA to sulfonamides and trimethoprim.** In MRSA strains, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) contain point mutations that weaken their affinity to sulfonamides and trimethoprim, respectively. The synthesis of THF can thus normally proceed. The abbreviations stand for: PABA – *p*-aminobenzoic acid, DHF – dihydrofolate, THF – tetrahydrofolate. Inspired by Bermingham & Derrick, 2002 and created with BioRender.com using the PDB structures 2VEF (Levy *et al.,* 2008) and 2HM9 (Feeney *et al.,* 2011).

3.5 Protein synthesis

Bacterial protein synthesis is targeted by a plethora of ATBs. Thanks to the knowledge of the 3D structure of the bacterial ribosome (Ban *et al.,* 2000; Schluenzen *et al.,* 2000; Wimberly *et al.,* 2000; Yonath *et al.,* 1986), the mode of their action could be unveiled and most of them were found to interfere with the elongation phase of translation (Figure 7) (Wilson, 2014). Despite their abundance, ribosome-targeting agents are usually not sufficient for the treatment of MRSA and VRSA infections as they readily induce resistance in these strains (Rayner & Munckhof, 2005).

The scope of staphylococcal resistance mechanisms to translation-inhibiting ATBs is truly enormous and the recent discovery of ribosome splitting-mediated resistance in Firmicutes suggests that many other mechanisms are waiting to be discovered (Duval *et al.,* 2018). For instance, aminoglycosides can be rendered inactive by their phosphorylation, acetylation or adenylation (Schmitz *et al.,* 1999). Macrolides and lincosamides, on the other hand, tend to induce the overexpression of efflux pumps or stimulate the methylation of ribosomes (Khodabandeh *et al.,* 2019). Tetracycline therapy has been associated with so-called ribosomal protection by TetO family of proteins. Mechanism of this process has been elucidated by cryo-electron microscopy, which exposed that TetO proteins act as allosteric modulators of ribosomes (Spahn *et al.,* 2001). By binding to the vicinity of the A site region, these proteins induce a conformational change that weakens the affinity to tetracyclines, which target the 30S subunit (Brodersen *et al.,* 2000).

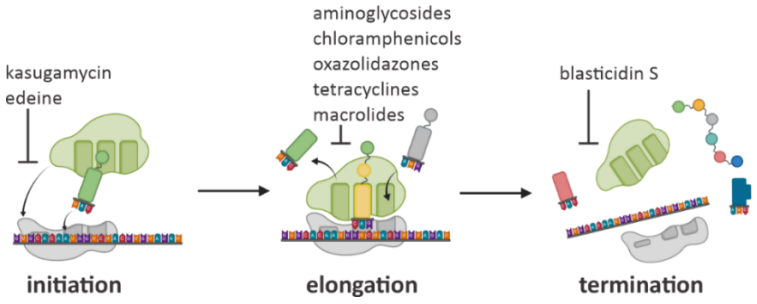


Figure 7: Examples of ATBs targeting the individual steps of the ribosomal polypeptide synthesis. Inspired by Wilson, 2014 and created with BioRender.com.

4. Bacterial transcriptional regulation as a platform for the development of a novel class of ATBs

Considering that conventional ATBs are undeniably failing to manage the spread of AMR, attempts have been made to identify novel antimicrobial agents. This ignited the search for anti-virulence compounds, which could possibly prevent AMR by interfering solely with the virulence of bacteria without affecting their viability (Dickey *et al.,* 2017). Attention has been drawn to virulence-associated TRs. In 1992, the first synthetic two-component system kinase inhibitors were described. These compounds aimed to interfere with the AlgR2 kinase and AlgR1 response regulator of *Pseudomonas aeruginosa* involved in alginate production that endangers patients with cystic fibrosis (Roychoudhury *et al.,* 1993). Since then, a myriad of TR-interfering compounds active across all bacterial strains have been discovered, providing a much-needed hope in combating AMR.

To convey how these inhibitors might exactly help in fighting multi-drug resistant *S. aureus*, a brief general description of bacterial transcriptional regulation will be provided, followed by a more in-depth focus on the regulation in MRSA.

Bacterial transcriptional regulation

In order to adapt to environmental and physiological changes, bacteria fine-tune their transcription by a repertoire of TRs. Traditionally, these proteins are functionally divided into activators and repressors, albeit many of them exert dual function. The same TR can thus activate expression of genes that are currently required whilst simultaneously repressing those that are not.

Activators enhance the recruitment of the RNAP to the promoter. This can be achieved either by a protein-protein interaction with the C-terminal domain (CTD) of RNAP (class I activation), a σ-factor (class II activation) or by contacting both components of the holoenzyme (Lloyd *et al.,* 2001).

Repressors can sterically hinder RNAP by occupation of its binding site, induce a spatially more extensive loop formation or block tandemly bound activators as an anti-activator (Figure 8) (Lloyd *et al.,* 2001).

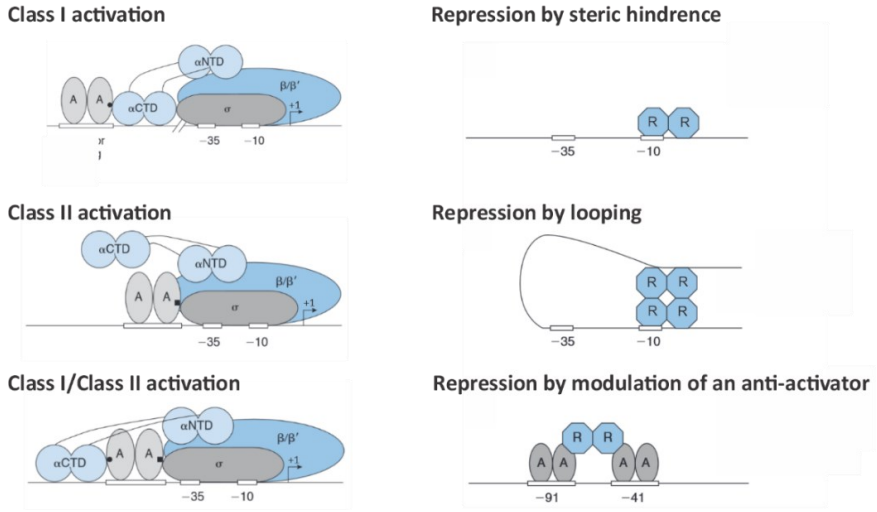


Figure 8: Mechanisms of transcriptional activation and repression. The abbreviations stand for: A – activator, R-repressor, CTD – C-terminal domain, NTD - N-terminal domain, σ – sigma factor, α/ β / β′- subunits of RNAP. Adapted from and edited by Lloyd *et al.,* 2001.

In most cases, TRs consist of two well-defined domains – a DNA-binding domain (DBD) and an effector-binding domain (EBD), which is oftentimes involved in the oligomerization of TRs. DBDs bind to specific DNA regions called DNA operators, which are usually positioned in the vicinity of promoters. EBDs follow the environmental changes via binding of a small molecule called an effector, such as a metabolite or an ion (Bagg & Neilands, 1987). The binding of the effector to the EBD induces a conformational change, which is transferred to the DBD and subsequently alters the affinity towards the operator (de Peredo *et al.,* 2001).

Intriguingly, although there are thousands of bacterial TRs, it has been estimated that about 95% of them possess the helix-turn-helix (HTH) motif or its variations (Aravind *et al.,* 2005; Sidote *et al.,* 2008). The canonical HTH comprises two α-helices which usually contact the major groove of DNA. The simplest DBD architecture comprising HTH is a tri-helical bundle. Among the elaborations of this simple domain are, for example, the helix-loop-helix, tetra- and multi-helical HTH, the ribbon-helix-helix or the winged-HTH (wHTH) containing a β-hairpin wing which provides additional interactions by wedging into the minor groove of DNA (Aravind *et al.,* 2005). A minority of DBDs features non-HTH-based motifs such as the leucine zipper (Lau *et al.,* 1997) or the LytTR motif rich in β-strands, which will be described in detail in the chapter 4.2.1.1 (Nikolskaya, 2002; Sidote *et al.,* 2008).

TR-based signal transduction systems can be divided into two categories of one- and two-component systems. The one-component system comprising a TR, its cognate DNA, and the effector molecule, has already been described above. More complex two-component systems (TCSs) consist of a membrane-bound histidine kinase (HK) and its cognate DNA-binding response regulator (RR). Upon binding of a signaling molecule, the HK autophosphorylates its conserved histidine residue and transfers the phosphate group to an aspartate residue of the RR. Subsequently, the RR undergoes a conformational change that increases its affinity to the cognate DNA-binding site, and thereby activates or represses transcription (Figure 9) (Jacob-Dubuisson *et al.,* 2018). Occasionally, TCS

crosstalk can also be observed. In *S. aureus*, for instance, GraS HK can *in vivo* phosphorylate a non-cognate RR ArlR, although the phosphorylation of its cognate RR, GraR, is notably favorable. Despite that they both control bacterial autolysis, this phenomenon is one-sided as GraR cannot be activated by non-cognate ArlS HK (Villanueva *et al.,* 2018).

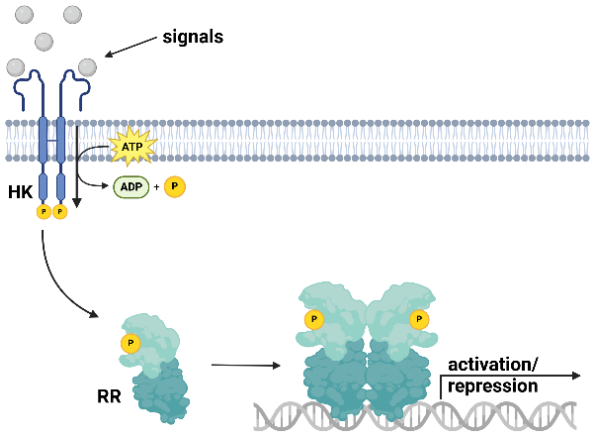


Figure 9: A schematic representation of TCS signal transduction. A signal is sensed by an N-terminal domain of HK, resulting in autophosphorylation of its C-terminal domain enabled by the hydrolysis of ATP. The phosphate is then transferred to an N-terminal receiver domain of the RR, leading to upregulation or downregulation of controlled genes. Inspired by Jacob-Dubuisson *et al.,* 2018 and created with BioRender.com.

