Untersuchungen zur Wirkungsweise von Cervimycin

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CONTENTS

1. Summary

Due to the increasing antibiotic resistance new antimicrobial substances are urgently needed. According to the World Health Organization, *Staphylococcus aureus* remains a high priority pathogen with regard to the need for new antibacterial compounds. The two parts of this work include (i) the whole genome sequencing and phenotyping of the model strain *S. aureus* SG511 Berlin, and (ii) investigations on the mode of action of the antibiotic cervimycin.

In the following the publications included in this thesis are summarized:

The first publication "**The hypersusceptible antibiotic screening strain *Staphylococcus aureus* SG511-Berlin harbors multiple mutations in regulatory genes" (Chapter 4.1)** describes the whole genome sequencing of *S. aureus* SG511 Berlin which revealed mutations in numerous regulatory genes in addition to a formerly known *graS* loss-of-function mutation, affecting the sensing of cationic antibiotic peptides. Since there is no single optimal strain for studying *S. aureus*, and even the probably most studied isolate *S. aureus* NCTC 8325 harbors mutations in regulatory genes, mapping of these mutations will be helpful to choose an isolate for future studies and to interpret experimental outcomes. *S. aureus* SG511 Berlin might also be a tool for screening of antibiotic lead structures and studying natural compounds with limited availability.

Cervimycins are such compounds, an antibiotic complex of polyketide glycosides with bactericidal activity against Gram-positive bacteria which is only active when the tetracycline-type aglycone is fully substituted with six sugar molecules. The second publication "**Cervimycin-Resistant *Staphylococcus aureus* Strains Display Vancomycin-Intermediate Resistant Phenotypes" (Chapter 4.2)** describes the phenotype-based approach to elucidate the mode of action of cervimycin. A serial passaging experiment for cervimycin-resistant *S. aureus* SG511 Berlin mutants yielded in vancomycin-intermediately resistant strains with loss-of-function mutations in the Clp protein degradation system (*clpC*/*clpP*) and the essential autolysin regulating WalRK two component system (*walK*). Nevertheless, a direct interaction of cervimycin with these proteins was not observed, indicating an indirect effect of the mutations.

The last chapter "**The unusual mode of action of the polyketide glycoside cervimycin C (Chapter 4.3)"** is a paper manuscript on the complex effects observed in cervimycin treated bacteria. Cervimycin caused cell division and chromosome segregation defects in Gram-

positive bacteria and omics analyses corroborated a dual de-repression of the protein stress response and the autolysin genes. Interestingly, cell lysis was impaired, cell walls were thickened, and a cell wall stress response was not observed under cervimycin treatment, contradicting a cell lysis mechanism. The cervimycin stress response was reminiscent of the ribosome-targeting aminoglycosides, but experimental data are needed to test this hypothesis.

2. Introduction

The discovery of penicillin by Alexander Fleming (Fleming, 1946) and the subsequent introduction of antibiotics into clinical use revolutionized medical practices and are a pre-requisite for most modern treatment procedures, like operations of the abdomen, organ transplantations, or neonatal medicine. Antibiotics either stop bacterial growth or kill the bacteria, without harming the human host. Five major modes of action of antibiotics are distinguished:

I) The bacterial **cell envelope** is a well-proven target for antibacterial action, including inhibition of cell wall precursor biosynthesis, blockage of the transport of peptidoglycan monomers across the cytoplasmic membrane, or blockage of peptidoglycan transpeptidation or transglycosylation (Silver, 2006).

II) DNA **topoisomerases** are enzymes which can redundantly relax supercoiled DNA throughout the cell cycle, but the DNA gyrase (topoisomerase II) is unique and essential in introducing negative supercoils into the DNA. Fluoroquinolones and aminocoumarins are two antibiotic classes which act as topoisomerase inhibitors (Gellert *et al*., 1976; Gilbert & Maxwell, 1994; Sato *et al*., 1986).

III) Ansamycins, like rifampin (rifampicin), target the RNA polymerase and prevent the **transcription** from DNA to mRNA (Wehrli, 1983).

IV) The subsequent **translation** of mRNA at the ribosome is also targeted by different antibiotic classes which either interfere with the large (chloramphenicol, MLS_B antibiotics) or the small ribosomal subunit (tetracyclines, aminoglycosides) or prevent the formation of the initiation complex by both subunits (linezolid) (Poehlsgaard & Douthwaite, 2005).

V) Disruption of the bacterial **membrane** is also detrimental for the bacterial cell, though much less antibiotics with this mode of action are in clinical use (Ledger *et al*., 2022).

Antibiotic activity and antibiotic resistance are tightly connected processes and the inevitable evolution of antibiotic resistance necessitates the constant development of novel antibiotics which overcome these resistance mechanisms. Alternative therapies, other than the development of novel antibiotics, are also under investigation, including antimicrobial oligonucleotides, monoclonal antibodies, and phage therapy (Streicher, 2021), and the use of antibiotic combinations (Foster, 2017). In a simplified model, all antibiotic resistance

mechanisms are can be assigned to three classes (Figure 1): either (i) the antibiotic is enzymatically inactivated or modified, (ii) the target is modified so that the affinity of the antibiotic towards the target is reduced, or (iii) the access to the target is restricted (modulation of influx and/or efflux). In this regard, Gram-negative bacteria are particularly challenging, because the additional outer membrane (OM) of these bacteria efficiently blocks the influx of many antibiotics. Extrusion of antibiotics by multidrug efflux complexes further synergizes with this diffusion barrier (Nikaido, 1996).

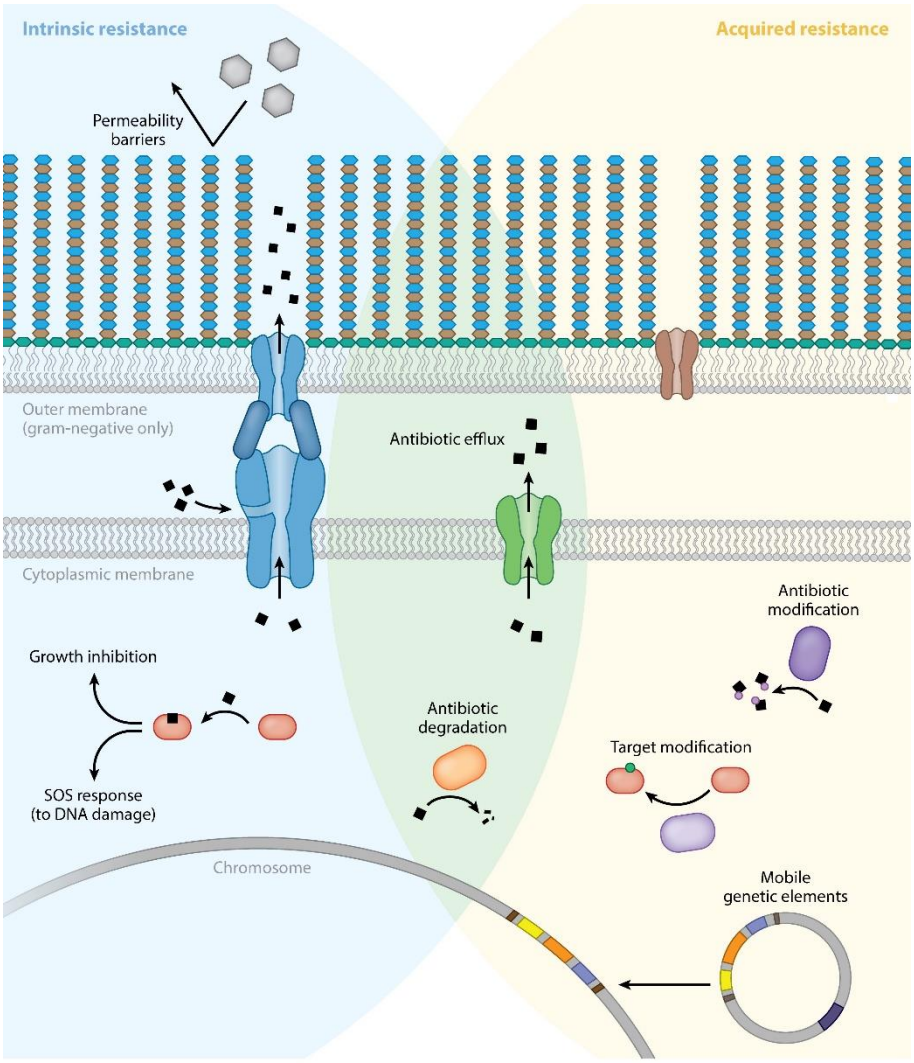

Figure 1: Bacterial resistance mechanisms against antimicrobials (Surette & Wright, 2017). Intrinsic (blue background) and acquired resistance mechanisms (yellow) are distinguished. The most common mechanisms of antibiotic resistance in bacteria are: reduced influx, e.g., due to the outer membrane of Gram-negative bacteria; increased efflux or enzymatic inactivation/hydrolysis of the antibiotic; antibiotic target modification or enzymatic modification of the antibiotic itself (group transfer, redox process).

2.1 Antimicrobial resistance (AMR) crisis

Apart from intrinsic antibiotic resistance, acquired resistance mechanisms evolve as countermeasures to particular antibiotics or scaffolds. Highly effective defense mechanisms often pre-exist in antibiotic producers or their competitors and are acquired by other species via horizontal gene transfer (HGT) of mobile genetic elements (Nesme & Simonet, 2015). These mobile elements (e.g., plasmids and transposons) offer facile routes of HGT that often do not respect species or genus boundaries (Surette & Wright, 2017). The tight interconnection between antibiotic action and antibiotic resistance development is also reflected by the occurrence of antibiotic resistant isolates after clinical approval of specific antibiotic classes (Figure 2).

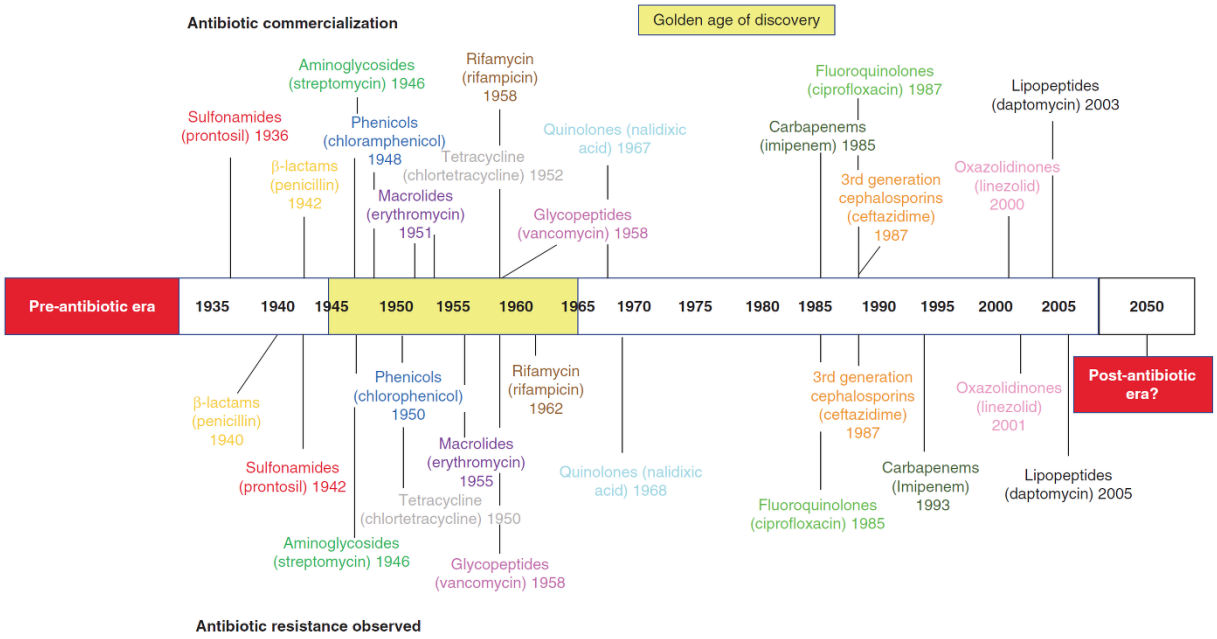

Figure 2: Timeline of the commercialization of the major classes of antibiotics (above the timeline) and their first reported case of resistance (below) (Stephens *et al*., 2020). Most antibiotic classes were discovered during the "golden age of discovery" (yellow). Only few novel antibiotic classes were approved afterwards which is referred to as "discovery void".

During the golden era of drug discovery, which was characterized by the commercialization of natural product antibiotics, the relatively quick isolation of antibiotic resistant clinical isolates was overcome by the approval of new antibiotic classes. Later, chemical modification of lead compounds was used, but fewer antibiotics came into clinical use. Despite of methodical progresses, like testing purified proteins with compounds *in vitro* or *in silico* "docking" simulations, the imbalance between effective antibiotics in clinical use and resistance development progressed to the detriment of effective antibiotics and led to the current

antimicrobial resistance crisis. Beneath the acceleration of antibiotic resistance development, due to misuse and overuse of antibiotics, also the lacking interest of pharmaceutical companies thwarts the development of new antimicrobials (Chaudhary, 2016) jointly worsening the antibiotic resistance crisis. The mis- and overuse of antibiotics also includes the excessive use of the same or closely related antibiotics in veterinary medicine and animal husbandry as those used in human (Foster, 2017). Consequently, new terms for the description of antibiotic resistant germs were introduced: multidrug-resistant (MDR, i.e., resistant to three or more classes of antimicrobials), extensively drug resistant (XDR, i.e., resistant to all but one or two classes), and pandrug-resistant (PDR, i.e., resistant to all available classes) (Magiorakos *et al*., 2012). Indeed, treatment options are occasionally already exhausted due to infections with pan-resistant bacteria, such as pan-resistant *Acinetobacter baumannii* (Nichols, 2019). As a corollary, some antibiotic classes cannot be used with guaranteed success due to the widespread resistance to these antibiotic classes and the antibiotic pipeline is running dry. The World Health Organization (WHO) warned against a post-antibiotic era, where there are no drugs available to prevent or treat bacterial infections (Reardon, 2014, Figure 2).

Table 1: Priority list of pathogens released by the WHO (modified after WHO (2017)). Pathogens are arranged according to the urgency of need for new antibacterial compounds to guide research and development of such substances.

Priority	Pathogens	Gram	Resistance towards
Critical	*Acinetobacter baumannii*	-	carbapenems
	Pseudomonas aeruginosa	-	carbapenems
	Enterobacteriaceae	-	carbapenems, most β-lactams (ESBL*)
High	*Enterococcus faecium*	+	vancomycin
	Staphylococcus aureus	+	methicillin, vancomycin (VRSA and VISA)
	Helicobacter pylori	-	clarithromycin
	Campylobacter spp.	-	fluoroquinolones
	Salmonellae	-	fluoroquinolones
	Neisseria gonorrhoeae	-	cephalosporins, fluoroquinolones
Medium	*Streptococcus pneumonia*	+	penicillin (non-susceptible)
	Haemophilus influenzae	-	ampicillin
	Shigella spp.	-	fluoroquinolones

*ESBL, extended spectrum β-lactamase.

Although Gram-negative bacteria are of highest priority for research and development of new antibacterial compounds according to the WHO, also Gram-positive bacteria like *Staphylococcus aureus* are an area of concern (Table 1). This is also underlined by the prioritization of the so-called **ESKAPE** pathogens, which stands for ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* species, to overcome antimicrobial resistance, because these pathogens are responsible for the majority of nosocomial infections (Rice, 2008).

Recently, European Antimicrobial Resistance Collaborators estimated the AMR burden in the WHO European region in the year 2019 and seven leading pathogens were responsible for about 457,000 deaths associated with resistance in 53 countries of this region (European Antimicrobial Resistance Collaborators, 2022). These pathogens were, in descending order of mortality, *Escherichia coli, S. aureus, K. pneumoniae, P. aeruginosa, E. faecium, Streptococcus pneumoniae*, and *A. baumannii* (Figure 3) (European Antimicrobial Resistance Collaborators, 2022), confirming the continuing importance of the ESKAPE pathogens. Globally, five leading pathogens among 33 bacterial genera or species investigated were responsible for 54.9% of the deaths in 2019: *S. aureus, E. coli, S. pneumoniae, K. pneumoniae*, and *P. aeruginosa*, each involved in more than 500,000 deaths in this year (Antimicrobial Resistance Collaborators, 2022).

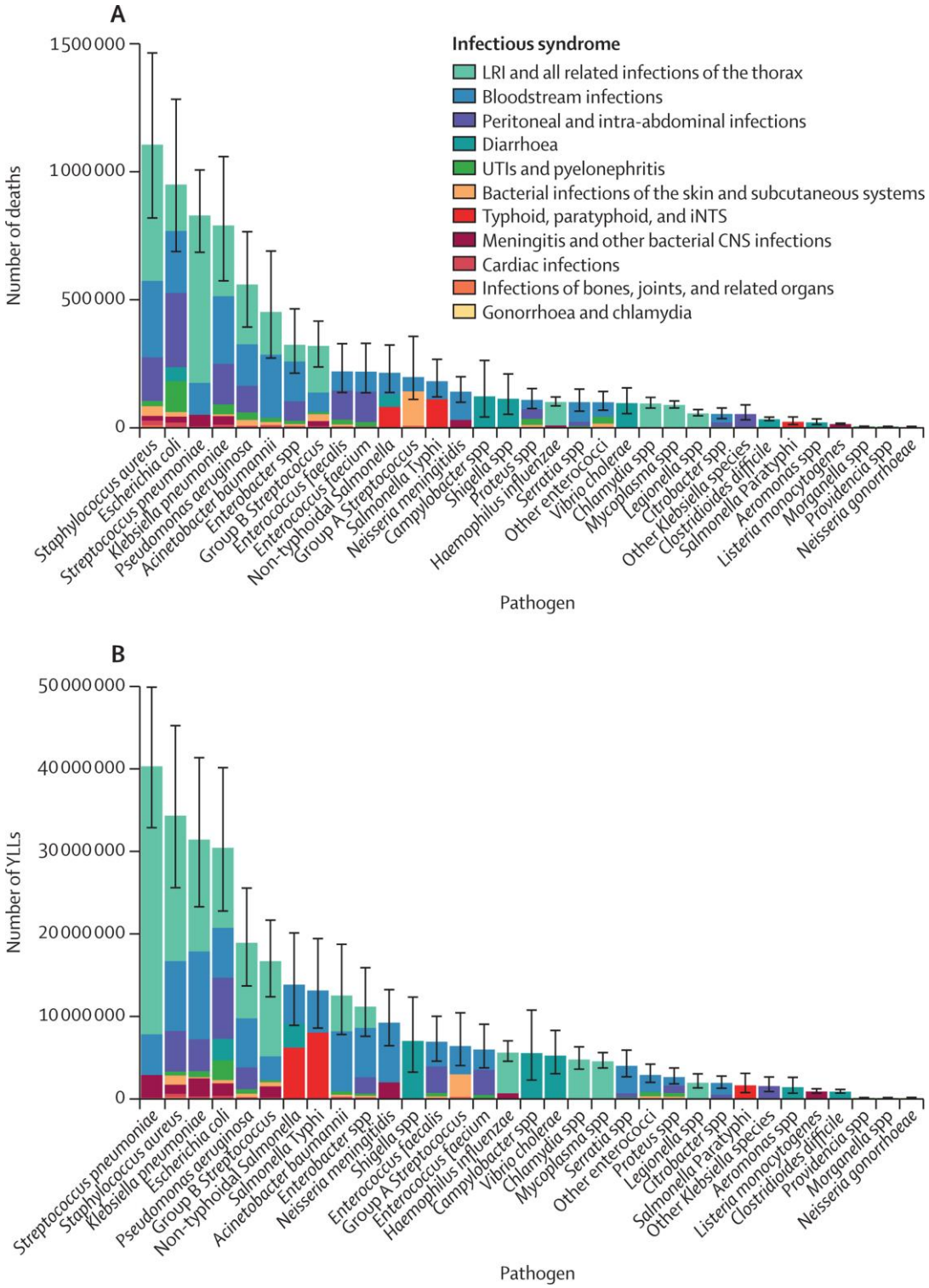

Figure 3: *S. aureus* was the leading cause for fatal infectious syndromes in 2019 (Antimicrobial Resistance Collaborators, 2022). (**A**) Global number of deaths and (**B**) years of life lost (YLLs) by pathogen and infectious syndrome. Columns show total number of deaths for each pathogen, with the bars split into infectious syndromes; LRI=lower respiratory infection; iNTS=invasive non-typhoidal *Salmonella; Salmonella* Typhi=*Salmonella enterica* serotype Typhi; *Salmonella* Paratyphi=*Salmonella enterica* serotype Paratyphi; UTI=urinary tract infection.

2.2 The high priority pathogen *Staphylococcus aureus*

S. aureus is a Gram-positive, coagulase- and catalase-positive, and facultative anaerobic bacterium. These small spherical bacteria (~ 1 µm cell diameter) divide sequentially in three orthogonal planes over three consecutive division cycles (Tzagoloff & Novick, 1977). Humans are a natural reservoir of *S. aureus* (Lowy, 1998), where the skin and mucosa, especially of the anterior nares, are colonized. Approximately 30% of individuals are intermittently colonized with *S. aureus* (Gordon & Lowy, 2008). Its adaptability allows the resistance to environmental stresses, including reactive oxygen species, heat, acid, and osmotic stress (Clements & Foster, 1999). As an opportunistic pathogen, *S. aureus* is the cause of diverse infections, including wound infections, bacteremia-sepsis, endocarditis, pneumonia, osteomyelitis, arthritis, and skin diseases (Dayan *et al*., 2016). In 2019 *S. aureus* was the leading bacterial cause of death in 135 countries and was also associated with the most deaths in individuals older than 15 years globally (Antimicrobial Resistance Collaborators, 2022). In the WHO European region 83,325 deaths were attributed to *S. aureus* in 2019 and methicillin-resistant *S. aureus* (MRSA) was shown to be the leading pathogen–drug combination in 27 countries for deaths attributable to AMR (European Antimicrobial Resistance Collaborators, 2022).

Human staphylococcal isolates are categorized into hospital- (HA) or community-acquired (CA) isolates and separated from the livestock-associated (LA) *S. aureus*. Genotyping, especially sequencing of housekeeping genes (MLST-typing: multilocus sequence typing), provides further information about the relatedness of staphylococcal isolates, yielding clonal complexes (CCs) which are sub-divided into sequence-types (STs). The staphylococcal genome encodes diverse virulence factors, contributing to its pathogenicity and immune evasion mechanisms, including toxins (cytotoxins, enterotoxins, exfoliatines, hemolysins, toxic shock syndrome toxin), enzymes (proteases, lipases, hyaluronidases), surface proteins (protein A, coagulase, clumping factor), and microcapsules (Lowy, 1998; Tuffs *et al*., 2018).

Beginning with penicillin in the 1940s, the alternating pattern of introduction of novel antibiotics into clinical use and the subsequent isolation of antibiotic resistant isolates is also apparent for *S. aureus*. Penicillin is a β-lactam antibiotic which binds to and inhibits the penicillin-binding proteins that are essential for the last stage of bacterial cell wall synthesis. However, quickly after introduction of penicillin into the clinics, penicillin-resistance spread, based on penicillinases, enzymes which hydrolyze the β-lactam ring of the antibiotic. Luckily, methicillin was developed as an alternative in the 1950s, an isoxazolyl penicillin which is

resistant towards penicillinases. Here, another resistance mechanism was developed. Methicillin-resistance is based on the alternative penicillin-binding protein PBP2a which is encoded in the SCC*mec* cassette and binds penicillin less efficiently, leading to methicillin-resistant *S. aureus* (MRSA) isolates in contrast to methicillin-susceptible (MSSA) ones (Deurenberg *et al*., 2007). Unfortunately, some SCC*mec* cassettes (type II and III) determine multi-resistance due to the integration of additional drug resistance genes in the resistance cassette (Deurenberg *et al*., 2007). In these cases, glycopeptide antibiotics remained as treatment option. Vancomycin, which inhibits the peptidoglycan biosynthesis by binding to the D-Ala-D-Ala terminus of the cell wall precursor lipid II (Perkins, 1969), was frequently used to treat MRSA infections (Appelbaum, 2007). However, vancomycin-intermediately resistant (VISA) (Hiramatsu *et al*., 1997) and vancomycin-resistant *S. aureus* strains (VRSA) were frequently isolated (Chang *et al*., 2003). While vancomycin-resistance is mediated by the extrachromosomal *van* gene cluster, which encodes a machinery to produce an alternative lipid II terminus (D-Ala-D-Lac) when glycopeptide antibiotics are present (Chang *et al*., 2003), vancomycin-intermediate resistance is mediated by the accumulation of single nucleotide polymorphisms (SNPs), especially in two-component systems (TCSs) like VraSR, GraSR and WalKR (Hafer *et al*., 2012). These mutations are thought to increase the cell wall biosynthesis of *S. aureus*, while autolysis is simultaneously decreased, leading to an increase of un-cross-linked peptidoglycan monomers which impedes the diffusion of vancomycin towards the division septum (Cui *et al*., 2000). More recently, the membrane active lipopeptide antibiotic daptomycin came into clinical use, which is one of the last resort antibiotics against MRSA (Ledger *et al*., 2022), but still treatment options remain limited. Other options include the synthetic oxazolidinones linezolid and tedizolid, which are protein biosynthesis inhibitors (Stevens *et al*., 2002), and the 5th generation cephalosporins that inhibit the cell wall biosynthesis (Lin *et al*., 2013).

Thus, both sides must always be taken into account, the development of new resistance-breaking antibiotic classes and, on the other hand, the trends in antibiotic resistance. For the latter, Ventola (2015) summed up some strategies to overcome, or at least significantly reduce, antibiotic resistance, such as adopting an antibiotic stewardship program (correct administration of antibiotics), improving diagnosis, tracking and prescribing practices, optimizing therapeutic regimens (shorter treatment periods are often sufficient), and preventing infection transmission.

Production and purification of natural compounds is a complex process, often yielding only trace amounts of the product (Hug *et al*., 2018). Consequently, especially antibiotic susceptible

bacterial strains are used to characterize these molecules. One isolate, *S. aureus* SG511 Berlin, turned out to be hypersusceptible to some antibiotics, making it a popular standard strain for antimicrobial susceptibility testing and basic research (Sass & Bierbaum, 2009). This hypersusceptibility was attributed to a loss-of-function mutation in the *graS* kinase gene of *S. aureus* SG511 Berlin (Sass & Bierbaum, 2009), but further contributors remained elusive, since the genome was not fully sequenced. GraS is part of a five-component cationic antimicrobial peptide (CAMP) sensing and resistance network in *S. aureus* (Falord *et al*., 2012, Figure 4).

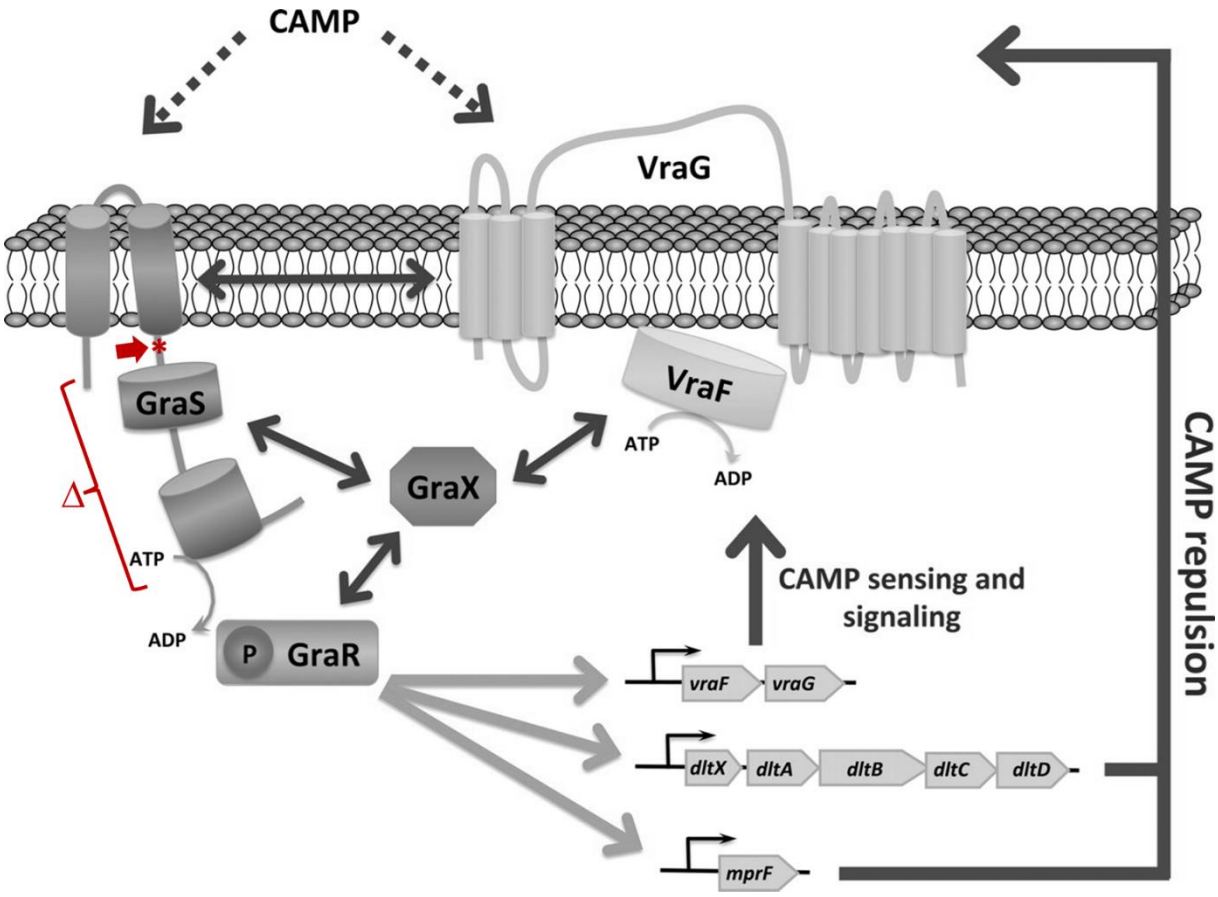

Figure 4: The five-component signal transduction network controlling CAMP sensing and resistance in *S. aureus* (modified after Falord *et al*. (2012)). Because of a pre-mature stop-codon in the *graS* kinase gene of *S. aureus* SG511 Berlin (*), the entire cytoplasmic part of the protein is deleted (Δ) (Sass und Bierbaum 2009), interrupting the CAMP feedback mechanism.

CAMPs would first be sensed by the VraFG ABC transporter and/or the GraS kinase, the former case includes the signal transduction from the VraG permease to GraS, followed by autophosphorylation of GraS, and transphosphorylation of the GraR response regulator. Subsequently, GraR acts as a transcription factor which induces the transcription of *vraFG*, the *dlt* operon and *mprF*, leading to D-alanylation of wall teichoic acids (WTAs) and lipoteichoic

acids (LTAs) and lysinylation of membrane phosphatidylglycerol, increasing the cell surface positive charges and conferring resistance through electrostatic repulsion of CAMPs (Falord *et al*., 2012, Figure 4). Reconstitution of a functional GraS kinase in *S. aureus* SG511 Berlin significantly decreased the susceptibility to the lantibiotics mersacidin, Pep5, and nisin (Sass & Bierbaum, 2009), proving the relevance of this CAMP sensing and resistance system in *S. aureus*.

Taken together, a better understanding of the cellular mechanisms underlying susceptibility and resistance to clinically applied antibiotics remains a prerequisite in antibiotic research.

2.3 Polyketide antibiotics

Polyketides are a group of structurally diverse compounds produced by bacteria, fungi, and plants which is classified by their synthesis via the polyketide pathway (Schneider, 2005). These molecules are produced through the successive condensation of small carboxylic acids and their structural diversity confers a variety of biological properties, including: antibacterial, antifungal, antitumor, and anthelmintic properties, association with sporulation or other developmental pathways, a combination of pharmacological functions, or a so far unknown function (Katz & Donadio, 1993). Structurally, polyketides are divided into complex and aromatic molecules, while the latter are further sub-divided by the number, arrangement, and occurrence of rings. Aromatic polyketides are mainly built from acetate units through a reiterative process wherein the β-carbonyl groups formed after each condensation cycle are mainly left unreduced (Katz & Donadio, 1993). The polyketide backbone is then converted into the multicyclic scaffold and often modified afterwards, giving rise to the remarkable diversity of secondary metabolites (Schneider, 2005). This process is catalyzed by type II polyketide synthases (PKSs) which are composed of 5–10 individual enzymes, each encoded by a distinct gene (Schneider, 2005).

Despite the fact that polyketide producers are diverse, some prominent polyketides, like the antibiotics tetracycline and erythromycin and the cancer drug doxorubicin, share actinomycetes as producer organisms. Actinomycetes are filamentous bacteria and the major producers of known natural product antibiotic classes (64%) (Hutchings *et al*., 2019). Prominent antibiotic classes produced by actinomycetes include the above mentioned tetracyclines, aminoglycosides, macrolides, glycopeptides, and lipopetides (Hutchings *et al*., 2019). In spite of the barrier of rediscovering already known antibiotics, actinomycetes are still a promising source of novel antibiotics.

Hug *et al*. (2018) summarized some promising approaches to identify novel antibiotic classes from actinomycetes (which are similarly applicable to other microbes):

1. **Metabolomics** approach. Improvement of the conventional cultivation, extraction, and identification technique by the systematic assessment of all primary and secondary metabolites in a biological sample; simplified by more sensitive and discriminative detection techniques.
2. The potential of **unculturable organisms**. Upgrade of cultivation techniques to identify new species and/or usage of culture-independent **metagenomic approaches** to gain access to the hidden reservoir of secondary metabolite encoding sequences.
3. **Genome mining**. In conjunction with the metagenomic approach, unravel the silent biosynthetic potential by whole genome sequencing of (potential) antibiotic producer strains and automatic identification and analysis of biosynthetic gene clusters.
4. Exploring **new habitats**. A strategy to avoid rediscovery of the already exploited terrestrial *Streptomyces* strains; sampling of marine habitats, extreme environments, endophytic species, and on non-*Streptomyces* actinomycetes.

The latter approach led to the identification of the antibiotic complex of cervimycins, polyketide glycosides produced by a *Streptomyces* strain isolated from a cave which had been left undisturbed since ancient time (Herold *et al*., 2005).

2.2.1 The antibiotic complex of cervimycins – state of the art

Cervimycins were named after the locality of their first discovery, the cave *Grotta dei Cervi* in Italy, where the producer strain *Streptomyces tendae* HKI 0179 was isolated (Herold *et al*., 2005). Cervimycins A to D (Figure 5) were isolated as main components of the antibiotic complex from a fermentation of the producer strain *S. tendae* HKI 0179. Interestingly, Malmierca *et al*. (2018) isolated a second cervimycin producer, *Streptomyces sp.* CS113 (whole genome sequence in NCBI: KZ195574.1), from the integument of ants of the Tribe *Attini* and identified a complete biosynthesis gene cluster (BGC) (Figure 7).

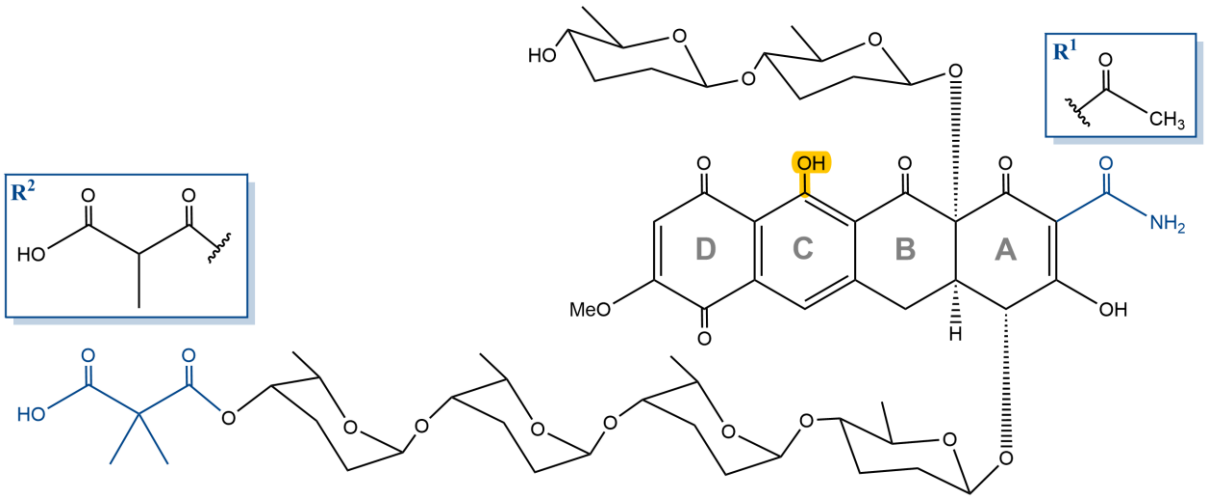

Figure 5: Structure of Cervimycins according to the findings of Herold *et al*. (2005); for simplicity in relative configuration and not in accordance with IUPAC-nomenclature. Components A to D of the antibiotic complex differ in the acetyl and carbamoyl ring substituent (R^1) and the methylmalonyl or dimethylmalonyl units at the end of the longer sugar chain (R^2). The phenol group (marked in yellow) in vicinity to the quinoid system forms phenolate salts under alkaline conditions, making the cervimycins pH-sensitive.

The aglycone, the sugar-free backbone of the cervimycins, is reminiscent of but not identical with tetracycline which differs in the quinoid system on the outer ring of the cervimycins (ring D) and the inversion of the keto group and the hydroxy group (Figure 6, marked in yellow) relative to classical tetracyclines. Tetracyclines prevent the attachment of aminoacyl-tRNA to the ribosomal acceptor site, thus inhibiting the bacterial protein biosynthesis (Chopra & Roberts, 2001). Strikingly, the C4 dimethyl-amino group, which is essential for the bioactivity of tetracyclines (Nelson, 1998), is missing in cervimycin (Figure 6, grey highlighted). Also in contrast to tetracycline, cervimycins are highly glycosylated with six sugar moieties, divided into two sugar side chains (Herold *et al*., 2005).

The shorter sugar chain is composed of two α-amicetose molecules, while the longer sugar chain is composed of one α-amicetose molecule followed by three β-rhodinose molecules, ending in either a dimethylmalonyl (cervimycin A and C) or a monomethylmalonyl unit (cervimycin B and D) (Figure 5: R^2) (Herold *et al*., 2005). Cervimycins differ in the substitution of ring A (position C2) which is either an acetyl- (cervimycin A and B) or a carbamoyl-group (cervimycin C and D) (Figure 5: R^1) (Herold *et al*., 2005), yielding in different molecular weights of the specified molecules (cervimycin A: 1,226 Da; B: 1,212 Da; C: 1,250 Da; D: 1,213 Da).