Based on the extent of regulation, TRs can be either classified as local or global. Initially, it was believed that most TRs tend to be local, meaning that they control only one or a small subset of related genes (for example an operon of a specific metabolic pathway). Nevertheless, the majority of TRs acts on a global scale by modulating the expression of multiple, and even functionally distinct, genes (Ishihama *et al.,* 2016). Thanks to this, the regulon of one TR is interconnected with regulons of many other TRs, which gives rise to an elaborate hierarchical regulatory network. For more consistent classification, a system of TR families was installed, with main respect to the structure and position of the DBD which correlates with their function and mechanism of action. This system, originally consisting of 63 families based on the regulon of *Escherichia coli* (*E. coli*), keeps expanding to this day (Ishihama, 2012; Novakova *et al.,* 2022; Weickert & Adhya, 1992).

4.1 Transcriptional regulation in MRSA

Comparative genomic sequence analysis has revealed that transcription in MRSA is orchestrated by 135 TRs which can be classified into 36 different families (including two still uncharacterized TRs). The most abundantly found family is the MarR (multiple-antibiotic resistance regulator) family encompassing 18 protein members, including those of the Sar (Staphylococcus accessory regulator) subfamily (Ibarra *et al.,* 2013). The MarR family, and therefore also the Sar subfamily, is associated with the regulation of virulence determinants, such as toxins, adhesins, efflux pumps and biofilm formation (Chien *et al.,* 1999; Sulavik *et al.,* 1995). Intriguingly, most members of the Sar subfamily (9

out of 11) seem to be specific for staphylococci, which predestines them to be excellent candidates for targets of species-specific ATBs (Balamurugan *et al.,* 2017; Ibarra *et al.,* 2013).

Out of 16 TCSs, only the WalKR system regulating cell wall synthesis appeared to be vital for survival (Ibarra *et al.,* 2013). However, survival of WalKR mutants was observed in stationary phase, suggesting that WalKR is essential for cell division but not for cell arrest during starvation. Additionally, acquisition of an additional TCS has been recorded in some highly pathogenic MRSA strains (Villanueva *et al.,* 2018). While its specific function is elusive, this TR is sequentially homologous to the KdpDE TCS involved in extracellular toxin production (Villanueva *et al.,* 2018; Xue *et al.,* 2011).

In general, while the conserved TRs are central for cellular processes, the phylum-specific TRs have been found to be mainly involved in stress response and virulence (Ibarra *et al.,* 2013). The production of virulence and stress determinants is predominantly regulated via the quorum sensing (QS) system (Figure 10) (Boles & Horswill, 2008; Cheung *et al.,* 1992; Recsei *et al.,* 1986). Components of QS thus represent prospective anti-virulence targets. In order to develop the most efficient compounds attacking this intricate network, it is crucial to understand the molecular mechanism of its function. To achieve such knowledge, the structural information about its components is essential to reveal. The succeeding chapters are therefore devoted to the structural and functional description of central QS components in *S. aureus*.

Gene	Encoded protein	Main function(s)	AgrA	SarA
hla	**α-hemolysin**	pore-forming toxin, disrupts ion transport and thereby induces apoptosis of targeted cells (Jonas *et al.,* 1994)	+	+
hlb	**β-hemolysin**	hydrolyses sphingomyelin, rendering host cells more susceptible to other toxins (e.g. PSMs) (Cheung *et al.,* 2012; Doery *et al.,* 1963)	+	+
hld	**δ-hemolysin**	may cause curvature or lysis of host cell membranes, induces the activation of mast cells together with PSMs (Hodille *et al.,* 2016; Verdon *et al.,* 2009)	+	NAN
psm	**Phenol-soluble modulins (PSMs)**	induces cell lysis, triggers inflammatory responses (e.g. cytokine expression) (Wang *et al.,* 2007)	+	NAN
pvl	**Panton-Valentine leukocidin**	pore-forming toxin that induces cell lysis, apoptosis of neutrophils and tissue necrosis (Genestier *et al.,* 2005; Lina *et al.,* 1999)	+	NAN
tst	**toxic shock syndrome toxin-1**	superantigen that crosslinks MHC-II glycoproteins of antigen-presenting cells, leading to an elevated production of cytokines (Fleischer & Schrezenmeier, 1988)	+	+
sbi	**second immunoglobulin-binding protein**	immune evasion factor, protects *S. aureus* from opsonophagocytosis (Smith *et al.,* 2011)	-	NAN
seb	**enterotoxin B**	superantigen, stimulates internalization of T-cell receptors (Niedergang *et al.,* 1995)	+	+
fnbA	**fibronectin binding protein-A**	mediates adhesion to fibronectin, fibrinogen and elastins and thus contributes to biofilm formation (McCourt *et al.,* 2014)	-	+
spa	**Staphylococcal protein A**	cell-surface protein, ensures phagocytosis avoidance by binding Fc fragments of mammalian immunoglobulins (Dossett *et al.,* 1969)	-	-

Figure 10: Key virulence determinants whose expression is directly regulated by the two main QS-associated TRs AgrA and SarA. The (+) sign stands for positive regulation, (-) for downregulation, (NAN) for none or unknown function for the particular gene. Inspired by Arya & Princy, 2013a.

4.2 Quorum sensing (QS) and virulence regulation

QS is a cell-to-cell communication system which allows bacteria to modulate gene expression in response to the population-density-dependent concentration of signaling molecules called autoinducers. Generally, gram-positive bacteria use oligopeptide autoinducers (AIPs) which are sensed by TCSs (Miller & Bassler, 2001). In *S. aureus*, four different types of AIPs (I – IV) have been described so far (Jarraud *et al.,* 2000). These molecules allow a mutual cross-activation between strains with the same type of AIP and cross-inhibition in distinct strains. Thus, QS signaling is not restricted to a given strain as different bacterial populations can crosstalk via their AIPs (Ji *et al.,* 1997). *S. aureus* employs two governing QS regulatory systems – *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator), which will be described in the following chapters (Figure 11) (Cheung *et al.,* 1992).

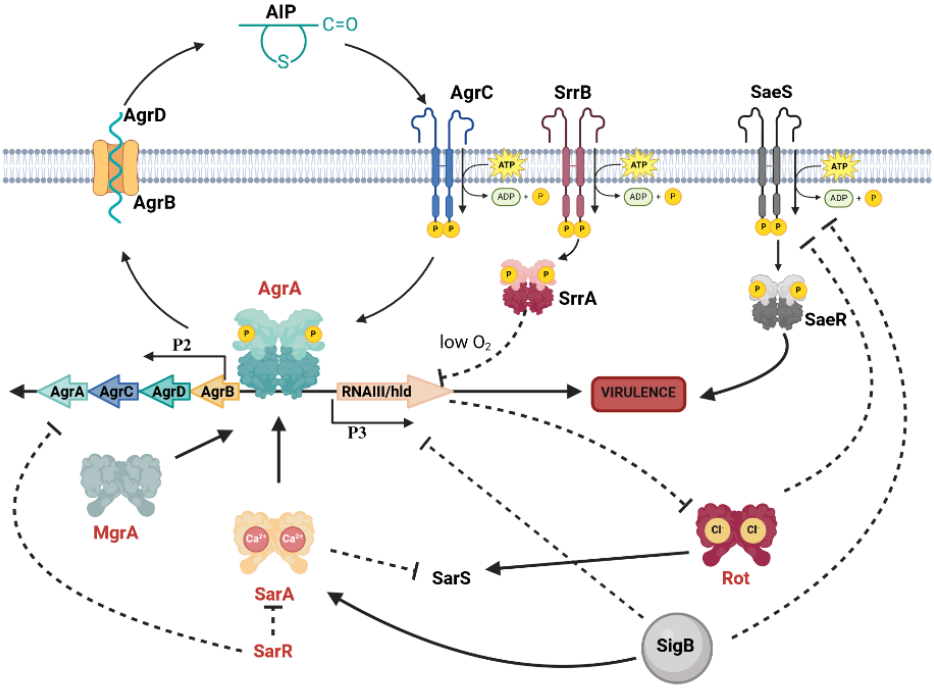


Figure 11: Scheme of the main QS-associated TRs in *S. aureus*. AgrA/AgrC, SrrA/SrrB and SaeR/SaeS are TCSs. MgrA, SarA, SarR, SarS and Rot represent single-component TRs. SigB is a stress-induced alternative σ-factor. TRs which will be described in the following chapters are highlighted in red. Sharp arrows represent positive regulation, negative regulation is depicted by blunt inhibitory arrows. Inspired by Cheung *et al.,* 2021 and created with BioRender.com.

4.2.1 Agr system

The *agr* QS system is regarded as the master regulator of virulence as it plays a central role in the production of virulence determinants (Figure 10 and 11). Particularly, *agr* is responsible for upregulation of extracellular virulence factors (VFs) and downregulation of cell surface virulence determinants. Among the many positively *agr*-regulated virulence determinants are hemolysins, phenol-soluble modulins, leukocidins, serine proteases and toxic shock proteins, whilst adherence factors and biofilm formation are mostly repressed (Peng *et al.,* 1988; Janzon *et al.,* 1989; Novick *et al.,* 1993; Lebeau *et al.,* 1994; Wang *et al.,* 2007). This allows *S. aureus* to simultaneously promote acute invasion

and impede chronic persistence. To enable chronic persistence, many MRSA strains get rid of functional *agr* by the acquisition of frameshift mutations in the locus. However, this advantage in installing chronic infection comes at the expense of toxin production (Seidl *et al.,* 2011; Shopsin *et al.,* 2008).

Furthermore, *agr* is involved in oxidative stress response, which hints that its full potential remains to be unveiled (Sun, Liang, *et al.,* 2012). In fact, transcription profiling-based studies of *S. aureus* revealed 138 *agr*-dependent genes, out of which 104 are upregulated and 34 downregulated by this system (Dunman *et al.,* 2001).

The *agr* locus comprises the *agrBDCA* and *hld* (*δ-hemolysin*) operon transcribed from P2 and P3 promoters, respectively (Figure 11). Transcription of *agrBDCA* yields an RNAII transcript encoding proteins of the QS cascade (Novick *et al.,* 1995). AgrD serves as a precursor of cyclic thiolactone AIP which is processed and exported by AgrB permease. Once fully matured AIP reaches sufficient concentration, it evokes signal transduction between AgrC HK and its cognate RR AgrA (Ji *et al.,* 1995). The phosphorylated AgrA then binds to the P2 and P3 promoters and induces their transcription. The basal production of AIP is ensured by the high affinity of P2 for non-phosphorylated AgrA, whereas P3 exhibits higher affinity for its phosphorylated form (Figure 12B) (Rajasree *et al.,* 2016). Transcription from the P3 promoter yields an RNAIII transcript that can either be translated into δ-hemolysin or act as a regulatory RNA (Janzon *et al.,* 1989; Novick *et al.,* 1993). The scope of RNAIII-mediated regulation is enormous as it negatively regulates production of coagulases and the human evasion protein Sbi (second immunoglobulin-binding protein) (Chevalier *et al.,* 2010; Chabelskaya *et al.,* 2014) and upregulates the production of toxins (e.g., toxic shock syndrome toxin-1), immunomodulatory α- and β-hemolysins or exoenzymes (lipases, proteases) (Patel *et al.,* 1992; Novick *et al.,* 1993; Boisset *et al.,* 2007). Moreover, RNAIII also blocks the translation of the *rot* (repressor of toxins) mRNA (Geisinger *et al.,* 2006).

Given its pivotal role in the production of VFs, AgrA has recently sparked interest in the development of anti-virulence compounds (Greenberg *et al.,* 2018). In order to understand their mechanism of action, profound knowledge of its 3D structure is required. The succeeding chapters will therefore focus on the 3D structure of the AgrA domains and briefly describe the effects of discovered inhibitors.

4.2.1.1 AgrA

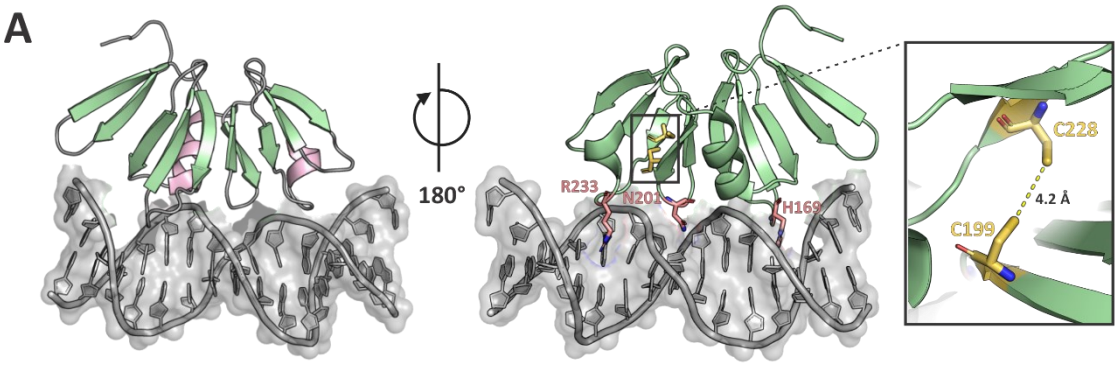
AgrA is a monomeric RR protein consisting of 238 amino acid residues (Bateman *et al.,* 2023) which form two domains connected via a flexible linker: the N-terminal EBD (residues 1 – 130) and the C-terminal DBD (residues 138 – 238). The DBD features a unique LytTR fold of the AlgR / AgrA / LytTR family, which is mostly associated with TRs that regulate the production of virulence determinants (Figure 12A) (Nikolskaya, 2002). Albeit not structurally resolved, the EBD presumably contains a flavodoxin fold (Bateman *et al.,* 2023) typical for the CheY-like family of proteins (Wolanin *et al.,* 2003).

DNA-binding domain

The LytTR motif comprises ten-stranded β-fold arranged into three anti-parallel β-sheets with an interspaced α-helix and a short 3_{10} helix. Three loops between the β-strands interact with two consecutive major grooves and an intervening minor groove of DNA. The recognition is facilitated both by base-specific and sugar-phosphate-backbone readout interactions. Base-specific interactions are mediated by His169 and Arg233 in the major grooves and Asn201 in the intervening minor groove (Figure 12A). None of these three residues are strictly conserved among the LytTR family (Sidote *et al.,* 2008). This is quite surprising as the residues that facilitate base-specific interactions are usually conserved in members of the same family (Liu *et al.,* 2006).

AgrA binds as a dimer to a set of two imperfect 9-bp direct repeats separated precisely by 12-bp, which are located upstream to the promoters P2 and P3 transcribed in the opposite direction (Figure 12B). Intriguingly, the dimerization is solely facilitated by the N-terminal domain and follows a translational symmetry where the monomers bind to the same face of DNA in the same orientation (Sidote *et al.,* 2008).

Besides facilitating DNA recognition, DBD also works as an oxidative stress sensor. During oxidative stress, Cys199 and Cys228 undergo oxidation and form a disulfide bond, which imposes a steric clash that eliminates the DNA binding activity of AgrA (Figure 12A). Without functional AgrA, RNAIII is downregulated, which leads to a derepression of the *bsaA* gene encoding glutathione peroxidase BsaA. BsaA is crucial for withstanding oxidative stress, hence C199S mutants exhibit increased susceptibility to H_2O_2 exposure. This corroborates the crucial role of AgrA in the protection against oxidative stress (Sun, Liang, *et al.,* 2012). In addition, Cys199 can be reversibly modified by Coenzyme A via CoAlation. This modification is observed both under oxidative stress and nutritional (particularly glucose and nitrogen) deprivation and gets removed once the stress ceases. CoAlation decreases the affinity of AgrA to the P2 promoter about 10-fold and even more to the P3 promoter (Baković *et al.,* 2021). While the exact function of CoAlation is unclear, one possible explanation is that it helps *S. aureus* save energy and halt *agr*-mediated VF production when being faced with adverse conditions.



B

	K_D (x10^{-8}M)		
	AgrA	AgrA~P	binding site 1 / binding site 2 / -35 / -10 / +1
P1	19.2	9.92	GT**ATCGAAATACCCGAT**GAAGTA**AGTAGCATTAACTTG**AAT-ATGATC-[20]-TATTATTCTTGATAA
P2	6.59	4.02	AT**TTAACAGTTAAGTAT**TTATTT**CCTACAGTTAGGCAA**TATAATGATA-[18]-TATAATGACAGTGAG
P3	15.3	2.09	AA**TTTACAGTTAAGAAT**AAAAAA**CGACTAGTTAAGAAA**AA--TTGGAA-[20]-TTTAATATAACT-AG

Figure 12: (A) **A cartoon representation of the crystal structure of the C-terminal AgrA DBD (residues 138 – 238) interacting with a pentadecamer DNA operator fragment.** α-helices and β-sheets are distinguished by color – pale pink and pale green, respectively. The model rotated by 180° highlights the residues mediating base-specific DNA contacts represented by salmon sticks (1.6Å resolution, PDB code: 3BS1; Sidote et al., 2008). The oxidative stress-sensitive cysteine residues are shown in yellow stick representation and highlighted in a close-up view. The measured distance between them (dotted line) is 4.2Å (Sun, Liang, et al., 2012). Created with PyMOL (version 2.5.2, Schrödinger, USA). (B) **AgrA operator regions of the promoters P1-P3 with different affinities**. Inspired by Rajasree *et al.,* 2016 and created in the Corel editor.

Effector-binding domain

Although the structure of the N-terminal EBD has not been experimentally resolved yet, it is sequentially homologous to the CheY-like domain family (Bateman *et al.,* 2023), named after the chemotaxis protein CheY from *E. coli*. The CheY-like domain comprises a flavodoxin-like fold – a three-layer α / β / α sandwich made of five parallel β-strands surrounded by five α-helices (Wolanin *et al.,* 2003). This description is also consistent with the model of full-length AgrA predicted by AlphaFold (Figure 13) (Jumper *et al.,* 2021).

Apart from being essential for signal transduction, the EBD also mediates oligomerization. Upon activation of the AgrC HK, the phosphate group is transferred from the His39 to the highly conserved Asp59 of AgrA. The transfer of the phosphate group induces a conformational change that favors dimerization, which enhances the affinity of AgrA to the sub-optimal P3 promoter (Rajasree *et al.,* 2016). Similarly to the DBD, the EBD plays a role in response to oxidative and nutritional stress through CoAlation of Cys6 (Baković *et al.,* 2021).

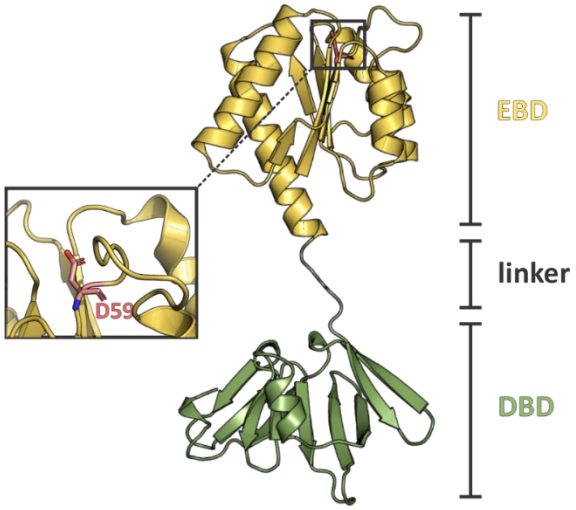


Figure 13: A cartoon representation of the AlphaFold model of a full-length AgrA monomer. The Asp59 residue crucial for the phosphorylation by AgrC is shown as a salmon stick in the close-up view. Created with PyMOL (version 2.5.2, Schrödinger, USA) using a model from AlphaFold (Jumper *et al.,* 2021).