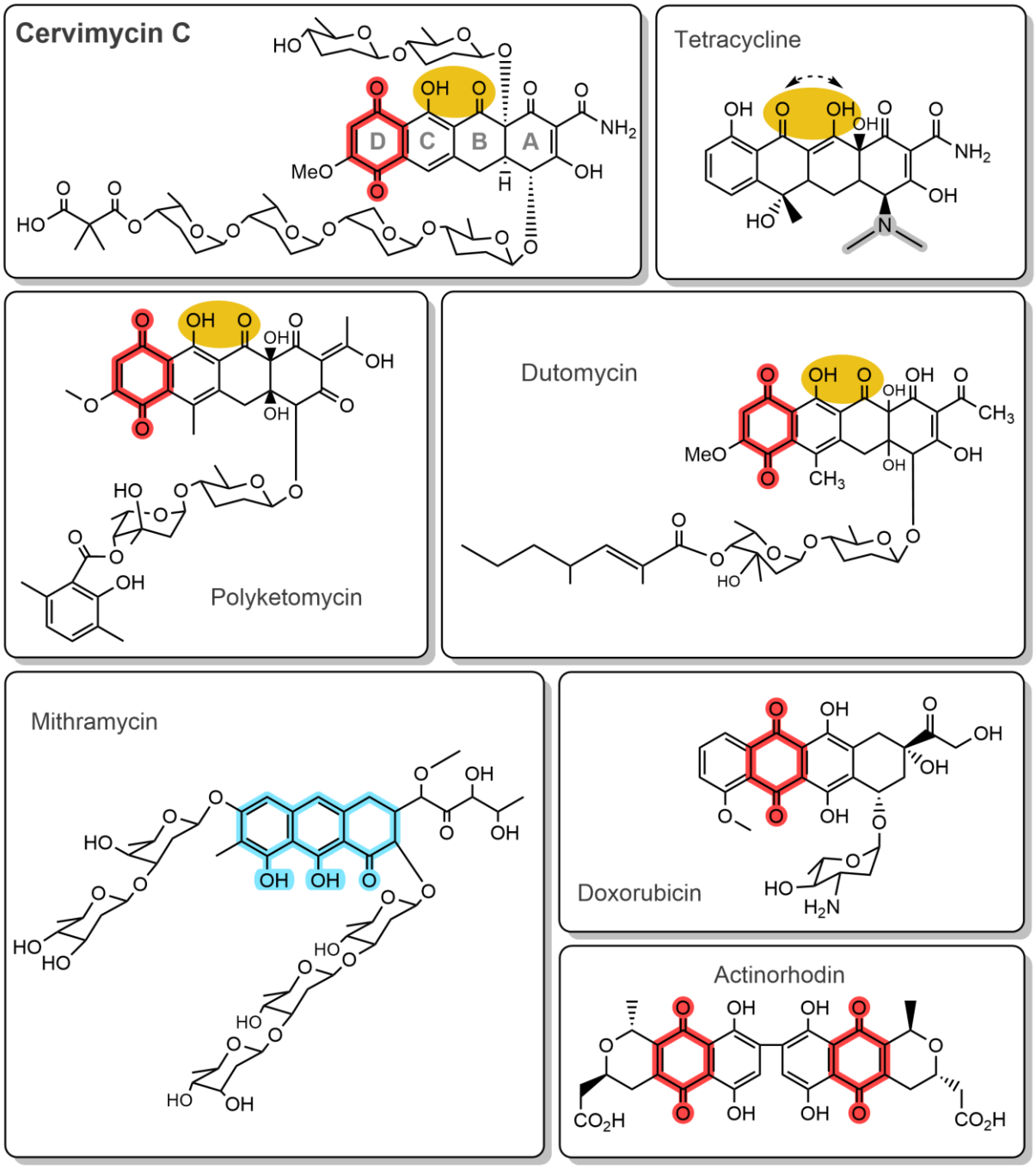

Figure 6: Comparison of cervimycin C with structurally similar compounds (created with ChemDraw Professional V 21.0.0.28; modified after Nelson (1998); Herold *et al.* (2005); Daum *et al.* (2009); Sun *et al.* (2016); Bhave *et al.* (2015); Agudelo *et al.* (2014); Mak and Nodwell (2017)). The sugar-free backbone of the cervimycins is reminiscent of the protein biosynthesis inhibitor tetracycline, but glycosylated like the antiproliferative agents dutomycin and doxorubicin. Major similarities and differences are color-highlighted.

In summary, cervimycins are glycosylated polyketides which resemble tetracyclines, but lack the C4 dimethyl-amino group that is essential for the bioactivity of tetracyclines. In addition to that, the sugar side chains significantly increase the molecular weight of the cervimycins in comparison to the tetracyclines, resembling the substitution pattern of the

anticancer drugs dutomycin and mithramycin. Interestingly, polyketomycin, whose substitution pattern resembles the one of dutomycin, possesses antibacterial activity, but the mode of action remains to be elucidated (Momose *et al*., 1998). In fact, the presence of all tridesoxyhexoses is obligatory for the antibacterial activity of cervimycin, characterizing both saccharide side chains as pharmacophore groups (Herold, 2005). The quinoid system (Figure 6, red highlighted) might cause reactive oxygen species, as described for actinorhodin and doxorubicin (Agudelo *et al*., 2014; Mak & Nodwell, 2017). A planar ring system, like in mithramycin (Figure 6, light blue), confers a DNA intercalation activity (Kersten *et al*., 1966), however, this was not observed for cervimycins (Herold, 2005).

Initial investigations on the cervimycin biosynthesis in *S. tendae* HKI 0179 were undertaken, using feeding experiments and PCRs with degenerate primers, targeting the ketosynthase subunits of aromatic polyketide synthases (Herold *et al*., 2004). Feeding experiments with 2-^{13}C-, 1,2-^{13}C-, and 1,2,3-^{13}C-malonate probes were successful in revealing a ^{13}C incorporation comparable with tetracycline-type polyketides, where malonamate undergoes eight Claisen condensations (Herold *et al*., 2004). The resulting polyketide aglycone is then subject to a series of tailoring reactions, such as methylation, oxygenation, glycosylation with the tridesoxy-sugars, and acylation with an unprecedented monomethylmalonyl or dimethylmalonyl unit (Herold *et al*., 2004). The latter is indeed derived from valine which undergoes a transamination-decarboxylation-carboxylation sequence and is then transferred onto the glycoside by the CerJ ketosynthase (Herold *et al*., 2004). CerJ is capable of transferring malonyl, methylmalonyl, and dimethylmalonyl units onto the glycoside *in vitro* (Bretschneider *et al*., 2011). Consequently, a deletion of the enzyme, or an exchange of one of the amino acids composing the catalytic triad of CerJ (cysteine-valine-histidine) with alanine, leads to cervimycins lacking that malonyl side chain, with the surprising effect of an even increased antibiotic action. Furthermore, the nucleotide sequence of *cerJ* (B9W62_31950; Accession Nr: JN001183) was the first sequence information on the cervimycin BGC available (Bretschneider *et al*., 2011).

Malmierca *et al*. (2018) identified the complete ~40 kbp cervimycin biosynthesis gene cluster in *Streptomyces sp.* CS113 using the "antibiotics and Secondary Metabolite Analysis Shell" (antiSMASH v.4) (Blin *et al*., 2021) (Figure 7) (Position in the genome: 7,078,663 – 7,118,727 nt.; total: 40,065 nt, BGC0001439).

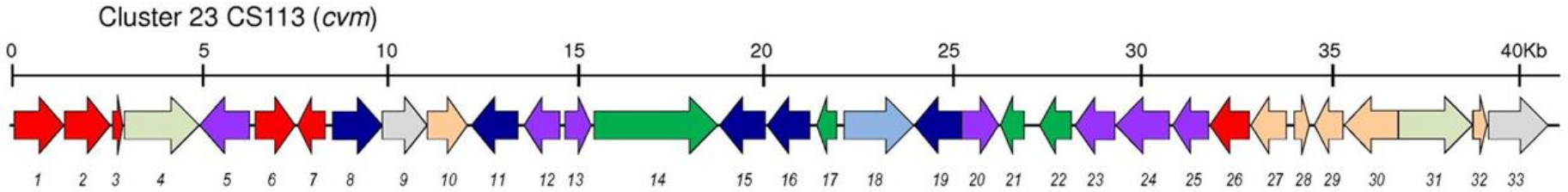

Figure 7: Cervimycin biosynthesis gene cluster (BGC) from *Streptomyces sp*. CS113 (modified after Malmierca *et al*. (2018)). A total of 33 genes was assigned to the cervimycin BGC: six polyketide synthase (PKS) encoding genes (red), six post-PKS genes (salmon), seven sugar biosynthesis genes (purple), five glycosyltransferase encoding genes (dark blue), one transporter encoding gene (light blue), four regulatory genes (dark green), two genes putatively involved in the biosynthesis of an "asparagine unit" (light green), and two genes of unknown function (grey).

The biosynthesis cluster included 33 genes and, computationally, no resistance markers were detected. According to BLAST analysis, the genes of unknown function also encode enzymes participating in cervimycin biosynthesis and encode an acyl-CoA dehydrogenase (B962_31965; gene 9) and an acetyl-CoA carboxylase (B962_32085; gene 33). Nevertheless, the transport-associated gene (B9W62_32010, gene 18) might function as resistance factor in the producer strain. Interestingly, no homolog for this transporter was found in the *Bacillus*/*Staphylococcus* taxon, using the protein BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul *et al*. (1990)), while some *E. coli* MFS transporters resembled the *Streptomyces* transporter (alignment score ≥200) which fits to the observed activity spectrum of cervimycin (see below). However, no experimental data on the participation of these efflux pumps on cervimycin resistance were collected and various factors contribute to the insusceptibility of Gram-negative bacteria towards numerous antibiotics (**Chapter 2.**, above).

Sequence comparisons of the cervimycin BGC with other secondary metabolite biosynthesis clusters using the MIBIG tool (Kautsar *et al*., 2020) yields other polyketides, but with quite low sequence similarity (25–39%) (Figure 8). Due to the low similarity to other BGCs a sequence-based study seems not promising to unveil aspects of the mechanism of action of cervimycin.

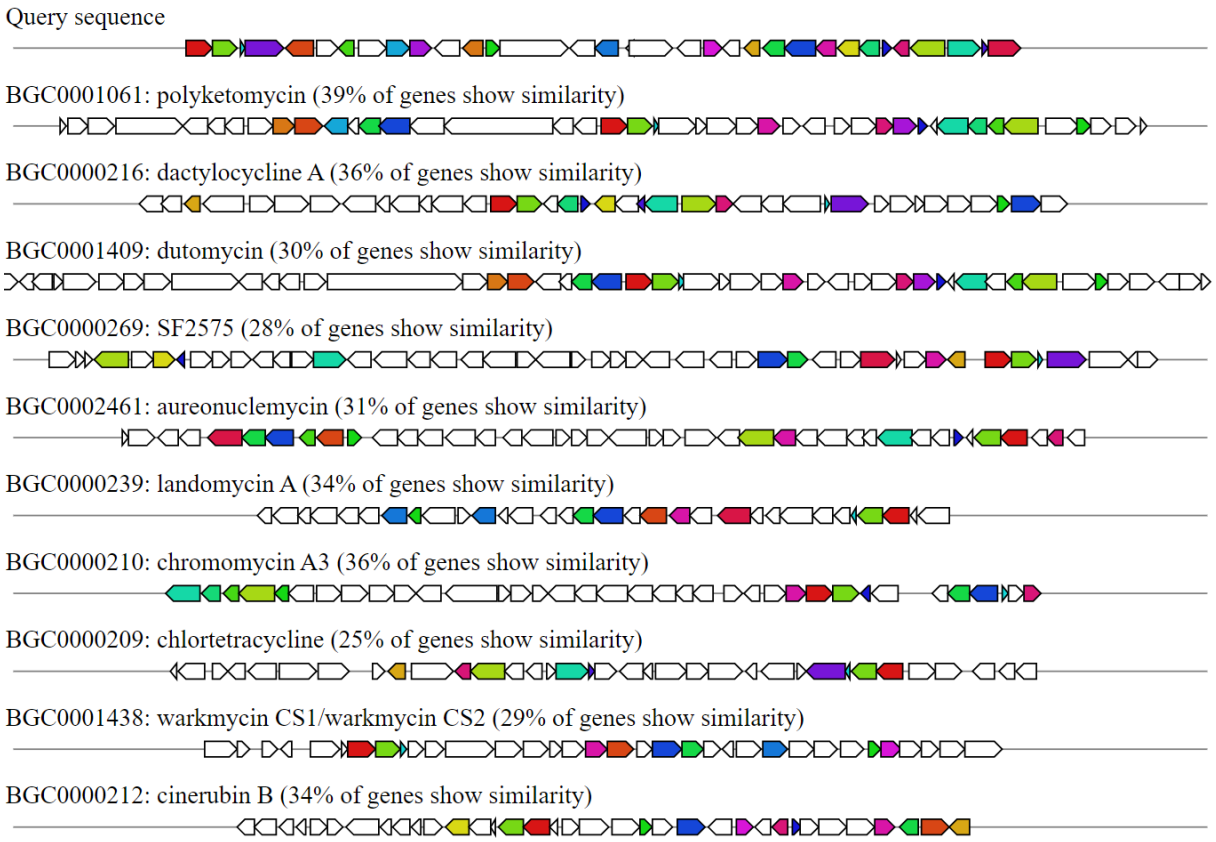

Figure 8: Sequence comparison of the cervimycin biosynthesis gene cluster with other secondary metabolite biosynthesis clusters using the MIBIG tool (Kautsar *et al*. 2020).

In general, cervimycins show antibiotic activity against a range of Gram-positive pathogens, including MSSA and MRSA and vancomycin-resistant *Enterococcus faecalis*, with minimal inhibitory concentrations (MICs) between 3.1 and 25 µg/mL (Herold *et al*., 2005). Because of the structural similarity of the compounds cervimycin A to D, a congruent mode of action of all cervimycins was assumed. Two different initial approaches were used to unravel the mode of action of cervimycin. First, metabolite incorporation tests with radioactively labeled precursors were used to measure the influence of cervimycin on the DNA, RNA, protein, and cell wall biosynthesis (Herold, 2005). Surprisingly, up to 4-times MIC cervimycin A did not affect the precursor incorporation in *Bacillus subtilis* 168 (Herold, 2005). However, higher cervimycin concentrations impaired the thymidine incorporation into the DNA with a considerable delay (35 min) and subsequently the RNA metabolism (Herold, 2005). Nevertheless, DNA intercalation was excluded as mechanism of action (Herold, 2005). Secondly, cervimycin C-resistant *B. subtilis* mutants were generated in a passaging experiment which led to a 10-fold increased MIC (Krügel *et al*., 2010). Genome sequencing elucidated an efflux-based resistance mechanism of the resistant *B. subtilis* strains, by the constitutive over-expression of the BmrA ABC transporter (Krügel *et al*., 2010).

Table 2 summarizes the state of the art on cervimycins before this study in comparison to structurally similar compounds.

Table 2: State of the art of cervimycins and structurally related compounds.

Compound	**Cervimycin**	**Polyketomycin**	**Tetracycline**	**Doxorubicin**	**Plicamycin/ Mithramycin**	**Actinorhodin**
Producer	*Streptomyces tendae* HKI 0179, *S. sp.* CS113	*Streptomyces sp.* MK277-AF1, *S. diastatochro-mogenes* Tü6028	*Streptomyces spp.*	*Streptomyces peucetius var. caesius*	*Streptomyces plicatus*	*Streptomyces coelicolor*
Classification/ structure	Aromatic polyketide glycoside	Aromatic polyketide glycoside	Aromatic polyketide	Aromatic polyketide glycoside	Aromatic polyketide glycoside	Aromatic polyketide
Details	Bis-glycosylated tetracyclic quinone	Mono-glycosylated tetracyclic quinone	Tetracene	Mono-glycosylated anthracycline	Bis-glycosylated anthracycline	Benzoisochromane quinone dimer
Molecular weight [g/mol]	1,212 – 1,250	864.8	444.44	543.52	1085.15	634.55
Antibacterial activity	Gram+, MRSA & VRE	Gram+, MRSA	Gram+ & Gram−	Gram+	Gram+, MRSA & VRE	Gram+, MRSA
Bacteriostatic/ bactericidal	?	?	Bacteriostatic	?	?	Bacteriostatic
Special features	?	–	Metal chelator	May generate ROS	At physiological pH in anionic form	pH-responsive (most potent under acidic conditions)
Additional activities	Not cytotoxic/ antiviral	Cytotoxic	Various: "chemically promiscuous"	Antitumor	Antitumor	Organo-catalyst

Compound	Cervimycin	Polyketomycin	Tetracycline	Doxorubicin	Plicamycin/ Mithramycin	Actinorhodin
Antibiotic resistance	Efflux-based (BmrA *in B. subtilis* 168; 10× MIC)	?	Efflux, ribosomal protection, enzymatic inactivation	?	?	*walRKHI* mutations (*S. aureus* ATCC 29213; 2 to 16× MIC)
DNA intercalation	−	hypothesized	−	+	−	-
^{3}[H]-labelled precursor incorporation	(DNA, RNA ↓)	?	Protein↓	DNA ↓	RNA ↓	DNA, RNA ↓ cell wall ↑
Target	?	DNA synthesis?	30S ribosomal subunit	DNA intercalation	DNA groove binder	ROS
Responsible structural features	Sugar moieties	Anthracycline core?	C4 dimethyl-amino group	Planar anthracycline nucleus	Aglycone hydroxyl groups + deoxysugars, Mg^{2+} dependent	Quinone groups
Secondary targets	?	?	Photosensitization	DNA topoisomerases	Core histone binding	DNA gyrase, topoisomerase IV, cell wall?
Responsible structural features	?	?	Naphthacene nucleus and lower peripheral region	Planar anthracycline nucleus	Mg^{2+} independent	?
References	Herold (2005); Herold *et al*. (2005); Malmierca *et al*. (2018)	Momose *et al*. (1998); Daum *et al*. (2009)	Taylor and Chau (1996); Nelson (1998)	Bodet *et al*. (1985); Chen *et al*. (1998); Pommier *et al*. (2010); Agudelo *et al*. (2014)	Lombó *et al*. (2006); Banerjee *et al*. (2014); Bhave *et al*. (2015)	Mak and Nodwell (2017)

MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant Enterococci; ROS, reactive oxygen species; +, positive; − negative; ?, unknown/not tested

The comparison of cervimycin with other aromatic polyketides produced by *Streptomyces* species underlines the variety of this class of natural compounds and reveals the partially incomplete knowledge on the mechanisms of action of these compounds (Table 2). Aromatic polyketides occur in un-glycosylated as well as in mono- and bis-glycosylated forms, leading to major differences in the molecular weight of these compounds. Independent of the target structure, the immense size of cervimycin and other compounds like mithramycin alone might prevent the antimicrobial activity against Gram-negative bacteria due to the impermeability of the outer membrane. Consequently, the antibacterial activity of these compounds is often restricted to Gram-positive bacteria, with exception of the relatively small un-glycosylated tetracyclines.

In addition, the mode of action of these compounds is quite frequently associated with the DNA, but is not known for all molecules or was only addressed for one biological function. E.g., the antitumor activity of mithramycin is quite well studied, while little is known about its antibiotic activity. Matching structural features to specific biological activities might be helpful to draw conclusions about the mode of action. For instance, the planar ring system (ring B, C, and D) of anthracyclines (Patel *et al*., 1981) and the C4 dimethyl-amino group of tetracyclines (Nelson, 1998) are essential for the interaction with the target structure. The lack of these features in the cervimycin structure point towards an alternative mode of action of this antibiotic complex which likely involves the sugar moieties (Herold, 2005), but not necessarily the terminal methyl malonyl group (Bretschneider *et al*., 2011). The poor response of *B. subtilis* towards cervimycin in the precursor incorporation tests complicates the comparison with the other substances. The results might either point towards a DNA associated mechanism or towards a target outside the tested metabolic pathways, which would be promising for a resistance breaking novel mode of action. Also, the test system might not be suitable to detect a mechanism within one of these metabolic pathways, like for the aminoglycosides that target the protein biosynthesis, but primarily lead to mistranslation and not translation inhibition, which is not detected in the precursor incorporation assay or only at very high antibiotic concentrations (Moreau *et al*., 1984).

Interestingly, for some of the listed compounds the generation of reactive oxygen species (ROS) was associated with the mode of action, and, in case of actinorhodin, linked to the quinone groups (Mak & Nodwell, 2017) which are also present in cervimycin. Furthermore, a general involvement of ROS in the mechanism of action of bactericidal antibiotics was assumed (Foti *et al*., 2012; Kohanski *et al*., 2007; Kohanski *et al*., 2010), but has recently been seriously

questioned (Fang, 2013; Keren *et al*., 2013; Liu & Imlay, 2013).

Antibiotic resistant bacteria might also help to unveil the target structure of a compound, if the resistance mechanism involves the modification of the target site. On the other hand, overexpression of efflux pumps also effectively protects from antibiotics, leaving the antibiotic target site elusive (Figure 1, Table 2).

Taken together, the mechanism of action of cervimycin still remains to be elucidated.

3. Aim of the thesis

Antibiotic resistance of bacteria is an emerging problem in hospital and community surroundings. As a corollary of the antimicrobial resistance crisis, some antibiotic classes cannot be used with guaranteed success in a calculated therapy and the antibiotic pipeline is running dry. Increasing resistance rates due to the strong selective effects of antibiotic therapy, misuse of antibiotics, as well as the lack of new drug development contribute to this process. At best, countermeasures against the antimicrobial resistance crisis include a dual strategy, limiting resistance development and transmission and the development of new antimicrobial compounds. The former comprises a profound understanding of the pathogens including potential antibiotic target structures, pathogenicity, and host interaction. The development of novel antibiotics is even more complicated, because new compounds with resistance-breaking mechanisms need to be identified and the pharmaceutical industry has to restart this neglected section.

Aims of this thesis were:

- Genomic and phenotypic characterization of the standard lab strain *Staphylococcus aureus* SG511 Berlin
- Elucidation of the resistance mechanism of cervimycin-resistant mutant strains derived from *S. aureus* SG511 Berlin
- Detailed analysis of the mode of action of cervimycin C as lead compound of the cervimycin antibiotic complex

4. Publications included in this thesis

4.1 The hypersusceptible antibiotic screening strain *Staphylococcus aureus* SG511-Berlin harbors multiple mutations in regulatory genes

- The study was published in the **International Journal of Medical Microbiology** in **2021** (https://doi.org/10.1016/j.ijmm.2021.151545). Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ijmm.2021.151545.
- **Authors:** Alina Dietrich (A.D.), Ursula Steffens (U.S.), Peter Sass (P.S.), Gabriele Bierbaum (G.B.)
- **Contributions:**
    - **A.D.:** Conceptualization of the study; funding acquisition; computational annotation of the full-length genome of *S. aureus* SG511 Berlin; bioinformatic analyses; generation of antibiotic susceptibility profiles of *S. aureus* SG511 Berlin, MRSA252 and *S. aureus* HG001; data visualization; writing the original draft and review & editing.
    - **U.S.:** DNA purification and preliminary analyses of DNA sequences.
    - **P.S.:** Microarray-based gene expression profiling of *S. aureus* SG511 Berlin versus strain SA137/93 A; review & editing.
    - **GB:** Conceptualization; funding acquisition; supervision; review & editing.

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a prominent pathogen with persistently high mortality (Turner *et al*., 2019). Whole genome sequencing of *S. aureus* isolates provides a detailed insight into the evolutionary processes leading to strains of differing virulence and drug-resistance potential (Holden *et al*., 2004). The staphylococcal genome is divided into core and accessory components. While the core genome covers about 75% of the whole genome, and refers to those genes present in all isolates (generally containing essential genetic information related to cellular metabolism and replication), the determinants for antibiotic resistance, immune evasion, and virulence are predominantly encoded in the accessory genome (remaining ~25%) which includes plasmids, bacteriophages, transposons, cassette chromosomes, and genetic islands (Turner *et al*., 2019). These mobile genetic elements have a vital role in bacterial adaptability, virulence, and survival (Turner *et al*., 2019).

S. aureus SG511 Berlin was already in use in the 1950s (Poehn, 1960), is susceptible to most clinically used antibiotics, and is used for antibiotic screening. Despite of the long-term use, little was known about the genetic background of this strain. Only in 2009, a loss-of-

function mutation in the *graS* kinase gene of *S. aureus* SG511 Berlin was identified and, consequently, a hypersusceptibility towards the antimicrobial peptides nisin, Pep5 and mersacidin (Sass & Bierbaum, 2009).

This study aimed at the identification of further factors which render *S. aureus* SG511 Berlin susceptible to antibiotics or even hypersusceptible towards some substances. Accordingly, the first chapter of this thesis describes the whole genome sequencing of *S. aureus* SG511 Berlin, its bioinformatic analyses, and interlinking with the generated antibiotic susceptibility profile in comparison to the related methicillin-resistant MRSA252 and the more distantly related methicillin-susceptible (MSSA) *S. aureus* HG001.

The 2.74 Mbp single-contig genome sequence of *S. aureus* SG511 Berlin was generated by long-read sequencing by GATC Biotech (Ebersberg, Germany), annotated, and compared with two other staphylococcal isolates, MRSA252 and *S. aureus* HG001. MRSA252 was included in the study because of the relatedness to *S. aureus* SG511 Berlin (Sass & Bierbaum, 2009). *S. aureus* HG001 was added to compare the sequences to another MSSA isolate. Obviously, the major difference between *S. aureus* SG511 Berlin and MRSA252 was the lack of the SCC*mec* cassette in the MSSA strain, but also other mobile elements and resistance determinants were rare in the genome of *S. aureus* SG511 Berlin, and a large deletion (~43 kb) comprising the important virulence factor SpA occurred in this strain. Susceptibility testing showed that *S. aureus* SG511 Berlin was indeed susceptible to most of the tested antibiotics of different classes and, in addition to that, on average 1 – 2 titer steps more susceptible towards cell wall and cell membrane-targeting agents than *S. aureus* HG001. In depth comparative genome analysis verified the loss-of-function *graS* mutation and a SNP in *pbp4* described before (Finan *et al*., 2001; Sass & Bierbaum, 2009). Further mutations were detected in the lysyltransferase gene *mprF*, the structural giant protein gene *ebh*, and the regulator genes *codY, gdpP, rnY, rsbV, saeR,* and *sarZ,* all of which might contribute to antibiotic susceptibility. In addition, an insertion element in the quorum sensing kinase gene *agrC* abolishes Agr-activity in *S. aureus* SG511 Berlin and a truncation of the FarE efflux pump leads to a sensitization towards the antimicrobial fatty acid linoleic acid.

By correlating genome sequence data to phenotypic antibiotic susceptibility, our study enables a better interpretation and understanding of test and screening results obtained with *S. aureus* SG511 Berlin. The interplay of multiple mutations affecting the cell envelope morphology together with the lack of mobile resistance genes may explain the hypersusceptibility of this strain towards most cell wall-targeting agents. Strikingly, the

accumulation of mutations in the regulatory *codY*, *gdpP*, *rnY*, *rsbV*, *saeR* and *sarZ* genes may interfere with the fine-tuned regulation system and contribute to the susceptibility of this strain towards numerous antibiotics.

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The hypersusceptible antibiotic screening strain *Staphylococcus aureus* SG511-Berlin harbors multiple mutations in regulatory genes

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ABSTRACT

The genetic plasticity of *Staphylococcus aureus* has facilitated the evolution of many virulent and drug-resistant strains. Here we present the sequence of the 2.74 Mbp genome of *S. aureus* SG511-Berlin, which is frequently used for antibiotic screening. Although *S. aureus* SG511 and the related methicillin-resistant *S. aureus* MRSA252 share a high similarity in their core genomes, indicated by an average nucleotide identity (ANI) of 99.83%, the accessory genomes of these strains differed, as nearly no mobile elements and resistance determinants were identified in the genome of *S. aureus* SG511. Susceptibility testing showed that *S. aureus* SG511 was susceptible to most of the tested antibiotics of different classes. Intriguingly, and in contrast to the standard laboratory strain *S. aureus* HG001, *S. aureus* SG511 was even hyper-susceptible towards cell wall and membrane targeting agents, with the exception of the MurA-inhibitor fosfomycin. In depth comparative genome analysis revealed that, in addition to the loss of function mutation in the antibiotic sensor histidine kinase gene *graS*, further mutations had occurred in the lysyltransferase gene *mprF*, the structural giant protein gene *ebh*, and the regulator genes *codY* and *saeR*, which might contribute to antibiotic susceptibility. In addition, an insertion element in *agrC* abolishes Agr-activity in *S. aureus* SG511, and the *spa* and *sarS* genes, which encode the surface protein SpA and its transcriptional regulator, were deleted. Thus, the lack of mobile resistance genes together with multiple mutations affecting cell envelope morphology may render *S. aureus* SG511 hyper-susceptible towards most cell wall targeting agents.

1. Introduction

Gram-positive bacteria, particularly Gram-positive cocci like coagulase-negative staphylococci, *Staphylococcus aureus*, and *Enterococcus* spp., are notorious pathogens in the hospital environment (Rice, 2006). Multidrug-resistant *S. aureus* is one of the leading causes of bacterial infections and mortality (Reddy et al., 2017). Although the number of serious infections due to resistant strains has decreased in recent years (Reddy et al., 2017), multidrug-resistant MRSA (methicillin-resistant *S. aureus*) remain a major healthcare issue (Hassoun et al., 2017). Therefore, a better understanding of the cellular mechanisms underlying susceptibility and resistance to clinically applied antibiotics remains a prerequisite in antibiotic research.

The antibiotic-susceptible strain *S. aureus* SG511-Berlin, which can be distinguished from *S. aureus* SG511-Hoechst (DSM 6247) and *S. aureus* SG511-Duesseldorf (Pöhn, 1960), has been extensively used in antibiotic screening and as a model strain in basic research on antibiotics (Henze and Berger-Bächi, 1995; Müller et al., 2018; Pag et al., 2008; Raafat et al., 2017; Sass et al., 2008; Wencewicz et al., 2013), as well as a control strain in routine tests (Pulverer and Grün, 1960). All three strains share a consistent spectrum of susceptibility to antibiotics. It is known that a loss of function mutation in *graS* renders *S. aureus* SG511-Berlin susceptible towards antimicrobial peptides (Sass and Bierbaum, 2009). Briefly, a nucleotide insertion mutation in *graS* leads to a premature stop codon, deleting the entire cytoplasmic part of the protein (Sass and Bierbaum, 2009). By controlling stress response and cell envelope structure, GraS acts as a resistance factor to counteract antimicrobial peptides (AMPs) (Falord et al., 2011; Herbert et al., 2007; Li et al., 2007; Sass and Bierbaum, 2009). The complementation of *graS* decreased the susceptibility of *S. aureus* SG511 towards the lantibiotics

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nisin, Pep5 and mersacidin, whereas the susceptibility to other cell wall active substances like vancomycin and bacitracin was hardly affected (Sass and Bierbaum, 2009).

Here, we report the complete genome sequence of *S. aureus* SG511-Berlin and its susceptibility to an array of antibiotics in comparison to *S. aureus* MRSA252 (Holden et al., 2004) as well as to *S. aureus* HG001, which is a *rsbU*-restored derivative of the laboratory strain *S. aureus* NCTC 8325 (Herbert et al., 2010). MRSA252 was isolated as a clinical *S. aureus* strain in 2004 (Holden et al., 2004), whilst the original *S. aureus* SG511 strain was distributed by the Hoechst company. However, the original SG511-Hoechst isolate is different from the SG511-Berlin strain, whose origin remains unclear (Pöhn, 1960). MLST (multilocus sequence typing) had demonstrated that *S. aureus* SG511 (ST30) and the clinical isolate MRSA252 (ST36) both belong to clonal complex 30 (Sass and Bierbaum, 2009). In this study, core genome MLST (Jünemann et al., 2013) revealed less than 400 SNPs between *S. aureus* SG511-Berlin and MRSA252, confirming the close relationship of both strains. In contrast, *S. aureus* HG001 is more distantly related to *S. aureus* SG511. By correlating genome sequence data to phenotypic antibiotic susceptibility, our study enables a better interpretation and understanding of test and screening results obtained with this widely used strain in antibiotic research.

2. Materials and methods

2.1. Phage typing

Phage typing was carried out with the international set of phages at routine test dilutions according to the subcommittee on phage typing of staphylococci of the International Association of Microbiological Societies.

2.2. Whole genome sequencing

Genomic DNA was isolated from wildtype *S. aureus* SG511-Berlin using the Master Pure™ Gram positive DNA Purification Kit (Epicentre Biotechnologies). All steps were performed according to the manufacturer's protocols, except for the addition of lysostaphin solution (30 ng/mL) during the lysis step. The genome of *S. aureus* SG511 was sequenced using long-read sequencing technology. As described previously, long-read sequences were obtained using the Pacific Biosciences RS II platform from a single molecule in real-time (SMRT) (Davies et al., 2015). Genome sequences were assembled using the SMRTpipe version v2.1.0 using the Hierarchical Genome Assembly Process (HGAP) and Quiver for post-assembly consensus validation. A single contig was achieved, with a mean coverage of 95.24%. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/, last access: 16.08.2021) (Tatusova et al., 2016).

2.3. Data availability

The genome sequence of *S. aureus* SG511-Berlin was deposited in NCBI GenBank under accession number CP076660.

2.4. Comparative genomics

Comparison of the genome sequence of *S. aureus* SG511-Berlin with the reference genomes of *S. aureus* HG001 (Herbert et al., 2010) and MRSA252 (Holden et al., 2004) was performed with Geneious R10.0.9 (https://www.geneious.com, last access: 16.08.2021). Potential lysogenic phages were identified with the PHASTER search tool (Arndt et al., 2016) (http://phaster.ca/, last access: 16.08.2021), potential pathogenicity islands were detected with the Island viewer tool (Bertelli et al., 2017) (http://www.pathogenomics.sfu.ca/islandviewer/upload/, last access: 16.08.2021), and potential resistance determinants were identified with the ResFinder tool (Bortolaia et al., 2020) (https://cge.cbs.dtu.dk/services/ResFinder/, last access: 16.08.2021) and the ARG-ANNOT database (Gupta et al., 2014) (https://ifr48.timone.univ-mrs.fr/blast/arg-annot_nt.html, last access: 16.08.2021). Additionally, single amino acid exchanges were compared with the data of Hyun and colleagues (Hyun et al., 2020) to identify known antimicrobial resistance mutations. Intrinsic resistance genes of *S. aureus* were identified by searching the AureoWiki database (Fuchs et al., 2018) (https://aureowiki.med.uni-greifswald.de/Main_Page, last access: 16.08.2021). The average nucleotide identity of the genomes was calculated with the ANI calculator (Rodriguez-R and Konstantinidis, 2014) (http://enve-omics.ce.gatech.edu/ani/, last access: 16.08.2021).

2.5. Bacterial strains, growth conditions, and antimicrobial susceptibility testing

Bacterial strains and plasmids are listed in Table 1. *S. aureus* strains were cultured in cation-adjusted Müller Hinton (MH) medium (Thermo Fisher, Dreieich, Germany) at 37 °C with aeration unless indicated otherwise. *S. aureus* strains harboring the pTX-vector, which comprises a xylose-inducible promoter, or its derivatives were grown in the presence of 12.5 µg/mL tetracycline, and 0.5% of xylose was routinely used to induce expression from the plasmid. Determination of minimal inhibitory concentrations (MICs) was performed in polystyrene round-bottom microtiter plates (Greiner, Frickenhausen, Germany) using cation-adjusted MH broth. An inoculum of 5×10^5 CFU/mL was employed in the arithmetic broth microdilution method. For MIC testing of vancomycin and daptomycin (Vancomycin CP LILLY®, Lilly GmbH, Bad Homburg, Germany; Cubicin®, Novartis Pharma GmbH, Nürnberg, Germany), $CaCl_2$ was added to all cultures to a final concentration of 1.25 mM (Müller et al., 2018) and 50 mg/L according to CLSI standards, respectively. The MIC was defined as the lowest concentration of the antibiotic that inhibited visible growth after 24 h incubation at 37 °C. Susceptibility testing of *S. aureus* strains SG511, HG001, HG003 and MRSA252 towards linoleic acid, vancomycin and teicoplanin was performed on BHI gradient agar. Material of several colonies was resuspended to an optical density at 600 nm wavelength of 0.125 in 0.9% NaCl and the gradient agar plates were inoculated using a cotton swab.

2.6. Biofilm assays

Biofilm assays were carried out in triplicates as previously described (Singh et al., 2012). Briefly, *S. aureus* cultures were grown in flat bottom microtiter plates in TSB containing 3% NaCl and 0.5% glucose at 37 °C for 24 h without shaking. Afterwards, wells were washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.6 mM KH_2PO_4, 10.1 mM Na_2HPO_4 x 2 H_2O, pH 7.4) and the adherent biofilm was fixed with 200 µL of ethanol and stained with crystal violet followed

Table 1
Bacterial strains used in this study.

Strain	Relevant characteristic (s)	Reference (s) / source
Staphylococcus aureus		
SG511-Berlin	Antibiotic-susceptible strain (CP076660, locus tag prefix KQU62_)	RKI Berlin, Germany
SG511 pTX16	*S. aureus* SG511 with derivative of pTX15 lacking the lipase gene downstream of *xylAR*	Sass and Bierbaum (2009)
SG511 pTX-*graS*	*S. aureus* SG511 with pTX15 derivative with *graS* under the control of *xylAR*	Sass and Bierbaum (2009)
MRSA252	UK hospital-acquired methicillin-resistant *S. aureus* strain (NC_002952, locus tag prefix SAR)	Holden et al. (2004)
HG001	*S. aureus* NCTC 8325-derivative; *rsbU* repaired; *tcaR*	Herbert et al. (2010)
HG003	*S. aureus* NCTC 8325-derivative; *rsbU* repaired; *tcaR* repaired	Herbert et al. (2010)

by three washes with PBS. The crystal violet stain was dissolved in absolute ethanol at room temperature for 10 min and the absorbance representing the relative cell densities of biofilms was measured at 595 nm using the Tecan Infinite M Plex multimode plate reader (Tecan Group Ltd.).

2.7. CAMP assay - assessment of δ-hemolysin activity

Delta-hemolytic activities of *S. aureus* strains SG511, MRSA252 and *S. aureus* HG001 were determined by cross-streaking test strains on Columbia blood agar perpendicularly to strain RN4220, which is a producer of β-hemolysin but does not produce α-hemolysin (Traber et al., 2008; Traber and Novick, 2006).

2.8. Microarray-based gene expression profiling

Detailed information on RNA extraction, CyDye-3 and CyDye-5 labeling, the microarray platform, and the hybridization procedure as well as the corresponding processed and raw microarray data of *S. aureus* strain SG511-Berlin versus strain SA137/93 A have been deposited in NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/, last access: 16.08.2021) under the GEO Series GSE12016 (Sass, 2009). Differential gene expression data connected to the loss of function mutation of the GraS kinase, that have been published before (Sass and Bierbaum, 2009), are marked as such.

3. Results and discussion

3.1. Characteristics of the S. aureus SG511-Berlin genome

In-depth knowledge on the molecular and regulatory principles underlying the susceptibility of pathogens towards antibiotic treatment remains a prerequisite in antibiotic research and new antibacterial development. *S. aureus* strain SG511-Berlin, subsequently referred to as *S. aureus* SG511, has been used as a standard strain in antibiotic susceptibility testing for decades, however, so far neither the genome sequence nor a comprehensive antibiotic susceptibility profile is available. Therefore, to close this gap, we sequenced the genome of this strain, using long-read sequencing technology and 150,292 polymerase reads with an average read length of 9,544 bp were assembled to a single contig. Because different *S. aureus* strains were called *S. aureus* SG511 (Pöhn, 1960), the identity of *S. aureus* SG511-Berlin was verified via phage typing. As expected, *S. aureus* SG511-Berlin only reacted with type I phages. Characteristic features of the genome, as presented in Table 2 and in Fig. 1, comprise an overall length of 2.74 Mbp with a GC ratio of 32.86% and 2,689 annotated open reading frames (ORFs), four genomic islands (νSaα, νSaβ, νSaγ, νSa4), two prophages and 22 IS elements. Sequencing data had previously shown that *S. aureus* SG511 (ST30) and MRSA252 (ST36) belong to clonal complex 30 (Sass and Bierbaum, 2009) together with *S. aureus* WKZ-1. ST36 harbors an exchange in the *pta* housekeeping gene and is a single locus variant of ST30, the predicted ancestor of the clonal complex 30, and, additionally, MRSA252 contains a SCC*mec* type II cassette (Enright et al., 2002). Sequence comparison of the core genome with the Ridom SeqSphere+ SERVER (version 8.0.1, cgMLST) (Jünemann et al., 2013) revealed only 398 SNPs between *S. aureus* SG511-Berlin and MRSA252. With a mean nucleotide substitution rate of 1.42×10^{-6} substitutions per site per year within CC30 (McAdam et al., 2012), a 2.74 Mbp genome and an estimated time span of 45 years between the isolation of MRSA252 and *S. aureus* SG511, a large number of SNPs can already be explained. Of note, *S. aureus* HG001, an *rsbU*-restored derivative of the susceptible standard laboratory strain *S. aureus* NCTC 8325 (ST8, CC8) (Berglund et al., 2005; Herbert et al., 2010), that is used for comparison of susceptibility profiles in this study, harbors 1,720 SNPs when compared to *S. aureus* SG511.

Table 2
Genomic features of *S. aureus* SG511.

Core genome	
Length	2,739,564 bp
GC content	32.86%
Protein coding sequences (ORFs)	2,689
– assigned to known functions	2,268
– assigned to unknown functions	343
– pseudogenes	88
– pseudogenes also found in MRSA252	52
16 S rRNA genes	5
23 S rRNA genes	5
5 S rRNA genes	6
tRNA genes	58
Genomic islands	4
νSaα	*set, lpl*
νSaβ	*hysA, spl, seg, sen, seu, sei, sem, seo*
νSaγ	*hlY, ssl*12, *ssl*13, *ssl*14, *psmβ1*
νSa4	*sel, sec3, tst*
Mobile elements	
Prophages	2
PT1028 (similar to SAPI4)	–
ΦSa3	*sak, scn, chp*
Plasmids	0
IS elements	22
– IS*3*	6
– IS*21*	1
– IS*30*	7
– IS*1182*	8

3.2. Mobile elements other than IS-elements are rare in the genome of S. aureus SG511

Determinants for antibiotic resistance and virulence are predominantly encoded in the accessory genome of *S. aureus*, which represents approximately 25% of the whole genome and includes plasmids, bacteriophages, transposons, cassette chromosomes and genetic islands (Lindsay and Holden, 2004). Two prophage sequences of 24.7 kb and 42 kb, respectively, were identified in the genome of *S. aureus* SG511. The first prophage sequence was similar to phage PT1028 (NC_007045, pairwise identity of 67.4%, 69,970–94,635 bp), but also shared similarity with the pathogenicity island SAPI4 found in MRSA252 (pairwise identity 72.9%).

Sequence comparisons also revealed the presence of a ΦSa3-like element, that is inserted into the hemolysin b gene of strain SG511 (KQU62_08740), which shows a pairwise identity of 86.7% to ΦSa3 of MRSA252 and encodes the genes for the staphylokinase (*sak*, KQU62_08470/ SAR2039), the complement inhibitor SCIN (*scn*, KQU62_08455/ SAR2035) and the chemotaxis inhibitory protein CHIPS (*chp*, KQU62_08460/ SAR2036). However, enterotoxin A (SAR2043) located on ΦSa3 of MRSA252 (Holden et al., 2004) is not present in SG511.

The three genomic islands νSaα, νSaβ and νSaγ (Fig. 1 and Table 2) were identified in both strains *S. aureus* SG511 and MRSA252 with pairwise identities of 90.4%, 99.9% and 100% respectively. νSaα harbors several staphylococcal exotoxin (*set*) genes (Williams et al., 2000) and lipoproteins (Baba et al., 2002), νSaβ includes *hysA*, encoding a hyaluronate lyase, several *spl* genes, encoding putative serine proteases (Kuroda et al., 2001), and the minor enterotoxins in both strains (*seg, sen, seu, sei, sem, seo*), and νSaγ contains, *hlY* (KQU62_04195), which encodes the alpha hemolysin, the staphylococcal superantigen like genes *ssl*12, *ssl*13, *ssl*14, and one phenol soluble modulin *psmβ*1 (Table 2). Additionally, the genomic island νSa4 was identified in *S. aureus* SG511, which was absent from MRSA252. νSa4 from *S. aureus* SG511 showed similarity to the νSa4 genomic island found in *S. aureus* N315 (pairwise identity of 73.2%) and high similarity to a genomic island in *S. aureus* AR_0471 (CP029652, pairwise identity of 99.4%), encoding enterotoxin L (*sel*), enterotoxin C (*sec3*) and the toxic shock syndrome toxin (*tst*) (Baba et al. 2002).

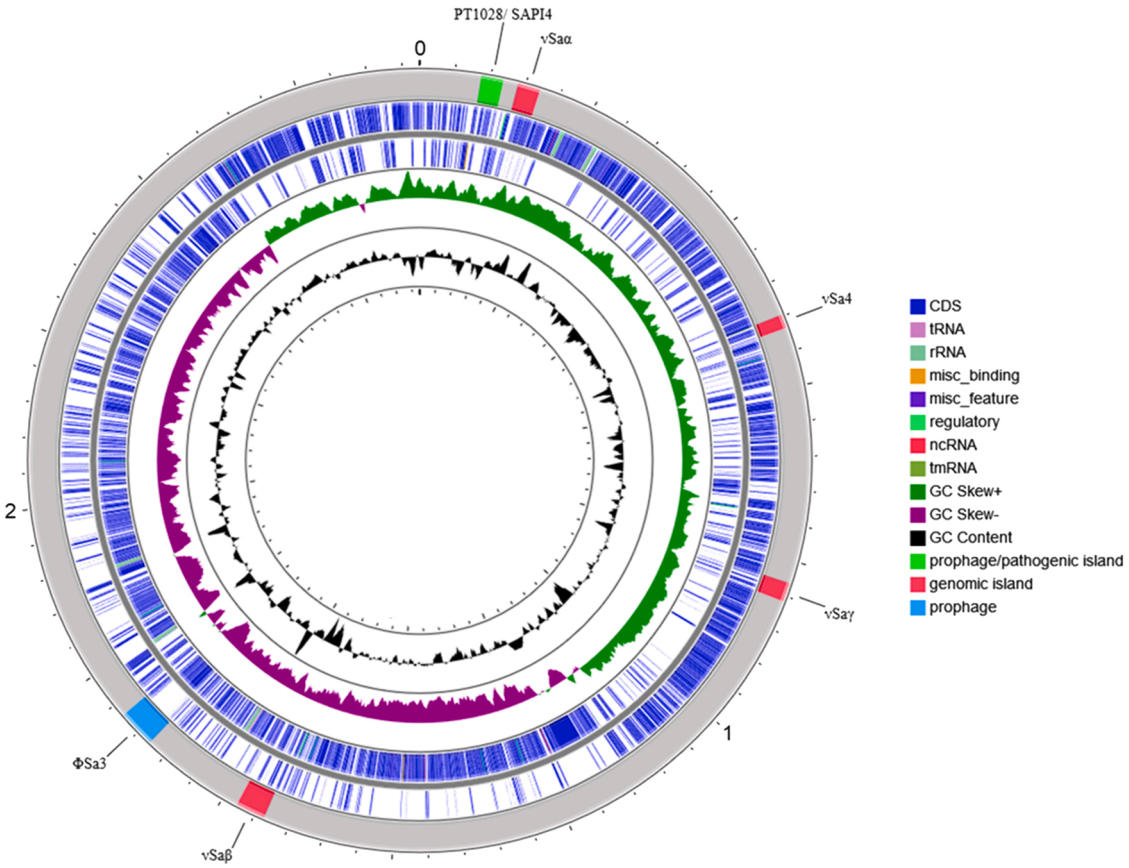

Fig. 1. Schematic circular diagram of the *S. aureus* SG511 genome. The schematic diagram was created on the CGView Server (http://cgview.ca/, last access: 19.10.2021). Where appropriate, categories are shown as pairs of concentric circles representing both coding strands. The outer colored segments on the gray outer ring represent genomic islands and horizontally acquired DNA. The rings from outside to inside represent scale in Mbp, annotated CDS (blue) (colored according to predicted function), tRNA (pink) and rRNA (green); percentage of GC content (black), and GC deviation (> 0%, green; < 0%, purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. IS element insertions and a recombination lead to the deletion and low expression of virulence factors in S. aureus SG511

Three coding reading frames are disrupted by insertion elements; the second copy of the oligoendopeptidase F-coding *pepF* gene (KQU62_05400), the *opp-4A* gene (KQU62_03275), which encodes a less conserved oligopeptide ABC transporter substrate-binding protein that is also disrupted in MRSA252 (Aurelia Hiron et al., 2007), and, probably most important, the *agrC* gene (KQU62_08830). The accessory gene regulator (*agr*) is the master quorum sensing system of *S. aureus*, and regulates the expression of most virulence factors and genes involved in biofilm formation (Kavanaugh and Horswill, 2016). MRSA252 and SG511 belong to *agr* type III, which are medium biofilm builders (Cafiso et al., 2007). *S. aureus* HG001 belongs to *agr*-I; this group includes weak and medium biofilm builders (Cafiso et al., 2007). In strain SG511, however, the insertion of an IS*1182*-like element, encoding an IS*Sau3* family transposase, in the 5' end region of *agrC* truncates the regulator protein. Moreover, strain SG511 showed a significantly decreased δ-hemolysis and biofilm formation compared to the control strains MRSA252 and HG001 (Fig. 2). To further address this aspect, we re-evaluated our data on differential gene expression of strain SG511 versus *S. aureus* SA137/93 A, a vancomycin intermediate- and methicillin-resistant *S. aureus* strain (Reipert et al., 2003), that had been obtained in one of our previous studies (Sass and Bierbaum, 2009). Here,

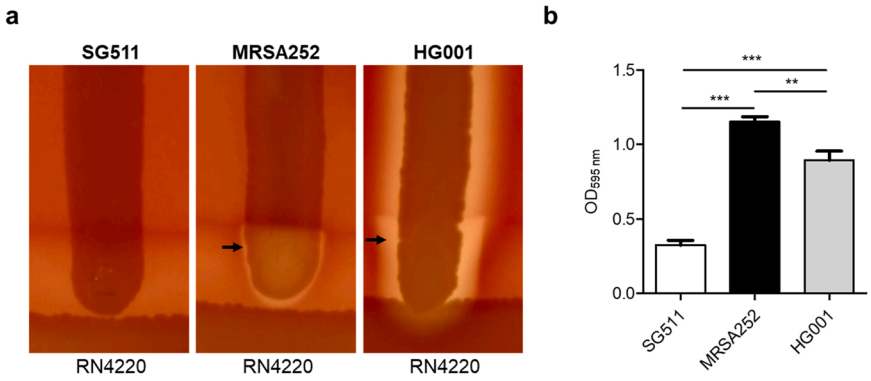

Fig. 2. Due to the insertion of IS*1182* into *agrC*, *S. aureus* SG511 is Agr-negative. (a) Hemolytic activities determined by cross streaking perpendicularly to RN4220, which produces only β-hemolysin, thereby enhancing lysis by δ-hemolysin but inhibiting lysis by α-hemolysin. Black arrow marks the area of the synergistical effect of β- and δ-hemolysin of *S. aureus* RN4220 and MRSA252 or HG001, respectively. (b) Biofilm formation in microtiter plates. Absorbance representing the relative cell densities of biofilms was measured at 595 nm; ** $p < 0.0017$, *** $p < 0.0001$.

inactivation of the Agr system in *S. aureus* SG511 is further corroborated by the low expression of *agrC* (Sass and Bierbaum, 2009) (0.08-fold) in SG511 compared to SA137/93 A. Additionally, the previously observed increased level of *rot*-expression (4.32-fold; Sass and Bierbaum, 2009) can now be explained by the inactivation of *agrC*, as *agr*, via RNAIII, is a negative regulator of *rot* (Geisinger et al., 2006; Novick, 2003). Similar insertions into *agrC* have been also described for IS*256* (Botelho et al., 2016; Vuong et al., 2004), indicating that *agrC* may represent a hotspot for the insertion of mobile elements, as seen for *S. epidermidis* populations (Both et al., 2021). However, although the Agr system regulates the expression of virulence factors, about 10.9% of all clinical isolates appear *agr* negative in mass spectrometry (Josten et al., 2014; Traber et al., 2008).

S. aureus SG511 lacks the insertion of the SCC*mec* type IV cassette as well as its flanking regions otherwise found in MRSA252. Quite the contrary, in comparison to *S. aureus* HG001, a 43 kb deletion encompasses the region between the *adsA* gene (KQU62_12435) and the iron ABC transporter permease encoding *sirC* gene (KQU62_12445). Thus, the genome of *S. aureus* SG511 has lost a total of 34 genes in this region, including the multidrug efflux system encoding genes: SAR0094 (uncharacterized transporter subunit) and SAR0109 (*norC*). The efflux pump NorC is involved in low-level fluoroquinolone resistance, though wild-type expression levels are not sufficient to affect susceptibility towards these antibiotics (Truong-Bolduc et al., 2006). Thus, an influence of the *norC* deletion in strain SG511 may not significantly affect the susceptibility profile of strain SG511 compared to other *S. aureus* strains expressing NorC at wild-type level, such as strain HG001 (Mäder et al., 2016). Furthermore, the deletion of the region between *adsA* and *sirC* comprises *spa* (SAR0114) and *sarS* (SAR0115), the key regulator in the complex regulatory network of *spa* expression (Cheung et al., 2001; Tegmark et al., 2000). Instead, an IS*30*-element is localized between *adsA* and *sirC*, and a recombination of two IS*30* elements may have occurred, as previously observed for other *S. aureus* strains (McGavin et al., 2012; Ohtsubo and Sekine, 1996). Of note, our microarray data also verified the missing expression of these genes. Although protein A is an important virulence factor, *spa*-deficient strains are also found in clinical isolates, and are still able to cause invasive disease, because the redundancy of *S. aureus* virulence factors may conceal the lack of protein A (Baum et al., 2009).

3.4. Acquired resistance genes

Mobile elements other than insertion elements, like plasmids and exogenously acquired resistance genes were not found in *S. aureus* SG511. However, the chromosome of SG511 encodes a putative fosfomycin resistance gene (*fosB*) which is also present in MRSA252 (Holden et al., 2004), a gene that is annotated only in 67% of all sequenced isolates included in the AureoWiki database (Fuchs et al., 2018). In contrast, MRSA252 contains multiple acquired antibiotic resistance determinants, most strikingly the 58.8 kb SCC*mec* type IV cassette with an integrated copy of pUB110 with bleomycin and kanamycin resistance genes, two copies of Tn*554* with erythromycin and spectinomycin resistance genes, and a Tn*552* transposon encoding the BlaI, BlaR and BlaZ components of the inducible *S. aureus* β-lactamase system (Holden et al., 2004). Hence, while *S. aureus* MRSA252 encodes a large repertoire of antibiotic resistance genes rendering this strain resistant towards a considerable number of clinically important antibiotics, *S. aureus* SG511 mostly lacks equivalent resistance determinants, which should also be reflected by its antibiotic susceptibility profile. *S. aureus* SG511-Berlin was already in use in the 1950s (Pöhn, 1960), therefore, it is not clear, whether the lack of resistance determinants is due to the early isolation of this strain (in comparison, MRSA252 was isolated more than 45 years later), before introduction of numerous antibiotic classes to the clinics, or due to the fact, that *S. aureus* SG511 was not clinically associated.

3.5. S. aureus SG511 is susceptible towards protein biosynthesis inhibitors, DNA gyrase inhibitors and antibacterial fatty acids

In order to evaluate the phenotypic antibiotic susceptibility profile of *S. aureus* SG511, we compared its susceptibility towards a range of antibiotics to that of strain MRSA252 as well as of the laboratory methicillin-susceptible *S. aureus* (MSSA) standard strain HG001 (ANI of 97.57%). The antibiotic susceptibility profiles of all three strains are shown in Table 3 and amino acid exchanges unique for *S. aureus* SG511, which potentially correspond to the results of susceptibility profiles, are summed up in Table 4. *S. aureus* SG511 and HG001 were equally susceptible towards the protein biosynthesis inhibitors erythromycin and clindamycin, and comparably susceptible towards spectinomycin, and no resistance determinants for these antibiotics were detected in their genomes. However, the presence of the intrinsic resistance determinants, *aph-Stph* (KQU62_07260; aminoglycoside-resistance (Han et al., 2017)), *emrB* (KQU62_10475; ampicillin and heavy metals (Zhang et al., 2016)), and *tet38* (tetracycline (TET) and fosfomycin (FOS)-resistance when overproduced (Truong-Bolduc et al., 2005, 2018)) was confirmed for all three strains (Table S1). As all three strains were susceptible towards tetracycline and gentamicin, the latter representing the only tested aminoglycoside not affected by other acquired resistance determinants in MRSA252, these genes were probably not upregulated in all three strains and, therefore, did not confer clinical resistance. According to our microarray data, the expression of *tet38* was even decreased in *S. aureus* SG511 compared to *S. aureus* SA137/93 A (Table S2). All strains were susceptible towards the oxazolidinone antibiotic linezolid, although an uncommon amino acid exchange in RplC (A98V) in *S. aureus* SG511 might have affected the MIC towards this substance. However, the *rplC* mutation is not localized in the peptidyl transferase center (PTC) on the ribosome, which is the binding site of this antibiotic (Locke et al., 2009; Long and Vester, 2012; Miller et al., 2008).

In addition, the susceptibility towards two different classes of DNA gyrase inhibitors was tested. Here, *S. aureus* SG511 and HG001 were highly susceptible towards the fluoroquinolones ciprofloxacin and norfloxacin as well as the aminocoumarin novobiocin, with *S. aureus* HG001 being most susceptible. In contrast, MRSA252 was resistant towards ciprofloxacin and norfloxacin, known to be due to an amino acid substitution in the subunit A of the DNA gyrase (S84L) (Hyun et al., 2020), however, MRSA252 was highly susceptible to novobiocin. All three *S. aureus* strains contain several efflux pumps that can confer resistance to quinolones and other antibiotic compounds when upregulated (Table S2). Fifteen drug transporters were identified in all three genomes, however, the *lmrS* gene was disrupted in MRSA252 and the *norC* gene was deleted in *S. aureus* SG511 (Table S2). Identical nucleotide exchanges were found for the transporter-coding genes *mdeA, mepA, msrA1, msrA3, norA, emrB*, and *sdrM* in the genome of MRSA252 and SG511, but not in HG001. Notably, the expression of *norA* and *sdrM* was very low in *S. aureus* SG511 compared to strain SA137/93 A (Sass and Bierbaum, 2009) (Table S2). Also, unique nucleotide exchanges were identified in *norB* and *SAV1866* in the genome of *S. aureus* SG511.

S. aureus SG511 harbors a shortened version of the multidrug efflux pump FarE, which lacks amino acids 751–777 compared to its homolog in MRSA252. FarE, the effector of fatty acid resistance, is regulated by the FarR regulator, and belongs to the RND family of multidrug efflux pumps (Alnaseri et al., 2015). FarE promotes efflux of antimicrobial fatty acids like linoleic acid (Alnaseri et al., 2015, 2019). As demonstrated by agar diffusion assays (Fig. 3), *S. aureus* SG511 is hyper-susceptible towards linoleic acid when compared with MRSA252 and HG001, which supports the idea of a dysfunctional FarE transporter in *S. aureus* SG511.

Regarding inhibitors of folic acid synthesis, *S. aureus* SG511, the related MRSA252 and *S. aureus* HG001 were susceptible towards sulfadiazine and sulfamethoxazole, according to CLSI standards (MIC $\leq$ 256 μg/mL), with SG511 being 1–2 titer steps more susceptible. Sulfadiazine and sulfamethoxazole are competitive inhibitors of the

Table 3
MIC determinations of *S. aureus* strains SG511, MRSA252, HG001, as well as the *graS* complemented *S. aureus* SG511 and the corresponding pTX16 control strain (μg/mL).

Target	Antibiotic	SG511	MRSA252	HG001	SG511 pTX16	SG511 pTX*graS*
Protein	AMK	0.5	16	4	n.d.	n.d.
	CHL	4	4	2	n.d.	n.d.
	CLI	0.125	1024	0.125	n.d.	n.d.
	ERY	0.5	> 4096	0.5	n.d.	n.d.
	FA	0.0625	0.0625	0.03125	n.d.	n.d.
	GEN	0.125	0.375	0.75	0.0625	0.125
	KAN	1	128	4	0,5	1
	LZD	3	6	4	n.d.	n.d.
	SPT	64	> 4096	32	n.d.	n.d.
	TET	0.5	0.5	0.125	n.d.	n.d.
DNA/RNA	CIP	1	32–64	0.5	0.5	0.5
	NOR	1	32	0.75	n.d.	n.d.
	NOV	0.25	0.125	0.0625	n.d.	n.d.
	RIF	0.0039	0.0039	0.0078	n.d.	n.d.
Folic acid	SD	32	64–128	128	n.d.	n.d.
	SMZ	16	32	32	n.d.	n.d.
Cell wall	AMP	0.5	512	1	1	1
	BAC	2	16	8	2	2
	CTX	1	1024	2	1	2
	FOS	64–128	32	32	n.d.	n.d.
	LYS	0.03125–0.0625	0.0078	0.0625	0.25	1
	LYZ	2048	> 4096	> 4096	2048	> 4096
	OXA	0.25	512	0.25	n.d.	n.d.
	PEN	0.0625	512	0.125	n.d.	n.d.
	TEC	0.125	1	2	0.0625	0.125
	VAN	0.5	1	2	0.5	1
Membrane	BENZ	1	16	2	n.d.	n.d.
	DAP	0.125	0.25	0.5	0.0625	0.125
	PMB	32	256	256	16	64

Abbreviations: AMK, amikacin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FA, fusidic acid; GEN, gentamicin; KAN, kanamycin; LZD, linezolid; SPT, spectinomycin; TET, tetracycline; CIP, ciprofloxacin; NOR, norfloxacin; NOV, novobiocin; RIF, rifampicin; SD, sulfadiazine; SMZ, sulfamethoxazole; AMP, ampicillin; BAC, bacitracin; CTX, cefotaxime; FOS, fosfomycin; LYS, lysostaphin; LYZ, lysozyme; OXA, oxacillin; PEN, penicillin G; TEC, teicoplanin; VAN, vancomycin; BENZ, benzalkonium chloride; DAP, daptomycin; PMB, polymyxin B; n.d., not determined.

Table 4
Mutations with possible influence on resistance phenotype identified in the genome of *S. aureus* SG511.

Gene	Function	Mutation/amino acid exchange	Impact	Possible influence on susceptibility	Relative transcript level*
agrC	autoinducer sensor AgrC	Insertion IS*1182*	loss of function	biofilm formation	0.08
codY	global regulator	E252G	unknown	unknown	0.85
ebh	(extracellular matrix)-binding protein homolog	frameshift; stop AA 8,985	disruption of C-terminal end	OXA, TEC	1.12
farE	Multidrug efflux pump	Δ751–771	deletion of 27 AA	Linoleic acid	5.64
fosB	bacillithiol-S-transferase	R23K	unknown	FOS	0.72
gdpP	c-di-AMP degradation	SNP; R602C	important for substrate interaction	OXA	1.21
graS	Sensor histidine kinase	frameshift; pre-mature stop at position 64	loss of function	Antimicrobial peptides	0.36
mprF	Multiple Peptide Resistance Factor	SNP; F657I	synthase domain	VAN, DAP, GEN	0.40
pbp4	penicillin-binding protein 4	G375R	probably no impact since over-expression confers PEN-resistance	PEN	0.99
rny	ribonuclease Y	I490V	not in functional domain	unknown	1.66
rplC	50 S ribosomal protein L3	A98V	binding site of tRNA	linezolid, pleuromutilin	1.11
rsbV	regulator of SigB	G59V	2 AA from phosphorylation site	Cell wall active substances	not significant
saeR	TCS, regulator of virulence genes	L108I, R154C	loop region, arrangement of domains	β-lactams, CLI	1.16
sarZ	global regulator	Frameshift, 3 AA extension	dimerization domain	VAN, CHL	0.71
tcaR	transcriptional regulator	M1I	unknown	VAN, TEC, OXA	0.43

*Transcript levels of *S. aureus* SG511-Berlin compared to *S. aureus* SA137/93A indicated as mean of the 'median of ratios'. Data in part previously published in Sass and Bierbaum (2009).
Abbreviations: OXA, oxacillin; TEC, teicoplanin; FOS, fosfomycin; VAN, vancomycin; DAP, daptomycin; GEN, gentamicin; PEN, penicillin G; CLI, clindamycin; CHL, chloramphenicol.

dihydropteroate synthase (DHPS), encoded by *folP*, which is part of the folate metabolism (Brown, 1962). We found 11 amino acid exchanges in the DHPS from *S. aureus* SG511, which were also present in MRSA252, when compared with *S. aureus* HG001 *folP*. Although the structure and function of the *S. aureus* DHPS have been elucidated, the role of individual residues remains unclear (Hampele et al., 1997).

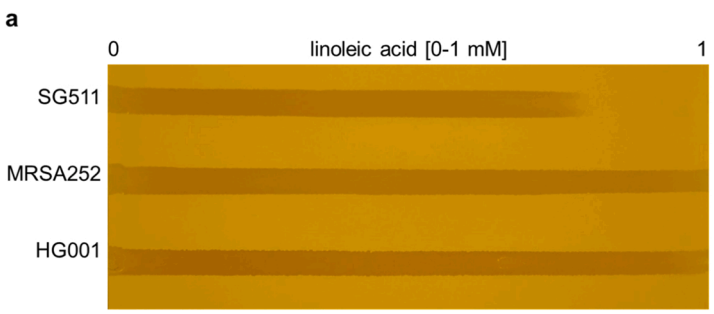

Fig. 3. *S. aureus* SG511 is highly susceptible towards linoleic acid. (a) Gradient plate with increasing concentration of linoleic acid. Concentrations up to 1 mM did not inhibit growth of *S. aureus* HG001 and MRSA252, though *S. aureus* SG511 was inhibited. (b) Agar diffusion test with 1 mg linoleic acid loaded on the filter disk, from top to bottom: *S. aureus* SG511, MRSA252, and HG001. Growth of *S. aureus* MRSA252 and HG001 and was hardly affected by linoleic acid, whilst *S. aureus* SG511 shows a clear inhibition halo around the filter disk.

3.6. S. aureus SG511 is hyper-susceptible to the majority of cell wall and cell membrane targeting substances

Although *S. aureus* SG511 had already shown a rather susceptible profile in MIC tests as described above, the effect of cell wall and cell membrane targeting antibiotics was considerably more pronounced. On average, *S. aureus* SG511 was 1–2 titer steps more susceptible towards cell wall and cell membrane targeting agents than *S. aureus* HG001 (Table 3). A loss of function mutation in *graS* disrupts the function of the GraS kinase in *S. aureus* SG511 and renders this strain highly susceptible towards antimicrobial peptides, including nisin, Pep5 and mersacidin (Sass and Bierbaum, 2009). In addition, our present data shows that *S. aureus* SG511 was eightfold more susceptible towards the polypeptide polymyxin B (PMB) than MRSA252 and HG001, and resistance was nearly completely restored by complementation with *graS* from MRSA252 (Table 3). This is likely due to the restored expression of *graR, vraG* (Herbert et al., 2007; Meehl et al., 2007; Vestergaard et al., 2017) and of the *dlt* operon that confers the incorporation of positively charged D-alanine residues into the teichoic acids (Peschel et al., 1999; Sass and Bierbaum, 2009). Similar effects were observed with the cell wall targeting metallo-endopeptidase lysostaphin and the muramidase lysozyme after complementation with *graS* (Table 3). On the one hand, lysozyme acts as a muramidase, which hydrolyses bonds within the peptidoglycan (PGN)-network, and *S. aureus* protects itself by *O*-acetylation of the C6-position of *N*-acetyl muramic acid by OatA (Bera et al., 2005). On the other hand, lysozyme (pI 11.0) also is a cationic antimicrobial peptide (CAMP), which is sensed by GraS and stimulates esterification of the wall teichoic acids by Dlt, which inhibits binding of lysozyme to the cell envelope (Herbert et al., 2007). Optimal binding and highest hydrolytic activity can be displayed by lysozyme only if both modifications are missing (Bera et al., 2007). Since mature lysostaphin has a basic pI of 10.4, it can be assumed that here as well the missing GraS activity will sensitize *S. aureus* SG511 to lysostaphin (Wadstrom and Vesterberg, 1971). It was recently shown, that the interaction between the extra-cytoplasmatic loop (EL) of the VraG membrane permease and the GraS kinase, inhibits the sensing of GraS (Cho et al., 2021). The lysine residues within the EL were important for this interaction. However, only a single amino acid exchange was found in *S. aureus* SG511 VraG (L313S) in comparison to MRSA252.

On the other hand, only minor or no alterations were observed for the susceptibility of *S. aureus* SG511 towards ampicillin, cefotaxime, teicoplanin, vancomycin, daptomycin, and the protein biosynthesis inhibitors gentamicin and kanamycin, when *graS* was complemented (Table 3). Although GraSR positively regulates the expression of the two ABC transporters VraDE and VraFG, that function in bacitracin efflux (Yoshida et al., 2011), the complementation of *S. aureus* SG511 with a functional GraS did not alter the susceptibility towards this substance (Table 3). The low influence of *graS* reconstitution regarding the vancomycin MIC was also observed before (Sass and Bierbaum, 2009).

The Multiple Peptide Resistance Factor gene *mprF* is also regulated by GraSR (Nishi et al., 2004) and, consequently, *mprF* is expressed at a lower level in *S. aureus* SG511 compared to SA137/93 A (Sass and Bierbaum, 2009) (Table 4). We further identified an F657I amino acid substitution in the MprF protein of strain SG511 that is located in the C-terminal synthase domain, which was not described as mutational 'hot spot' before. Although added effects cannot be excluded, the roles of the decreased expression of MprF and the amino acid substitution are hard to distinguish in *S. aureus* SG511.

As a further cell wall associated variation, a frameshift-mutation disrupts the *ebh*-gene, which encodes the ECM (extracellular matrix)-binding protein homolog, and deletes the lysine and arginine-rich C-terminal end as well as the cell wall spanning region of the 1.1 megadalton protein. This giant protein is thought to span the peptidoglycan layer and the plasma membrane and by that to act as a cytoskeletal element (Cheng et al., 2014) which maintains the structural homeostasis of the cell (Kuroda et al., 2008). Truncation mutations in *ebh* were often found in strains that had been propagated for years in the laboratory prior to genome sequencing, like in *S. aureus* Newman, which also encodes a C-terminally truncated Ebh (Cheng et al., 2014). Mutations that disrupt the *ebh* reading frame are associated with increased oxacillin and teicoplanin susceptibility (Cheng et al., 2014; Kuroda et al., 2008), which might explain the 16-times lower teicoplanin-MIC of *S. aureus* SG511 in comparison to HG001.

The MarR-like transcriptional regulator TcaR has been postulated to be a regulator of virulence determinants in *S. aureus*, more precisely a regulator of the surface-associated protein gene *sasF* and the *spa*-regulating gene *sarS* (McCallum et al., 2004). Deletion of the *tcaRAB* operon raised teicoplanin- and vancomycin-MICs (Brandenberger et al., 2000). However, this outcome was mainly attributed to the absence of *tcaA* (Maki et al., 2004). The non-canonical start-codon (ATA) of the TcaR^{M1I} regulator observed in strain SG511 might lower expression levels of *tcaR* (Belin et al., 1979; Cheung et al., 2014; Köpke and Leggatt, 1991), but should not affect the susceptibility to vancomycin and teicoplanin, since *S. aureus* HG001, which harbors a pre-mature stop codon in *tcaR*, and HG003, in which this pre-mature stop codon was repaired (Herbert et al., 2010), did not differ in their susceptibility towards teicoplanin or vancomycin (Fig. 4). In addition, the difference in the teicoplanin MIC between *S. aureus* HG001 and SG511 was smaller in the agar diffusion assay than in the broth dilution (compare Table 3 and Fig. 4). This systematic trend of lower glycopeptide MICs in broth dilution in comparison to other methods complicates the detection of glycopeptide intermediate resistant strains (Vaudaux et al., 2010).

S. aureus SG511 was susceptible towards all tested cell wall and membrane targeting agents, with the exception of fosfomycin. FosB is a Mn^{2+}-dependent bacillithiol-S-transferase which inactivates the epoxide antibiotic fosfomycin (Roberts et al., 2013; Thompson et al., 2014). For this process, the FosB-substrate bacillithiol (BSH) is needed, which in turn is made by BshA, BshB2 and BshC (Posada et al., 2014). Of note, BSH-production is disrupted in *S. aureus* strains of the NCTC 8325-lineage, because of an insertion mutation in the *bshC* gene (Pöther et al., 2013), which includes *S. aureus* HG001 and renders this strain fosfomycin-susceptible, regardless of the *fosB* gene. We identified an amino acid substitution at position 23 of the amino acid sequence of FosB in *S. aureus* SG511, resulting in FosB^{R23K}, and determined an

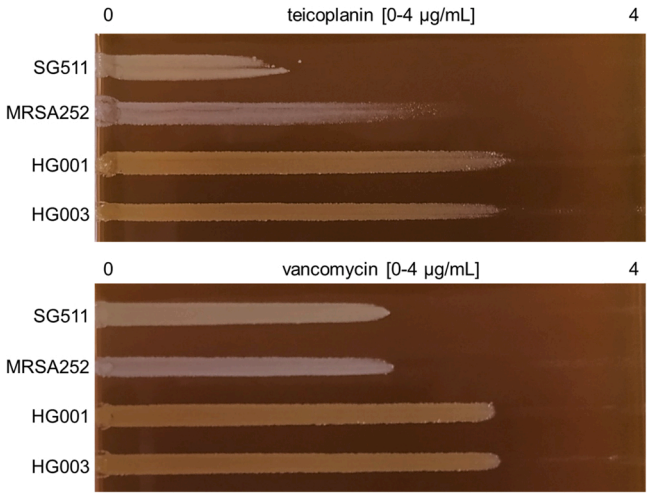

Fig. 4. Gradient plate with increasing concentrations of teicoplanin (top) or vancomycin and 1.25 mM $CaCl_2$ (bottom). *S. aureus* strains SG511 and MRSA252 showed similar susceptibility to vancomycin, but SG511 was clearly more susceptible to teicoplanin than MRSA252. *S. aureus* HG001 was least susceptible towards both antibiotics. *S. aureus* HG001 harbors a pre-mature stop codon in the *tcaR* gene (Herbert et al., 2010). However, repair of the *tcaR* gene in *S. aureus* HG003 did not alter the susceptibility towards teicoplanin or vancomycin compared to the parent strain HG001.

elevated fosfomycin MIC (1- to 2-fold), in comparison to MRSA252 and *S. aureus* HG001, suggesting a role of the R23K substitution in fosfomycin resistance of *S. aureus* SG511. However, *S. aureus* strains with different genetic backgrounds reached much higher fosfomycin-MICs (312.5–2500 µg/mL), which were reduced 16- to 60-fold when *fosB* was deleted (Posada et al., 2014). Thus, the R23K substitution in SG511-FosB plays a rather minor role in fosfomycin resistance.

A mutation in the penicillin binding protein 4 in *S. aureus* SG511 ($PBP4^{G375R}$) has previously been noticed (Finan et al., 2001) and is localized in the C-terminal domain of the protein, which is thought to play a role in determining the preference for transpeptidase or carboxypeptidase activity (Welsh et al., 2017). As β-lactam and cephalosporin-resistance are mediated by PBP2a and PBP4 in MRSA (Alexander et al., 2018), this mutation might affect the susceptibility of *S. aureus* SG511 towards these antibiotic classes. However, Henze and Berger-Bächi (1996) selected a penicillin-resistant mutant of *S. aureus* SG511 which had maintained the mutation in *pbp4* and had simply increased its PBP4 expression level by a mutation in the promoter region upstream of *pbp4-abcA*. Together with the fact that *pbp4* deletion sensitized *S. aureus* SG511 for ampicillin, cefoxitin, and methicillin in their study, this supports the idea that the SG511 PBP4 variant is at least partially functional.

3.7. Multiple mutations in global regulators

Further analysis also revealed amino acid exchanges in six global regulators of *S. aureus* SG511, namely $GdpP^{R602H}$, $RsbV^{G59V}$, $CodY^{E252G}$, $SaeR^{L108I/R154C}$, Rny^{I490V}, and 9 amino acid exchanges at the C-terminal end of SarZ. GdpP (GGDEF domain protein containing phosphodiesterase) controls the level of the nucleotide messenger c-di-AMP, which is essential for survival under standard laboratory conditions (Moscoso et al., 2016). The mutation ($GdpP^{R602H}$) is localized in the DHH DHHA1 domain, which is largely responsible for the binding of the substrate c-di-AMP (Corrigan et al., 2011; Galperin et al., 2010; Rao et al., 2010) and R602 directly interacts with the phosphate group of the nucleotide (Corrigan and Gründling, 2013). Interestingly, a $GdpP^{R602H}$ was previously described to convert a heterogeneous MRSA into a homogeneously resistant strain by increasing the expression of PBP2a (Pozzi et al., 2012), but PBP2a is not encoded in the genome of *S. aureus* SG511.

We also found an amino acid substitution in close proximity of the phosphorylation site of RsbV, namely G59V, in *S. aureus* SG511. It remains elusive, whether $RsbV^{G59V}$ behaves differently in the SigB-regulation system, but 60% of the predicted SigB-regulon (Pané-Farré et al., 2006) were down-regulated in *S. aureus* SG511 when compared with SA137/93 A. The SigB-system largely contributes to cell wall properties, which determine the susceptibility to various cell wall active substances, as SigB-depletion sensitizes *S. aureus* for these substances (Morikawa et al., 2001).

Another amino acid exchange was found in the global transcriptional regulator CodY of *S. aureus* SG511, $CodY^{E252G}$. So far, only R61 and G129 were reported to be important for the activity of *S. aureus*-CodY, but the GTP-binding domain has not been localized yet (Waters et al., 2016), which impedes the prediction of the effect of the E252G amino acid substitution in *S. aureus* SG511. CodY acts as a master regulator of pathogenesis by tying nutrient availability to virulence gene expression, e.g., hemolysis and biofilm formation (Mlynek et al., 2018), affecting amino acid biosynthesis, transport of macromolecules, and virulence (Majerczyk et al., 2010).

CodY also regulates the expression of *saeR* (Majerczyk et al., 2010), and SG511-SaeR harbors two amino acid exchanges in comparison to MRSA252-SaeR. These two amino acid exchanges were both localized in the C-terminal domain of SaeR, $SaeR^{L108I/R154C}$, representing a loop region of the protein which plays an important role in DNA substrate binding and/or in SaeR domain arrangement (Liu et al., 2016). Although SaeSR mainly controls the expression of virulence factors (Liu et al., 2016) and the deletion of the TCS in *S. aureus* N315 did not alter MICs, *saeSR* transcription was upregulated by β-lactam treatment and down-regulated by low concentrations of the protein biosynthesis inhibitor clindamycin (Kuroda et al., 2007). Thus, secondary effects on the antibiotic susceptibility profile of *S. aureus* SG511 cannot be ruled out.

In addition, ribonuclease Y (Rny^{I490V}) was mutated in *S. aureus* SG511. Rny is a membrane-associated part of the degradosome (Koch et al., 2017), which controls the stability of specific mRNAs (Marincola et al., 2012). Regulatory effects of Rny include genes involved in pathogenicity, proteolysis, transport, and metabolic processes (Marincola et al., 2012). The amino acid substitution in SG511-Rny is localized at the very C-terminal end of the protein, outside of the known functional domains. Thus, the impact of this amino acid substitution on antibiotic susceptibility of strain SG511 remains elusive.

Further, a 4 bp deletion in the *sarZ* gene leads to a frameshift mutation at amino acid position 137, resulting in the mutation of the last eleven C-terminal amino acids and an extension by 3 amino acids of the protein in *S. aureus* SG511. SarZ belongs to the Sar family of transcriptional regulators (Cheung and Zhang, 2002) and is a redox active global regulator controlling metabolic switching, antibiotic resistance, peroxide stress defense, virulence, and cell wall properties (Kaito et al., 2006; Poor et al., 2009). The frameshift-derived mutations found in SG511-SarZ are localized in the α-helix six of the regulator and might affect the dimerization capacity of the protein (Poor et al., 2009). Because of the influence of SarZ on multiple cell wall properties (Kaito et al., 2006), the frameshift mutation in this gene might well contribute to the high susceptibility of *S. aureus* SG511 towards vancomycin and other cell wall active compounds.