AgrA inhibitors

Although all components of the *agr* QS system could be theoretically targeted by anti-virulence compounds, AgrA appears to be the most well-suited candidate. This is predominantly due to its conserved structure among individual strains and high solubility that eases further experiments. In comparison, AgrB and AgrC are transmembrane proteins that tend to be difficult to purify and are much more polymorphic as they mediate the processing and recognition of the four different types of strain-specific AIPs, respectively (Sidote *et al.*, 2008; Tan *et al.*, 2022).

To date, several inhibitors of AgrA have been synthesized and tested for their anti-virulence properties. Among the most potent ones is the biaryl hydroxyketone F19 (Figure 14A) (Greenberg *et al.,* 2018), that was designed based on the AgrA DBD crystal structure (PDB code: 3BS1; Sidote *et al.,* 2008). Molecular docking and site-specific mutagenesis studies have revealed that the hydrophobic compound F19 binds to Val235 and Ile238 (Figure 14A), which abolishes the DNA binding activity of AgrA and leads to a diminished expression of Hla, PSMα, RNAIII and other AgrA-controlled VFs. Remarkably, F19-treated murine models subjected to *S. aureus*-induced bacteremia exhibited a 100% survival rate and full recovery after seven days, whilst negative controls without any treatment exhibited 70% mortality. Moreover, F19 raised the efficacy of conventional ATBs (e.g., cephalosporins and fluoroquinolones) to which MRSA normally is not susceptible. In combination with F19, these ATBs proved to be more effective than the golden standard vancomycin. F19 thus provides strong evidence that anti-virulence strategies can be sufficient for treating severe bacterial infections. This might not be enough for the treatment of immunocompromised patients, however, that could possibly be overcome by the usage of combined ATB therapy (Greenberg *et al.,* 2018).

A significant drawback of AgrA inhibitors is the fact that many gram-positive bacteria possess AgrA homologs, which is inconvenient for the development of species-specific anti-virulence

compounds. Indeed, F19 was shown to be effective not only against other staphylococci, but also against bacilli and streptococci (Greenberg *et al.,* 2018). However, another AgrA inhibitor, savirin, was proved to be non-detrimental to *Staphylococcus epidermidis* (*S. epidermidis*). This can be explained by its different mode of binding as it makes π-stacking interactions with Tyr229 (Figure 14B), which is replaced with phenylalanine in *S. epidermidis*. Nonetheless, this probably will not be enough for sparring other bacteria of the human microbiome. While AgrA inhibitors are not fully species-specific, they still bring hope in fighting AMR as multiply passaged cells treated with savirin did not acquire resistance in both *in vitro* and *in vivo* experiments (Sully *et al.,* 2014).

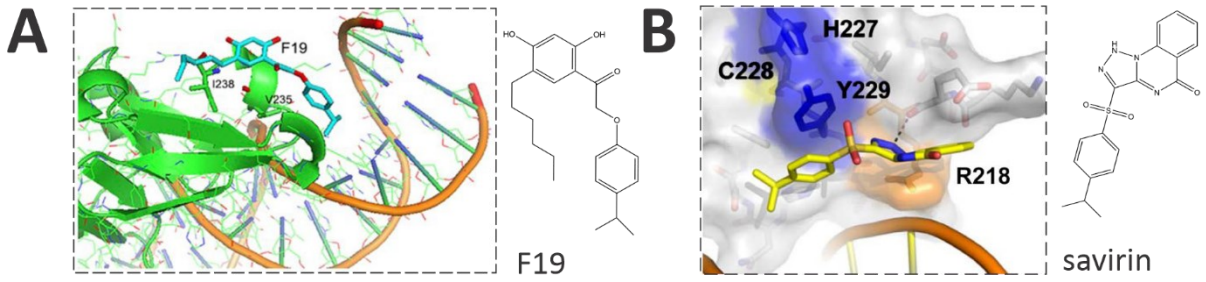


Figure 14: Structural formulas of F19 (A) and savirin (B) with molecular docking models. In (A) F19 is represented as a cyan stick, (B) highlights savirin in yellow stick representation. The structural formulas were created with the ChemDraw editor and the docking models were adapted from Greenberg *et al.,* 2018 and Sully *et al.,* 2014, respectively.

4.2.2 Sar system

The *sar* system is the second global regulatory locus in charge of QS and virulence in *S. aureus* (Cheung *et al.,* 1992). *Sar* is intricately linked to *agr* as it can regulate the expression of virulence determinants in both *agr*-dependent and *agr*-independent fashion (Chien *et al.,* 1999). The main TR responsible for this regulation is SarA, which governs the expression of 120 genes (with 76 being upregulated and 44 downregulated) (Dunman *et al*., 2001). Conversely to AgrA, SarA is highly conserved solely among staphylococci and mostly upregulates both extracellular and cell-wall associated VFs (Cheung *et al.,* 1992; Ibarra *et al.,* 2013).

Apart from upregulating the *agr* operon (Heinrichs *et al.,* 1996), SarA also promotes the production of α- (Hla) and β-hemolysins (Hlb), toxic shock syndrome toxin-1 (Tst), staphylococcal enterotoxin B (Seb), fibronectin-binding protein (Fnb) and many other virulence determinants, whilst downregulating staphylococcal protein A (Spa) and proteases (Cheung *et al.,* 1992; Heinrichs *et al.,* 1996; Chan & Foster, 1998). Additionally, SarA can directly influence mRNA turnover by binding to mRNA transcripts. For example, SarA can bind the *spa* transcript and block its translation as well as degradation by RNase III at the same time. The repressed transcript is thus protected and can be released when its product is needed (Morrison *et* al., 2012).

By virtue of its specificity and role in the regulation of the production of VFs and biofilm, SarA represents a prospective target for the development of highly selective anti-virulence compounds. The following paragraphs are devoted to its structure, mechanism of action and its inhibitors, which are

currently under development. Furthermore, other clinically relevant SarA homologs will be briefly described.

SarA family

SarA belongs to the eponymous family of TRs that shares a sequence homology with the MarR family and is henceforth occasionally referred to as the MarR subfamily (Cheung *et al*., 2004). *S. aureus* employs eleven TRs belonging to the SarA family – SarA, -R, -S, -T, -U, -V, -X, -Y, -Z, MgrA and Rot. The majority of them, excluding MgrA and SarZ, are highly specific for staphylococci, with only a few exceptions (Ibarra *et al.,* 2013). Members of this family are characterized by a conserved wHTH motif and pleiotropic functions ranging from the production of various VFs, biofilm formation, degradation, and efflux of toxic substances (including ATBs) to mediating stress responses (Cheung *et al.,* 2008).

In most cases, TRs of the SarA / MarR family form homodimers, albeit Spa protein-activating SarS has a monomeric structure (Li *et al.,* 2003). Based on their sequence similarity and structural arrangement, the SarA proteins can be further divided into three subfamilies: single-domain (SarA, -R, -T. -V, -X and Rot), double-domain (SarS, -U and -Y) and MarR homologs (MgrA and SarZ) (Cheung *et al.,* 2008).

4.2.2.1 SarA

SarA is a 124 amino-acid-long single-domain protein (Bateman *et al.,* 2023) encoded by three overlapping transcripts of the *sarA* locus, whose transcription is driven by three separate promoters (P1, P2 and P3). Whilst P1 and P2 are predominantly active in the early exponential phase of growth, the expression driven by P3 peaks in the late stationary phase (Bayer *et al.,* 1996).

The crystal structure of SarA has revealed a homodimer, with each monomer comprising five α-helices and three anti-parallel β-strands. The α1-helices form the dimerization interface, whilst helices α3 and α4 form the wHTH together with a β-hairpin (formed by β2 and β3 strands separated with a loop). SarA contains the DNA-binding and activation sites, which are located opposite to each other. The DNA-binding site constitutes a highly conserved wHTH motif, whereas the activation site features a pocket for the binding of Ca^{2+}, an ion necessary for the oligomerization and function of SarA (Figure 15A) (Liu *et al.,* 2006).

Sequence alignment of different SarA homologs revealed several conserved residues (Figure 15B). Among them is a highly conserved DER tripeptide (residues 88 - 90) within the wing region, out of which only Arg90 is essential for DNA binding. Other highly conserved residues include basic residues of the α3- and α4-helices (Lys63 and Lys69) and semi-conserved Asp8 and Glu11 of the Ca^{2+} binding pocket (Liu *et al.,* 2006).

Although SarA typically forms homodimers, electrophoretic mobility shift assay (EMSA) has implied that SarA can form multimers upon DNA binding. The crystal structure showed a plausible mechanism of its tetramerization, which is mediated by Asn7 and the semi-conserved Asp8 and Glu11

residues. Each SarA monomer, together with an equivalent monomer from the neighboring asymmetric unit, coordinates a Ca^{2+} ion via these three residues, which thus brings the dimers together (Figure 15C). Nonetheless, the genuine oligomerization state of SarA which is necessary for transcription modulation is unknown (Liu *et al.,* 2006).

Similarly to AgrA, a cysteine residue of SarA (Cys9) can also undergo oxidation and phosphorylation, both of which lead to the dissociation of SarA from DNA. The phosphorylation is mediated by the eukaryotic-like kinase Stk1 and removed by the phosphatase Stp1. Interestingly, Stk1 can be inhibited by some cell-wall targeting ATBs (vancomycin and the cephalosporine ceftriaxone), leading to a decreased phosphorylation of Cys9. Moreover, this effect is highly redox-dependent as Cys9 can only be phosphorylated in its reduced form, representing another level of regulation (Sun, Ding, *et al.,* 2012). Other residues, particularly serines and threonines, can be phosphorylated as well, with similar effects on DNA binding. Nonetheless, their precise positions remain unmapped as the phosphorylation was uncovered solely by assays with radioactively labeled ATP and subsequent protein hydrolysis whose products were visualized by SDS-PAGE (Didier *et al.,* 2010).

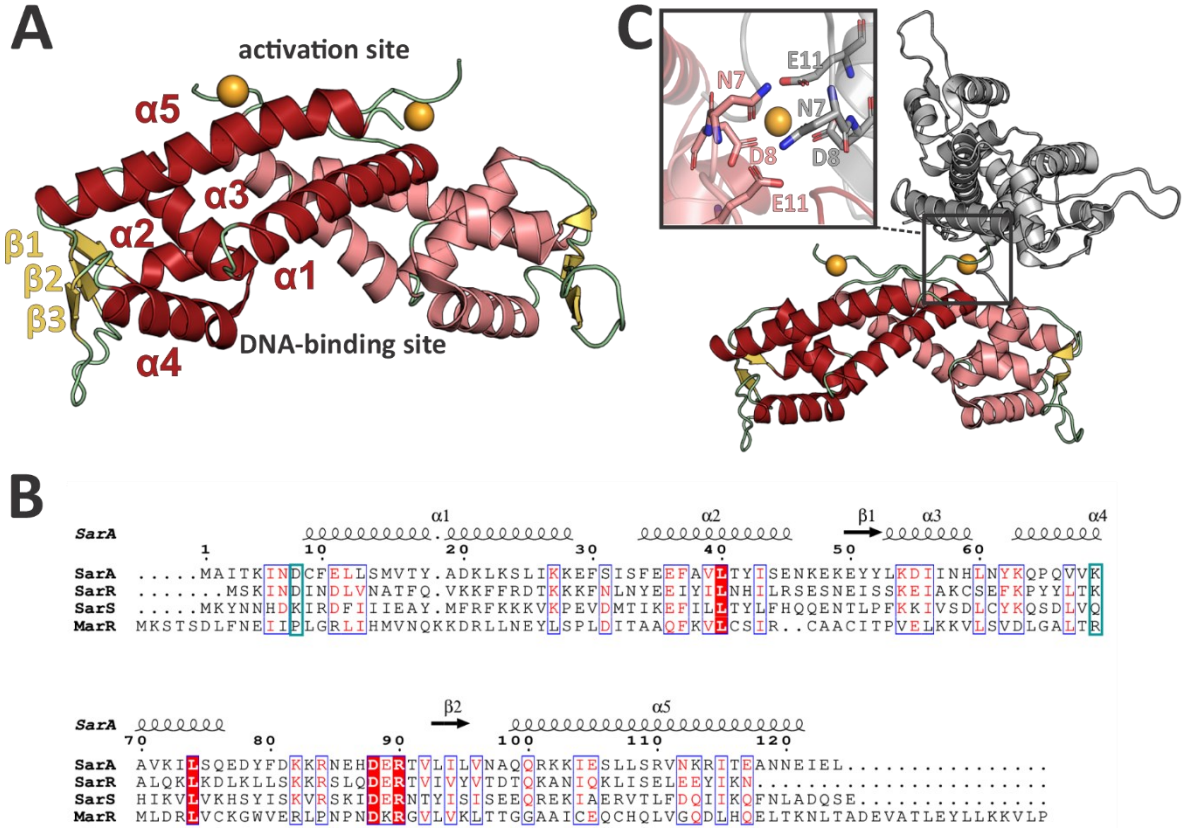


Figure 15: (A) **A cartoon representation of the SarA homodimer**. The monomers are distinguished by color, dark red and salmon, with β-sheets colored yellow and loops pale green (2.5 Å resolution, PDB code: 2FRH; Liu *et al.,* 2006). The picture shows the DNA-binding site and the activation site which coordinates Ca^{2+} ions (orange spheres). (B) **Multiple sequence alignment of selected SarA homologs.** The upper row depicts the position of secondary structures in SarA (PDB code: 2FRH; Liu *et al.,* 2006). Strictly conserved residues are highlighted in red boxes, residues with a similarity global score higher than 0.7 are shown in red font. Cyan frames highlight the crucial semi-conserved residues mentioned in the main text. The alignment was carried out using ClustalW (Thompson *et al.,* 1994), generated in ESPript 3.0 (Robert & Gouet, 2014) and adjusted in the Corel editor. Inspired by Liu *et al.,* 2006. (C) **SarA tetramer with a close-up view of Ca^{2+} coordinating residues**. One

homodimer is shown in the same color code as in panel A, the dimer of the neighboring asymmetric unit is in grey. The coordinating residues are shown as salmon (homodimer 1) and grey (homodimer 2) sticks. Created with PyMOL (version 2.5.2, Schrödinger, USA).

SarA binds to a wide range of operators, including a 29-bp consensus sequence in between the P2 and P3 promoters of the *agr* operon (Chien & Cheung, 1998) (Figure 16A). It has been predicted that the binding of SarA induces an 80° bend in the *agr* promoter, which brings two AgrA dimers together and effectively enhances the transcription from the P2 and P3 promoters (Reyes *et al.,* 2011). DNase I footprinting assays have revealed that SarA also recognizes homologous 26-bp consensus sequence, called the SarA box, in the promoters of *hla, spa, fnb* and *sec* genes, allowing SarA-mediated *agr*-independent regulation (Figure 16B) (Chien *et al.,* 1999). However, this cognate sequence does not fit all the regulated genes, such as the *cna* (collagen-binding adhesin) promoter. For this reason, an extensive sequence alignment of 56 SarA-regulated genes, which included *cna*, was performed. This reduced the SarA box to a 7-bp consensus sequence ATTTTAT with one allowed mismatch, which is found within 46 of the 56 tested sequences (Sterba *et al.,* 2003).

Figure 16: (A) **29-bp DNA operator of SarA within the *agr* promoter**. (B) **26-bp SarA box consensus sequence** (for *hla, spa, fnbA, fnbB* and *sec*). The upper-case and lower-case letters represent nucleotides conserved in at least 67% and 50% of tested promoters (including *agr*), respectively. Multiple nucleotides were listed in non- or semi- conserved positions. Inspired by Chien & Cheung, 1998 and Chien *et al.,* 1999, created in the Corel editor.

Only one crystal structure of the SarA-DNA complex had been reported so far (Schumacher *et al.,* 2001). Nonetheless, a subsequently determined structure of the SarR homolog revealed substantial inaccuracies in this model, as well as a model of the apo-form of SarA published by the same authors, (Schumacher *et al.,* 2001) and both structures were retracted from the PDB database (Davis *et al.,* 2008). The exact mode of DNA binding thus remains unknown. However, mutagenesis studies pinpointed critical amino-acid residues for DNA binding. Intriguingly, only two mutated residues located within the wing region (Arg84 and Arg90) completely abolished the DNA-binding activity of SarA. Mutations of other residues that contribute to binding (Cys9, Tyr18, Glu29, Glu36, Lys54, Leu74, and Phe80) reduced the affinity to DNA but the binding activity was still detectable (Liu *et al.,* 2006).

SarA inhibitors

Only a few SarA inhibitors have been developed so far, which might be due to the ongoing research of its regulatory network and the lack of structural studies of its precise DNA-binding activity.

The first selective SarA inhibitors were discovered by structure-assisted computational drug design. Several inhibitors were synthesized in order to competitively bind the highly conserved DER residues of the wHTH motif (Arya & Princy, 2013b). This led to the discovery of 2-[(Methylamino)methyl]phenol, which exhibits both anti-virulence and anti-biofilm forming activity (Figure 17A) and, aside from that, it significantly lowers the minimal inhibitory concentration (MIC) of conventional ATBs. Considering that the bactericidal effect of this small-molecule inhibitor appeared only at concentrations higher than 1.5 mM, this compound seems to be an excellent choice for alleviating AMR. Moreover, SarA deletion mutants were not affected at all by this treatment, proving its high selectivity (Balamurugan *et al.,* 2017). The disadvantage of this compound might lie in its dosage as the inhibitory effect on the biofilm formation positively correlates with increasing dose only up to the concentration of 1.25 µM and then steeply decreases. The proposed explanation of this finding is that after reaching the threshold concentration, the compound starts to aggregate and cannot pass into the cells (Balamurugan *et al.,* 2017). This could be a problem as the threshold dose might not be enough for ameliorating virulence.

The same research group further identified another hit-compound 4-[(2,4-diflurobenzyl)amino] cyclohexanol (SarABI) (Figure 17B), which is predicted to make hydrogen bonds with Asp88 and Arg89. This molecule was able to reduce 50% of biofilm deposition at the concentration of 200 µg/ml when applied to *S. aureus* cultures. This dosage was thus further tested on rat models that exhibited no sign of vascular graft infection despite enormous bacterial load. The cytotoxicity of SarABI was evaluated on the HepG-2 (human epithelial type 2) cell line, which revealed that even higher concentrations than 200 µg/ml do not show any signs of toxicity (Arya *et al.,* 2015).

In comparison with AgrA, SarA seems to be a more sensible choice for the development of novel anti-virulence compounds for two main reasons. First, unlike AgrA, SarA belongs to a highly conserved family found solely among staphylococci, which is ideal for the development of species-specific ATBs (Balamurugan *et al.,* 2017; Ibarra *et al.,* 2013). Second, SarA positively regulates both the production of virulence determinants and biofilm formation. AgrA, on the other hand, inhibits biofilm formation, which means that AgrA-inhibiting anti-virulence compounds might induce the overproduction of biofilm (Boles & Horswill, 2008). It is plausible that ATBs would not be able to penetrate the thick layer of biofilm, rendering them virtually ineffective (Anderl *et al.,* 2000).

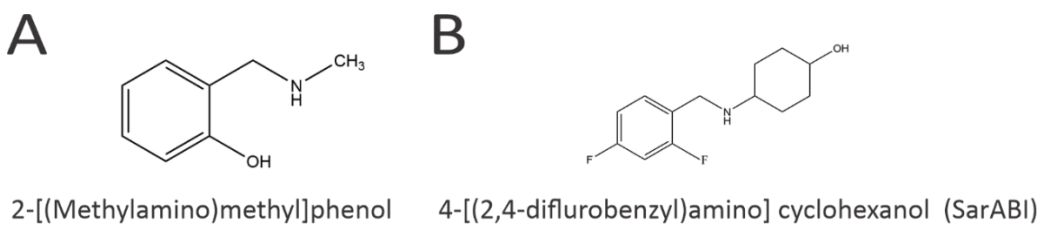


Figure 17: SarA inhibitors. Inspired by Arya & Princy, 2013b and Arya *et al.,* 2015, created with the ChemDraw editor.