4. Conclusions

Infections caused by *S. aureus* still play an important role in human and animal diseases as known antibiotics are no longer effective due to increasing resistance (Montanaro et al., 2016). The large number of complete genomes now available for *S. aureus* provides a detailed insight into the evolutionary processes leading to strains of differing virulence and drug-resistance potential (Holden et al., 2004). Here we present the complete genome of *S. aureus* SG511 (CP076660), which was compared to its relative MRSA252 and the antibiotic-susceptible *S. aureus* HG001. *S. aureus* SG511 was observed to be susceptible towards inhibitors of

protein, DNA/RNA and folic acid biosynthesis comparable to *S. aureus* HG001. Beyond that, *S. aureus* SG511 turned out to be hyper-susceptible towards cell wall and cell membrane targeting agents with exception of fosfomycin. In addition to the already known loss of function mutation in *graS*, we identified numerous mutations in the genome of *S. aureus* SG511, which potentially contribute to this phenomenon. Inter alia, amino acid substitutions were identified in the global regulators CodY and SarZ, the transcriptional regulators TcaR and SaeR, the SigB-regulator RsbV, the phosphodiesterase GdpP, and in the ribonuclease Rny. The accumulation of mutations in regulatory genes in the genome of *S. aureus* SG511-Berlin may interfere with the fine-tuned regulation system and contribute to the susceptibility of this strain towards numerous antibiotics. In *S. aureus* SG511, also the giant structural protein Ebh was found to be C-terminally truncated, the virulence factor *spa* was lost, and SG511 turned out to be *agr*-negative due to an insertion element in *agrC*. Hence, *S. aureus* SG511 comprises several mutations compared to the related antibiotic-resistant MRSA252, which individually or in a synergistic manner may contribute to the extraordinary susceptibility of this standard test strain for antibiotic lead discovery and development. This study therefore provides the basis for further work on the particular effects of each single mutation to shed further light onto their decisive consequences and potential interconnections.

CRediT authorship contribution statement

A.D. and G.B.: Conceptualization. **G.B.**: Funding acquisition. **A.D., U.S., P.S.**: Investigation, Methodology. **G.B.**: Supervision. **A.D.**: Writing. **G.B. and P.S.**: Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ijmm.2021.151545.

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4.2 Cervimycin-Resistant *Staphylococcus aureus* Strains Display Vancomycin-Intermediate Resistant Phenotypes

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- **Contributions:**
  - **A.D.:** Conceptualization of the study; funding acquisition; serial passaging of *S. aureus*; allelic exchange experiments; phenotyping of *S. aureus* strains (sample preparation for whole genome sequencing, electron microscopy, omics analysis; antimicrobial susceptibility testing; lysis experiments; zymography; stress assays); heterologous protein expression and activity assays (auto-phosphorylation of WalK variants with cervimycin, activity of ClpP variants); data validation, curation and visualization; writing the original draft; review & editing.
  - **U.S.:** First serial passaging experiments including phenotyping of *S. aureus* strains (sample preparation for whole genome sequencing and antimicrobial susceptibility testing).
  - **M.G.:** First activity testing of WalK; review & editing.
  - **A.-L.B.:** Testing of auto-phosphorylation activity of WalK variants from *S. aureus*.
  - **J.K.D.:** Antibiotic susceptibility tests with glycopeptides and daptomycin.
  - **C.S.:** Testing of antibacterial activity of cervimycins against different Gram-positives.
  - **P.S.:** Provided *Bacillus clpP* mutants; conceptualization; review & editing.
  - **I.T.M.:** Support with ClpP activity assays.
  - **J.B.:** Support with MIC determinations of *clpP* mutants.
  - **L.R.:** Firefly luciferase assays with *Bacillus* reporter strains.
  - **B.M., M.F.-W.:** Funding acquisition; proteomics; review & editing.
  - **K.N., T.A.H.:** Funding acquisition; transcriptomics; data curation; review & editing.
  - **H.B.-O.:** Funding acquisition; conceptualization; supervision; review & editing.

- **K.S., C.H.:** Purification of cervimycin; review & editing.
- **H.-G.S.:** Funding acquisition; conceptualization; supervision; review & editing.
- **G.B.:** Funding acquisition; project administration; conceptualization; supervision; review & editing.

Due to the antimicrobial resistance crisis novel antibiotics are urgently needed, preferably with resistance breaking modes of action. Because of their diverse biological activities, secondary metabolites from *Streptomyces* species represent a promising source for such compounds (Katz & Donadio, 1993). Following this approach, the antibiotic complex of cervimycins was isolated from *Streptomyces tendae* HKI 0179. Cervimycins are bi-glycosylated polyketide antibiotics, ring-substituted with either a carbamoyl or an acetyl moiety, bear either a dimethylmalonyl or a monomethylmalonyl residue attached to the longer sugar side chain, and display activity against Gram-positive bacteria. Initial mode of action studies on the antibiotic complex considered the DNA metabolism as possible target due to the inhibition of incorporation of radiolabeled thymidine at high cervimycin concentrations, but excluded a DNA intercalation mechanism (Herold, 2005). This study aimed to close this gap on the mode of action of cervimycin. Accordingly, the second chapter of this thesis describes the selection for and characterization of cervimycin-resistant *S. aureus* mutants.

Pre-screening of different *S. aureus* isolates had revealed that *S. aureus* SG511 Berlin was very susceptible towards cervimycin, especially towards cervimycin C (CmC). As explained in **Chapter 4.1 (*p*. 25)**, the extensive lack of resistance determinants in a combination with mutations in regulatory genes and in the kinase gene *graS* renders *S. aureus* SG511 Berlin susceptible to most antibiotics and especially susceptible to cell envelope-targeting substances. Here, CmC and cervimycin D (CmD) were used as lead compounds of the antibiotic complex for a serial passaging experiment with *S. aureus* SG511 Berlin, generating nine **c**ervi**m**ycin-**r**esistant (CmR) mutants. The CmR strains were highly cervimycin-resistant, also in presence of the ABC transporter inhibitor reserpine. Strikingly, cross-resistances towards the glycopeptides vancomycin (intermediate resistance) and teicoplanin, the lipopeptide daptomycin, and the ClpP activating acyldepsipeptides (ADEPs) were detected. Whole genome sequencing of the CmR mutants revealed two mutational hotspots, first the non-essential Clp system with the *clpC* ATPase and the *clpP* caseinolytic protease genes and, second, the kinase gene *walK* which is part of the essential WalRK two-component system. Interestingly, all CmR mutants harbored a combination of mutations in either *clpC* or *clpP* and in the *walK* gene. The combination of *walK* and *clpP* mutations and the resulting protection from cell envelope-

targeting agents like vancomycin and daptomycin had been described for some staphylococcal isolates (Shoji *et al*., 2011; Song *et al*., 2013).

The amino acid exchanges in ClpP and ClpC were characterized as loss-of-function mutations, impeding the natural function of the Clp system in protein homeostasis and regulation (Brötz-Oesterhelt & Sass, 2014). ClpP is the target of ADEP antibiotics which activate ClpP, leading to uncontrolled proteolysis, inhibition of bacterial cell division, and eventually cell death (Brötz-Oesterhelt *et al*., 2005). A homologous mechanism for cervimycins was excluded, since CmC did not alter the *in vitro* SsrA-eGFP degradation activity by ClpXP and *clpP* deletion did not alter the susceptibility of *B. subtilis* 168 towards cervimycin. In addition, reversion of resistance was observed for strains with secondary mutations in *walK*.

All CmR strains encoded amino acid exchanges in the essential WalK kinase. The WalRK two-component system, which comprises WalK and its cognate response regulator WalR, positively controls global autolytic activity, particularly via AtlA and the LytM endopeptidase and functions in the regulation of peptidoglycan maturation, cell wall turnover, cell separation and protein secretion, as well as biofilm formation (Dubrac *et al*., 2007). Phenotyping of the CmR strains indicated a decreased WalK activity due to a low autolysin level and cell lysis and thick cell walls, fitting to the decreased glycopeptide susceptibility. However, a clear clustering of the amino acid exchanges around a possible cervimycin binding site was not observed. Also, an interference of cervimycin with the *in vitro* WalK auto-phosphorylation activity could not be proven, since cervimycin formed phenolate salts under the test conditions.

The global impact of the above-mentioned mutations was also seen on the omics level, with major shifts in the heat shock response, metal ion homeostasis, carbohydrate metabolism, and the autolysins. Since a direct interaction of cervimycin was neither seen with ClpXP nor with WalK, we assumed an indirect effect of the mutations in the CmR strains, suggesting a target structure associated with growth, cell wall biosynthesis or the central metabolism, or protection of the target site from cervimycin by a thickened cell envelope.

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RESEARCH ARTICLE

Cervimycin-Resistant *Staphylococcus aureus* Strains Display Vancomycin-Intermediate Resistant Phenotypes

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ABSTRACT Resistance to antibiotics is an increasing problem and necessitates novel antibacterial therapies. The polyketide antibiotics cervimycin A to D are natural products of *Streptomyces tendae* HKI 0179 with promising activity against multidrug-resistant staphylococci and vancomycin-resistant enterococci. To initiate mode of action studies, we selected cervimycin C- and D-resistant (CmR) *Staphylococcus aureus* strains. Genome sequencing of CmR mutants revealed amino acid exchanges in the essential histidine kinase WalK, the Clp protease proteolytic subunit ClpP or the Clp ATPase ClpC, and the heat shock protein DnaK. Interestingly, all characterized CmR mutants harbored a combination of mutations in *walK* and *clpP* or *clpC*. *In vitro* and *in vivo* analyses showed that the mutations in the Clp proteins abolished ClpP or ClpC activity, and the deletion of *clpP* rendered *S. aureus* but not all *Bacillus subtilis* strains cervimycin-resistant. The essential gene *walK* was the second mutational hotspot in the CmR *S. aureus* strains, which decreased WalK activity *in vitro* and generated a vancomycin-intermediate resistant phenotype, with a thickened cell wall, a lower growth rate, and reduced cell lysis. Transcriptomic and proteomic analyses revealed massive alterations in the CmR strains compared to the parent strain *S. aureus* SG511, with major shifts in the heat shock regulon, the metal ion homeostasis, and the carbohydrate metabolism. Taken together, mutations in the heat shock genes *clpP*, *clpC*, and *dnaK*, and the *walK* kinase gene in CmR mutants induced a vancomycin-intermediate resistant phenotype in *S. aureus*, suggesting cell wall metabolism or the Clp protease system as primary target of cervimycin.

IMPORTANCE *Staphylococcus aureus* is a frequent cause of infections in both the community and hospital setting. Resistance development of *S. aureus* to various antibiotics is a severe problem for the treatment of this pathogen worldwide. New powerful antimicrobial agents against Gram-positives are needed, since antibiotics like vancomycin fail to cure vancomycin-intermediate resistant *S. aureus* (VISA) and vancomycin-resistant enterococci (VRE) infections. One candidate substance with promising activity against these organisms is cervimycin, which is an antibiotic complex with a yet unknown mode of action. In our study, we provide first insights into the mode of action of cervimycins. By characterizing cervimycin-resistant *S. aureus* strains, we revealed the Clp system and the essential kinase WalK as mutational hotspots for cervimycin resistance in *S. aureus*. It further emerged that cervimycin-resistant *S. aureus* strains show a VISA phenotype, indicating a role of cervimycin in perturbing the bacterial cell envelope.

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Gram-positive cocci like coagulase-negative staphylococci, *Staphylococcus aureus*, and *Enterococcus* spp., are important pathogens in the hospital environment (1). *S. aureus* is a leading cause of bacterial infections and mortality (2). Although the number of serious infections due to resistant strains has decreased in recent years (2), methicillin-resistant *S. aureus* (MRSA) remains a major health care issue (3). Therefore, new antibacterial agents are needed, preferably with novel modes of action to overcome resistance. *Streptomyces* species are a promising source of secondary metabolites, especially polyketides, with diverse biological activity (4).

Cervimycins belong to this group and the substances were named after the location of their first discovery, the cave *Grotta dei Cervi* in Italy. The cervimycin antibiotic complex consists of four main components A to D, and the minor components E to K, produced by the actinomycete *Streptomyces tendae* HKI 0179 (5–7). Interestingly, the color of cervimycin is pH-dependent, yellow or violet, a typical property of naphthoquinoid systems that form phenolate salts under alkaline conditions (6). The structures of cervimycins are fully resolved (8) (Fig. 1); cervimycins are bi-glycosylated polyketides, ring-substituted with either a carbamoyl or an acetyl moiety and bear either a dimethylmalonyl or a monomethylmalonyl residue attached to the longer sugar side chain. The unusual di- and tetrasaccharide chains, composed of the trideoxysugars β-D-amicetose and α-L-rhodinose are pharmacophore groups of the molecules, connected via a 1,4-O-glycosidic linkage. The common structural feature of these molecules is the quinoid system on ring D of the naphthacene core, which is inverted relative to classical tetracyclines (8). Tetracyclines, that bear some structural resemblance, are inhibitors of protein biosynthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (9). However, the cervimycins did not inhibit protein biosynthesis in a ribosome target test assay, indicating that the mode of action of these antibiotics is different (6).

Earlier precursor incorporation tests rather indicated the DNA metabolism as a possible target, but only at high concentrations (8× MIC) and with a considerable delay (35 min), whereas cell wall biosynthesis was not affected (6). Furthermore, a selection for cervimycin-resistant *Bacillus subtilis* mutants revealed an efflux-mediated resistance mechanism, but did not unveil the target structure of the antibiotic complex (10).

To close this gap, we investigated single nucleotide polymorphisms (SNPs) found in cervimycin-resistant *S. aureus* strains and the cervimycin resistance mechanisms. To this end, cervimycin-resistant *S. aureus* strains were generated by a serial passaging experiment. Interestingly, the transporter inhibitor reserpine only marginally affected cervimycin susceptibility in these strains, indicating an efflux-independent resistance mechanism. Genome sequencing of the cervimycin-resistant (CmR) strains revealed mutations in the nonessential caseinolytic protease gene *clpP* and its cognate ATPase gene *clpC*, and in the essential histidine kinase gene *walK*, which is part of the cell wall regulatory two-component system WalRK. Moreover, in one strain, an additional mutation in the nonessential heat shock protein 70 (HSP70) gene *dnaK* was observed. Importantly, the combination of *walK* and *clpP* or *clpC* mutations simultaneously increased the cervimycin and the vancomycin MICs of *S. aureus*, indicating the ClpCP complex or the Gram-positive cell envelope as possible targets of this novel antibiotic.

RESULTS

Cervimycin C is active against MRSA and VRE. Extended testing of cervimycin C (CmC) and cervimycin D (CmD) confirmed earlier reports on the activity of cervimycin against MRSA and VRE (5) (Table 1). Analysis of the minimal bactericidal concentrations (MBCs) validated the bactericidal activity of CmC and CmD, with MBCs of one or two titer steps above the MIC values (Table S1). In contrast, mycobacteria and the Gram-negative bacteria *Neisseria sicca* and *E. coli* were hardly affected by the cervimycins. Even an *E. coli* strain with a defective outer membrane (*E. coli* MB5746) was not susceptible to cervimycin. The susceptibility of

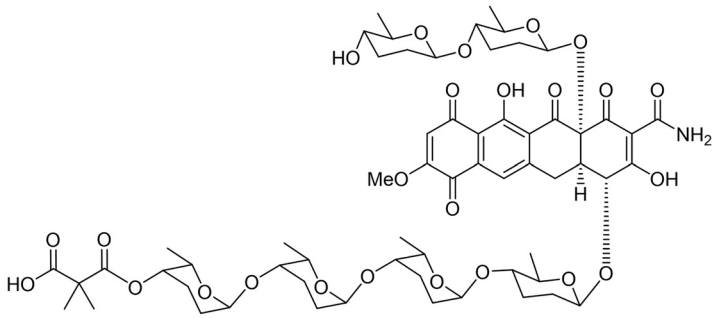

FIG 1 Structure of cervimycin C; relative configuration. Cervimycins are bi-glycosylated polyketides, decorated with unusual di- and tetrasaccharide chains. Cervimycin C is ring-substituted with a carbamoyl moiety and bears a dimethylmalonyl residue attached to the longer sugar side chain.

E. coli MB5746 was also not increased by the addition of polymyxin B nonapeptide (PMBN), which induces outer membrane permeability in Gram-negative bacteria (11). In contrast, *E. coli* MB5746 was further sensitized toward erythromycin (2-fold) and novobiocin (4-fold) by the addition of PMBN. Of note, the activity of cervimycin turned out to be pH-responsive, as the alkalization of the medium abolished the antibacterial activity (Table 2). This loss of antibacterial activity was accompanied by a color change from yellow to red-violet.

Also, as shown in Table 1, the methicillin-susceptible *S. aureus* SG511 Berlin was very susceptible toward CmC, but less susceptible toward CmD, which was generally less active than CmC. Therefore, all further tests were conducted with CmC, the major component of the antibiotic complex, unless stated otherwise. The mode of action of CmC is unknown. In *B. subtilis* firefly luciferase reporter strains (12) only the *yorB* promoter was activated, indicating a LexA-dependent DNA stress response. *yorB* encodes a protein of unknown function, that belongs to the LexA regulon of *Bacillus* and is strongly induced by substances, which produce double-strand breaks, while noncovalent DNA binding agents yield no signal induction. However, the induction level of the *yorB* promoter by CmC did not reach significance, remaining below a 2.5-fold increase above the baseline level (Fig. S1). Notably, the cell envelope stress reporters (*ypuA* and *lial*) and the translation stalling reporter (*bmrC*) were not stimulated by cervimycin (Fig. S1).

TABLE 1 MIC values (μg/mL) for cervimycin C (CmC) and D (CmD)[a]

Strain	CmC	CmD
Gram-positive		
Bacillus subtilis 168	0.25	0.5
Corynebacterium xerosis VA 167198	0.125	4
Enterococcus faecium BM 4147-1 (vancomycin-susceptible)	2	16
E. faecium BM 4147 (vancomycin-resistant)	2	4
Listeria welshimeri DSM 20650	0.25	4
Micrococcus luteus ATCC4698	0.5	1
Mycobacterium smegmatis	32	32
Staphylococcus aureus SG511 Berlin (methicillin-susceptible)	2	32
S. aureus N315 (methicillin-resistant)	4	64
Staphylococcus haemolyticus 655-2 (vancomycin-susceptible)	1	64
S. haemolyticus 655-2 R16 (vancomycin-resistant)	1	64
S. haemolyticus 655-2 R32 (vancomycin-resistant)	1	64
Staphylococcus simulans 22	8	64
Streptococcus agalactiae B Ku	0.5	0.25
Streptococcus pyogenes Ku	0.25	4
Gram-negative		
Escherichia coli MB5746 (*lpxC*, *tolC*:Tn*10*)	>64	>64
E. coli MB5746 (*lpxC*, *tolC*:Tn*10*) + 4 μg/mL PMBN[b]	>64	ND
Neisseria sicca Ku	64	64

[a]Broth dilution method was used in Müller Hinton medium and samples were incubated for 24 h.
[b]Polymyxin B nonapeptide (PMBN) is a derivative of polymyxin B and induces outer membrane permeability in Gram-negative bacteria.

TABLE 2 MIC values (μg/mL) of *S. aureus* SG511 Berlin for cervimycin C (CmC) at different pH values[a]

pH	MIC (μg/mL)
5	0.5
5.5	2
6	4
6.5	16
7	32
7.5	64
8	>64
8.5	>64

[a]Broth dilution method was used in Mäller Hinton medium with indicated pH values and samples were incubated for 24 h.

Whole-genome sequencing of cervimycin-resistant *S. aureus* strains reveals mutations in *walK* and *clpCP*. To further explore, how cervimycin acts on bacterial cells, we generated cervimycin-resistant mutants by serial passaging of *S. aureus* SG511 with gradually increasing concentrations of CmC or CmD. In total, six cervimycin C-resistant (CmR-01 to CmR-03, resulting from 11 passages, and CmR-04 to CmR-06, from another 13 passages) and three cervimycin D-resistant *S. aureus* strains (CmR-07 to CmR-09, 22 passages) were isolated. Revertants (REV) were obtained by passaging of the CmR strains for several generations in nonselective medium (Fig. S2).

Sequencing of resistant mutants and their revertants showed that all CmR strains harbored a combination of mutations in the essential histidine kinase gene *walK*, and in the genes encoding the nonessential Clp system, either in *clpP* or in *clpC* (Table 3 and Table S4). Only one strain (CmR-02) harbored an additional mutation in the heat shock protein gene *dnaK*. To investigate the effects of the single mutations and their possible participation in the resistance mechanism, SNPs were introduced into the genome of the *S. aureus* SG511 Berlin parent strain by markerless allelic replacement. *S. aureus* SG511 WalK^{A243V} was generated by reverting the *clpP* mutation in *S. aureus* CmR-01. Susceptibility testing toward commonly used antibiotics revealed a cross-resistance toward the lipopeptide daptomycin, and a reduced susceptibility of the CmR strains toward the glycopeptide antibiotics vancomycin and teicoplanin (Table 4, Fig. 2), but no further resistances toward other clinically used antibiotics, including tetracycline. In contrast, CmR-01, CmR-02, and CmR-03 were resistant toward the ClpP activating acyldepsipeptides (ADEPs). ADEPs bind to ClpP, the proteolytic core of the ClpP protease, leading to uncontrolled proteolysis, inhibition of bacterial cell division, and eventually cell death (13). As expected, the amino acid exchange in ClpP in CmR-02,

TABLE 3 Comparative whole-genome sequencing of *S. aureus* SG511 Berlin, the cervimycin resistant mutants (CmR), and the respective cervimycin-susceptible revertants (REV)[a]

Strain	MIC CmC	*clpP*	ClpP	*walK*	WalK
CmR-02[b]	128	A →T	I29F	C →T	A243V
02REV	2	A →T; G → C	I29F; M31I	C →T; C →T	S191L; A243V
CmR-03	128	A →T	I29F	C →T	A243V
03REV	2	A →G	I29V	C →T; C →T	S191L; A243V
	MIC CmC	***clpC***	**ClpC**	***walK***	**WalK**
CmR-04	128	4,561 bp Del.	Deletion	ΔCAA	ΔQ371
04REV	32	4,561 bp Del.	Deletion	ΔCAA; G →A	ΔQ371, A554T
CmR-05	128	C →T	T215I	C →T	A243V
05REV	16	C →T	T215I	C →T; C →T	A243V, T217M
	MIC CmD	***clpC***	**ClpC**	***walK***	**WalK**
CmR-09	107.5	C →A	P204H	T → G	Y549D
09REV	16	C →A	P204H	T → G; G →A	Y549D, R555H

[a]Sequence comparisons revealed a combination of amino acid exchanges in the protease ClpP or the cognate Clp ATPase ClpC, and the essential histidine kinase WalK.
[b]CmR-02 harbors an additional mutation in the heat shock protein gene *dnaK* (G → C), leading to the amino acid exchange A112P.

TABLE 4 MIC values of *S. aureus* strains in Müller Hinton medium or brain heart infusion broth[a]

S. aureus strain	Tet	VA	DAP	Teico	ADEP 4
SG511	0.5	1	0.25	0.5	0.25–0.5
CmR-02	0.0625	**4**	**2**	**8**	ND
CmR-04	0.25	**4**	**1**	**4**	ND
CmR-05	0.25	**4**	**1**	**4**	ND
CmR-09	0.25	**2**	**1**	**2**	ND
02REV	0.25	1	0.25	0.5	ND
04REV	0.25	1	0.125	0.5	ND
05REV	0.25	1	0.25	0.25	ND
09REV	0.25	**2**	0.25	**2**	ND
WalK^{A243V}	0.5	**4**	**1**	**4**	0.5
ClpP^{I29F}	0.125	1	0.5	1	**16**
ClpC^{T215I}	0.25	1	0.25	1	0.125
USA300 JE2	0.5	**2**	**1**	**2**	0.5
USA300 JE2 ΔclpP	0.125	**2**	**1–2**	**2**	**>32**

[a]The tetracycline (Tet) and ADEP 4 MICs were determined in MH broth; the glycopeptide MICs were determined in BHI broth (VA, vancomycin; DAP, daptomycin; Teico, teicoplanin). 1.25 mM $CaCl_2$ was added for MIC determinations with VA and DAP. According to the EUCAST guideline (published 01/2022), breakpoints for the tested antibiotics are as follows: Tet > 2 μg/mL; VA > 2 μg/mL; DAP ≥ 1 μg/mL; and Teico > 2 μg/mL. MICs exceeding the EUCAST breakpoints and significantly elevated ADEP-MICs are shown in bold. ND, no data. Grey shading is to distinguish between CmR mutants, revertants and allelic exchange mutants.

which is also present in CmR-01 and CmR-03, or the deletion of the entire *clpP* (14), but not the mutations in the *clpC* gene (CmR-04 to CmR09) led to ADEP 4 resistance in *S. aureus* (Table 4). This suggested that the detected *clpP* mutations affected either the activity of ClpP or the binding of ADEPs to their target.

CmR-01, CmR-02, and CmR-03 strains harbor loss-of-function mutations of ClpP. Since MIC determinations of *S. aureus* SG511, CmR-02, 02REV, and 03REV revealed remarkable differences in ADEP-susceptibility, we wanted to explore this effect further. Three CmR strains showed cross-resistance to ADEP 2 (CmR-01, CmR-02, CmR-03: MIC >32 μg/mL; wild type: MIC 2 μg/mL), an effect which was alleviated in two revertants (01REV, 02REV; MIC 16 μg/mL) and reversed in one revertant (03REV; MIC 1 μg/mL). MIC determinations of *clpP* deletion strains in different genetic backgrounds of *S. aureus* revealed a 4-fold increased resistance to CmC compared to the respective wild-type *S. aureus* strains (Table 5). CmR-01, CmR-02, and CmR-03 carried a nucleotide exchange in *clpP*, yielding ClpP^{I29F}. Interestingly, amino acid 29 is located within the hydrophobic pocket of ClpP, a major regulatory site of the protein, which is also known as the binding site of the ADEPs (15, 16). Furthermore, two different suppressor mutations were observed in the cervimycin-susceptible revertants, an additional exchange at position 31, yielding ClpP$^{I29F/M31I}$, and a conservative exchange yielding ClpP^{I29V}.

This together with a severe growth defect (17) of the *clpP*-mutation-containing strains CmR-01, CmR-02, and CmR-03 under anaerobic conditions (Fig. 3A and B) indi-

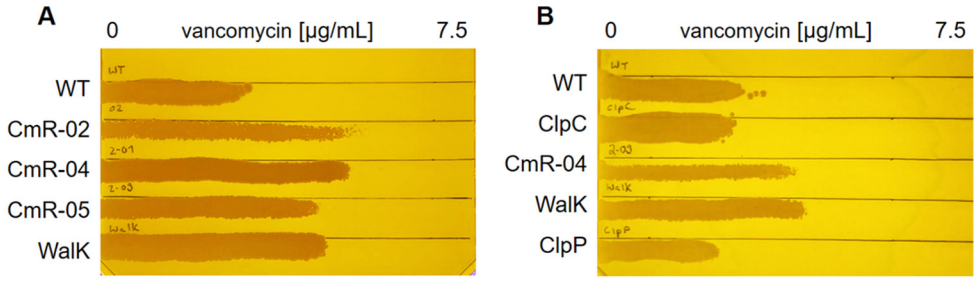

FIG 2 Gradient plates with increasing concentrations of vancomycin and 1.25 mM $CaCl_2$. The CmR strains showed a decreased susceptibility toward vancomycin (A), which probably relied on the *walK* mutations in these strains, because the *walK* mutation alone decreased the vancomycin susceptibility (B). WT, *S. aureus* SG511; WalK, *S. aureus* WalK^{A243V}; ClpC, *S. aureus* SG511 ClpC^{T215I}; ClpP, *S. aureus* SG511 ClpP^{I29F}.

TABLE 5 MIC values (μg/mL) for cervimycin C (CmC) in strains that contain only single exchanges in the parental background or knockout mutants[a]

Strain	CmC
S. aureus SG511	2
S. aureus SG511 ClpC^{T215I}	8
S. aureus SG511 ClpP^{I29F}	4
S. aureus SG511 DnaK^{A112P}	2
S. aureus SG511 WalK^{A243V}	16
S. aureus NCTC 8325-4	16
S. aureus NCTC 8325-4 Δ*clpP*	64
S. aureus USA300 JE2	16
S. aureus USA300 JE2 Δ*clpP*	64
B. subtilis 168	0.25
B. subtilis 168 Δ*clpP*	0.25
B. subtilis 168 Δ*clpC*	0.125
B. subtilis 168 Δ*clpX*	0.125
B. subtilis JH642	1
B. subtilis JH642 Δ*spx*	0.5
B. subtilis JH642 Δ*spx* Δ*clpP*	2

[a]Broth dilution method was used in Müller Hinton medium and samples were incubated for 24 h.

cated a loss of ClpP function in these strains. To verify this, the intrinsic catalytic activity and ADEP-responsiveness of wild-type and ClpP variants, as well as the activity of the ClpP variants in association with the cognate Clp ATPase ClpX were characterized. In principle, ClpP alone can degrade small peptides like Suc-LY-AMC (18) and the activity of all ClpP variants was decreased with this substrate (Fig. S3). Larger substrates like FITC-casein normally cannot be degraded by ClpP alone (15), but degradation can be induced by the addition of ADEPs. ClpP^{I29F} and ClpP$^{I29F/M31I}$ were not activated by ADEP 2 in the FITC-casein assays, whereas ClpP^{I29V} retained good ADEP responsiveness (Fig. 3C and D). To further study the effect of the SNPs on the interaction of ClpP with the cognate Clp ATPase ClpX, we used eGFP-SsrA as model substrate for the natural function of ClpP together with the *S. aureus* ClpX ATPase. In accordance with the casein degradation data, ClpP^{I29F} and ClpP$^{I29F/M31I}$ did not lead to ClpX-mediated degradation of eGFP-SsrA. In contrast, ClpP^{I29V} nearly possessed wild-type activity. Hence, our results show that I29 of ClpP is important for ADEP- and ClpX-binding, which leads to a loss-of-function of the entire ClpP protease.

It may be hypothesized that cervimycin directly interferes with ClpP, leading to its deregulation, similar to ADEPs. However, we assumed this to be rather unlikely since CmC did not affect the activity of wild-type ClpXP in a GFP-SsrA assay (Fig. S4). In addition to that, MIC determination (Table 5) of a *B. subtilis* 168 *clpP* deletion mutant yielded the same susceptibility as seen in wild-type cells. However, in *Bacillus* the deletion of ClpP leads to an eventually toxic accumulation of the Spx protein, which is a ClpXP substrate. In order to eliminate this effect, a *spx* deletion mutant was also assayed. Here, the deletion of *spx* alone led to a sensitization of the mutant toward cervimycin because Spx activity can, by its regulatory function, also confer resistance to a wide range of stressors and thereby increase tolerance to certain antibiotics (19). The double *spx clpP* mutant showed an increased resistance to cervimycin and confirmed the results obtained with *S. aureus*.

CmR-04 to CmR-09 harbor inactive ClpC variants. We detected a 4,561-bp deletion of the genes *clpC* and *radA* in CmR-04, and other resistant mutants carried SNPs within *clpC*, leading to ClpC^{T215I} (CmR-05 and CmR-06) or ClpC^{P204H} (CmR-07 to CmR-09) (Table S4). The amino acid exchanges were localized within (T215I) or in proximity (P204H) to the first ATP-binding Walker A motif of the ClpC ATPase. Functionality of the ClpC ATPase was tested in a heat shock assay. The loss of *clpC* leads to a growth defect at 45°C (20), which was true for all strains with the mutated ClpC variants, indicating a loss of ClpC function in these strains (Fig. 3E).

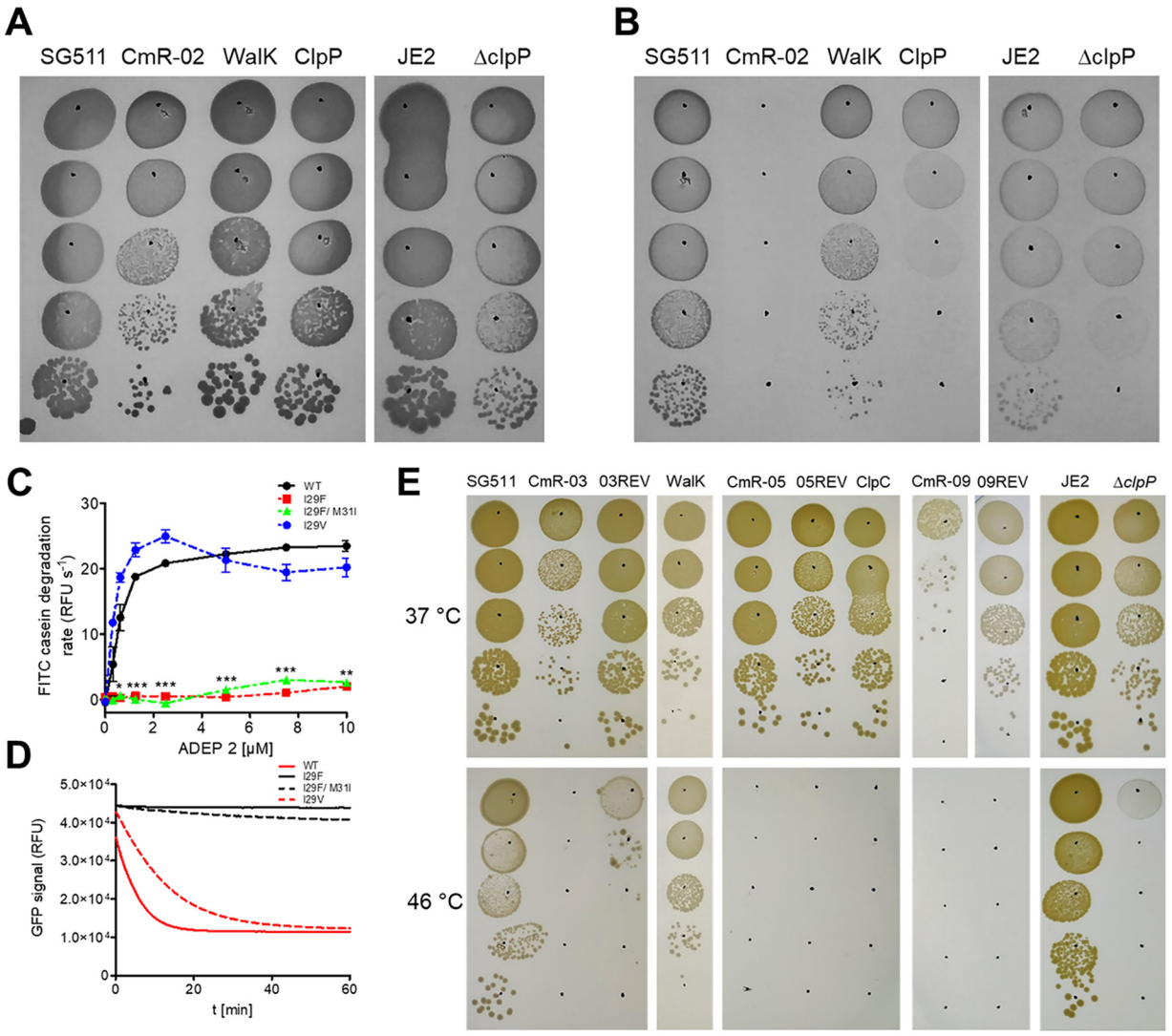

FIG 3 CmR strains harbor loss-of-function mutations in *clpP* or *clpC*. (A and B) A loss-of-function mutation in *clpP* abolishes growth of *S. aureus* under anaerobic growth conditions. Stationary-phase *S. aureus* strains were serially diluted 10-fold in 0.9% NaCl (10^{-1}- to 10^{-5}-fold) and 10 μL of each dilution was spotted on TSA plates. The plates were incubated under aerobic (A) or anaerobic (B) growth conditions. (C) FITC-casein (30 μM) degradation activity of wild-type and mutant SaClpP (1 μM) with increasing concentrations of ADEP 2 (0–10 μM). ADEP responsiveness of two ClpP variants was significantly decreased (*, *P* value $\leq$ 0.027; **, *P* value $\leq$ 0.015; ***, *P* value $\leq$ 0.0004). (D) SaClpXP eGFP-SsrA assay. The natural function of four ClpP-variants (2.8 μM: $ClpP^{WT}$, red line; $ClpP^{I29F}$, black line; $ClpP^{I29F/M31I}$, black dashed line; $ClpP^{I29V}$, red dashed line) was tested with SsrA-tagged eGFP-substrate (0.36 μM) and the ClpX ATPase (2.4 μM). The decrease of the fluorescence signal indicates unfolding or degradation of the substrate. The mutated ClpP variants displayed a consistently decreased GFP degradation activity, compared to the wild type (comparison of the initial 3 min: ***, *P* value $\leq$ 0.0002). (E) A loss-of-function mutation in *clpC* abolishes growth of *S. aureus* at elevated temperatures. Cervimycin-resistant and susceptible strains were grown in TSB at 37°C until OD_{600} ~0.5, serially diluted 10-fold in 0.9% NaCl (10^{-1}- to 10^{-5}-fold), then 10 μL of each dilution was spotted on TSA plates. The plates were incubated at indicated temperatures. WalK, *S. aureus* $WalK^{A243V}$; ClpP, *S. aureus* SG511 $ClpP^{I29F}$; ClpC, *S. aureus* SG511 $ClpC^{T215I}$.

Effects of cervimycin resistance mutations on the essential WalK histidine kinase. All cervimycin-resistant *S. aureus* strains carried mutations in the essential histidine kinase gene *walK*, yielding either $WalK^{A243V}$, $WalK^{\Delta Q371}$ (cervimycin C-resistant strains), or $WalK^{Y549D}$ (cervimycin D-resistant *S. aureus* strains). Zymographic analysis showed that the autolysin (Atl) activity was severely restricted in the CmR strains, with the exception of CmR-04, and that Triton X-100 induced cell lysis was significantly reduced in all strains (Fig. 4B–E), which might be due to alterations of WalK activity. Electron microscopy of one cervimycin-resistant model strain (CmR-02) and its revertant (02REV), in comparison to the wild-type strain *S. aureus* SG511, confirmed the VISA phenotype, as CmR-02 possessed a significantly thickened cell wall (wild type: 34.91 $\pm$ 5.93 nm, CmR-02: 58.7 $\pm$ 13.93 nm, 02REV: 36.41 $\pm$ 5.64 nm) (Fig. 4B and C).

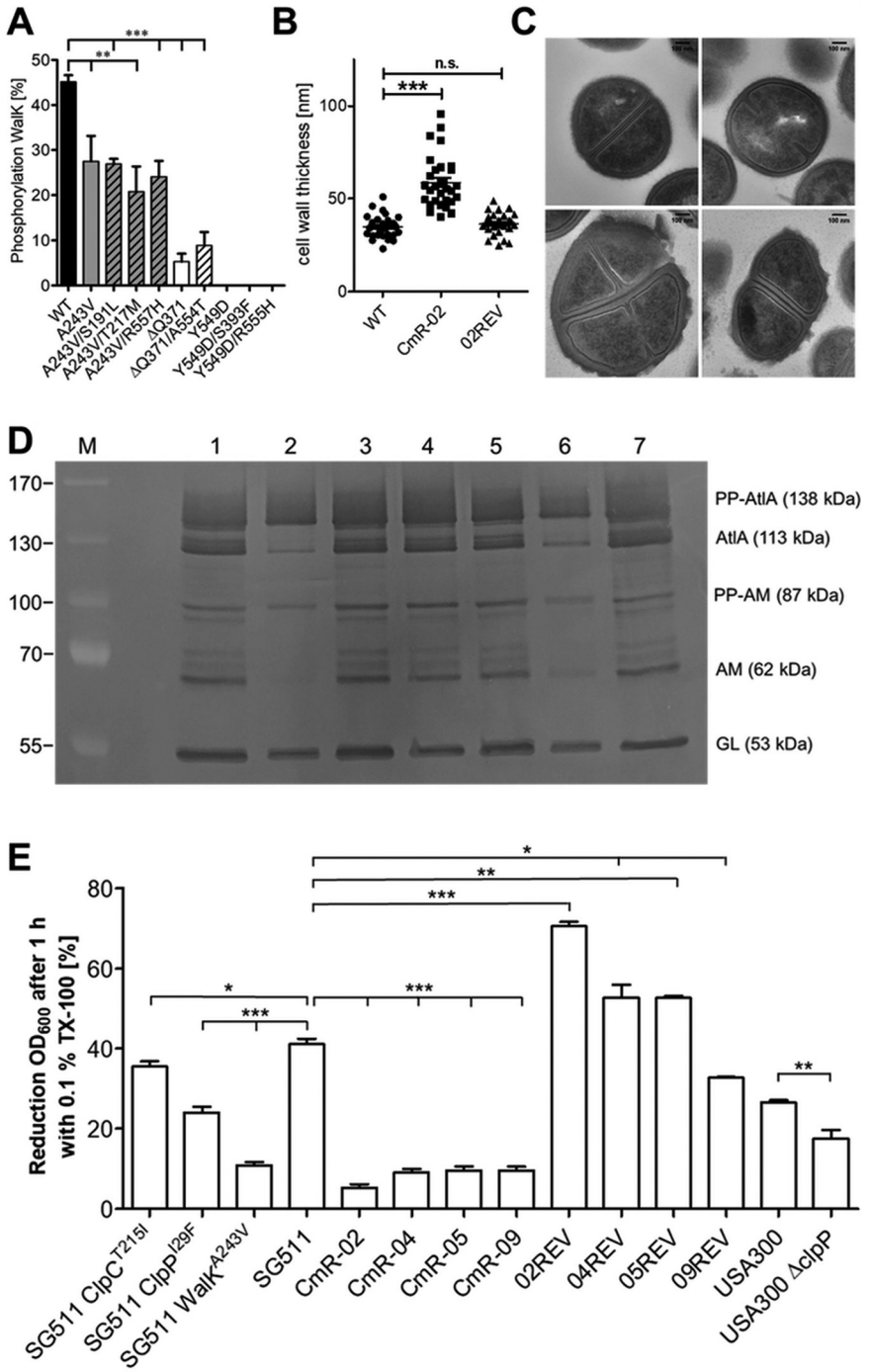

FIG 4 CmR-02 exhibits phenotypic characteristics of a vancomycin-intermediate resistant *S. aureus* strain. (A) The autophosphorylation of WalK variants from CmR strains and revertants is significantly reduced *in vitro* (**, $P < 0.018$; ***, $P \leq 0.0001$). (B) Quantification of the cell wall thickness in *S. aureus* SG511 (WT), CmR-02, and 02REV reveals a significant cell wall thickening in CmR-02 (***, *P* value < 0.0001). (C) Scanning transmission electron microscopy (STEM) of exponentially growing *S. aureus* strains: top left *S. aureus* SG511, top right 02REV, bottom CmR-02. (D) Zymogram of LiCl extracts from *S. aureus* strains SG511 (1), CmR-02 (2), 02REV (3), CmR-04 (4), 04REV (5), CmR-09 (6), and 09REV (7). Numbers to the left of the gel indicate the molecular weights of size standard (PageRuler Plus prestained protein ladder). Dark bands indicate regions of hydrolase activity of the major *S. aureus* hydrolase AtlA, which appears in different subunits (PP, propeptide; AM, amidase; GL, glucosaminidase). (E) Percentage reduction of the OD_{600} after 60 min of exponential *S. aureus* cells in PBS with 0.1% Triton X-100. The OD_{600} was normalized to the starting value (* $P \leq 0.0272$; ** $P \leq 0.0063$; *** $P \leq 0.0001$).

TABLE 6 Downregulation of the histidine kinase gene *walK* or the response regulator gene *walR* increases the susceptibility of *S. aureus* RN4220 to cervimycin C[a]

Altered gene expression	Function	Fold increase diam inhibition zone
Decreased gene expression		
Heat shock response		
dnaK	Heat shock protein 70	1.10
Cell wall metabolism		
pbp3	Penicillin-binding protein 3	1.00
smc	Chromosome segregation protein	1.00
ftsZ	Cell division protein	1.08
walK	Cell wall metabolism sensor histidine kinase	1.24
walR	DNA-binding response regulator	1.30
Δ*atl*	Bifunctional autolysin	1.00
Increased gene expression		
ssaA	Secretory antigen precursor	1.10
lytM	Glycyl-glycine endopeptidase	1.65
walR	DNA-binding response regulator	1.10
walR^{C}	DNA-binding response regulator, constitutively active (D55E amino acid exchange)	1.24

[a]Expression levels of indicated genes were increased/decreased with the pEPSA5-system (95). CmC susceptibility was tested in agar diffusion assays in comparison to an empty vector control.

We next determined the autophosphorylation activity of the WalK variants as the proportion of autophosphorylated WalK kinase after 30 min under activating conditions. WalK variants from the CmR strains displayed a significantly decreased WalK activity, with WalK^{Y549D} being completely inactive in the *in vitro* test system (Fig. 4A). Surprisingly, the additional *walK* mutations from the revertant strains had no extra effect on WalK autophosphorylation *in vitro*, and resembled the activity of the respective WalK variants from the cervimycin-resistant mutants (Fig. 4A). Unfortunately, we were not able to obtain reproducible results of the influence of cervimycin on the phosphorylation activity of WalK, neither with the Phos-tag activity assay, nor with a ^{32}P liposome system. The naphthoquinoid system of cervimycin formed phenolate salts under the assay conditions (pH 8, 5 mM dithiothreitol [DTT]), indicated by a color change from yellow to violet, and in MIC assays the violet form of cervimycin showed poor activity. However, in an agar diffusion assay, induced downregulation of *walK* or *walR* acted synergistically with CmC (Table 6). SsaA and LytM expression were shown to restore cell viability in the absence of WalRK (21). However, overexpression of SsaA or LytM from the xylose-inducible vector pEPSA5 did not promote cervimycin resistance, but acted synergistically with CmC, as well as the overexpression of a constitutively active WalR variant WalR^{D55E} (Table 6). Hence, a lower and a higher WalRK activity increased the susceptibility of *S. aureus* toward cervimycin.

The *dnaK* mutation has no effect on cervimycin susceptibility. One CmR strain (CmR-02) carried a nucleotide polymorphism in *dnaK*. The nucleotide exchange C358G in *dnaK* of *S. aureus* CmR-02 led to an amino acid exchange from alanine to proline at position 112 in the N-terminal nucleotide-binding domain of the protein. However, the reconstitution of the A112P amino acid exchange into the parent strain and the downregulation of *dnaK* using the pEPSA5 system showed no effect on CmC susceptibility (Table 5 and 6).

Transcriptomic and proteomic analyses reveal major alterations in the cervimycin-resistant mutant CmR-02. Mutations found in the CmR strains raised both the cervimycin and the vancomycin MICs. Because the regulatory aspect of these mutations is of interest, and also to obtain a more global view on the effect of the mutations found in the cervimycin-resistant mutants, transcriptomic and proteomic analyses of one CmR strain, its susceptible revertant, and the wild-type *S. aureus* SG511 were performed.

While only minor alterations occurred in the transcriptome of the revertant 02REV in comparison to the *S. aureus* SG511 wild type, a large number of genes was differentially expressed in the resistant strain CmR-02 (Table 7, Fig. 5A, Fig. S5). This was also

TABLE 7 Differential expression/translation of genes/proteins in *S. aureus* CmR-02 and its revertant 02REV in comparison to *S. aureus* SG511 Berlin[a]

	CmR-02		02REV	
Sample	Transcriptome	Proteome	Transcriptome	Proteome
Total	1993	565	1315	439
Up	370 (18.57%)	78 (13.81%)	56 (4.26%)	38 (8.66%)
Down	442 (22.18%)	73 (12.92%)	100 (7.61%)	21 (4.78%)
Sum	812 (40.74%)	151 (26.73%)	156 (11.86%)	59 (13.44%)

[a]Shown are the total numbers of genes/proteins with a significant detection level (total, P value ≤ 0.05), as well as the numbers of significantly upregulated (up, $\log_2$ fold change ≥ 1) and downregulated (down, $\log_2$ fold change ≤ -1) genes/proteins.

confirmed by the proteomic analysis and revealed the broad impact of the mutations in *clpP* and *walK* in CmR-02 (and possibly *dnaK*), which was substantially alleviated by the additional mutations in the revertant 02REV.

In CmR-02 the expression of several ABC transporters was repressed, as well as the expression of purine biosynthesis genes, of several regulons of the amino acid biosynthesis (ArgR regulon, S-box), of major parts of the carbohydrate metabolism (MalR, GapR, CcpA-regulon), and of the CodY-regulon (Fig. S6). On a transcriptomic level, also the *lac* operon was repressed. On the other hand, several metabolic pathways were upregulated, like the riboflavin-biosynthesis pathway (FMN-box regulon) and the last four genes of the staphyloxanthin (STX) biosynthesis cluster (KQU62_11530 to KQU62_11545), which might contribute to the intense coloring of CmR-02 on blood agar (Fig. S2).

As expected, major alterations were also detected in the expression of the CtsR/HrcA operon (including *clpP* and *dnaK*) and the WalR regulon (regulated by WalRK). *In vitro* and *in vivo* experiments indicated that the I29F amino acid exchange led to a loss of ClpP function in CmR-02. Expression of the CtsR/HrcA operon, which encodes ClpP and DnaK, was completely derepressed in CmR-02, and the Clp machinery and the chaperones were also more abundant on the protein level (Fig. 5B and Fig. S6), which is in accordance with the observations in the *clpP* mutant of Michel et al. (17). Transcription of *clpX* and *trfA* (sometimes also called *mecA*), which are not encoded in the CtsR/HrcA operon, was also increased (Table S5).

The WalRK TCS positively controls global autolytic activity, particularly via AtlA and the LytM endopeptidase (22). The *walK* mutation of CmR-02 is located in the HAMP-domain coding region, which is a signal transduction domain (23). The reduced autolytic activity in the zymography of CmR cell extracts, together with the reduced cell lysis and the thickened cell walls observed via electron microscopy, suggest a hampered WalK function due to the detected mutation. However, the expression profile of the WalR regulon in CmR-02 was not as explicit as for the CtsR/HrcA operon (Fig. 5C and Fig. S6). Six genes of the WalR regulon were significantly upregulated, the expression of *sle1* was repressed, and the reduced abundance of the major autolysin Atl and the Sle1 amidase was confirmed on the protein level. Interestingly, WalJ (YycJ), which is encoded downstream of the *walRKHI* operon but functionally unrelated and transcribed from a separate promoter (24), was more abundant in CmR-02. However, the consequences of the increased abundance of WalJ remain to be elucidated. Although functions in mismatch repair and chromosome segregation have been described for the genus *Bacillus* (25, 26), a *walJ* knockout mutant did not show any growth related phenotype in *S. aureus* (24).

In the suppressor mutant, the expression of three hydrolases was increased, and *sceD* expression was decreased. The altered expression of autolysins in 02REV in comparison to CmR-02 indicates an effect of the additional *walK* mutation in the revertant strain, which was not detectable in the *in vitro* activity assay. An upregulation of the VraS regulon, indicating increased cell wall biosynthesis (27) in the resistant mutant, was not observed.

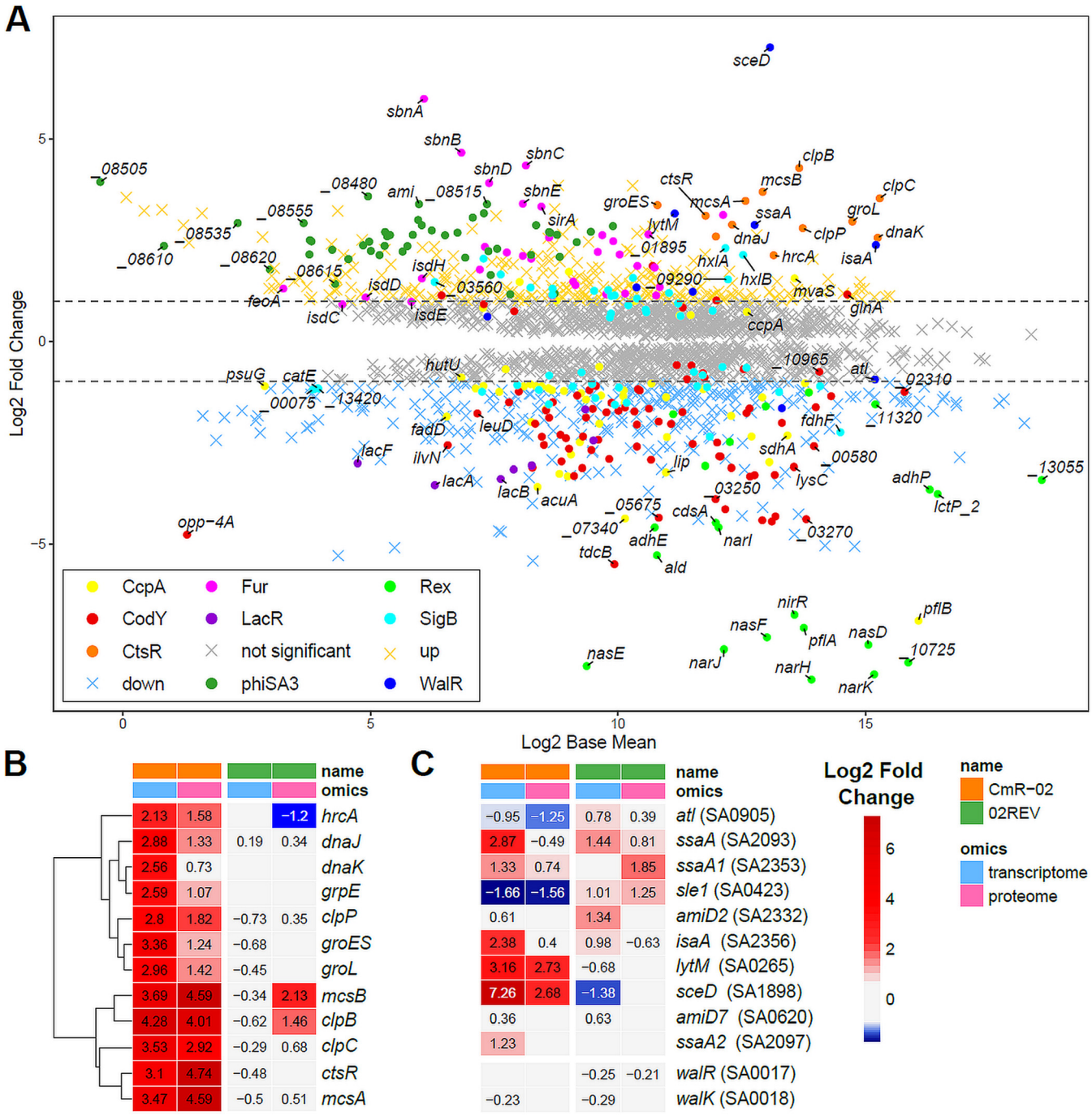

FIG 5 RNA-Seq transcriptomics of *S. aureus* CmR-02. (A) The expression profile is shown as ratio/intensity scatterplot (M/A-plot: M value, $\log_2$ fold change; A value, $\log_2$ base mean), which is based on the differential gene expression analysis. Colored symbols indicate significantly induced or repressed transcripts (M value ≥ 1 or ≤ -1; *P* value ≤ 0.05). Colors refer to the annotated regulator (based on the AureoWiki Database [118]). Regulons with at least seven differentially expressed genes and the phage ΦSA3 encoding genes are shown. Light gray symbols denote transcripts with similar expression levels in comparison to the wild-type *S. aureus* SG511 Berlin (*P* value ≤ 0.05, $\log_2$ FC ≤ 1 or ≥ -1). Genes of interest with at least 1.5-fold differential expression are also color-coded (e.g., *atl*). The RNA-seq data of differential transcription of all genes and regulons of CmR-02 are listed in Table S5. Expression profiles of the CtsR/HrcA heat shock operon (B) and the WalR regulon (C) together with the *walRK* genes of *S. aureus* CmR-02 (orange) and its revertant 02REV (green) reveal major alterations. Up-regulation ($\log_2$ FC ≥ 1, *P* value ≤ 0.05; red color) and downregulation ($\log_2$ FC ≤ -1; blue color) on the transcriptomic (light-blue) and proteomic level (light-pink) are compared.

DISCUSSION

Streptomyces tendae HKI 0179 produces the antibiotic complex of cervimycin, which possesses promising activity against Gram-positive organisms, especially against MRSA and VRE (8). Earlier incorporation studies indicated disturbance of the

DNA metabolism, due to cervimycin treatment, although at high concentrations and time-delayed (6). The anthracycline antibiotics dutomycin (28) and polyketomycin (29) are structural homologs of cervimycin, and it has been assumed that these substances might intercalate into DNA, as described for other anthracyclines (30), an activity which was not seen for the cervimycins (6).

We selected cervimycin-resistant strains from *S. aureus* SG511 Berlin. Cross-resistances to other antibiotics were observed for vancomycin, daptomycin, and teicoplanin for all strains and for acyldepsipeptides (ADEPs) for some strains. Genome sequencing revealed SNPs in the caseinolytic protease gene *clpP* or its cognate ATPase gene *clpC*, the HSP70 gene *dnaK*, and the essential histidine kinase gene *walK*.

ClpP is the proteolytic core of the caseinolytic protease and possesses a key function in protein homeostasis, generally maintaining protein quality, and tightly controlling key regulatory proteins (31), affecting a large number of processes. ClpP forms two-component proteases together with ATPases like ClpX and ClpC (32). This proteolytic machinery degrades various substrates in an ATP-dependent manner (32), allowing adaptation to multiple stresses by degrading accumulated and misfolded proteins (17). The ClpC ATPase forms a complex with the ClpP protease for protein degradation under thermal stress conditions (20). In addition, ClpC is thought to act as a chaperone for the CtsR repressor, which blocks transcription of the CtsR/HrcA operon, encoding *clpCP*, *dnaK*, *groESL*, and more (20). DnaK is a HSP70 chaperone, which catalyzes the folding of newly synthesized polypeptides and the refolding of stress-denatured and aggregated proteins in an ATP-dependent manner (33–35). Although ClpP is not essential, proteolysis of "unemployed," disintegrated and inactive proteins is a fundamental process of cellular regulation in all organisms (36). Substrates larger than small peptides are degraded by a complex of ClpP and a Clp ATPase, i.e., ClpX or ClpC in *S. aureus* (37), which also determines the substrate specificity for protein degradation (38, 39).

The ClpP^{I29F} amino acid exchange is located in the N-terminal hydrophobic pocket, which is a major regulatory site of the protease (15, 16). The expression of the Clp machinery is autoregulated, since the CtsR repressor is a substrate of ClpXP (40). However, the CtsR repressor seems to accumulate in an inactive form in CmR-02, an effect which was also seen in a *clpP* deletion mutant (17) and a daptomycin-resistant mutant with a SNP in *clpP* (41), leading to a derepressed transcription of the CtsR/HrcA operon. Several additional characteristics of a *clpP* deletion mutant (17) were also detected in the transcriptome of CmR-02: (i) the derepression of the Fur regulon and the downregulation of the MntR regulon, which are both involved in metal ion homeostasis; (ii) increased expression of the urease operon; and (iii) reduced expression of the ArcR, NreC, and Rex regulons, which are important in anaerobic growth. In addition, the enhanced expression of the *sirABC* and the *clpC* operons was shared with the previously described *clpP* mutant (42). The loss of ClpP function was also reflected on the proteomic level, because proteins of the CtsR/HrcA operon accumulated in CmR-02, and proteins of the purine/pyrimidine metabolism were depleted, which was also described for *clpP* mutants of different genetic backgrounds (43, 44). The transcriptome and the proteome of the revertant 02REV showed only little similarity to the *clpP* mutant and resembled the wild type.

ClpP activity is an important mechanism to regulate gene expression in *S. aureus*, which might be the reason for the occurrence of *clpP* mutations in VISA strains (42, 45). Inactivation of either ClpX (46–48) or ClpP (48–50) were found in either vancomycin or daptomycin, and cefotaxime-resistant Gram-positives. In addition to that, a multitude of SNPs was found in ClpP, conferring mostly glycopeptide, β-lactam, or ADEP resistance (41, 42, 50–52). Bæk et al. (48) revealed that *S. aureus* may under some circumstances benefit from shutting down the Clp proteolytic activity during treatment with antibiotics that target the cell wall, due to the accumulation of ClpP target substrates participating in cell wall metabolism. However, on MIC level, the *clpP* mutation had a lower impact than the *walK* mutation (42), which was also seen for CmR-02 (Table 4). Still, the

combination of the *clpP* and the *walK* mutation had a synergistic effect on glycopeptide/daptomycin resistance, with CmR-02 being more resistant than *S. aureus* WalK^{A243V} (Table 4). Such a strong synergy was not observed for the combination of the same *walK* mutation with the loss-of-function *clpC* mutation in CmR-05 (Table 4).

Three different mutations were identified in the ClpC ATPase gene of different CmR strains, all leading to a complete deletion or an inactivation of the ClpC ATPase (Table 3, Table S4, Fig. 3E). ClpC is important under thermal stress conditions (20) and affects the expression of genes and/or proteins of gluconeogenesis, the pentose-phosphate pathway, pyruvate metabolism, the electron transport chain, nucleotide metabolism, oxidative stress, metal ion homeostasis, stringent response, and programmed cell death (53–56). In addition, ClpCP is responsible for degradation of SsrA-tagged proteins in *S. aureus*, proteins that arise from stalled ribosomes and may contain translation errors (57); however, ClpXP is also capable to degrade SsrA-tagged substrates *in vitro* (15, 52, 58). In *B. subtilis*, ClpXP degrades SsrA-tagged proteins (59). Hitherto, the precise role of ClpC in response to drug exposure and antibiotic resistance is not well defined (60). Although *clpC* mutants were generated and extensively studied (20, 53, 54, 61–64), antibiotic resistant mutants with *clpC* mutations have not yet been described for *S. aureus*. However, in mycobacteria, ClpC1 is the molecular target for the lassopeptide lassomycin and the cyclic peptide cyclomarin A, which activate the ATPase activity, or the proteolysis mediated by the ClpP protease, respectively (65–67). However, these compounds act specifically on the N-terminal domain of mycobacterial ClpC1, while other Gram-positive and Gram-negative bacteria are resistant (65, 66), possibly because of the protective role of ClpC adapter proteins (67). Here, *Mycobacterium smegmatis* showed poor susceptibility toward cervimycin. So far, a direct interaction between cervimycin and ClpC or ClpP similar to the ADEP antibiotics seems unlikely. Although a *clpP* deletion in *S. aureus* conferred low-level CmC resistance, a *clpP* mutant in *B. subtilis* was still susceptible to cervimycin, and cervimycin did not interfere with the degradation of the model substrate eGFP-SsrA in *in vitro* assays using *S. aureus* ClpXP.

Clinically relevant resistance is often built up through multiple steps, each of which contributes to an increase in resistance (68). The loss-of-function mutation in *clpP* or *clpC* conferred only a two- to 4-fold decrease in cervimycin susceptibility (Table 5). Possible additional contributors were the HSP70 DnaK and the histidine kinase WalK. However, the introduction of the *dnaK* mutation in an antibiotic-susceptible background did not alter the cervimycin susceptibility on the MIC level, and *dnaK* was only mutated in one CmR strain.

Depletion of functional ClpP alters cell wall composition, thickness, and cross-linking (48), which are also extensively influenced by the WalK histidine kinase (22). The WalRK two-component system, which comprises WalK and its cognate response regulator WalR, functions in the regulation of peptidoglycan maturation, cell wall turnover, cell separation and protein secretion, as well as biofilm formation, and positively controls global autolytic activity, particularly via AtlA and the LytM endopeptidase (22). Combinations of mutations in *clpP* and *walK* have been described before and were identified after selection for vancomycin- (42) or daptomycin-resistant mutants (41). Shoji et al. (42) described a laboratory-derived VISA (LR5P1-V3) strain with an N-terminal truncation of ClpP and an amino acid deletion in WalK. Interestingly, the very same amino acid deletion was also detected in a cervimycin-resistant mutant (CmR-04: WalKΔQ371). The nucleotide exchanges found in the *walK* gene of the CmR strains affected the HAMP-domain (A243V), the region between the intracellular PAS-domain and the HisKA-domain (ΔQ371), or the HATPase_c-domain (Y549D) of the essential kinase. The *in vitro* activity of these variants was significantly decreased, although the inactivity of WalK^{Y549D} is doubtful, due to the essentiality of the WalK kinase in *S. aureus* (69) and the fact that the activating signal for WalK is still unknown and, therefore, could not be incorporated into the *in vitro* assays. There is a high diversity of SNPs in the *walRK* locus, which were mainly identified in VISA strains or daptomycin-resistant mutants (27, 41, 42, 51, 70–72), but were also induced by the lassopeptides siamycin-I

and streptomonomicin (73, 74), the membrane active compound chlorhexidine (75), or the redox-active antibiotic actinorhodin (76). No clear clustering of mutations in specific functional regions of these genes has been apparent (77). Numerous mutations were already detected in the HAMP-domain of WalK (27, 78, 79), including amino acid exchanges on position 243 (42, 77, 80). In these clinical VISA, the *walK* mutation was frequently accompanied by mutations in other two-component systems, like VraSR or GraSR, that influence biosynthesis and composition of the cell envelope (42, 77, 80). Mutations in the HATPase_c domain of WalK are more likely to occur in laboratory-derived antibiotic resistant *S. aureus* strains (72, 80, 81), but are also seen in clinical isolates (27).

The multitude of SNPs observed across all functional WalRK domains leads to the assumption that WalRK activity is reduced in VISA strains (27, 78, 82, 83). This was supported by the *in vitro* activity tests with the WalK variants from the CmR strains, the increased lysis resistance and increased cell wall thickness. WalK depletion also caused this phenotype (22, 82). However, intermediate vancomycin resistance was also seen with a hybrid promoter that increased *walRK* transcription (84), but a constant WalR activation failed to produce a VISA phenotype (82).

In *walHI* deletion mutants, which exhibit reduced WalRK activity, the expression of *atlA* and *sle1* was decreased (83), which is consistent with the gene expression in CmR-02. Also, the upregulation of *isaA* was seen in another *S. aureus* strain with a mutation in the HAMP-domain of WalK (27). However, most autolysins under the control of WalRK were overexpressed in CmR-02. This contradicts the theory of a decreased WalK activity in CmR-02, but actually an elevated expression of the minor autolysins was also seen for other VISA/glycopeptide-intermediately resistant *Staphylococcus aureus* (GISA) strains (81, 85–87), and these proteins are most probably not involved in cell lysis but in growth and confer plasticity to the cell envelope (21).

Taken together, the activity measurements and phenotype of the CmR strains indicate a reduced WalK activity. However, the identified mutations do not cluster around a possible interaction site between WalK and cervimycin, and tests of WalK activity did not yield consistent results in the presence of cervimycin *in vitro*. The uncoupling of autolysin expression from WalRK through homologous expression of LytM or SsaA was shown to restore cell viability in *S. aureus* (21). However, neither overexpression of these autolysins nor downregulation of WalK did abrogate the bactericidal effect of CmC in *S. aureus* RN4220 (Table 6). Therefore, a direct interaction between cervimycin and the kinase seems unlikely as antibiotic mode of action, in contrast, a carefully regulated activity of WalK seems to support survival in the presence of CmC.

In conclusion, we studied the mode of action of the naphthoquinone antibiotic cervimycin, which is bactericidal and exclusively active against Gram-positive bacteria. A selection for cervimycin-resistant *S. aureus* mutants revealed loss-of-function mutations in the caseinolytic protease gene *clpP* or its cognate Clp ATPase gene *clpC*, and mutations in the essential histidine kinase gene *walK*, eventually leading to a VISA phenotype. However, a direct interaction with cervimycin was neither seen for ClpP nor for WalK, leaving the definitive target of cervimycin to be determined. Omics data of cervimycin treated *S. aureus* might give further insight into what happens inside the cell in response to cervimycin, and whether there are analogies to antibiotics with known mode of action. Nevertheless, the combination of *clp* and *walK* mutations in the cervimycin-resistant mutants might suggest either a target structure associated with growth and cell wall biosynthesis, like lipid II, which is the target of vancomycin, or protection of the target site from cervimycin by a thickened cell envelope. The VISA phenotype, based on the mutations in *walK*, might also have a compensatory effect toward cervimycin activity, either by slowing down growth, or by modulation of the cell wall and central metabolism.

MATERIALS AND METHODS

Construction of cervimycin-resistant mutants. *S. aureus* SG511 Berlin was serially passaged by subculturing the strain in 1/100 dilutions each day in medium supplemented with cervimycin C or D (for

15–28 days). Cervimycin was added at 0.5× MIC (1 μg/mL) and increased 1.5- to 2-fold each day up to 64× MIC. This culture was plated on TSA with 120 μg/mL cervimycin and incubated overnight at 37°C. Ten sibling clones of three different passaging experiments were isolated and three clones (CmR strains, CmR-01 to CmR-09) of each lineage were analyzed thoroughly. Revertants (01REV–09REV) were generated by passaging the CmR strains in the absence of cervimycin for several times.

Genomic DNA was isolated from nine CmR mutants and their respective revertants using the Master Pure Gram-positive DNA purification kit (Epicentre Biotechnologies) with one modification: lysostaphin solution (30 ng/mL) was added during the lysis step. Whole-genome sequencing of the wild-type strain *S. aureus* SG511 (PacBio) was previously described (88). Illumina technology was used for resequencing of the mutant strains, according to Kehl et al. (89). Genomes of all sequenced mutants were assembled *de novo* and were aligned to the parent strain *S. aureus* SG511 Berlin (NZ_CP076660) using the Geneious mapping and assembly tool. SNPs were called on the mapped reads by comparing the mutant genomes with the parent strain using Geneious SNP caller (Geneious R10.2.6; https://www.geneious.com [90]). Mutations were verified via PCR and Sanger sequencing. Bacterial strains and primers used in this study are listed in Tables S2 and S3.

Allelic exchange using the pMAD-system. The pMAD-vector is a temperature-sensitive shuttle vector for markerless allelic replacement in Gram-positive bacteria (91). The pMAD-system was used to introduce SNPs identified in cervimycin-resistant mutants into the genome of *S. aureus* SG511. Fragments of the respective genes (500 bp) were amplified via PCR (primers are listed in Table S3), cloned into the pMAD-vector, transformed into *E. coli* DC10B, and verified via sequencing. The constructs were then transformed into *S. aureus* RN4220, and afterwards transduced into *S. aureus* SG511 or CmR-01 using phage 85. For the allelic exchange, a blue colony of *S. aureus*, containing the respective pMAD-vector, was inoculated into 5 mL brain heart infusion (BHI) broth with 10 μg/mL erythromycin and grown at 30°C. Subsequently, the temperature was shifted to the nonpermissive temperature (42 or 44°C) or a 1:10 dilution was directly plated on BHI agar containing erythromycin and X-gal (300 mg/L) and incubated at the nonpermissive temperature. Then, a blue colony was inoculated into 5 mL BHI without antibiotic and the procedure was repeated. This time the culture was diluted 1:10,000-fold and plated on BHI agar containing only X-gal. The presence of specific mutations in white colonies was verified via PCR and sequencing. Whole-genome sequencing was performed for *S. aureus* WalK^{A243V} and *S. aureus* SG511 ClpP^{I29F} using Illumina technology as described above.

Phenotyping of CmR strains and revertants. The colony morphology of the different *S. aureus* isolates was analyzed after plating of serially diluted stationary cells on Columbia blood agar and overnight incubation at 37°C. The colony size was quantified using the ImageJ Software (92). Growth of *S. aureus* at different temperatures was carried out as described earlier (93). Briefly, TSB was inoculated with 1% of a *S. aureus* overnight preculture and incubated at 37°C until reaching an optical density at 600 nm wavelength (OD_{600}) of 0.5. Serial dilutions (0.9% NaCl, 10-fold dilutions, 10 μL each) of the culture were spotted on TSA plates. The agar plates were incubated at different temperatures until visible growth occurred, usually overnight. Anaerobic growth conditions were generated using the GasPak system (Becton, Dickinson, Heidelberg, Germany).

Electron microscopy. For scanning transmission electron microscopy (STEM) sample preparation, BHI was inoculated with 1% of a *S. aureus* SG511, CmR-02, or 02REV preculture and incubated with aeration at 37°C until reaching an OD_{600} of 1.0. Cells were harvested (8,000 × *g*, 5 min, room temperature (RT)), washed with 1 mL ultrapure water, resuspended in fixation buffer (4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, pH 7.4) and incubated at 4°C overnight. After fixation, bacteria were washed with cacodylate buffer before treatment with osmium (1% osmium tetroxide, 0.8% ferricyanate in 0.1 M cacodylate buffer) for 2 h at room temperature. Bacteria were pelleted and dehydrated in ethanol. During dehydration, at 70% ethanol, 0.5% uranyl acetate was added for 1 h. After dehydration in ethanol, samples were incubated in propylene oxide before embedding in EPON. Ultrathin sections were collected onto Formvar/Carbon coated copper slot grids, and counter stained with uranyl acetate and lead citrate. Imaging was done using a Zeiss Crossbeam 550 at 30 kV with STEM detector.

Firefly luciferase assay. Construction and validation of whole-cell antibiotic biosensors was reported earlier (12). The assay allows a high-throughput diagnosis of antibiotic interference in the major metabolic pathways of bacteria and was performed as described previously (94). Briefly, the promoter and upstream regions of certain genes induced selectively by reference antibiotics were fused to the firefly luciferase reporter gene in *B. subtilis* 1S34. Specifically, the promoter regions of *ypuA*, *lial*, *bmrC* (*yhel*), *helD*, and *yorB* signaled cell membrane stress, interference with the undecaprenyl-phosphate cycle, translation stalling, inhibition of RNA synthesis, and interference with DNA synthesis or structure, respectively. Overnight cultures grown in LB medium with 5 μg/mL erythromycin at 37°C with shaking were diluted to an OD_{600} of 0.05 in LB or BMM (*bmrC*) with 5 μg/mL erythromycin and were grown as before to an OD_{600} of approximately 0.8 or 0.4 (*bmrC*), respectively. Serial 2-fold dilutions of cervimycin C (0.002–4 μg/mL) were inoculated with suspensions of the reporter strains in white 96-well flat-bottom polystyrene microtiter plates and incubated at 37°C for different periods of time depending on the induction kinetics of the respective strain (12). Subsequently, flash luminescence was measured in a Tecan M200 microtiter plate reader after injecting 60 μL of a 2 mM luciferin solution.

Antimicrobial susceptibility testing. Determination of MICs was performed in polystyrene round-bottom microtiter plates (Greiner, Frickenhausen, Germany) using cation-adjusted Müller Hinton (MH) broth or BHI broth. An inoculum of 5×10^5 CFU/mL was employed in the arithmetic broth microdilution method. Due to the high mutation rate, only 1×10^5 CFU/mL were used for ADEP MICs. For dilution of *Mycobacterium smegmatis*, 0.05% Tween 80 was added to the MH broth. For MIC testing of vancomycin and daptomycin (Vancomycin CP LILLY, Lilly GmbH, Bad Homburg, Germany; Cubicin, Novartis Pharma

GmbH, Nürnberg, Germany), $CaCl_2$ was added to all cultures to a final concentration of 1.25 mM (51). The MIC was defined as the lowest concentration of the antibiotic that inhibited visible growth after 24 h of incubation at 37°C. Susceptibility testing of *S. aureus* strains against vancomycin was performed on BHI gradient agar supplemented with 1.25 mM $CaCl_2$. Material of several colonies was resuspended to an optical density at 600 nm wavelength of 0.1 in 0.9% NaCl and the gradient agar plates were inoculated using a cotton swab.

Antisense-based susceptibility profiling. The vector-based antisense clones were created in *S. aureus* RN4220 by Forsyth et al. (95) and provided by Merck (USA). The pEPSA5 vector is xylose-inducible and selected by 34 μg/mL chloramphenicol. Antisense strains were inoculated into LB broth and grown at 37°C without shaking. Next, 20 μL of the cell suspension were diluted in 4 mL 0.9% potassium chloride, poured on LB agar with chloramphenicol and xylose, allowed to settle for 2 min, and surplus suspension was removed. The inocula of antisense strains and the empty vector control were adjusted to yield similar inhibition zones with the control antibiotics. Antibiotic discs with 5 μg CIP, 30 μg VA, and 25 μg cervimycin were added. Inhibition zones were measured after overnight incubation at 37°C. The same procedure was used to measure the effect of protein-overexpression on antibiotic susceptibility. The primers used to construct the *lytM*- and *ssaA*-overexpression strains are listed in Table S3. The pEPSA5-ssaA vector was cloned using *S. aureus* VC40 as the template (96), and the SNP present in this strain was repaired using the QuikChange lightning kit (Agilent) and the primers listed in Table S3.

Zymographic analysis of autolysin extracts and Triton X-100-induced autolysis. Zymographic analyses of autolysin extracts were performed according to Gajdiss et al. (97). For Triton X-100-induced lysis experiments, *S. aureus* strains were grown in tryptic soy broth (TSB) with aeration at 37°C. At an OD_{600} of 0.6, the cells were cooled on ice and harvested by centrifugation. The cell pellets were washed once with ultrapure water, resuspended in phosphate-buffered saline (pH 7), and autolysis was induced by the addition of 0.1% Triton X-100. The cells were incubated at 37°C and lysis was observed photometrically at 600 nm every 30 min.

Transcriptomics. For RNA extraction, cultures of *S. aureus* SG511, CmR-02, and 02REV were grown in TSB at 37°C with shaking until an OD_{600} of 1.0 was reached. Next, 20 mL cultures were harvested (5 min, 8,000 $\times$ *g*, RT), the cells were resuspended in 1 mL RNA protect buffer (New England Biolabs, Frankfurt am Main, Germany) and incubated for 5 min at RT. The cells were resuspended in 200 μL Tris-EDTA (TE) buffer with 25 μg lysostaphin and cell lysis was achieved by incubation at 37°C for 30 min. RNA was extracted using the Monarch total RNA miniprep kit (New England Biolabs) following the manufacturer's instructions and stored at −80°C. Quality and quantity of total RNA were determined by agarose gel electrophoresis. Per replicate, a total amount of 400 ng RNA was subjected to rRNA depletion. For rRNA depletion and cDNA library construction, the Illumina Stranded Total RNA Prep Kit and the Ribo-Zero Plus Kit were used according to the manufacturer's instructions. Libraries were sequenced as single-read (101 bp read length) on a NovaSeq 6000 platform (Illumina) at a depth of 10.1 to 24.9 million reads each. Library preparation and sequencing procedures were performed by the same individual and a design aimed to minimize technical batch effects was chosen. RNA sequencing was performed by the Institute for Medical Microbiology (part of the NGS Competence Center NCCT (Tübingen, Germany)) while data management, including data storage of raw data for this project were done by the Quantitative Biology Center (QBiC, Tübingen, Germany).

Sequencing statistics, including the quality per base and adapter content assessment of resulting transcriptome sequencing data were conducted with FastQC v0.11.5 (98). All reads mappings were performed against the reference strain of *S. aureus* SG511 Berlin (RefSeq ID NZ_CP076660.1). The mappings of all samples were conducted with HISAT2 v2.1.0 (99). Spliced alignment of reads was disabled and library type was set to reverse (HISAT2 parameter –no-spliced-alignment and –rna-strandness R). The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.9 (100). Mapping statistics, including percentage of mapped reads and fraction exonic region coverage, were conducted with the RNA-Seq module of QualiMap2 v2.2.2-dev (101). Gene counts for all samples were computed with featureCounts v1.6.4 (102) based on the annotation of the respective reference genome, where the selected feature type was set to transcript records (featureCounts parameter -t transcript). To assess variability of the replicates of each condition, a principal-component analysis (PCA) was conducted with the DESeq2 package v1.20.0 (103).

For the computation of genes differentially expressed between the wild-type strain and the strains *S. aureus* SG511, CmR-02, and 02REV, respectively, DESeq2 v1.20.0 (103) was applied to the absolute gene counts as computed with featureCounts. Genes with low counts (less than 10 reads) over all replicates in both media were filtered prior to differential expression analysis. For differences between each strain, genes with an adjusted *P*-value (false discovery rate [FDR]) $<$ 0.05 and absolute $\log_2$ fold change (FC) $\geq$ 1 were reported as differentially expressed.