4.2.2.2 SarR

The native regulator of SarA is the SarR protein. In late exponential and stationary phases, the expression of SarR peaks, allowing the negative regulation of SarA and downstream regulated genes. Apart from repressing SarA, SarR also downregulates the *agr* operon (Manna & Cheung, 2006) and positively regulates the expression of *aur* (aureolysin metalloprotease) and *sspA* (V8 serine protease) genes (Gustafsson & Oscarsson, 2008).

SarR is a 115 amino-acid-long TR (Bateman *et al.,* 2023) that forms homodimers. The monomers are composed of five α-helices and three antiparallel β-sheets (Liu *et al.,* 2001). Analogously to all SarA family proteins, SarR contains the hallmark wHTH motif which differs from the canonical wHTH by its prolonged W1 loop region and the replacement of W2 by the α5-helix (Figure 18) (Liu *et al.,* 2001; Gajiwala & Burley, 2000).

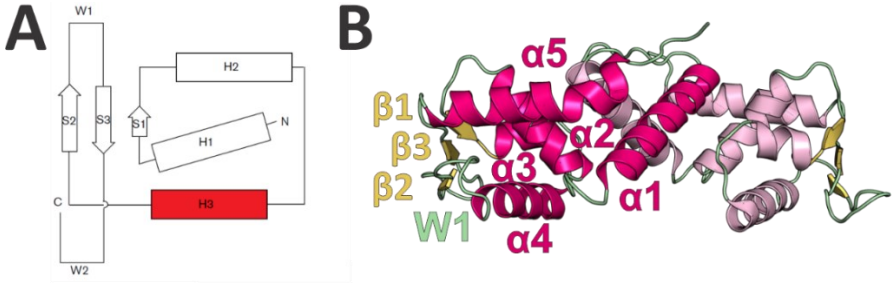


Figure 18: (A) **A topology diagram of the structural arrangement of the canonical wHTH**. The recognition α3-helix that wedges into the major groove of DNA is highlighted in red. Adapted from Gajiwala & Burley, 2000. (B) **A comparison of the structural arrangement of the SarR protein.** α-helices are colored pink, β-sheets yellow and loops pale green. The W1 is significantly prolonged and the W2 is replaced by the α5-helix. In this case, the recognition of the major groove of DNA is mediated by the α4-helix. (2.3 Å resolution PDB code: 1HSJ; Liu *et al.,* 2001). Created with PyMOL (version 2.5.2, Schrödinger, USA).

The crystallographic model of the SarR structure (PDB code: 1HSJ) published by Liu *et al.,* 2001 reported a low electron density in the initial 2Fo-Fc map and high temperature factors in the region of HTH, suggesting its high flexibility. Contrarily, helices α1, α2 and α5 were shown to be incredibly rigid, owning to their extensive hydrophobic interactions. Apart from ensuring stability, hydrophobic interactions also facilitate the dimerization. Particularly, Leu10, Ile7 and Ile4 from one monomer and Phe20, Ile35, Leu109 and Ile13 from the second monomer form two extensive hydrophobic cores, with the interface buried solvent-accessible area of 1,500Å^2. Dissociation of the two monomers is sterically blocked by the L-resembling structure of α1 and a stretch of the N-terminal residues. Thus, only extremely harsh conditions, such as 4 M urea, can disrupt the dimer (Liu *et al.,* 2001).

While the protein-DNA structure has not been resolved yet, a highly structurally homologous CAP (catabolite activator protein) was identified (PDB code: 1CGP, 3 Å resolution; Schultz *et al.,* 1990) with the aid of the DALI server (Holm & Rosenström, 2010). CAP has a highly similar structural arrangement of the HTH (Figure 19A), with the root mean square deviation (RMSD) of 1.8 - 2.1 Å for the superposition of the HTH Cα atoms. Superposition of the two proteins uncovered SarR residues that could possibly interact with DNA bases (Lys61 and Lys67) and the sugar-phosphate backbone of DNA

(Lys52, Lys56, Lys71, Arg82). Furthermore, SarR was superposed with the CAP-DNA complex (Figure 19B), which hinted that the flexible loop within the wing region can be repositioned in such a way that it extensively interacts with the minor groove of DNA (plausibly via Asp86, Glu87 and Arg88). Considering that the binding of the CAP dimer induces a 90° bend in the DNA, it is quite possible that SarR might promote the bending of at least some of its cognate promoters (Liu *et al.,* 2001; Schultz *et al.,* 1990).

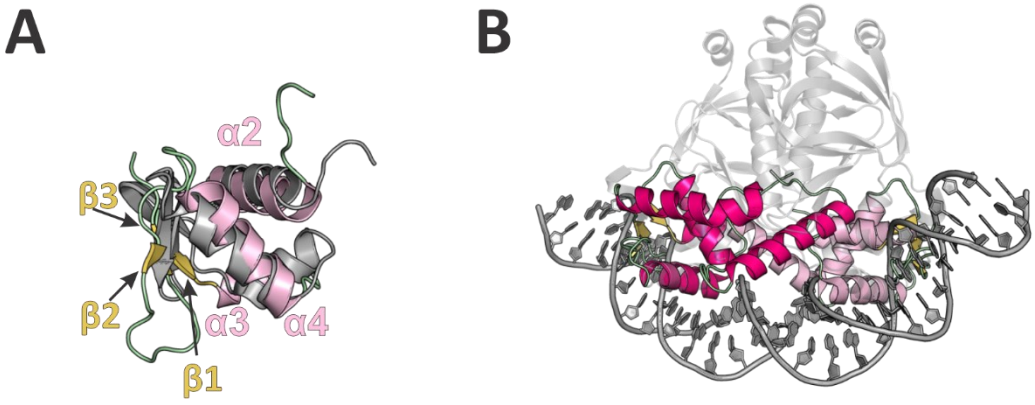


Figure 19: (A) **Alignment of the wHTH motif of SarR and the DNA binding HTH of CAP.** SarR is represented by pale pink α-helices, yellow β-sheets and pale green loops, CAP is depicted in grey. (B) **SarR-DNA model based on the superposition with the CAP-DNA crystal structure.** The SarR protein was crystallized in fusion with a maltose-binding protein (2.3 Å resolution, PDB code: 1HSJ; Liu *et al.,* 2001). For clarity, the picture only shows the SarR part of the fusion protein (residues 370 - 485). One SarR monomer is shown in magenta, the second one in pale pink, following the color code of panel A. CAP is shown in a grey transparent cartoon model (3 Å resolution, PDB code: 1CGP; Schultz *et al.,* 1990). Created with PyMOL (version 2.5.2, Schrödinger, USA).

Although SarR can bind all three *sarA* promoters, the repression is mostly mediated from the P1 promoter. The identification of SarR operators within the *sarA* locus is significantly complicated as EMSA analysis uncovered atypical laddering pattern. SarR can thus conceivably form multimers upon binding or bind to multiple binding sites within each of the three promoters. DNAse I footprinting assay was able to localize a 28-bp SarR protected sequence within the P2 promoter, which is extremely rich in AT base pairs (a 96% content). Attempts have been made to identify a consensus sequence shared by all *sarA* promoters, which lead to the discovery of an 8-bp motif TAAATTAN (with the last nucleotide being variable). Albeit this sequence is found within all three *sarA* promoters (and once in the *agr* operon), it is too small to accommodate the protein, making it insufficient for the formation of the SarR-DNA complex (Manna & Cheung, 2001).

SarR also represses the transcription from the P2 promoter of the *agr* operon. Conversely to SarA, SarR modules the P2 transcription without the induction of DNA bending. Moreover, EMSA analysis has revealed that SarR has about 4-fold higher affinity (K_D ~ 50 nM) to the P2 promoter and can even displace SarA from the promoter as their recognition sequences overlap (Reyes *et al.,* 2011).

While the *agr* operator of SarR is still unknown, site mutagenesis studies were able to pinpoint critical DNA-binding residues using a 33-bp *agr* operon fragment. A complete defect in DNA binding

was observed in substitution mutants of Lys52, Lys80, Lys82, Arg88 and Leu105. Some of these residues (Arg82 and Arg88) have conserved SarA residues (Arg84 and Arg90) that are also crucial for DNA binding, while the remaining residues differ. This demonstrates that SarA and SarR have different recognition patterns for the shared *agr* operon (Manna & Cheung, 2006).

4.2.2.3 MgrA

MgrA is a sequence homolog of SarA, yet it structurally more resembles the MarR regulator of *E. coli* of the MarR family, which is mainly associated with gram-negative bacteria (Chen *et al.,* 2006). This global regulator controls over 350 genes with diverse functions, including the production of efflux pumps (e.g. NorB and Tet38), upregulation of capsule polysaccharides and downregulation of the Spa protein, α-toxins, coagulases and proteases (Luong *et al.,* 2003; Truong-Bolduc *et al.,* 2005). Importantly, MgrA downregulates the production of FnbA that facilitates internalization of bacteria into host cells, and thereby hinders cell invasion (Lei *et al.,* 2019). Nonetheless, thanks to its enormous regulatory spectrum, the disruption of MgrA in mouse models of infection led to a 100- and 100,000-fold reduction of CFUs (colony forming units) in livers and kidneys, respectively (Chen *et al.,* 2006).

MgrA structure

MgrA is composed of 147 amino-acid residues (Bateman *et al.,* 2023) that form seven α-helices and three β-strands. Like most SarA-family proteins, MgrA forms homodimers. The crystal structure of its truncated form (residues 6 – 143; 2.8 Å resolution, PDB code: 2BV6) (Figure 20) revealed that helices α1, α6 and α7 form the dimerization domain, which is connected via α5 to the three-stranded wHTH DBD (Chen *et al.,* 2006). The α4-helices are suspected to be the recognition ones which wedge into the two consecutive major grooves of DNA. Nonetheless, the structure of the MgrA-DNA complex has not been determined yet, so the DNA-binding mechanism remains unmapped. Moreover, though the MgrA consensus sequence of (A/T)GTTGT has been unveiled, the recognition sites are separated by spacers of a hitherto unknown length (Manna *et al.,* 2004; Crosby *et al.,* 2016).