Proteomics. Total protein extraction was performed from *S. aureus* cultures in TSB using the same cultivation conditions as for total RNA extraction. Cells were harvested (6,000 $\times$ *g*, 10 min, 4°C) and washed with 1 mL ice-cold ultrapure water. The cells were resuspended in 1 mL SDS buffer (4% wt/vol SDS, in 100 mM Tris-HCl, pH 8.0), incubated at 95°C for 10 min, chilled on ice for 5 min, transferred into bead beating tubes with 0.1 mm glass beads (Carl Roth GmbH, Karlsruhe, Germany), and disrupted via bead beating (3 $\times$ 30 s, 5,000 rpm with pauses of 90 s). Cell debris was removed via centrifugation (11,000 $\times$ *g*, 5 min, 4°C) and the supernatant was treated with 10 mM DTT (45 min, 650 rpm, RT) and 5.5 mM iodoacetamide (45 min, 650 rpm, RT). Debris was removed (12,000 $\times$ *g*, 15 min, RT) and proteins were precipitated with eight volumes of ice-cold acetone/methanol (8:1 vol/vol mixture) at −20°C overnight. Precipitated proteins were harvested by centrifugation (13,000 $\times$ *g*, 10 min, RT) and washed 3 times with 1 mL 80% aqueous acetone. Residual acetone was evaporated, protein pellets were resuspended in denaturation buffer (6 M

urea, 2 M thiourea in 10 mM Tris-HCl, pH 7.5), and protein concentration was determined using the Bradford method. Ten micrograms of proteins per sample were purified on a NuPAGE 12% gel (Invitrogen) and Coomassie-stained gel pieces were digested in gel with trypsin (104). Desalted peptide mixtures (105) were separated on an Easy-nLC 1200 system coupled to a quadrupole Orbitrap Exploris 480 mass spectrometer (all Thermo Fisher Scientific) as described previously (106) with slight modifications: peptides were separated using a 57-min segmented gradient from 10% to 33, 50, and 90% of HPLC solvent B (80% acetonitrile in 0.1% formic acid) in HPLC solvent A (0.1% formic acid) at a flow rate of 200 nl/min. The mass spectrometer was operated in data-dependent mode, collecting MS spectra in the Orbitrap mass analyzer (60,000 resolution, 300–1,750 *m/z* range) with an automatic gain control (AGC) set to standard and a maximum ion injection time set to automatic. The 20 most intense precursor ions were sequentially fragmented with a normalized collision energy of 28 in each scan cycle using higher energy collisional dissociation (HCD) fragmentation. In all measurements, sequenced precursor masses were excluded from further selection for 30 s. MS/MS spectra were recorded with a resolution of 15,000, whereby fill time was set to automatic.

Acquired MS spectra were processed with MaxQuant software package v1.6.14.0 (107) with integrated Andromeda search engine (108). Database search was performed against a *Staphylococcus aureus* database obtained from Uniprot (https://www.uniprot.org/; 2.889 protein entries), and 286 commonly observed contaminants. Endoprotease trypsin was defined as protease with a maximum of two missed cleavages. Oxidation of methionine, and protein N-terminal acetylation were specified as variable modifications. Carbamidomethylation on cysteine was set as fixed modification. Initial maximum allowed mass tolerance was set to 4.5 ppm for precursor ions and 20 ppm for fragment ions. Peptide, protein and modification site identifications were reported at a FDR of 0.01, estimated by the target-decoy approach (109). The Intensity Based Absolute Quantification (iBAQ) and Label-Free Quantification (LFQ) algorithms were enabled, as was the "match between runs" option (110, 111). The generated data were analyzed using the LFQ-Analyst (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/).

Cloning, overexpression, and purification of *S. aureus* WalK and ClpXP. Cloning and purification of the full-length WalK histidine kinase (112, 113) and the ClpX ATPase (114) was described before. WalK- and ClpP variants (listed in Table 3) were created using the same protocol and the primers listed in Table S3. Shortly, the genes of interest were cloned into pET22bΔpelB, transformed into *E. coli* C43 (DE3) pREP4groESL(MT) for expression of membrane proteins, or *E. coli* BL21 LOBSTR (115) for expression of cytoplasmic proteins, and overexpressed overnight at 30°C. Cells were harvested, lysed by ultrasonication, and membrane proteins were removed from the membrane using n-dodecyl-β-D-maltoside (DDM). Proteins were purified via Ni-NTA affinity chromatography. The WalK proteins were dialyzed (50 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 200 mM KCl, 50% [vol/vol] glycerol, pH 8) using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific), supplemented with glycerol to a final concentration of 50% and stored at −20°C. ClpX and ClpP were dialyzed in sodium phosphate buffer (25 mM sodium phosphate pH 8.0, 100 mM KCl, 5% glycerol [vol/vol]).

ClpP activity assays. The ClpP peptidase activity, ClpP FITC-casein, and SaClpXP assays were performed in triplicates as previously described (52). For the ClpP peptidase activity assay, ClpP (final concentration 2 μM) was preincubated in assay buffer (100 mM HEPES, pH 7.0, 100 mM NaCl) at 32°C. Then, 50 μL Suc-Leu-Tyr-7-amino-4-methylcoumarin (Suc-LY-AMC) was added to a final concentration of 200 μM and a total volume of 100 μL. The reaction was measured every minute using an Infinite M200Pro plate reader (Tecan), following the increase of fluorescence (excitation, 380 nm; emission, 440 nm). Fluorescence was plotted over time and slopes of the initial segment of the fluorescence-time plot were calculated via linear regression.

The ClpP FITC-casein assay was performed in black flat-bottomed 96-well plates (Sarstedt). Next, 1 μL ADEP 2 (final concentration 0 to 10 μM) was incubated with 49 μL ClpP (final concentration 1 μM) in assay buffer at 37°C. FITC-casein was added to a final concentration of 30 μM and a total volume of 100 μL. The reaction was measured using an infinite M200Pro plate reader (Tecan), following the increase of fluorescence (excitation, 494 nm; emission, 521 nm). The initial 300 s were used for data analysis. A casein solution without ClpP served as reference.

The SaClpXP assay was performed in PZ buffer (PZ: 25 mM HEPES, 200 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10% [vol/vol] glycerol, pH 7.6) in a white flat-bottom well plate (Greiner, Frickenhausen, Germany) with a 100 μL reaction volume at 30°C. The reaction contained 2.8 μM ClpP, 2.4 μM ClpX, 0.36 μM eGFP-SsrA, and an ATP regeneration system (4 mM ATP, 16 mM creatine phosphate, 20 U/mL creatine phosphokinase). eGFP-SsrA degradation was measured in an Infinite M200 Pro plate reader (Tecan) with an exciting wavelength of 465 nm and a measured emission of 535 nm. eGFP-SsrA unfolding activity was derived as initial slopes in fluorescence time courses.

Detection of WalK phosphorylation by Phos-tag SDS-PAGE or radioactively labeled ATP. Autophosphorylation and phosphorylation detection of WalK was performed in Triton X-100 micelles and via Phos-tag PAGE or in phospholipid liposomes with radioactively labeled ATP as described in Gajdiss et al. (113).

Data availability. The whole-genome sequencing data of the CmR strains, the revertants, and the allelic exchange mutants *S. aureus* $WalK^{A243V}$ and *S. aureus* SG511 $ClpP^{I29F}$ were deposited in NCBI under the BioProject number PRJNA852436. All high-throughput RNA-sequencing data in this publication have been deposited in NCBI's Gene Expression Omnibus (116) and are accessible under accession number GSE206309. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (117) partner repository with the data set identifier PXD034970.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.5 MB.

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4.3 The unusual mode of action of the polyketide glycoside cervimycin C

Natural products represent a rich source of compounds with highly diverse structures and wide-ranging bioactivities and play a pivotal role in drug discovery. Current strategies to find and characterize novel natural products include metagenomic approaches, genome mining, metabolomics, and the exploration of new habitats (Hug *et al*., 2018). Following the latter approach, the antibiotic complex of cervimycins was isolated from the walls of an ancient cave. Cervimycins are polyketide glycosides with a yet unknown mode of action (Herold *et al*., 2005). Polyketides are a group of structurally diverse natural compounds, which is classified by their synthesis via the polyketide pathway (Schneider, 2005), and confer a variety of biological properties, including antibacterial activity (Katz & Donadio, 1993). As described in **Chapter 4.1 (*p*. 39)**, a serial passaging experiment for cervimycin-resistant (**CmR**) *S. aureus* mutants yielded in vancomycin-intermediately resistant strains, with mutations in either the ATPase gene *clpC* or the protease gene *clpP* and the essential kinase gene *walK*. Nevertheless, a direct interaction of cervimycin was neither seen with the Clp protein degradation system, nor with the WalRK two component system (**TCS**), indicating an indirect effect of these mutations. Also, a *Bacillus clpP* deletion mutant exhibited the same growth defects under cervimycin treatment as the *Bacillus* wild type (microscopy performed by Dr. Ursula Steffens). This was underlined by the globally altered gene expression in a CmR mutant in comparison to its revertant strain and the wild-type strain. In this paper manuscript, we used a multidisciplinary approach to directly address the effects of cervimycin in bacterial cells, namely *B. subtilis* and *S. aureus*.

Structurally, cervimycins are reminiscent of tetracyclines, but lack the C4 dimethyl-amino group which is essential for the bioactivity of tetracyclines at the ribosome (Nelson, 1998). Also, cervimycins efficiently killed *B. subtilis* and *S. aureus*, while tetracyclines are bacteriostatic. In addition to that, the sugar moieties, which are not present in the tetracyclines, seem to play a key role in the mode of action of cervimycin (Herold, 2005).

Microscopy studies with cervimycin treated *B. subtilis* revealed a cell division and chromosome segregation defect, with filamentous, curved cells, and ghost cells devoid of DNA. Cervimycin treatment also disturbed multiple processes in *S. aureus*, indicated by unevenly separated cells, misshapen cells and septa, possibly due to a chromosome segregation defect, and thickened cell walls with a rough cell surface. Taking these phenotypes together with a previously observed inhibitory effect on thymidine incorporation into the DNA (Herold, 2005),

a DNA associated target structure was taken into account. Interestingly, cervimycin acted synergistically with the down-regulation of the B subunit of the DNA gyrase and high cervimycin concentrations inhibited the DNA gyrase supercoiling activity *in vitro*. However, the cervimycin concentrations that resulted in gyrase inhibition were many times higher than the MIC and the inhibitory concentrations of characterized gyrase inhibitors. Also, CmR *S. aureus* mutants displayed no mutations in the topoisomerase genes and the DNA stress response was not induced by cervimycin treatment.

In contrast, the most differentially expressed genes in cervimycin treated *S. aureus* included the CtsR/HrcA heat shock operon and most autolysin genes. Interestingly, the essential WalRK TCS plays a central role in the regulation of autolysin expression in *S. aureus* and was a mutational hotspot in the CmR strains. Nevertheless, cell lysis was impaired rather than induced by the addition of cervimycin and a cell wall stress response was not observed in the omics analysis. In fact, the cervimycin stress response was reminiscent of another class of antibiotics, namely the mistranslation inducing aminoglycosides, like streptomycin, again opening the ribosome as possible target site. Aminoglycosides are the only bactericidal ribosome-targeting agents which is thought to rely on membrane damage caused by misread membrane proteins (Davis *et al*., 1986). Thus, whether and how cervimycin interacts with the ribosome will be addressed in future studies.

The unusual mode of action of the polyketide glycoside cervimycin C

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Running Head: Cervimycins disturb multiple cellular processes

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antibiotic · polyketide · gyrase · chromosome · septum · mode of action · transcriptome · proteome · ribosome

Abstract

Cervimycins A–D are bi-glycosylated polyketides produced by *Streptomyces tendae* HKI 0179 with bactericidal activity against Gram-positive bacteria. Microscopically, cervimycin C (CmC) treatment caused a spaghetti-like phenotype in *Bacillus subtilis* 168, with elongated curved cells which stayed joined after cell division and exhibited a chromosome segregation defect, resulting in ghost cells without DNA. Electron microscopy of cervimycin treated *S. aureus* revealed swelling of some cells, misshapen septa, cell wall thickening, and a rough cell wall surface. Incorporation tests in *B. subtilis* indicated an effect on the DNA metabolism at high cervimycin concentrations. Indeed, the down-regulation of the B subunit of the DNA gyrase (*gyrB*) acted synergistically with cervimycin and, in high concentrations, the antibiotic inhibited the DNA gyrase supercoiling activity *in vitro*. To obtain a more global view on the mode of action of CmC, transcriptomics and proteomics of cervimycin treated versus untreated *S. aureus* cells were performed. Interestingly, cervimycin did not induce the SOS response in *S. aureus*, which would indicate disturbance of the DNA gyrase *in vivo* comparable to fluoroquinolones. Instead, cervimycin induced the expression of the CtsR/HrcA heat shock operon and the expression of autolysins, exhibiting similarity to the ribosome-targeting aminoglycoside gentamicin. In summary, we identified the DNA gyrase as one target of cervimycin, but electron microscopy and omics data revealed a more complex mode of action. Cervimycin induced the protein stress response and the expression of cell wall hydrolyzing enzymes, similar to gentamicin, characterizing cervimycin as a possibly novel class of protein biosynthesis interfering agent.

Importance

Antibiotic resistance of Gram-positive bacteria is an emerging problem in hospital and community surroundings, resulting in a pressing need to find new antibiotics with novel modes of action. Secondary metabolites from *Streptomyces* species are known as a source of diverse natural products, like the antibiotic complex of cervimycins produced by *Streptomyces tendae* HKI 0179. By characterizing the phenotypic response of *Bacillus subtilis* and *Staphylococcus aureus* towards cervimycin, a chromosome-segregation and septum-formation defect became obvious. This effect, which was seen at high antibiotic concentrations, was at first attributed of an interaction between cervimycin and the DNA gyrase. However, omics data

of cervimycin treated versus untreated *S. aureus* cells indicated a different mode of action, because the extensive stress response did not include the SOS response, but resembled the response towards ribosome-targeting agents like gentamicin or puromycin.

Introduction

The progressive spread of infections caused by antibiotic resistant bacteria represents a serious health threat worldwide. Hence, new antibacterial agents are urgently needed, preferably with novel resistance breaking modes of action. Especially methicillin-resistant *Staphylococcus aureus* (MRSA) strains and vancomycin-resistant enterococci (VRE) belong to a group of high priority pathogens with urgent need for new antibiotics (WHO, 2021). In this regard, *Streptomyces* species are a promising origin of secondary metabolites including anti-MRSA drugs (Kemung *et al*., 2018).

The cervimycins are produced by *Streptomyces tendae* HKI 0179 – and show potent activity against Gram-positive pathogens, including VRE and MRSA (Herold *et al*., 2005). They are polyketide glycosides that are highly substituted with six sugar moieties and the components of the antibiotic complex differ in their substitution patterns (Herold *et al*., 2005). Polyketides find application in numerous clinical and agricultural applications, e.g., the immunosuppressants FK506 and rapamycin, antitumor agents like mithramycin, and antibiotics like erythromycin, tylosin, rifamycin, and the tetracyclines (McDaniel *et al*., 2005).

The anthracyclines dutomycin (Xuan *et al*., 1992) and polyketomycin (Momose *et al*., 1998) are structural homologs of cervimycin. Although DNA intercalation was thought to be the primary mechanism of action of anthracyclines in general, drug interference with the function of DNA topoisomerase II was attributed to doxorubicin and closely related anthracyclines (Pommier, 2013). A DNA intercalation activity was not seen for cervimycins and only very high cervimycin concentrations inhibited the incorporation of radioactively labeled precursors into the DNA (Herold, 2005).

The selection for cervimycin C-resistant *B. subtilis* yielded an efflux-based resistance mechanism based on the overexpression of the BmrA ABC transporter (Krügel *et al*., 2010). A selection for cervimycin C- and D-resistant *S. aureus* strains resulted in vancomycin-intermediately resistant strains with loss-of-function mutations in the caseinolytic protease gene *clpP* or its cognate Clp ATPase gene *clpC* and mutations in the essential histidine kinase gene *walK* (Dietrich *et al*., 2022). ClpP is the proteolytic core of the caseinolytic protease and possesses a key function in protein homeostasis, generally maintaining protein quality and tightly controlling key regulatory proteins (Brötz-Oesterhelt & Sass, 2014), affecting a large

number of processes. Cognate Clp ATPases control the proteolytic activity of ClpP, in *S. aureus* ClpX and ClpC, whereby ClpC is needed under thermal stress conditions and for the modulation of respiratory growth (Frees *et al*., 2004; Mashruwala *et al*., 2019). The WalK kinase is part of a two-component system, together with response regulator WalR, that functions in the regulation of peptidoglycan maturation, cell wall turnover, cell separation and protein secretion, as well as biofilm formation, and positively controls global autolytic activity, particularly via AtlA and the LytM endopeptidase (Dubrac *et al*., 2007). However, the alterations in *clpCP* and *walK* might exert compensatory effects, as a direct interaction with cervimycin was neither seen for ClpP nor for WalK, leaving the definitive target of cervimycin to be determined (Dietrich *et al*., 2022).

Interestingly, the WalK kinase shares a common ATP-binding domain – the so-called Bergerat fold – with the DNA topoisomerase II, one target of the above-mentioned doxorubicin (Pommier *et al*., 2016). This ATP-binding motif was first described in type II topoisomerases (Bergerat *et al*., 1997) and is found in the DNA gyrase, heat shock protein 90, histidine kinases, MutL mismatch repair protein (GHKL) superfamily.

Having this in mind, we continued the search for the target of cervimycin. Initial results indeed suggested the DNA gyrase as target of cervimycin. Cervimycin treated *Bacillus* exhibited a chromosome condensation and segregation defect, the down-regulation of the DNA gyrase B subunit acted synergistically with cervimycin, and the supercoiling activity was inhibited *in vitro*. However, the high cervimycin concentrations needed for gyrase inhibition together with the generated omics data pointed towards a different antibiotic target, the ribosome.

Results

Cervimycin C is a bactericidal antibiotic

Cervimycin C (CmC) kills Gram-positive bacteria, since colony-forming units (CFU) decreased upon cervimycin treatment, without recovery even after over-night incubation (Figure 9). The bactericidal activity of cervimycin was restricted to Gram-positive bacteria.

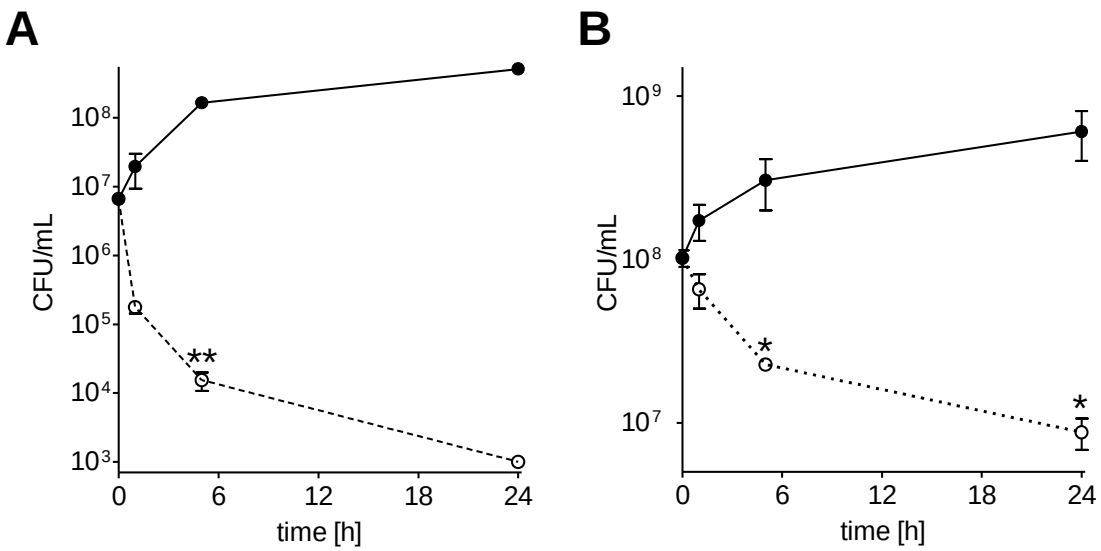


Figure 9: Cervimycin C (CmC) kills Gram-positive bacteria. Exponentially growing *Bacillus subtilis* 168 (**A**) or *S. aureus* SG511 Berlin (**B**) were treated with 6× MIC cervimycin C, respectively, or the same volume of DMSO (untreated), and dilutions of the cultures were plated on TSA after the indicated period of time; *, $P \leq 0.0366$; **, $P \leq 0.0055$; CFU, colony forming units.

Cervimycin C treatment leads to ghost cell formation in *B. subtilis*

For further insight into the effect of CmC *in vivo*, *B. subtilis* 168 was grown in presence and in absence of 8× MIC CmC and visualized by fluorescence microscopy. Cells were stained with DAPI (DNA) and Nile red (membrane) (Figure 10).

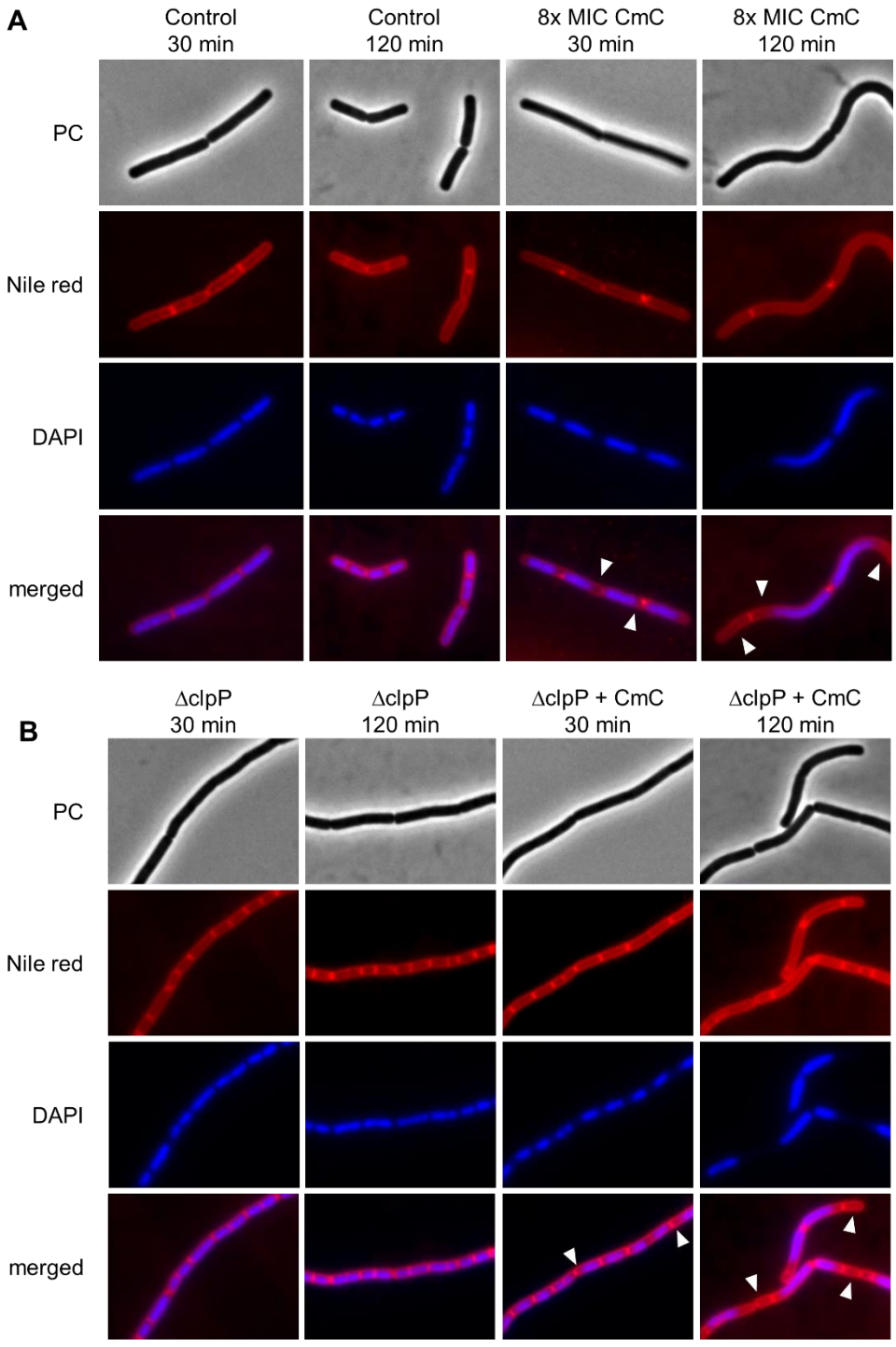


Figure 10: Fluorescence microscopy of cervimycin treated *Bacillus subtilis* 168 revealed a filamentation phenotype in combination with a chromosome segregation defect (microscopy performed by Dr. Ursula Steffens). (**A**) *B. subtilis* was grown in LB medium until early exponential growth phase and treated with 8× MIC CmC or the same volume of DMSO (solvent control). At indicated time-points, DNA was visualized with DAPI and the membrane was stained with Nile red. (**B**) A *B. subtilis clpP* deletion mutant exhibited the same chromosome segregation defect under CmC treatment as the wild type, but filamentation also occurred in the solvent control. PC, phase contrast; arrowheads, atypical partitioning of chromosome.

Cervimycin treatment caused a spaghetti-like phenotype in *B. subtilis*, with elongated curved cells which stayed joined after cell division. In addition, a chromosome condensation and a segregation defect occurred. The atypical partitioning of the chromosome caused 'ghost cells' devoid of DNA, especially after prolonged incubation (Figure 10: arrowheads).

Because the characterization of cervimycin-resistant *S. aureus* strains (CmR strains) had revealed loss of ClpC or ClpP activity (Dietrich *et al*., 2022), a *Bacillus clpP* mutant was also tested. Here, chromosome partitioning was still disturbed (Figure 10B), but the filamentation phenotype of the *clpP* mutant could not be attributed to cervimycin treatment, as filamentation was also observed in the DMSO treated control. This effect has been described before and is thought to rely on accumulation of protein substrates of ClpP involved in cell morphology or cell division (Gerth *et al*., 1998).

Cervimycin treatment disturbs septum formation in *S. aureus*

Electron microscopy of cervimycin treated *S. aureus* cells confirmed the cell division defect seen in *Bacillus* and revealed cell wall thickening after the treatment with cervimycin (Figure 11).

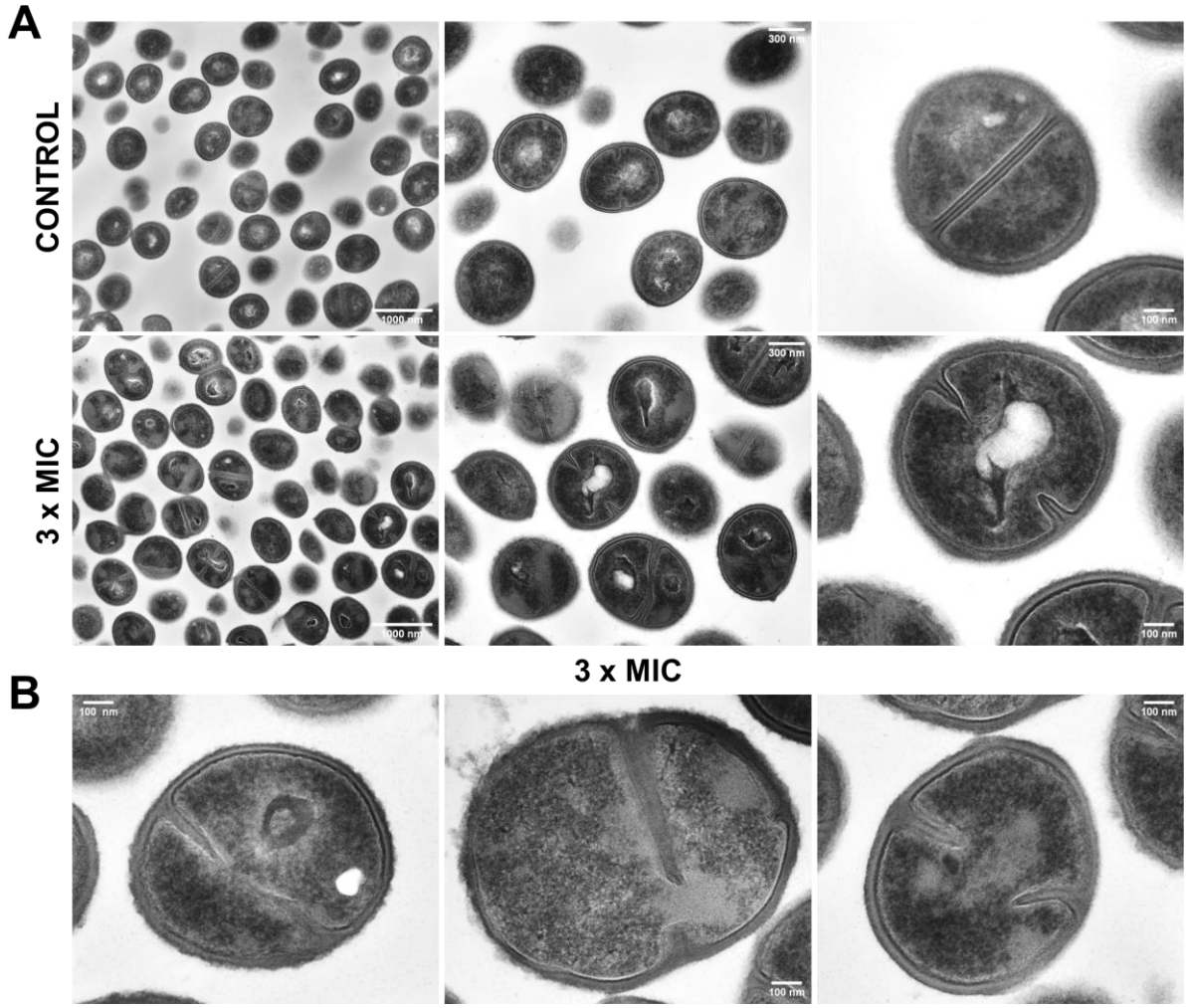


Figure 11: Scanning transmission electron microscopy (STEM) of *S. aureus* SG511 Berlin exposed to 3× MIC cervimycin C. (**A**) Comparison at different magnifications between unexposed (upper images) and exposed samples (3× MIC CmC, lower images). Note the regularly rounded shape of the control cells and septa compared with the irregular morphology of the exposed cells; scale bars: left 1000 nm, middle 300 nm, right 100 nm. (**B**) Close-ups of damaged cells exposed to 3× MIC CmC. Note the misshapen septa, the thickening of the cell wall, and the rough cell wall surface; scale bars: 100 nm.

Cervimycin treatment led to extensive defects in *S. aureus* cell morphology, causing irregularly shaped cells, including cell swelling, uneven separation of daughter cells, and sometimes a D shape after daughter cell separation was observed (Figure 11). Second, the cell wall was significantly thickened (control: 34.91±5.93 nm, 3× MIC CmC: 46.06±8.23 nm, Figure S1) and the cell surface seemed rougher under cervimycin treatment, indicating an imbalance between cell wall synthesis and cell wall hydrolysis. Third, various aspects of septum formation were disturbed under cervimycin treatment. Septa of irregular length, thickened septa, and thinning in the upper part of septa were visible. In some cases, septa were also bent, in contrast to the straight septa observed in the control. This effect might be caused by the presence of the

nucleoid which was still located between the ingrowing septa and the chromosome segregation effect observed in *Bacillus* supports this hypothesis.

Influence of cervimycin on proteins involved in cell wall growth and chromosome segregation

Localization and activity of cell wall hydrolases in *S. aureus* are mediated by teichoic acids (Biswas *et al*., 2012; Schlag *et al*., 2010). Targeting of the teichoic acid biosynthesis would explain the restriction of antibacterial activity of cervimycin to Gram-positive bacteria and the disturbance of autolytic activity. In this case, deletion of *tagO*, the initiator of wall teichoic acid biosynthesis, would render *S. aureus* cervimycin-resistant. However, *tagO* deletion only had a minor effect on the cervimycin MIC (Table 3).

Table 3: GyrB but not GyrA depletion sensitizes *S. aureus* to cervimycin. (**A**) Susceptibility of *tagO* and *recU* deletion strains was determined via broth dilution method in Müller Hinton medium after 24 h of incubation. (**B**) Expression-levels of tested genes were decreased by synthesis of antisense RNA using the pEPSA5-system (Forsyth *et al*., 2002). Cervimycin-susceptibility was tested in agar diffusion assays on LB agar in comparison to an empty vector control.

A)	**Strain**	**Description**	**MIC [µg/mL]**
	SA113	Wild type	6
	Δ*tagO*	Glycosyl transferase; initial step in WTA biosynthesis	3
	NCTC 8325-4	Wild type	4–8
	Δ*recU*	Holliday junction-specific endonuclease	4

B)	**Gene**	**Function**	**Xylose [mM] (IC_{50})**	**Fold increase of diameter of inhibition zone**
	gyrA	DNA gyrase subunit A	6.6	1.15
	gyrB	DNA gyrase subunit B	17	1.45
	parC	DNA topoisomerase IV subunit A	7.5	1.15
	parE	DNA topoisomerase IV subunit B	17	1.15
	topA	DNA topoisomerase I	6.6	1.12
	topB	DNA topoisomerase III	10.5	1.00
	divIVA	Cell division initiation protein DivIVA	10.5	0.86

The phenotype of cervimycin treated cells was reminiscent of the growth defect seen in *recU* depleted *S. aureus* cells. RecU is a Holliday junction resolvase, encoded in the same operon as penicillin-binding protein 2, is required for correct chromosome segregation and DNA damage repair, and its absence leads to the formation of cells with septa bisecting the DNA, compact nucleoids, and anucleate cells (Pereira *et al*., 2013). In spite of the similar phenotypic defects caused by cervimycin or by *recU* deletion, the *recU* deletion did not affect cervimycin-susceptibility (Table 3).

High concentrations of cervimycin inhibit *E. coli* and *S. aureus* DNA gyrase

Incorporation tests with radioactively labeled precursors (Herold, 2005) and a weak response of the *B. subtilis yorB* (Urban *et al*., 2007) firefly luciferase reporter strain (Dietrich *et al*., 2022) had indicated that cervimycin might interfere with the DNA metabolism. Taken together with the inhibitory effect of high cervimycin concentrations on the incorporation of radioactive labeled precursors into the DNA (Herold, 2005), DNA topoisomerases might constitute a possible target of cervimycin. DNA topoisomerases are essential enzymes which alter the topology of the DNA during the cell cycle. Topoisomerases can redundantly relax supercoiled DNA, but the DNA gyrase is unique and essential in introducing negative supercoils into the DNA in an ATP-dependent manner (Bates & Maxwell, 2007; Nöllmann *et al*., 2007). Indeed, and in contrast to other topoisomerases, down-regulation of the B subunit of the DNA gyrase (*gyrB*) sensitized *S. aureus* towards cervimycin (Table 3). The down-regulation of other topoisomerases only had a minor effect on cervimycin-susceptibility (Table 3). DNA gyrase inhibitors either bind the A subunit of the enzyme complex, like the fluoroquinolones ciprofloxacin and norfloxacin, leading to double strand breaks and the induction of the SOS response (Cirz *et al*., 2007; Sato *et al*., 1986), or bind the B subunit, like novobiocin, leading to relaxation of the DNA and increased expression of *gyrAB* (Gellert *et al*., 1976; Gilbert & Maxwell, 1994; Schröder *et al*., 2013; Schröder *et al*., 2014). We next tested the DNA gyrase supercoiling activity in absence and presence of cervimycin *in vitro* (Figure 12, Figure S2).

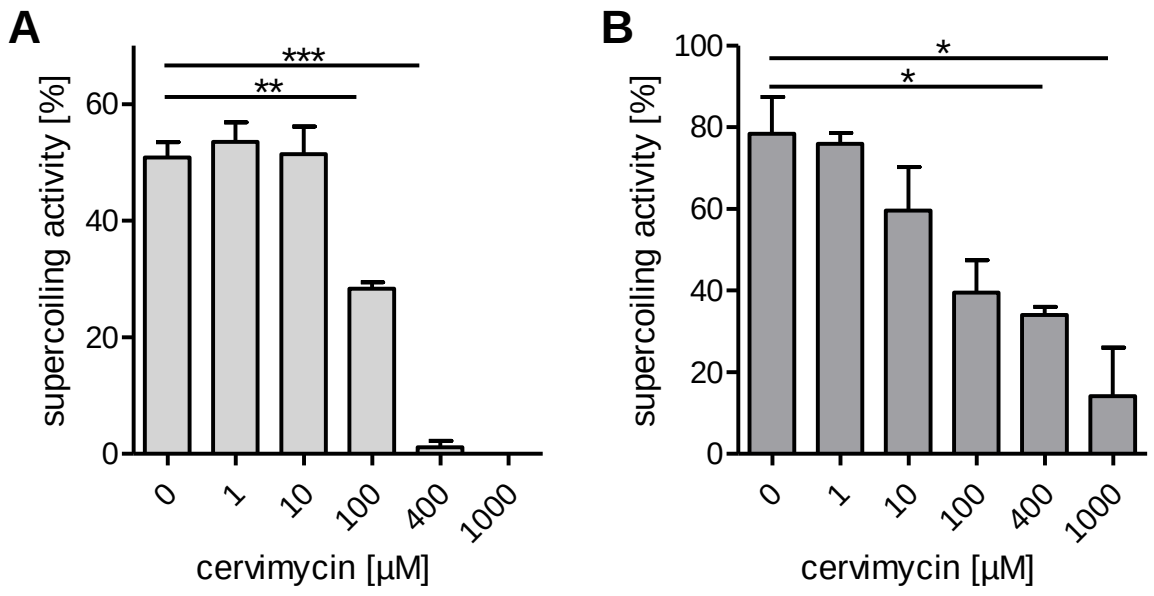


Figure 12: Cervimycin C (CmC) inhibits *S. aureus* and *E. coli* DNA gyrase supercoiling activity in a concentration-dependent manner. (**A**) *S. aureus* and (**B**) *E. coli* DNA gyrase supercoiling activity were tested with increasing concentrations of cervimycin (*, $P \leq 0.05$; **, $P \leq 0.0014$; ***, $P < 0.0001$). Exemplary results of the gel-based activity assays are shown in Figure S3**.** At least 100 µM cervimycin were necessary to significantly decrease the DNA gyrase activity, which corresponds to 125 µg/mL CmC or the 62.5× MIC of *S. aureus* SG511 Berlin.

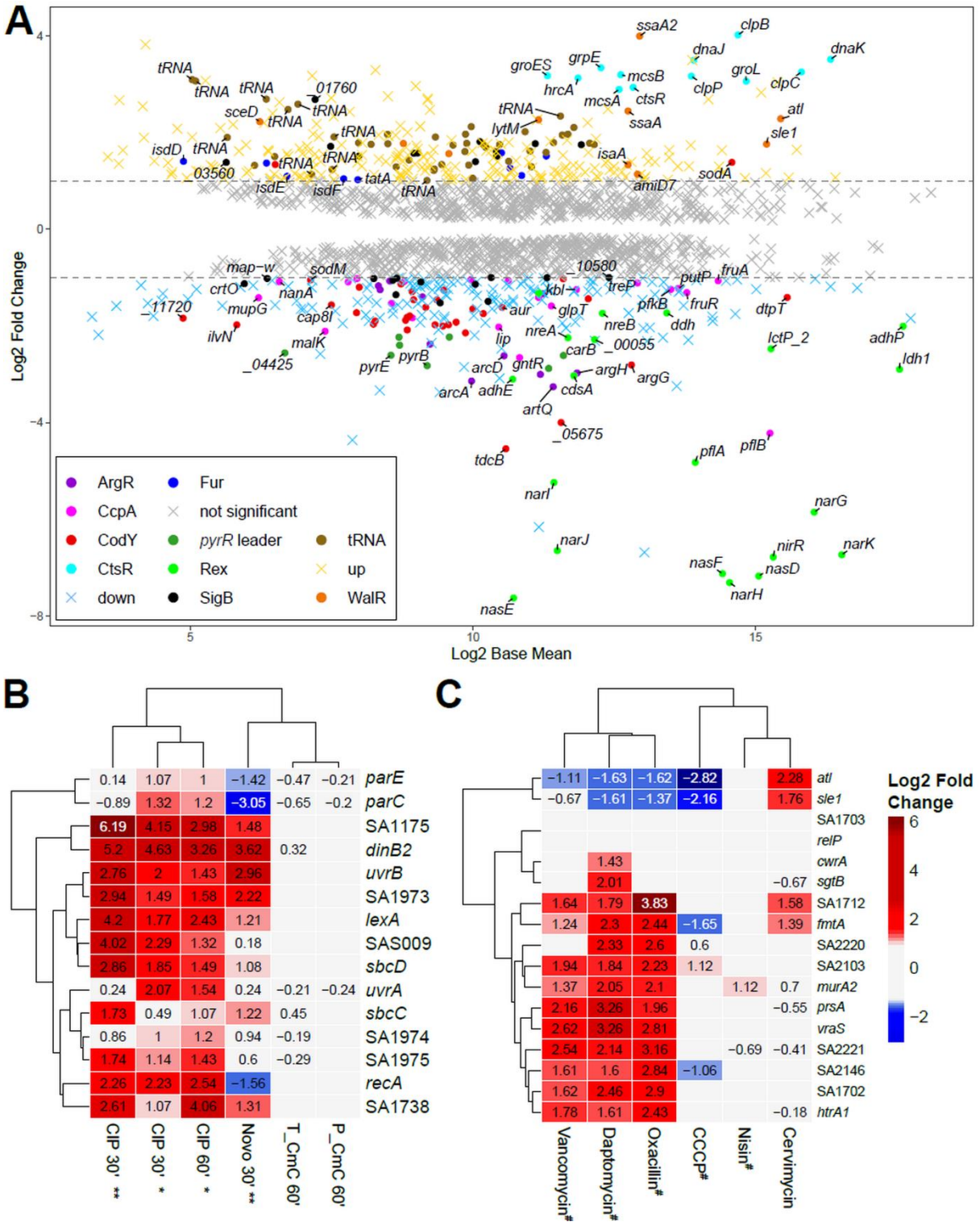
Interestingly, both, *S. aureus* and *E. coli* DNA gyrase were inhibited by high cervimycin concentrations, but *S. aureus* DNA gyrase was more susceptible to inhibition by cervimycin (Figure 12). However, norfloxacin completely abolished *S. aureus* DNA gyrase supercoiling activity at a concentration of 10 µM (corresponding to 6.34 µg/mL or the 25× MIC of *S. aureus* SG511 Berlin) (Figure S2). In contrast, 400 µM cervimycin was needed to achieve this effect (corresponding to 500 µg/mL CmC or the 250× MIC of *S. aureus* SG511 Berlin). Mutations in the DNA gyrase genes *gyrA* and *gyrB* were not detected during the selection of cervimycin-resistant *S. aureus* mutants (Dietrich *et al*., 2022), which contradicts the hypothesis of the DNA gyrase as primary target of cervimycin. We tested two *S. aureus* strains that were resistant towards norfloxacin or novobiocin due to amino acid exchanges in the DNA gyrase. However, these strains did not exhibit cross-resistance towards cervimycin (Table S1), indicating different binding sites of these antibiotics on the DNA gyrase.