Similarly to SarA, MgrA also possesses a cysteine residue (Cys12) located at the dimer interface, which can be oxidized and phosphorylated in the aforementioned manner (Chen *et al.,* 2006; Sun, Ding, *et al.,* 2012; Truong-Bolduc *et al.,* 2008). In its reduced form, Cys12 forms hydrogen bonds with Ser113 and Tyr38 of the second monomer (Figure 20). Under oxidative stress, oxidation of Cys12 disrupts the hydrogen bonds and induces the dissociation of MgrA from DNA (Chen *et al.,* 2006). This results in the activation of the appropriate defensive mechanisms that contribute to ATB resistance as ATBs tend to promote the production of reactive oxygen species (Kohanski *et al.,* 2007).

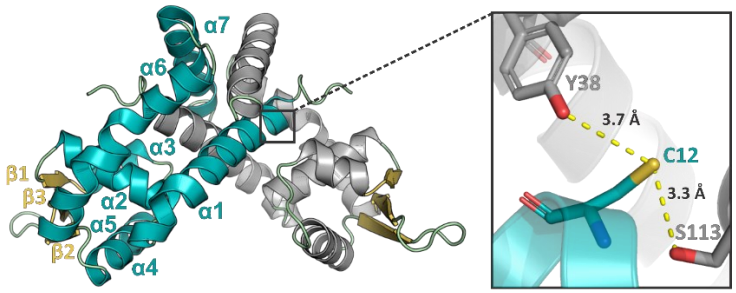


Figure 20: A cartoon representation of the MgrA crystal structure. One monomer is shown in teal, the second one in grey. β-strands and loops are colored yellow and pale green, respectively. The position of the oxidation-sensing Cys12 is indicated with a box. The close-up view highlights the interaction of Cys12 with residues of the second monomer. The dotted lines depict the distances of the hydrogen bonds measured in Å (2.8 Å resolution, PDB code: 2BV6; Chen *et al.,* 2006). Created with PyMOL (version 2.5.2, Schrödinger, USA).

MgrA inhibitors

One anti-virulence compound, 5,5′-methylenedisalicylic acid (MDSA) (Figure 21A), has been shown to target the phosphatase Stp1, resulting in an increased phosphorylation of not only MgrA, but also SarA and SarA-inactivating SarZ (Tamber & Cheung, 2009; Zheng *et al.,* 2015). It has been revealed that MDSA can also bind directly to MgrA. However, further studies need to be carried out in order to understand the mode of its binding mechanism as its position has only been mapped by molecular docking and a FRET (Förster resonance energy transfer)-based method. Treatment with MDSA led to a significant attenuation of virulence in both *in vitro* and *in vivo* studies. The MIC was estimated to 8 µM, whereas growth defects of *S. aureus* started appearing at 10 mM, proving that MDSA does not affect its viability. Nonetheless, MDSA is diacidic, which might cause problems in the crossing of the tissue-blood barrier. An optimized MDSA with methylated carboxyl groups was thus synthesized and injected into mice models of abscess formation through the retro-orbital route. Four days after the treatment, the CFUs were reduced to 10% compared to non-treated models (Sun *et al.,* 2011).

In 2024, an MgrA-selective compound, methylophiopogonanone (Mo-A), has been identified by virtual screening of flavonoid derivatives (Figure 21B). Fluorescence anisotropy and thermal shift assays have confirmed that this inhibitor disturbs the MgrA-DNA interaction. Molecular docking studies further pinpointed the critical MoA-binding residues Gln19, Tyr38 and Asn131. Upon the application of Mo-A, downregulation of genes associated with toxin production adhesion and immune evasion was observed. In mice models, a combination of Mo-A and vancomycin exhibited a 70% survival rate after 96 hours of post-infection. In comparison, the negative control group treated solely with vancomycin had an 80% mortality. Moreover, at the concentration of 64 µg/ml, Mo-A protected the A549 (adenocarcinomic human alveolar basal epithelial) cell line from destructive effects of infection and did not show any toxicity even at significantly higher concentrations (Guo *et al.,* 2024).

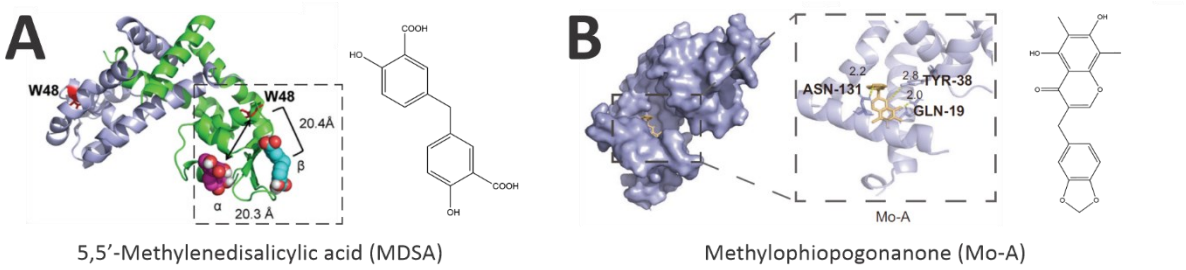


Figure 21: MgrA inhibitors with molecular docking models. (A) MDSA is shown in pink and cyan sphere representation docked to two potential binding sites labeled α and β. W38 highlights the tryptophan which was used for FRET experiments. (B) Mo-A is depicted in yellow stick model. The structural formulas were created with the ChemDraw editor and the docking models were adapted from Sun *et al.,* 2011 and Guo *et al.,* 2024, respectively.

4.2.2.4 Rot

The Rot (repressor of toxins) protein belongs to global regulators of the SarA family (McNamara *et al.,* 2000). Despite its designation, this TR holds a dual function by upregulating 86 and downregulating 60 genes. In general, it has an opposing effect to the regulation by AgrA (Saïd-Salim *et al.,* 2003). For instance, Rot represses genes encoding hemolysins (*hla* and *hlb*), lipases and urases, whilst activating transcription of genes for adhesins, clumping factors and the evasion Spa protein (Benson *et al.,* 2011; McNamara *et al.,* 2000; Saïd-Salim *et al.,* 2003). Additionally, Rot upregulates the production of SarS involved in positive Spa regulation (Saïd-Salim *et al.,* 2003). Rot is also directly linked to the *agr* system as the *rot* mRNA complementarily pairs with RNAIII, leading to the downregulation of Rot production (Geisinger *et al.,* 2006).

Rot structure

Rot is a 166 residues-long (Bateman *et al.,* 2023) single-domain TR comprising five α-helices and a two-stranded β-sheet that forms head-to-head homodimers coordinating two Cl^- ions. (Figure 22) (Zhu *et al.,* 2014). The crystal structure (PDB code: 4RBR; Killikelly *et al.,* 2015) showed the conserved wHTH of α3, α4, and a β-sheet and an extensive hydrophobic dimeric interface formed by α5- and α1-helices (buried surface area of 1,800 $Å^2$). Dimerization is mediated by hydrogen bonds between symmetry-related Gln124 residues and a water-mediated interaction between Glu16 of one monomer and Arg37 of the second monomer (Figure 22) (Killikelly *et al.,* 2015).

Although its structural arrangement does not differ from other members of the SarA family, Rot can be distinguished by the absence of cysteines and its exceptionally high acidic sequence (with a pI value of 5.1) (Cheung *et al.,* 2004). Its surface is therefore highly negatively charged, which indicates that the interaction of Rot with DNA is thus dependent on the basic residues of the wHTH motif. The electrostatic repulsion might be also diminished by other positively charged binding partners, such as the RNAP (Killikelly *et al.,* 2015).

The structure of the Rot-DNA complex has not been resolved yet, however, a protein-DNA crystal structure of a highly structurally homologous OhrR from *Bacillus subtilis* is available (PDB code:

1Z9C; Hong *et al.,* 2005). The superposition of this known complex with the crystal structure of the Rot apo-form enabled the prediction of amino-acid residues that might mediate interactions with DNA. The heptapeptide KPYKRTR (residues 64 - 70) was predicted to facilitate base-specific interactions with the major groove of DNA, whilst thirteen residues found across all domains are very likely to be involved in recognition of the sugar-phosphate backbone of DNA. Arg91, found at the tip of the wing, presumably forms a sequence-specific interaction with the intervening minor groove of DNA. The superposition also hinted at the function of the Cl^- ions as they were found to overlap with the sugar-phosphate backbone, indicating that they might play a crucial role in the DNA binding mechanism. The authors of the study suggest that the Cl^- ions dissociate upon DNA binding and leave the space for the phosphates of DNA (Killikelly *et al.,* 2015).

To further characterize critical DNA-binding residues, substitution mutants were constructed. These experiments revealed that Rot has distinct amino-acid recognition patterns for different promoters. Some mutations led to an altered transcription of all tested genes (*ssl7, spa, lukE* and *hla*), whilst other mutants affected only some of them. The crucial residues for recognition of all tested promoters were found to be Arg91 (within the conserved DER wing residues), Leu54 and Lys55 (in the recognition α3-helix), whose substitutions completely abolished the DNA binding activity (Figure 22). Based on the OhrR-assisted model, these residues most likely interact with the minor groove of DNA. Promoter-specific residues include Tyr66, Tyr71, Asn74, Ser36, Glu38 and Glu39. For instance, T66A mutation dramatically decreased the activation of *spa*, but had a mild effect on *ssl7* and *hla,* and exhibited no effect on *lukED*. These residues thus plausibly serve as filters of promoters. Overall, the differentiation of recognized promoters is mediated by residues located within the HTH as well as the helical core, whereas residues of the wing are crucial for DNA binding (Killikelly *et al.,* 2015).