Interestingly, GyrB shares the ATP-binding motif with another essential protein, namely the WalK kinase, and cervimycin-resistant *S. aureus* mutants carried single nucleotide polymorphisms (SNPs) in the essential *walK* gene. The WalRK two-component system, which comprises WalK and its cognate response regulator WalR, positively controls global autolytic

activity (Dubrac *et al*., 2007), and mutations in the *wal* locus are often observed in mutants resistant towards the cell envelope-targeting compounds vancomycin or daptomycin (Cameron *et al*., 2015; Howden *et al*., 2011; Kim & Lee, 2020; Müller *et al*., 2018; Peleg *et al*., 2012; Shoji *et al*., 2011; Song *et al*., 2013). Although a direct inhibition of WalK by cervimycin could not be demonstrated in earlier experiments (Dietrich *et al*., 2022), the cell wall thickening (Figure S1) and the rough cell wall surface under cervimycin treatment (Figure 11) might indicate that cervimycin causes cell wall damage.

Omics analyses reveal an extensive response of *S. aureus* towards cervimycin

To obtain a more global view on the effects of cervimycin on the bacterial cell, transcriptomic and proteomic analyses of cervimycin treated versus untreated *S. aureus* SG511 Berlin were performed. We used exponentially growing *S. aureus* cells treated with a growth inhibitory concentration of cervimycin C (3× MIC) for 1 hour. After cervimycin treatment, major alterations occurred on a transcriptomic level (564 differentially expressed genes), while minor differences occurred in the proteome (67 differentially abundant proteins) (Figure 13A, Table S2).


A
Log2 Fold Change
Log2 Base Mean
ArgR
CcpA
CodY
CtsR
down
Fur
not significant
pyrR leader
Rex
SigB
tRNA
up
WalR
B
parE
parC
SA1175
dinB2
uvrB
SA1973
lexA
SAS009
sbcD
uvrA
sbcC
SA1974
SA1975
recA
SA1738
CIP 30' **
CIP 30' *
CIP 60' *
Novo 30' **
T_CmC 60'
P_CmC 60'
C
atl
sle1
SA1703
relP
cwrA
sgtB
SA1712
fmtA
SA2220
SA2103
murA2
prsA
vraS
SA2221
SA2146
SA1702
htrA1
Vancomycin#
Daptomycin#
Oxacillin#
CCCP#
Nisin#
Cervimycin
Log2 Fold Change

▲

Figure 13: RNA-Seq transcriptomics of *S. aureus* SG511 treated with 3× MIC CmC for 60 min. (**A**) M/A-Plot. The expression profile is shown as ratio/intensity scatter plot (M/A-plot, M value $\log_2$ fold change, A value $\log_2$ base mean) which is based on the differential gene expression analysis. Colored symbols indicate significantly induced or repressed transcripts (M value ≥ 1 or ≤ -1; p value ≤ 0.05). Colors refer to the annotated regulator (based on the AureoWiki Database (Fuchs *et al*., 2018)). Regulons with at least eight differentially expressed genes and tRNA genes are color-highlighted. Light grey symbols mark transcripts with similar expression levels in comparison to the untreated *S. aureus* SG511 Berlin (p value > 0.05, $\log_2$ fc ≤ 1 or ≥ -1). Expression profiles of the SOS response genes (**B**) as induced by ciprofloxacin (*Data from Cirz *et al*. (2007), **Data from Jones *et al*. (2017)) and the cell wall stress response genes (**C**) as induced by different cell wall and membrane active compounds ($^{\#}$Data from McAleese *et al*. (2006)) are compared to the response to cervimycin. Up-regulation ($\log_2$ fc $\geq$ 1, red color) and down-regulation ($\log_2$ fc ≤ -1, blue color) on the transcriptomic level are shown.

However, neither induction of the SOS response or elevated *gyrAB* expression as expected from the gyrase inhibition experiments (Figure 13B) nor the cell wall stress response, as observed with β-lactams, vancomycin, or daptomycin, were detected (Figure 13C). Increased expression of the *dnaA* and *dnaD* chromosome replication genes, the *dnaN* DNA polymerase III β subunit gene, the *dinB* DNA polymerase IV gene, and the *nth* endonuclease III gene might indicate some DNA stress induced by cervimycin. On the other hand, several metabolic pathways were down-regulated, like the ArcR, NreC, and Rex regulons which are crucial for growth under anaerobic conditions (Makhlin *et al*., 2007; Pagels *et al*., 2010), the purine biosynthesis genes, parts of the carbohydrate metabolism like the CcpA regulon, the CymR-regulated cysteine metabolism, and major parts of the CodY regulon. Interestingly, the respiratory chain was also partially down-regulated, like the *qox* genes, encoding the quinol oxidase, *cydB*, encoding the cytochrome d ubiquinol oxidase subunit, and *ctaAB*, encoding a heme A synthase, and a protoheme IX farnesyltransferase, respectively. The SigB regulon was partially up- and down-regulated.

Cervimycin induces upregulation of most autolysins

Strikingly, the expression of WalR-regulated autolysins was increased under cervimycin treatment, which is also seen with gentamicin and other protein biosynthesis inhibitors (Figure 14A).

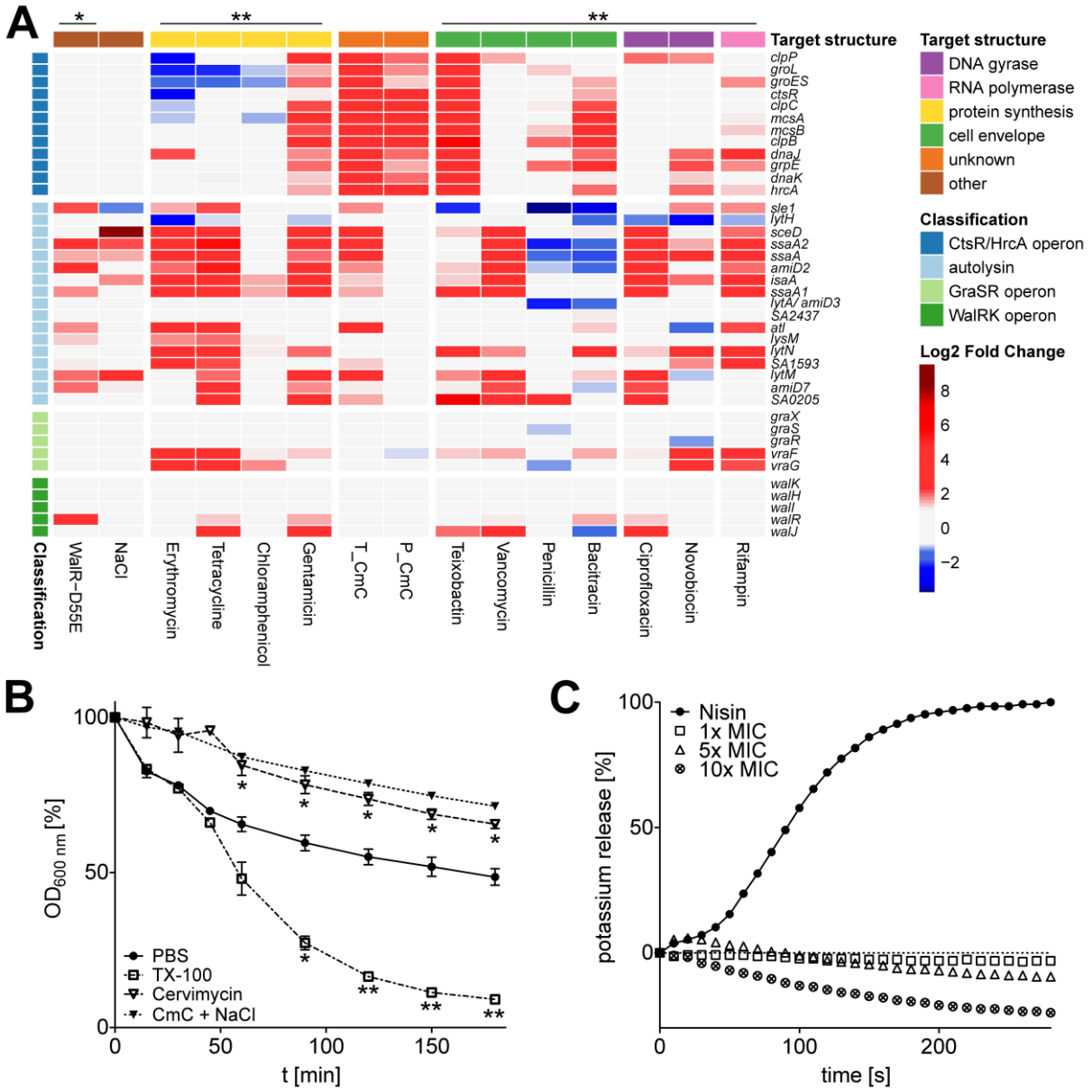


Figure 14: Cervimycin causes a unique induction of the heat shock stress response and autolysin expression without subsequent cell lysis. Cervimycin induced the expression of the CtsR/HrcA operon and of most autolysins (**A**), a response also seen for other antibiotic classes (comparison with data from Delauné *et al*. (2012) (*) and Jones *et al*. (2017) (**). Up-regulation ($\log_2$ fc ≥ 1, red color) and down-regulation ($\log_2$ fc ≤ -1, blue color) on the transcriptomic level are compared; T_CmC cervimycin transcriptome, P_CmC cervimycin proteome. (**B**) Triton X-100 (TX-100) leads to cell lysis in *S. aureus*, significantly reducing the optical density, while cervimycin treatment prevented cell lysis in absence (Cervimycin) and in presence of sodium chloride (CmC + NaCl); *P*: * ≤ 0.0423, **≤0.0059. (**C**) The release of potassium ions by nisin but not by cervimycin confirmed the lack of membrane activity.

Most autolysins in *S. aureus* are positively regulated by the WalRK TCS, which becomes apparent when WalR activity is constitutively activated by the phosphomimetic amino acid exchange D55E (Delauné *et al*. (2012), and Figure 14A). Except for *lysM*, autolysins regulated by the WalR response regulator were up-regulated in the cervimycin treated samples

(Figure 14A). These autolysins were not detected on a proteomic level, possibly due to the extracellular localization of mature autolysins. Interestingly, the addition of potassium chloride also increased autolysin transcription (Figure 14A) and the cervimycin MIC of *S. aureus* SG511 Berlin was decreased by two titer steps when 1 M NaCl was added (2 versus 0.5 µg/mL). Also, the overexpression of WalRK caused cell lysis of *S. aureus* in presence of increasing potassium chloride concentrations (Figure S4). However, two distinct cell lysis experiments confirmed that induction of autolysis is not a primary effect of cervimycin (Figure 14B+C).

Cervimycin induces upregulation of genes indicating protein damage

The modes of action of many antibiotics may be identified by comparing the expression levels of 69 *S. aureus* genes under the treatment with well characterized antibiotics (Jones *et al*., 2017). Testing the cervimycin stress response with this model, confirmed the clustering of well-known substances according to their mode of action (Figure S3). Surprisingly, the cervimycin stress response showed the strongest resemblance to protein biosynthesis inhibitors (Figure S3), but many genes that underwent major transcriptomic alterations due to cervimycin treatment were not included in the 69 gene subset. Comparing the whole transcriptome, cervimycin resembled gentamicin which induces mistranslation at the ribosome in a concentration-dependent manner (Tai & Davis, 1979), causing a strong protein damage response in *S. aureus* as revealed by induction of the CtsR and HrcA operons. Genes for the production of queuosine modified tRNAs and tRNA genes in general (43 of 58 genes, 74%) were also de-repressed by cervimycin treatment (Figure 13A).

Discussion

In this mode of action study, we characterized the effects of the polyketide antibiotic cervimycin C on the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, using a multidisciplinary approach. Both *Bacillus* and *S. aureus* displayed a chromosome and cell division defect after cervimycin treatment. While *B. subtilis* cells grew in filaments and chromosomes were condensed and inaccurately distributed, *S. aureus* cells of uneven size and with thickened cell walls occurred.

A filamentation phenotype is quite common in regulatory *Bacillus* mutants, like the *sigD* or *sin* mutant (Foster, 1992), or at elevated growth temperatures (48 °C) (Fan, 1970). Despite of a generally decreased autolysin level, the filamentous phenotype of various regulatory mutants could not be correlated to specific changes in the autolysin profile (Foster, 1992). Nevertheless, the heat-induced filamentation of *B. subtilis* was reversed by the addition of *Bacillus* autolysin extracts or lysozyme (Fan, 1970). Thus, the filamentation phenotype of cervimycin treated

Bacillus might indicate missing autolysin activity, which fits well to the cell wall thickening observed in *S. aureus*. The cell separation defect of the *Bacillus clpP* deletion mutant is not unicausal, but at least partially attributed to the accumulation of the ClpP substrate MurAA that catalyzes the first committed step in the peptidoglycan biosynthesis pathway (Kock *et al*., 2004).

In cervimycin treated *S. aureus* septa were misshapen, namely, septa of uneven length, curving, and thinning of the upper part was observed. Furthermore, the septum formation seemed to be impaired by the nucleoid. Chromosome replication and segregation are affected by various conditions, such as the lack of SpoIIIE and FtsK DNA translocases, the XerC or RecA recombinases, the RecU resolvase, or the presence of the gyrase and topoisomerase IV inhibitor nalidixic acid (Veiga & Pinho, 2017). The bacterial DNA gyrase is an essential type II topoisomerase which is involved in key cellular processes, like DNA replication and alteration of the topology of DNA, in particular the induction of negative supercoils that necessitates the cleavage of both DNA strands (Watt & Hickson, 1994). The DNA gyrase is composed of the A subunit, which cleaves and re-ligates the DNA, and the B subunit which is an ATPase. The GyrA binding fluoroquinolones induce the SOS response in *S. aureus*, increasing recombination, DNA-repair, lesion-bypass, and modify transcription of cell-cycle checkpoint proteins (Cirz *et al*., 2007). On the other hand, the GyrB binding aminocoumarin novobiocin leads to increased transcription of the *recF-gyrB-gyrA*, the *rib* and the *ure* operon, and decreased transcription of *arlRS, recA, lukA, hlgC,* and *fnbA* (Schröder *et al*., 2014). Indeed, precursor incorporation tests and *Bacillus* reporter strains had suggested a DNA related target of cervimycin (Dietrich *et al*., 2022; Herold, 2005) and high cervimycin concentrations inhibited the DNA gyrase supercoiling activity *in vitro*, but the cellular response of *S. aureus* towards cervimycin was not similar to the response of other gyrase-targeting agents (Figure 13B).

Interestingly, another polyketide antibiotic produced by *Streptomyces coelicolor* M510 – actinorhodin – displayed strong similarities to cervimycin, since it also inhibited the DNA gyrase with some degree of specificity, caused mutations in the *wal* operon in *S. aureus*, was more effective at a low pH, and only active against Gram-positive bacteria (Mak & Nodwell, 2017). In addition, both, cervimycin and actinorhodin, induced the protein damage response (CtsR/HrcA operon) and a specific DNA stress response (up-regulation of *dinB* and *nth*), but not the LexA-dependent SOS response. The authors concluded that actinorhodin most likely acts through oxidative damage to DNA, proteins, and the cell envelope

(Mak & Nodwell, 2017). However, in contrast to cervimycin, actinorhodin was bacteriostatic and autolysin genes were downregulated under actinorhodin treatment (Mak & Nodwell, 2017), pointing out some major differences between the modes of action of these antibiotics.

Juglone and lapachol, which are naphtoquinones like cervimycin, also cause oxidative stress in *S. aureus* and juglone is thought to additionally block replication and transcription by binding to the DNA (Linzner *et al*., 2020; Wang *et al*., 2016). For highly or fully substituted quinones redox-cycling activity rather than thiol alkylation was seen (Brunmark & Cadenas, 1989). Interestingly, ClpB and McsB from the CtsR operon accumulated in juglone treated *S. aureus* (Wang *et al*., 2016) and lapachol completely de-repressed transcription of the CtsR operon (Linzner *et al*., 2020). However, lapachol further induced oxidative stress regulons (PerR, HypR, QsrR, MhqR) and cell wall/general stress genes (SigB and GraRS regulons) (Linzner *et al*., 2020). Such an oxidative stress response was not seen after cervimycin treatment. The response of the GraS regulon, however, cannot be compared, because *S. aureus* SG511 Berlin harbors a truncated GraS kinase (Sass & Bierbaum, 2009).

The above mentioned *wal* operon encodes the WalRK two-component system, which comprises the WalK kinase and its cognate response regulator WalR, influences peptidoglycan maturation, cell wall turnover, cell separation and protein secretion, and biofilm formation (Dubrac *et al*., 2007). Salamaga *et al*. demonstrated that cell wall biosynthesis and cell wall hydrolysis need to be tightly controlled to allow cell growth (Salamaga *et al*., 2021). An unbalance of these processes, e.g., by down-regulation of WalRK activity or by vancomycin or methicillin treatment, leads to a detrimental cell wall thickening or induces cell lysis, respectively (Salamaga *et al*., 2021). This is underscored by the induction of cell lysis in a potassium chloride treated WalRK-overexpression strain (Figure S4). High WalRK-levels as well as high salt concentrations synergistically increased autolysin transcription (Figure 14A, Figure S4, Feng *et al*. (2022); Price-Whelan *et al*. (2013)) which finally outcompeted cell wall synthesis, leading to cell lysis. The increased autolysin transcription in cervimycin treated cells and the synergy of cervimycin and potassium chloride might have indicated a similar process, but, against our expectations, cervimycin combined with potassium chloride inhibited cell lysis in *S. aureus* (Figure 14). On the other hand, excessive cell wall biosynthesis might occur under cervimycin treatment, as indicated by the thickened cell walls of cervimycin treated *S. aureus*. However, the selection of cervimycin-resistant (CmR) *S. aureus* mutants yielded vancomycin-intermediately *S. aureus* (VISA) strains, which was mainly conferred by mutations in the essential kinase gene *walK* and a consequently decreased WalK activity (Dietrich *et al*., 2022),

conflicting with this hypothesis.

Tan *et al*. investigated the down-stream effects of a WalR mutation (T101M) which decreased WalRK activity. This caused a sensitization towards the TarO-inhibitor tunicamycin because of a limited MupG-dependent cell wall recycling pathway (Tan *et al*., 2022). Revertants harbored an additional mutation in the ATP-lid of WalK (R555C) which is thought to restore the WalRK activity (Tan *et al*., 2022). Interestingly, another suppressor mutation occurred in the protease gene *clpP* (C478T nucleotide exchange), introducing a TAA stop codon and probably leading to a truncation (Tan *et al*., 2022), indicating a link between the Clp system and WalRK. This yet unidentified link seems to be crucial in the CmR mutants that always carried a combination of mutations in the *wal* locus and the *clpP* or *clpC* gene (Dietrich *et al*., 2022).

Senges *et al*. established a proteomic response library of *B. subtilis* covering 91 antibiotics and comparator compounds of different modes of action (Senges *et al*., 2020). They identified marker proteins which specifically accumulate due to the impairment of cellular processes and structures (Senges *et al*., 2020). Tetracyclines inhibit protein biosynthesis and lead to the accumulation of ribosomal proteins like RpsB, RpsF, or RplJ or elongation factor Tu (TufA). In contrast, other antibiotics that target protein biosynthesis, i.e., puromycin, which leads to the premature termination of translation, aminoglycosides, which interfere with ribosomal decoding and proofreading, and acyldepsipeptides, which cause uncontrolled proteolysis by ClpP, elicit the upregulation of the chaperone systems GroEL/GroES and DnaK/DnaJ as well as the proteases ClpC and ClpE in *B. subtilis* (Senges *et al*., 2020). These proteins prevent the aggregation of misfolded proteins, facilitate refolding, or aid in the degradation of dysfunctional proteins and are also abundant under cervimycin treatment (Figure 13A).

In addition to that, cervimycin induced the transcription of eight ribosomal genes and numerous tRNA genes in *S. aureus* (Figure 13A). In a co-culture of *S. aureus* and *Pseudomonas aeruginosa* increased expression of tRNAs and ribosomal genes in *S. aureus* was attributed to a decrease in translation efficiency (Briaud *et al*., 2019). However, stalling of the ribosome by cervimycin was not displayed by the *bmrC* (former: *yheI*) bioreporter strain or the precursor incorporation tests in *B. subtilis* (Dietrich *et al*., 2022; Herold, 2005), and Sanger sequencing of the ribosomal genes of a cervimycin-resistant mutant (CmR-02) revealed no mutations at this site.

Nevertheless, the test system (*bmrC* bioreporter strain and also precursor incorporation tests) would be incapable of detecting aminoglycosides like streptomycin as translation stalling

agents (Urban *et al*., 2007), because they primarily induce misreading at the ribosome (at relatively low concentrations, 2× MIC), while much higher concentrations are needed to inhibit protein biosynthesis *in vivo* (35× MIC) (Goldemberg & Algranati, 1981; Moreau *et al*., 1984). The bactericidal effect of aminoglycosides is thought to rely on faulty membrane proteins causing membrane damage (Davis *et al*., 1986). Under salt stress membrane proteins like the ABC transporter MtsABC are needed to counteract the osmotic stress (Feng *et al*., 2022), which might be an alternative explanation for the synergism of cervimycin and potassium chloride, if cervimycin induces mistranslation at the ribosome in a similar manner.

Electron micrographs of aminoglycoside treated *S. aureus* resemble cervimycin treated cells, as cell walls are thickened and rough, and with amikacin also D shaped cells occurred (Santhana Raj *et al*., 2007), indicating lacking autolysin activity. In dividing cells, Atl localizes to the septal region, allowing separation of the daughter cells, a process which is controlled by teichoic acids (Biswas *et al*., 2006; Schlag *et al*., 2010). Immunogold labeling revealed a spatiotemporal disturbance of Atl localization in tetracycline treated *S. aureus*, where Atl was detected at the old septum but not on the new septal region (Yamada *et al*., 2001). This is thought to rely on the increased O-acetylation of the cell wall in cells treated with protein biosynthesis inhibitors, protecting the cell wall from hydrolysis (Yamada *et al*., 2001). Consequently, *atl* transcription was increased under tetracycline treatment (Ledala *et al*., 2006).

In conclusion, cervimycin treatment caused severe structural alterations in *B. subtilis* and *S. aureus*, with cell division, cell wall, and chromosome segregation defects. Cervimycin decreased the incorporation of radioactive labelled thymidine into the DNA of *B. subtilis* (Herold, 2005), and the DNA gyrase was inhibited by high concentrations of cervimycin. However, omics analysis revealed a dual de-repression of the heat shock response genes and the autolysin genes in *S. aureus*, while the DNA SOS response or the cell wall stress response were not induced. The expression profiles resembled the stress response towards the aminoglycoside antibiotic gentamicin, indicating the ribosome as possible target site which will be included in future studies on the mode of action of cervimycins.

Materials and Methods

Antimicrobial susceptibility testing

Determination of minimal inhibitory concentrations (MICs) was performed in polystyrene round-bottom microtiter plates (Greiner, Frickenhausen, Germany) using cation-adjusted MH broth. An inoculum of 5×10^5 CFU/mL was employed in the arithmetic broth microdilution method. The MIC was defined as the lowest concentration of the antibiotic that inhibited visible

growth after 24 h incubation at 37 °C. Cervimycins C and D were purified and provided by Dr. Kirstin Scherlach and Prof. Dr. Christian Hertweck. Bacterial strains and plasmids used in this study are listed in Table S3. The *recU* deletion strain was provided by Prof. Dr. Mariana Gomes de Pinho.

Time-kill kinetic studies

B. subtilis 168 or *S. aureus* SG511 Berlin were inoculated into fresh Müller Hinton broth and grown at 37 °C under aeration until reaching an OD_{600} of ~0.3. Then, 100 µL aliquots of the culture were transferred into a polystyrene round-bottom microtiter plate and treated with 6× MIC cervimycin (*Bacillus*: 1.5 µg/mL; *S. aureus*: 12 µg/mL) or the same amount of DMSO. At defined time-points (0, 1, 5 and 24 hours after cervimycin treatment) the cells were serially diluted in 0.9 % NaCl and 100 µL were plated on TSA, respectively. Plates were incubated at 37 °C over-night and then the number of colonies was determined.

Antisense-based susceptibility profiling

The vector-based antisense clones were created in *S. aureus* RN4220 by Forsyth *et al*. (Forsyth *et al*., 2002) and provided by Merck (USA). The pEPSA5 vector is xylose-inducible and selected by 34 µg/mL chloramphenicol. Antisense strains were tested as described earlier (Dietrich *et al*., 2022). Briefly, antisense strains were inoculated into LB broth, grown at 37 °C, diluted in 0.9% sodium chloride, poured on LB agar with chloramphenicol and xylose, allowed to settle for 2 min, and surplus suspension was removed. Antibiotic discs were added and inhibition zones were measured after overnight incubation.

Electron microscopy

For scanning transmission electron microscopy (STEM) sample preparation, BHI was inoculated with 1% of an *S. aureus* SG511 Berlin pre-culture and incubated with aeration at 37 °C until reaching an OD_{600} of 0.5. Then, the culture was either treated with 3 × MIC CmC (6 µg/mL) or the same amount of DMSO (solvent control) for 1 hour. The subsequent sample preparation and visualization was performed as described earlier (Dietrich *et al*., 2022). Briefly, cells were harvested and washed, fixated overnight, washed again, pelleted, dehydrated, and embedded in EPON. Ultra-thin sections were collected and imaged with a Zeiss Crossbeam 550 at 30kV with STEM detector.

Fluorescence microscopy

Fluorescence microscopy of cervimycin treated *Bacillus* and the *clpP* mutant were performed by Dr. Ursula Steffens at the Institute of Medical Microbiology, Immunology and Parasitology, Bonn. A pre-culture of *B. subtilis* 168 was grown in LB at 37 °C under shaking over-night,

inoculated into fresh LB medium and grown to an OD_{600} of ~0.2 at 37 °C, transferred to a polystyrene round-bottom microtiter plate (Greiner, Frickenhausen, Germany) and treated with 8× MIC CmC (2 µg/mL) or left untreated. *B. subtilis* 168 Δ*clpP* was grown in presence of 80 µg/mL spectinomycin hydrochloride. Samples of 25 µL were taken at different time points. Cells were stained with 0.25 mg/mL DAPI in order to visualize DNA and 0.01 mg/mL nile red (both Sigma-Aldrich, Taufkirchen, Germany) to visualize the cell membrane and analyzed by phase-contrast or fluorescence microscopy. To this end, 1 µL samples of the stained cells were placed onto a microscope slide covered with a thin layer of 1% agarose and analyzed using a Zeiss Axiovert 200M microscope equipped with a Photometrics CoolSnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany). Image acquisition was performed using the microscope software ZEN2011.

Cloning, overexpression and purification of *S. aureus* DNA Gyrase

The *gyrA* and *gyrB* genes from *S. aureus* SG511 Berlin were amplified using the oligonucleotide primers listed in Table S4, digested with the listed restriction enzymes, cloned into the corresponding sites of the pET19 (*gyrA*, Novagen) and pET21 vectors (*gyrB*, Stratagene), and transformed into *E. coli* JM109, respectively. Plasmid integrity was ensured via Sanger sequencing. *E. coli* BL21 LOBSTR (Andersen *et al*., 2013) was used for over-expression of the proteins and the *E. coli* chaperones GroESL were simultaneously expressed from pREP4groESL(MT) (Türck & Bierbaum, 2012) to facilitate protein folding. *E. coli* BL21 LOBSTR was grown in terrific broth at 30 °C with aeration, protein expression was induced with 1 mM IPTG over-night, and the temperature was lowered to 18 °C for expression of GyrB. Cells were harvested and lysed by ultrasonication. Proteins were purified via Ni-NTA affinity chromatography. GyrA and GyrB were dialyzed (100 mM Tris-HCl pH7.5, 2 mM EDTA, 100 mM NaCl, 2 mM DTT, 10% v/v Glycerol) using Slide-A-Lyzer™ Dialysis Cassettes (Thermo Fisher Scientific, Schwerte, Germany), supplemented with glycerol to a final concentration of 50%, and stored at −20 °C.

Inhibition of DNA gyrase supercoiling activity

E. coli and *S. aureus* DNA gyrase supercoiling activity was tested using the *E. coli* DNA Gyrase Drug Screening Kit (TopoGEN Inc., Port Orange, FL – TG2001G). Purified *E. coli* DNA gyrase was provided in the kit, *S. aureus* DNA gyrase was cloned and expressed using the pET-system as described above. DNA gyrase supercoiling was assayed following the provider's instructions. The assays were performed in either *E. coli* DNA gyrase supercoiling buffer (35 mM TrisHCl, pH 7.5; 24 mM KCl; 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine,

6.5% glycerol, 100 mg/mL BSA, and 1 mM ATP) or *S. aureus* DNA gyrase supercoiling buffer (75 mM TrisHCl, pH 7.5; 30 mM KCl; 7.5 mM $MgCl_2$; 7.5 mM DTT; 75 mg/mL BSA, and 2 mM ATP) (Chiriac *et al*., 2015). For the latter, 900 mM potassium glutamate were added to increase *S. aureus* DNA gyrase activity (Alt *et al*., 2011). The reaction was performed in a volume of 20 µL with 2 U DNA gyrase. Different concentrations of antibiotics or the corresponding amount of solvent were added to the reaction mix and pre-incubated at 37 °C for 10 min. The reaction was started by the addition of 0.125 mg relaxed DNA. The reactions were stopped after 1 h at 37 °C by the addition of 5 µL stop buffer (final concentration: 1% SDS, 5% glycerol, and 0.025% bromophenol blue), extracted with 20 µL chloroform:isoamylalcohol (24:1), briefly vortexed, centrifuged at 13,500 × *g* for 1 min, and the blue aqueous phase was analyzed by gel electrophoresis in a 2% agarose gel in TAE buffer (40 mM Tris-acetate and 0.01 M EDTA pH 8.3). DNA was stained with 0.5 mg/mL ethidium bromide, visualized with UV light, and photo-documented. The strength of the bands was quantified using the Gel Analyzer 2010a image analysis software.

Transcriptomics and proteomics

Cultures of *S. aureus* SG511 Berlin were grown in TSB at 37 °C with aeration until an OD_{600} of 0.5 was reached and treated with 3× MIC cervimycin C or the same volume of DMSO for 1 hour. Salt treated *S. aureus* was grown in TSB supplemented with 1 M NaCl. The subsequent sample processing was performed as described earlier (Dietrich *et al*., 2022), but RNA sequencing was performed by the Genewiz sequencing facility (Leipzig, Germany). Briefly, cells were harvested, and resuspended in RNA protect buffer, lysed with lysostaphin, and RNA was extracted using the Monarch™ Total RNA Miniprep Kit (New England Biolabs, Frankfurt am Main, Germany). Ribosomal RNA depletion, library preparation, and strand specific total RNA sequencing on an Illumina NovaSeq platform (2x150 bp sequencing, 10 M read pairs) were performed by the Genewiz sequencing facility.

For protein extraction, cells were harvested, washed, heated in SDS buffer, chilled on ice, and disrupted via bead beating. Cell debris was removed and the supernatant was treated with DTT and iodoacetamide. Debris was removed and proteins were precipitated and washed. The following sample preparation, recording of mass spectra and database search were performed by Prof. Dr. Boris Maček and Dr. Mirita Franz-Wachtel at the Proteome Center Tuebingen. Proteins were resuspended in denaturation buffer, purified on a NuPAGE gel, in gel digested with trypsin and desalted peptide mixtures were separated on a mass spectrometer. The most intense precursor ions were sequentially fragmented, generated spectra were processed with

MaxQuant software package, searched against a *Staphylococcus aureus* database, and analyzed using the LFQ-Analyst (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/, last access: 23.12.2022).

Triton X-100/cervimycin-induced autolysis of *S. aureus*

TSB medium or TSB medium supplemented with 1 M NaCl was inoculated with 1% *S. aureus* SG511 Berlin pre-culture and grown at 37 °C with aeration until reaching the exponential growth phase (OD_{600} ~0.6–0.8). Then, 1 mL of the culture was harvested (1,844 × *g*, 3 min), washed with 1 mL ice cold ultrapure water and re-suspended in 1 mL phosphate buffered saline (PBS), transferred into a polypropylene cuvette, and the initial OD_{600} was determined (=100 %). Afterwards, cervimycin C (3× MIC, 6 µg/mL) or the same volume of DMSO was added, cuvettes were sealed with parafilm, and inverted 3 to 6 times. The cuvettes were incubated at 37 °C and the OD_{600} was determined every 15 to 30 min after inverting the cuvettes 3 to 6 times. As a positive control Triton X-100 (0.1% final concentration), that is thought to remove lipoteichoic acids from the cell-envelope triggering autolysis (Raychaudhuri & Chatterjee, 1985), was used. Lysis curves were created with Microsoft Excel 2010. Results were normalized to the starting OD_{600}.

Potassium efflux from *S. aureus* cells

TSB medium was inoculated with 3% of an *S. aureus* SG511 Berlin pre-culture and grown at 37 °C with aeration until reaching the mid exponential growth phase (OD600 ~1–1.5). Cells were harvested (2,254 × *g*, 3 min, 4 °C), washed with 25 mL pre-cooled assay buffer (300 mM choline chloride, 30 mM MES monohydrate, 20 mM Tris, pH 6.5), and the optical density at 600 nm in assay buffer was adjusted to 30. The perfectION™ Combination Potassium Electrodes (Mettler-Toledo, Giessen, Germany) were equilibrated in assay buffer and calibrated in standard KCl solutions (1 mM, 0.1 mM, and 0.01 mM in assay buffer) for 40 s each, beginning with the lowest KCl concentration. For sample measurement, 200 µL *S. aureus* cells were diluted in 1.8 mL assay buffer and the baseline was measured for 3 min. Then, the 1×, 5×, or 10× MIC of cervimycin C (MIC = 2 µg/mL) was added and the potassium efflux was measured for 5 min. The addition of 1 µM nisin served as positive control and was assumed to release 100% of the intracellular potassium. Potassium efflux was calculated as ratio of the measured free potassium and the amount of potassium released by the addition of nisin. Initially free potassium (=baseline) was subtracted.

Determination of growth curves

Initial growth curves of *S. aureus* WalRK over-expression strains were determined by

Tanja Funk during her Bachelor thesis at the Institute of Medical Microbiology, Immunology and Parasitology, Bonn). *S. aureus* HG003 pTXvicRK and an empty-vector control were grown over-night in BHI medium to investigate the effect of WalRK over-expression on the growth behavior. *S. aureus* strains harboring the pTX-vector, which comprises a xylose-inducible promoter, or its derivatives were grown in the presence of 12.5 µg/mL tetracycline and 50 mM of xylose was routinely used to induce over-expression from the plasmid. Growth curves were measured in either BHI medium or BHI medium supplemented with sodium chloride (final concentration 0.1 or 0.3 M NaCl) and under un-induced (0 mM xylose) or induced (50 mM xylose) conditions. To this end, *S. aureus* over-night cultures were diluted in the above-mentioned media to an optical density at 600 nm wavelength of 0.05 and transferred into polystyrene round-bottom microtiter plates. Cells were grown at 25 °C in a Tecan Infinite M Plex multimode plate reader with aeration and the OD_{600} was measured every 20 min.

Sanger Sequencing of 23S rRNA genes

The 23S rRNA genes from *S. aureus* CmR-02 were amplified and sequenced using the oligonucleotide primers listed in Table S4.

Data availability

The high-throughput RNA-sequencing data in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible under accession number GSE209674 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE209674). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al*., 2021) partner repository with the dataset identifier PXD034970 (https://www.ebi.ac.uk/pride/archive/projects/PXD034970/).

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Tübingen for providing the *Bacillus subtilis clpP* deletion strain and the *S. aureus tagO* deletion strain, respectively.

Author contributions

Conceptualization: AD, HGS, GB; Data curation: AD, MFW; Funding acquisition: AD, BM, HGS, GB; Investigation: AD, US, TF, MFW; Methodology: AD, BM, MFW; Project administration: AD, GB; Resources: MGP, KS, CH; Software: BM, MFW; Supervision: HGS, GB; Validation: AD, US; Visualization: AD; Writing – original draft: AD; Writing – review & editing: AD, GB.

Supplementary Data

Figures

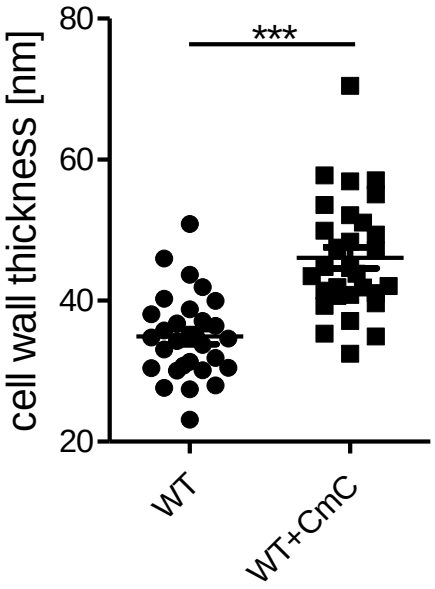

Figure S1: Cervimycin treatment of *S. aureus* SG511 Berlin (WT) leads to a significant increase in cell wall thickness. *S. aureus* was grown in BHI medium and after reaching the exponential growth phase treated with 3× MIC cervimycin C (6 µg/mL) for 1 h (WT+CmC) or left untreated (WT). Cells were fixed and visualized via scanning transmission electron microscopy. Analysis of the cell wall thickness using the ImageJ software (Schneider *et al*., 2012) revealed a significant increase in cell wall thickness after cervimycin treatment; ***, $P < 0.0001$.