Even though the Rot consensus sequence has not been established yet, the symmetry of the Rot dimer suggests that it might comprise a palindrome (Killikelly *et al.,* 2015). Fluorescence polarization assay with different DNA probes has revealed that the bound sequence is rich in AT base pairs (Zhu *et al.,* 2014). Moreover, the distance between the two Arg91 that are predicted to interact with the minor groove of DNA is 60 Å, which accounts for about 18-bp (Killikelly *et al.,* 2015). Additionally, in the OhrR homolog, the Arg91 residue interacts with an oxygen atom of a thymine (Hong *et al.,* 2005). Using this analogy on other highly conserved residues, other assumptions about the base-specific interactions with DNA could be plausibly made (Killikelly *et al.,* 2015). Nonetheless, these hypotheses need to be confirmed by profound experimental evidence.

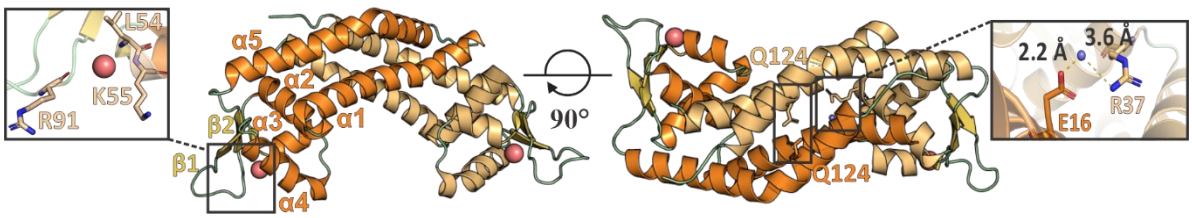


Figure 22: A cartoon representation of the Rot homodimer. One monomer is shown in dark orange, the second one in a lighter shade, β-sheets and loops are distinguished by yellow and pale green, respectively. The Cl^- ions are represented by salmon spheres. A close-up view shows residues expected to be the most critical for DNA recognition. The 90° rotated view highlights residues that facilitate interactions between the two monomers with a close-up view of the water-mediated (blue sphere) hydrogen bond (with the distance indicated by dotted lines and measured in Å) (1.7 Å resolution, PDB code: 4RBR) (Killikelly *et al.,* 2015). Created with PyMOL (version 2.5.2, Schrödinger, USA).

Rot as a therapeutic target?

Albeit no Rot-interfering anti-virulence agents have been reported so far, it might be only a matter of time. A study conducted on Δ*rot* mutants revealed that Rot acts as an inhibitor of various secreted proteases and especially all cysteine proteases. Deletion of *rot* was thus associated with decreased biofilm formation. This has been corroborated in murine models of catheter infection, where Δ*rot* mutant showed a substantial reduction in the production of biofilm. Nonetheless, some tested *S. aureus* clinical isolates (USA100 and USA400) were not affected by the deletion, suggesting that virulence and biofilm-associated regulatory pathways might vary among individual strains (Mootz *et al.,* 2015). The identification of all *S. aureus* transcriptional regulatory pathways is therefore significantly complicated, which hinders the development of TR-interfering anti-virulence compounds.

5. Targeting TRs to combat ATB resistance

Given their essential role in bacterial virulence, remarkable regulatory range as well as species or even strain specificity, TRs pose promising candidates for the development of unconventional and highly selective ATBs. Nonetheless, this type of intervention might also come with its drawbacks. The following paragraphs will thus attempt to elucidate the advantages and disadvantages that come with targeting TRs.

A major advantage of targeting bacterial TRs is that they lack homologous structures in human cells, which significantly reduces the chances of cytotoxicity of their inhibitors. This has been established by multiple experiments, which were mentioned in the preceding chapters (Arya *et al.,* 2015; Balamurugan *et al.,* 2017; Guo *et al.,* 2024). Considering that TRs play an indispensable role in the regulation of VFs, it is no wonder that these molecules are becoming increasingly popular among anti-virulence targets. By disarming bacteria from their virulence, AMR could be possibly prevented or at least significantly slowed down due to the diminished selection pressure (Dickey *et al.,* 2017). While this strategy needs to be tested at a larger scale, *in vivo* experiments have shown that even serially passaged cells treated with anti-virulence compounds (e.g. AgrA-targeting savirin) did not show any signs of resistance to given agents (Sully *et al.,* 2014). Apart from ameliorating AMR, anti-virulence treatment could also protect the human native microbiome by aiming solely at pathogenic bacteria (e.g. savirin does not affect normally harmless *S. epidermidis*) (Sully *et al.,* 2014). Anti-virulence compounds could also be used for the sole purpose of increasing the susceptibility to conventional ATBs (e.g. F19 inhibitor of AgrA that increases the susceptibility to cephalosporins and fluoroquinolones) (Greenberg

et al., 2018). This could also be achieved by targeting TRs associated with the production of efflux pumps (e.g. AdeRS TCS of *Acinetobacter baumannii* controlling the AdeABC efflux pump that confers the resistance to aminoglycosides, tetracyclines, fluoroquinolones, and many other ATBs) (Sun *et al.,* 2014).

In order to quench AMR, the inhibitors must aim at non-essential genes to lessen the selective pressure (Dickey *et al.,* 2017). However, targeting a single TR can consequently disrupt its whole regulon and thus affect all downstream regulated genes (Heinrichs *et al.,* 1996; Chien & Cheung, 1998). This can be advantageous as one molecule virtually represents multiple targets. Yet it can also be a major disadvantage as the affected TR can have a homolog in non-pathogenic species (Greenberg *et al.,* 2018) or be linked to essential genes, which would render its inhibitors bactericidal (González *et al.,* 2018). Aiming at non-essential genes might not be sufficient for eliminating infection, especially in immunocompromised patients, yet this could plausibly be overcome by the usage of combination therapy together with conventional ATBs or other anti-virulence compounds (Gaya, 1986; Greenberg *et al.,* 2018).

A significant drawback of this approach is the lack of knowledge. By virtue of their abundance, many TRs are not well studied and thus their structural and functional annotations are missing. Importantly, many TRs can have both beneficial and detrimental functions (e.g. inhibition of AgrA would decrease the production of Hla, Tst and other toxins, but also increase the formation of biofilm) (Boles & Horswill, 2008). Virulence-associated TRs are oftentimes global regulators regulating hundreds of genes, many of which are unspecified (e.g. MgrA regulating over 350 genes) (Luong *et al.,* 2003). Thus, in some cases, the effect of inhibition can be unpredictable (e.g. deletion of the SlyA regulator of *Enterococcus faecalis* that activates expression of hemolysins and adhesins resulted in increased virulence and persistence in mice models) (Michaux *et al.,* 2011). The selection of the right TRs used for the development of successful anti-virulence compounds is therefore a pivotal, yet immensely challenging, task.

Remarkably, one of the greatest achievements of a TR-based anti-virulence therapy is the development of a *Mycobacterium tuberculosis* (MTB) vaccine (MTBVAC). This vaccine is actually the very first live-attenuated MTB vaccine to enter clinical evaluation (Arbues *et al*., 2013) and has recently progressed into Phase 3 efficacy trials (Martín *et al.,* 2021). MTBVAC is based on a double *phoP* and *fadD26* deletion mutants (Arbues *et al.,* 2013). Whilst FadD26 is crucial for the synthesis of virulence lipids (Trivedi *et al.,* 2004), PhoP is a RR regulator of the PhoPR TCS, whose upregulation led to MTB outbreak, and which regulates the production of virulence determinants. Among the most crucial ones is ESAT-6 (early secreted antigenic target 6 kDa) responsible for cell invasion and induction of host cell apoptosis (Frigui *et al.,* 2008). This vaccine proved to be more efficient than the standard *Mycobacterium bovis*-based BCG (Bacillus Calmette-Guérin) vaccine even in murine and guinea pig models of infection. Moreover, MTBVAC vaccination of immunocompromised mice had a 100% survival rate, whilst the standard treatment with BCG resulted in 100% mortality 92 days post-infection

with MTB (Martin *et al.,* 2006; Arbues *et al*., 2013). This clearly demonstrates that the impact of targeting TRs is truly enormous and hopefully many other discoveries will soon unravel the full potential of the TR-interfering anti-virulence compounds.

6. Conclusion

The alarming spread of multi-drug resistant bacteria is a worldwide crisis that urgently calls for the development of novel ATBs. Despite their enormous potential, transcriptional regulators represent a hitherto unexploited group of anti-microbial targets. The aim of this thesis was to gather information about five promising candidates that could serve as potential targets of novel ATB compounds in the leading multi-drug resistant bacterium *S. aureus.* Special attention was given to structural information that could be utilized for the design of inhibitory molecules.

In conclusion, the most well explored is the AgrA response regulator, whose inhibition significantly attenuated the virulence of *S. aureus,* increased the susceptibility to conventional ATBs, and did not induce resistance in both *in vitro* and *in vivo* experiments. However, more studies need to be carried out to thoroughly investigate the effect of its disruption on the production of biofilm. Importantly, the 3D structure of the full-length protein and its protein-DNA complex is the missing puzzle piece in the comprehension of the molecular mechanism of AgrA and could plausibly uncover more sites for its inhibition.

SarA, SarR, MgrA and Rot all belong to the highly species-specific SarA family of transcriptional regulators and thus represent particularly attractive targets. Nevertheless, no experimental protein-DNA structures of the SarA family are available and this represents a knowledge gap for understanding how SarA family transcriptional regulators precisely modulate transcription and how they differentiate between their cognate operators. Moreover, 3D structures would also elucidate how various ligands, ranging from native effectors to inhibitory compounds, affect the DNA binding activity of given regulator. So far, the designed SarA family inhibitors could only take an advantage of the highly conserved DER residues of the DNA binding wHTH domain. 3D protein-DNA structures would undoubtedly reveal many other regions that could be targeted as well, and most likely even much more efficiently.

To sum up, although transcriptional regulator interference-based therapy could potentially revolutionize the treatment of infections caused by multi-drug resistant bacteria, there is still a long road ahead before we get to see, and hopefully utilize, the full potential of this strategy. The only way to move forward is to get a profound understanding about the precise molecular functions and mechanisms of given regulators. This cannot be revealed without the 3D structures of proteins as well as their complexes with DNA, native effectors, and inhibitory compounds. Further structural studies are thus an absolute necessity.

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