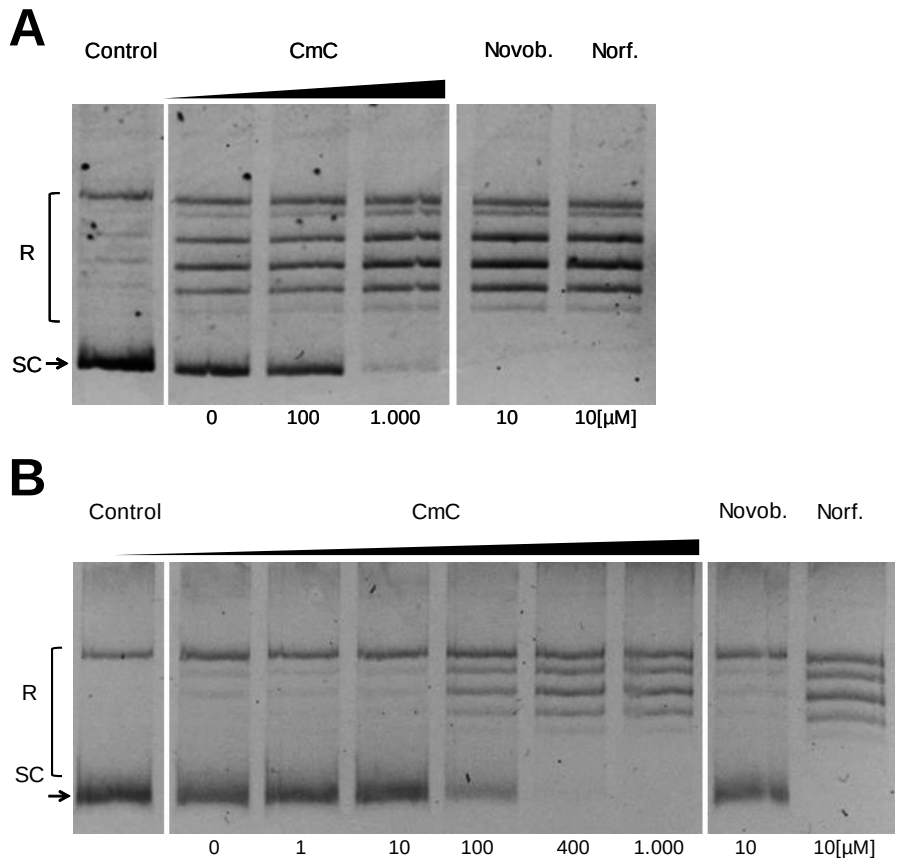

Figure S2: DNA gyrase supercoiling activity. *E. coli* (**A**) and *S. aureus* (**B**) DNA gyrase activity was measured as the proportion of supercoiled DNA after incubation of 2 U DNA gyrase with 0.125 mg relaxed plasmid DNA and cervimycin C (CmC) for 1 h at 37 °C in comparison to a control treated with the same amount of DMSO. DNA gyrase-targeting antibiotics novobiocin (Novob.) and norfloxacin (Norf.) served as positive controls for DNA gyrase inhibition.

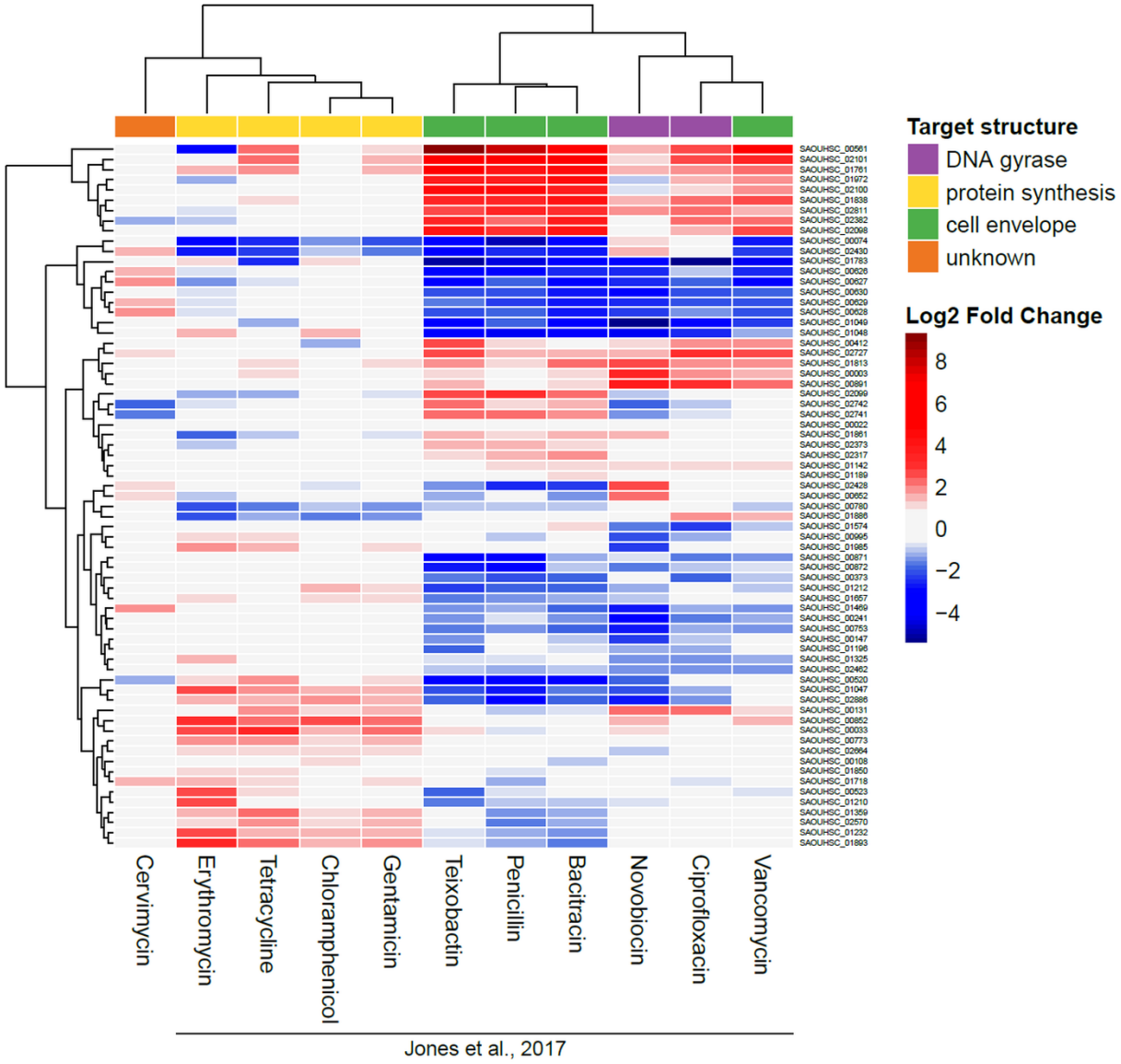

Figure S3: Heatmap of expression levels of 69 *S. aureus* genes predictive of antibiotic mode of action. The heatmap was generated from the data of Jones *et al*. (2017) and the cervimycin C RNA-Seq experiment (this study) using the ggplot2 R package (Wickham, 2016). Differential expression is displayed as $\log_2$ ratios generated from RPKM values (reads per kilobase of transcript per million mapped reads) for antibiotic and 2% DMSO-treated *S. aureus* cells. Only significant expression levels were included (p value ≤ 0.05). Red shading indicates higher expression of *S. aureus* genes compared to 2% DMSO treatment, blue shading indicates lower expression, grey shading indicates a non-significant expression level/a non-significant alteration (p value > 0.05).

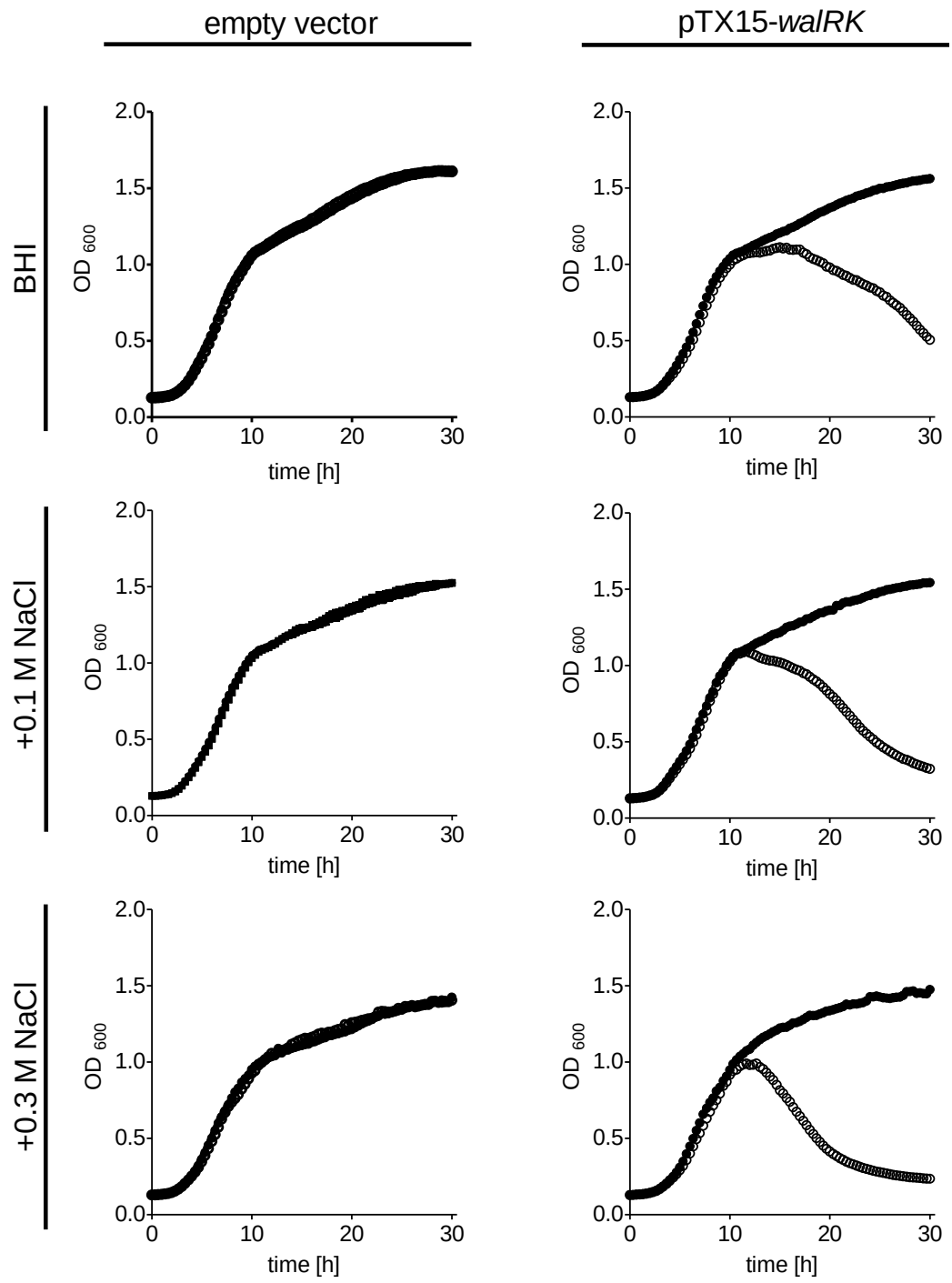

Figure S4: Growth curves of *S. aureus* HG003 pTX15 (empty vector) and *S. aureus* HG003 pTX15-*walRK* in BHI medium with increasing concentrations of sodium chloride. *S. aureus* strains were grown in BHI broth at 25 °C. Overexpression of WalRK was induced by the addition of 50 mM xylose (open circles), or measured without the addition of xylose (closed symbols). Increasing sodium chloride concentrations caused cell lysis of the WalRK overexpression strain at 25 °C, but not of the empty vector control.

Tables

Table S1: Ciprofloxacin- and novobiocin-resistance mediating gyrase mutations have no effect on cervimycin-susceptibility. Antibiotic susceptibility was tested in agar diffusion assays; VA, vancomycin (30 µg); CIP, ciprofloxacin (5 µg); Novo, novobiocin (25 µg); CmC, cervimycin C (25 µg).

	Diameter inhibition zone [mm]			
Strain	VA	CIP	Novo	CmC
S. aureus RN4220	16	22	23	11
S. aureus RN4220 $GyrA^{S84L}$	16	**9**	23	11
S. aureus RN4220 $GyrB^{G86S}$	16	22	**17**	11

Table S2: Differential expression/translation of genes/proteins in *S. aureus* SG511 Berlin treated with 3× MIC cervimycin C in comparison to untreated *S. aureus* SG511 Berlin. Shown is the total number of genes/proteins with a significant detection level (total, p value ≤ 0.05), as well as the number of significantly up-regulated (up, $\log_2$ fold change ≥ 1) and down-regulated (down, $\log_2$ fold change ≤ −1) genes/proteins.

Omics	**total**	**diff. expressed**	**up**	**down**
transcriptome	1210	564 (46.61%)	298 (24.63%)	266 (21.98%)
proteome	336	67 (19.94%)	32 (9.52%)	35 (10.42%)
transcriptome and proteome	1279	25 (1.96%)	15 (1.17%)	10 (0.78%)

Table S3: Strains and plasmids used in this study.

Strain	**Description/ Genotype**	**Source**
Bacillus subtilis		
B. subtilis 168	*trpC2*	Burkholder and Giles (1947); Spizizen (1958)
B. subtilis 168 Δ*clpP* (QB4916)	Derivative of *B. subtilis 168*; deletion of *clpP*; spectinomycin-resistant	Msadek *et al*. (1998)
Escherichia coli		
E. coli BL21 LOBSTR	Derived from *E. coli* BL21(DE3); modified copies of *arnA* and *slyD*	Andersen *et al*. (2013)
E. coli K12 JM109	*recA*1, *endA*l, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ^-, Δ(*lac-proAB*), [F', *traD*36, *proAB*, $iacI^qZ\Delta M15$]	Yanisch-Perron *et al*. (1985)
Staphylococcus aureus		
HG003	Derivative of NCTC 8325; $rsbU^+$, $tcaR^+$	Herbert *et al*. (2010)
NCTC8325-4	Laboratory strain; derivative of NCTC 8325; $rsbU^-$, agr^-, UV-cured of Φ11, Φ12, Φ13 prophages	Novick (1967)

Strain	Description/ Genotype	Source
NCTC8325-4 Δ*recU*	Derivative of *S. aureus* NCTC8325-4; *recU* mutant lacking initial 165 codons of the gene	Pereira *et al*. (2013)
RN4220	Laboratory strain; derivative of NCTC 8325-4; *rsbU* $^{-}$, *agr* $^{-}$, UV-cured of Φ11, Φ12, Φ13 prophages; restriction-negative	Kreiswirth *et al*. (1983)
RN4220 GyrA^{S84L}	Derivative of *S. aureus* RN4220; ciprofloxacin-resistant (GyrA Ser84Leu)	Chiriac *et al*. (2015)
RN4220 GyrB^{G86S}	Derivative of *S. aureus* RN4220; novobiocin-resistant (GyrB Gly86Ser)	Chiriac *et al*. (2015)
SG511 Berlin	Antibiotic-susceptible strain (CP076660, locus tag prefix KQU62_)	RKI Berlin, Germany
SA113 (*S. aureus* ATCC 35556)	Restriction-deficient mutant derived from strain NCTC 8325.	Iordanescu and Surdeanu (1976)
SA113 Δ*tagO*	Derivative of *S. aureus* SA113; erythromycin-resistant	Weidenmaier *et al*. (2004)
Plasmid	**Description**	**Source**
pEPSA5	Down-regulation of essential *S. aureus* genes; host *S. aureus* RN4220	Forsyth *et al*. (2002)
pET19-*gyrA*	Plasmid for over-expression of full-length GyrA gyrase subunit from *S. aureus* SG511 Berlin	this study
pET21-*gyrB*	Plasmid for over-expression of full-length GyrB gyrase subunit from *S. aureus* SG511 Berlin	this study
pREP4*groESL*(MT)	Plasmid encoding *E. coli* JM109 GroESL chaperones; facilitates folding and avoids formation of inclusion bodies during protein over-expression	Türck and Bierbaum (2012)
pTX15	Xylose-inducible vector, containing *xylA* and *xylR* for expression control, TetR	Peschel *et al*. (1996)
pTX15-*vicRK*	Derivative of pTX15 with *walRK* under control of XylR	Jansen *et al*. (2007)

Table S4: Oligonucleotide primers used in this study; n.d., not determined.

Primer	Sequence 5‘-3‘and restriction sites	T_A [°C]	Reference
gyrA-for	CTATACTCTAACTCGAGGCTGAATTACCTCA ATC (XhoI)	56.8	Saiki *et al*. (1999)
gyrA-rev	CATTACACATCCTCGAGTTATTATTCTTCATC TG (XhoI)	56.8	Saiki *et al*. (1999)
gyrB-for	CGCGGATCCAATTTTGTTTAACTTTAAGAAG GAGATATAGCATGGTGACTGCATTGTCA (BamHI)	65.0	Saiki *et al*. (1999)
gyrB-rev	TGCCTCGAGAGAACCCATGGTGAAGTCTAAG TTTGCATAAACTGC (XhoI)	65.0	Saiki *et al*. (1999)

Primer	Sequence 5‘-3‘and restriction sites	T_A [°C]	Reference
R1-for (V-448594)	TGAGGCAAACAAAACAACTCA	62.0	Berscheid (2014)
R1-rev	AGCCATCTTTCTTTGTGTTAACC	62.0	This study
R2-for (V-92795f)	AATTGAATAACGGGAAGTAGCTCAG	62.0	Berscheid (2014)
R2-rev	GTTTCGTCAGATTCAAACGTTTTC	62.0	This study
R3-for	GAATAAATGGTGGAGAATGAC	57.5	This study
R3-rev	CGCGTTATAATTAAATCTTGTAAG	57.5	This study
R4-for (V-98307r)	GCTTGCTTCTTTCCTCTCCTTC	62.0	Berscheid (2014)
R4-rev	ACCTCAGCCTTCCAAGCTGATG	62.0	This study
R5-for	CGTGTTACCGCCGTGAAAGG	58.4	This study
R5-rev_2	TTATTGTTATTGACTTCAATAATTAAATGAAG TAC	58.4	This study
V-450333	AGGTAGCCGTATCGGAGGT	n.d.	Berscheid (2014)
V-451191	GGAGGACCATCTCCTAAGGCTA	n.d.	Berscheid (2014)
V-452112f	GGGTTAGTCGGGTCCTAAGC	n.d.	Berscheid (2014)

5. Discussion

Antimicrobial resistance (AMR) is a natural phenomenon in antibiotic producing organisms and, presumably, in their close surroundings, but also a serious threat to global and public health, since it increases morbidity and mortality and is associated with high economic costs due to its health care burden (Majumder *et al*., 2020). *Staphylococcus aureus* remains the leading cause of mortality worldwide (Antimicrobial Resistance Collaborators, 2022; Vuong *et al*., 2016), and because of methicillin-resistant, vancomycin-resistant and vancomycin-intermediate resistant isolates, *S. aureus* is classified as a high priority pathogen by the World Health Organization (WHO) (WHO, 2017). The WHO is alerted by the high resistance rates to antimicrobials frequently used to treat common infections, such as urinary tract infections or some forms of diarrhea, indicating that the world is running out of effective ways to tackle these diseases which is further fueled by the inappropriate use of antibiotics during the COVID-19 pandemic (WHO, 2020). Antibiotic stewardship programs and alternative treatment options are necessary to reduce this disease burden (Majumder *et al*., 2020). Therefore, new antibiotics, preferably with resistance-breaking properties, need to be introduced into medicine. The other way round, the identification of new drug targets also offers options to develop new treatment strategies (Barh *et al*., 2011). Nevertheless, the subsequent approval by clinical trials is another bottleneck, being a costly and tedious process. The understanding of the antibiotic target in the biological context and in connection with the cellular function might also help to understand antibiotic susceptibility and resistance mechanisms in general.

An *S. aureus* model strain to study antibiotic susceptibility and antibiotic resistance. Comparative genomics uncovered many genome variations in closely related bacteria and revealed basic principles involved in bacterial diversification, improving our knowledge of the evolution of bacterial pathogens, especially by comparing pathogenic and non-pathogenic strains (Dobrindt & Hacker, 2001). Mechanisms like point mutations, DNA rearrangements, and horizontal gene transfer (HGT), contribute to this genome plasticity (Dobrindt & Hacker, 2001). Comparative genomics of *S. aureus* SG511 Berlin and MRSA252 revealed major differences in the accessory genomes with a considerably smaller accessory genome in *S. aureus* SG511 Berlin that might reflect a general trend between pathogenic and non-pathogenic strains (Dobrindt & Hacker, 2001). Nevertheless, the number of insertion elements was comparable between the two *S. aureus* strains. Insertion elements are transposable genetic elements conferring genome plasticity and adaptability (Dobrindt & Hacker, 2001).

Most of the characteristics identified in the SG511 Berlin genome were not unique – *spa*-deficiency, truncation of the *ebh* gene, loss of Agr-activity – but the accumulation of mutations in regulatory genes (*graS, codY, gdpP, rnY, rsbV, saeR* and *sarZ*) was unexpected and needs to be considered when *S. aureus* SG511 Berlin is under study. After the identification of the loss-of-function mutation in the *graS* gene of *S. aureus* SG511 Berlin, which alters the stress response towards cationic peptide antibiotics like mersacidin, the authors noted that *S. aureus* SG511 might not reflect the typical *S. aureus* response towards antibiotics (Sass & Bierbaum, 2009). Nevertheless, in a selection for daptomycin-resistant *S. aureus* isolates, although different routes of resistance development were seen for a *gra*-positive and a *gra*-negative background, the same resistance level was achieved (Müller *et al*., 2018). Although the single effects of each additional mutation in the regulatory genes of *S. aureus* SG511 Berlin were not part of this study, the fine-tuned regulatory system seems to be disturbed reflected by an antibiotic hypersusceptibility.

Herbert *et al*. compared the phenotype of nine *S. aureus* strains with minor and major differences in the genetic background (one or more regulatory features by which they differ from the others) that led to great interstrain variations in the phenotype (Herbert *et al*., 2010). Even the probably most studied *S. aureus* isolate, *S. aureus* NCTC 8325, which is enormously useful because of the incomparable wealth of knowledge that has accumulated since its isolation and the vast number of derivative strains, harbors mutations in two regulatory genes (Herbert *et al*., 2010). Thus, there is no single optimal strain of *S. aureus* for studying molecular genetics and pathobiology, also because clinical isolates differ from each other more or less as a result of permanent strain adaptation and selection in the course of colonization or infection (Herbert *et al*., 2010).

In summary, the hypersusceptibility of *S. aureus* SG511 Berlin towards cell envelope-targeting antibiotics might be helpful when studying natural compounds with limited availability. Exploiting this trait, we used *S. aureus* SG511 Berlin as model strain for the mode of action studies of the antibiotic complex of cervimycin.

Cervimycin C, the main component of the cervimycin antibiotic complex, disturbs various processes in Gram-positive bacteria

Elucidating how and when different antibiotics prevent the replication of bacteria or kill them will be useful for developing much needed new antibiotics, for designing protocols for the administration of existing antibiotics/combinations of antibiotics that are clinically effective, at the same time minimize the likelihood of emergence and rise of resistance to these drugs in

target bacteria or commensals, and minimize disturbance of the microbiota (Baquero & Levin, 2020). Efforts to develop novel antibiotics still involve natural products and, in this context, *Streptomyces* species as producers of diverse secondary metabolites are of interest. Flavonoids, terpenoids, and polyketides are structurally diverse secondary metabolites used widely as pharmaceuticals and most of these molecules exist in nature as glycosides, in which sugar residues act as a decisive factor in their architectural complexity and bioactivity (Vasudevan & Lee, 2020). Cervimycins are such compounds, an antibiotic complex of polyketide glycosides that is only active, if all six sugar residues are present (Herold, 2005). Cervimycins belong to a group of around 26 glycosylated tetracyclines produced by *Streptomyces* species, including polyketomycin, dutomycin, elloramycins, and tetracenomycins (Vasudevan & Lee, 2020). In contrast to the broad-spectrum bacteriostatic activity of the un-glycosylated tetracyclines (Nelson, 1998), the antibacterial activity of glycosylated tetracyclines is restricted to Gram-positive bacteria, probably due to the mere size of these molecules. Interestingly, the longer sugar side chain of cervimycin is further decorated with methylmalonyl or dimethylmalonyl units attached to the terminal sugar moiety (Herold *et al*., 2005) which involves the ketosynthase CerJ (Bretschneider *et al*., 2011). A *cerJ* deletion mutant produces the cervimycin variants cervimycin K1 and K2, lacking the manlonyl side chain (Bretschneider *et al*., 2011). Notably, cervimycin K1 is the most potent member of the cervimycin complex (Bretschneider *et al*., 2011), indicating that the malonyl side chain is not decisive for the antibacterial mode of action.

Cervimycin inhibits the DNA gyrase (topoisomerase II) in high concentrations. Beyond the importance of the sugar side chains as pharmacophore groups of cervimycin, little was known about the mode of action of these compounds. The incorporation of radiolabeled thymidine into the DNA of *Bacillus subtilis* was decreased by very high cervimycin A concentrations (8× MIC) (Herold, 2005) and, in this context, the DNA gyrase was identified as a target of cervimycin (detailed overview in **Chapter 4.3**, above). DNA gyrase (topoisomerase II) is a long-known antibiotic target, which is essential in introducing negative supercoils into the DNA, and gyrase inhibitors normally also target the topoisomerase IV (**Chapter 2.**, above). These enzymes are hetero-tetramers of A and B subunits, and inhibitors either bind to the A or the B subunit (Figure 15).

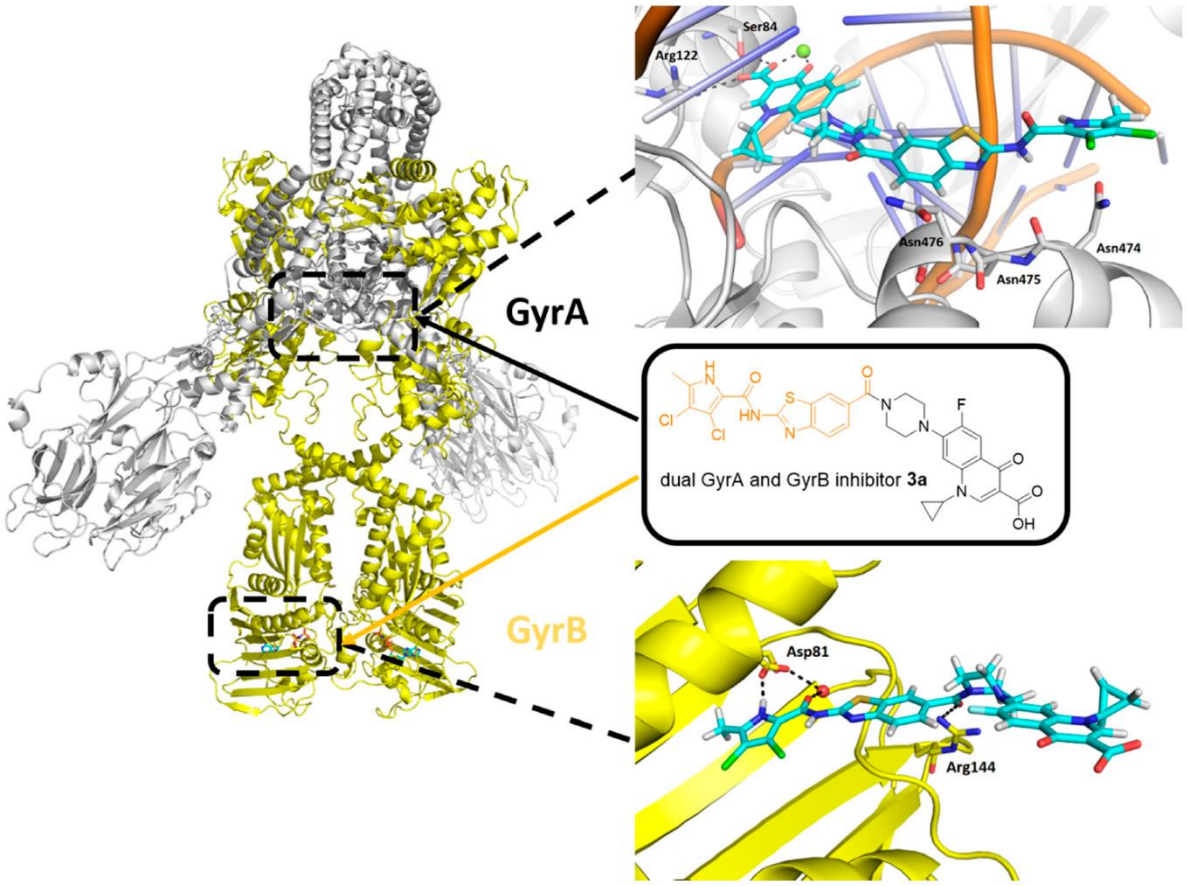

Figure 15: Binding sites of DNA gyrase-targeting antibiotics; docking at the *S. aureus* DNA gyrase (Durcik *et al*., 2020). While some DNA gyrase inhibitors either bind the catalytic site of the A subunit (gray) or the ATP-binding site of the B subunit (yellow), synthetic hybrid compounds like **3a** can target both sites (arrows).

Fluoroquinolones, like ciprofloxacin, which constitute a very successful synthetic antibiotic class, bind to the gyrase A subunit and cause double-stranded DNA breaks that cannot be repaired (Foster, 2017). The quinolone binding site is close to but distinct from the symocyclinone binding site (Edwards *et al*., 2009). Symocyclinones are natural bifunctional antibiotics comprising an aminocoumarin as well as a polyketide group and prevent DNA binding to the enzyme (Edwards *et al*., 2009). Aminocoumarins target the B subunit of the DNA gyrase (Figure 15). Notably, the *in vitro* inhibition activity (given as IC_{50}) of the fluoroquinolone ciprofloxacin and the aminocoumarin novobiocin mirror the *in vivo* activity of the compounds against Gram-positive bacteria (given as MIC) (Table 4). The effectiveness against Gram-negative bacteria was lower, probably due to a limited uptake through the membrane or the presence of an active efflux pump (Alt *et al*., 2011). Strikingly, resistance mutations towards these compounds occur in the DNA gyrase and/or the topoisomerase IV and are clinically relevant for the fluoroquinolones (Foster, 2017).

Table 4: Properties of the DNA gyrase inhibitors ciprofloxacin and novobiocin that target different subunits of the same enzymes.

Category		**Ciprofloxacin**	**Novobiocin**
Target		GyrA	GyrB
MW (g/mol)		331.35	612.62
Resistance mutations (apart from efflux-based resistance)		*gyrA, parC*	*gyrB, parE*
S. aureus	MIC [µg/mL]	0.2[1]	0.16[3]
	MIC [µM]	0.604	0.25[3]
	IC_{50} [µM]	1.2[2]	0.01[4]
E. coli	MIC [µg/mL]	12.5[1]	250[3]
	MIC [µM]	37.724	394[3]
	IC_{50} [µM]	0.73[2]	0.08[4]

[1]MICs of *S. aureus* RN4220 and *E. coli* O-19592, respectively; determined by Chiriac *et al*. (2015).
[2]Inhibition of the DNA gyrase supercoiling activity *in vitro*; determined by Chiriac *et al*. (2015).
[3]MICs of *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively; determined by Alt *et al*. (2011).
[4]Inhibition of the DNA gyrase supercoiling activity *in vitro*; determined by Alt *et al*. (2011).

Therefore, some serious differences between these characterized topoisomerase inhibitors and cervimycin can be noticed. First, in relation to the MIC much higher concentrations of cervimycin were needed to disturb the thymidine incorporation into the DNA or the *in vitro* gyrase activity. Second, the resistance mutations of the cervimycin-resistant (CmR) mutants did not occur in the topoisomerase genes (**Chapter 4.2**, above). And third, novobiocin and ciprofloxacin-resistant mutants showed no cross-resistance towards cervimycin. Because of these discrepancies, and to obtain a more global view on the effects of cervimycin on the bacterial cell, transcriptomics and proteomics analyses were performed.

Omics analyses dismiss the gyrase as primary target of cervimycin. Although cervimycin inhibited the DNA gyrase with some sort of specificity, the omics analyses of cervimycin treated *S. aureus* showed poor similarity towards the characterized gyrase inhibitors ciprofloxacin and novobiocin, because neither the SOS response nor *gyrAB* expression were induced. Nevertheless, down-regulation of the B subunit of the DNA gyrase acted synergistically with the bactericidal effect of cervimycin (**Chapter 4.3**, above), indicating some *in vivo* relevance of the observed *in vitro* gyrase inhibition. Interestingly, another polyketide antibiotic, actinorhodin, also inhibits the DNA gyrase with some sort of specificity without induction of the expected stress response (Mak & Nodwell, 2017). Actinorhodin is an un-glycosylated aromatic polyketide which shares the quinoid system with the cervimycin

structure (**Chapter 2.3, above**; Figure 6). Thus, gyrase inhibition might be attributed to the cervimycin aglycone, but the sugar side chains as pharmacophore groups seem to determine the primary target of the antibiotic.

Indeed, because of the chemical reactivity of actinorhodin, the compound was thought to belong to the Pan Assay INterference compoundS (PAINS) (Mak & Nodwell, 2017) which include compound classes defined by a common substructural motif, such as the quinone motif, that encodes for an increased chance of any member to register as a hit in any given assay (Baell & Nissink, 2018). Pan Assay Interference is based on reactivity with biological and bioassay nucleophiles, such as thiols and amines; photo-reactivity with any protein functionality; metal chelation; redox cycling and redox activity; physicochemical interference, such as micelle formation, or having photochromic properties that might interfere with typically used assay signaling, such as absorption and fluorescence (Baell & Nissink, 2018). Quinones have a high propensity to be redox active, as well as being reactive to nucleophiles present in the side chains of proteins, such as cysteine and lysine, and the quinone moiety can still display PAINS behavior, even if it is embedded in a drug and whether or not it is a natural product (Baell, 2016). Interestingly, the anthracycline anticancer drug doxorubicin (**Chapter 2.3, above;** Figure 6, Table 2), which exerts its activity at least in part as a topoisomerase II poison via DNA intercalation, is also redox active and reacts with biological nucleophiles, so the mechanism of its action is still not fully understood (Baell, 2016; Patel & Kaufmann, 2012). Notably, the reactivity of these compounds can be concentration dependent (Baell, 2016) which might explain the above seen discrepancy in the effects of cervimycin on the DNA gyrase.

Recently, Fritsch *et al*. (2019) identified the MarR-type regulator MhqR (SAOUHSC_02826) as quinone-sensing repressor of the *mhqRED* operon that is important for methylhydroquinone- (MHQ) resistance and resistance towards quinone-like antimicrobial compounds, including pyocyanin, ciprofloxacin, norfloxacin, and rifampicin (Fritsch *et al*., 2019). Indeed, *mhqR* expression is induced under cervimycin treatment (2.17-fold), but expression of MhqD, MhqE and the PerR regulon, that are quinone degradation enzymes and involved in reactive oxygen species (ROS) detoxification, respectively, was not significantly altered under cervimycin treatment but in MHQ treated *S. aureus*. Strikingly, both, cervimycin and MHQ, highly de-repressed expression of the CtsR/HrcA operon, encoding the CtsR-controlled Clp proteases and the HrcA-controlled chaperones, indicating protein stress.

Cervimycin strongly induces the heat shock/protein stress response in *S. aureus*. The ability to adapt to harsh environments is essential for staphylococcal persistence in infections or as a commensal (Morikawa *et al*., 2010). The heat shock response is such a mechanism of cellular protection against sudden adverse environmental growth conditions and results in the prompt production of various heat shock proteins (Roncarati & Scarlato, 2017). Several stress conditions are able to elicit the heat shock response, such as temperature variations, osmotic changes, desiccation, antibiotics, solvents, or heavy metals, that are sensed by biomolecules like DNA/RNA or proteins (Roncarati & Scarlato, 2017). In the following, the de-repression of the CtsR/HrcA operon is referred to as "heat shock response" or "protein stress response". In *S. aureus*, the tandem arrangement of the CtsR and HrcA operators leads to a dual heat shock regulation by these two repressors which synergistically act together to maintain low basal levels of expression of the *dnaK* and *groESL* operons in the absence of stress (Chastanet *et al*., 2003). High temperatures, SDS or ethanol induced the heat shock response in *S. aureus*, but not salt, alkaline, or acid stress (Chastanet *et al*., 2003). Interestingly, the CtsR operon was one of the mutational hotspots in the CmR mutants, inactivating either the ClpP protease or the ClpC ATPase (**Chapter 4.2**, above). Consequently, expression of the CtsR/HrcA operon was de-repressed in the CmR mutants and in cervimycin treated *S. aureus*. Also, other features were shared between these two samples, as indicated in Figure 16.

Figure 16 gives an overview of the significantly induced and depleted proteins which were shared between the CmR mutant CmR-02 (encoded amino acid exchanges: $ClpP^{I29F}$, $WalK^{A243V}$) and the cervimycin-treated *S. aureus* SG511 Berlin wild type (3× MIC for 60 min). As mentioned, chaperones and proteases from the CtsR/HrcA operon were more abundant. On the other hand, proteins from the purine metabolism were depleted. Intriguingly, the very same effect was seen in a *clpP* but not a *clpX* deletion mutant of *S. aureus* NCTC 8325 (Figure 17).

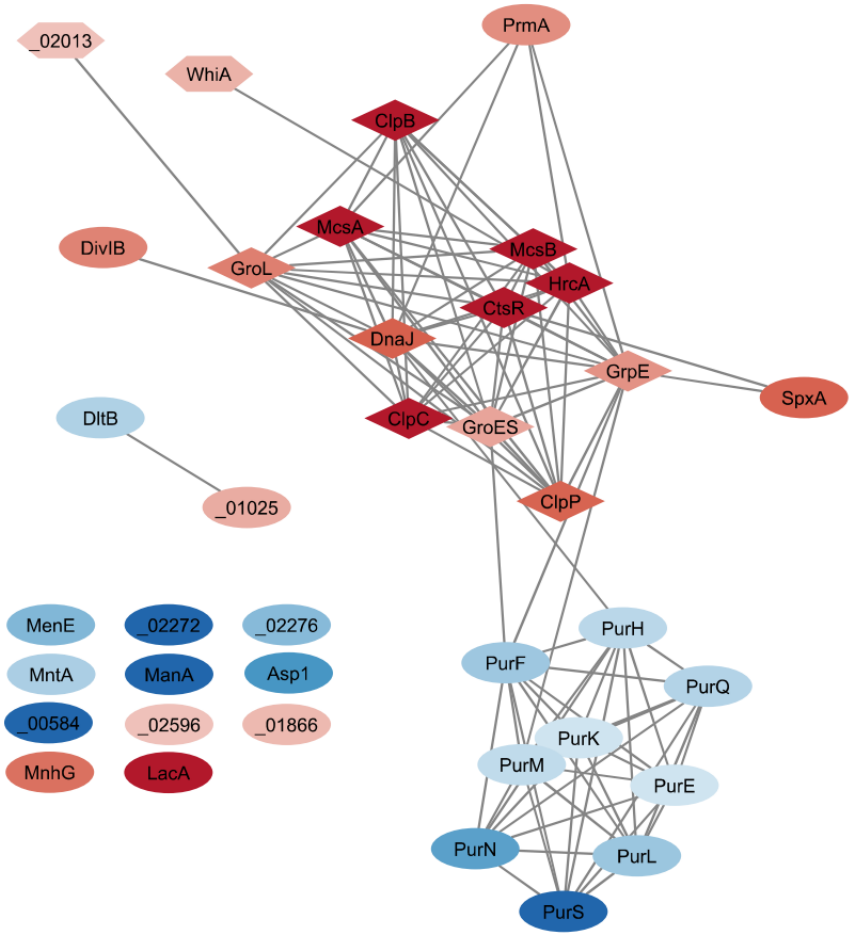

Figure 16: Protein network of congruously altered proteins in cervimycin treated *S. aureus* and in the cervimycin-resistant mutant CmR-02; created using Cytoscape v3.8.2 software (https://cytoscape.org/, last access 23.12.2022). Proteins with at least 2-fold differential expression in comparison to the untreated *S. aureus* SG511 Berlin (log2 fold change ≥ 1 or ≤ -1) and significant *p* value ($P \leq 0.05$) are shown. Induced proteins are marked in red, depleted proteins in blue (according to the detection level in cervimycin-treated *S. aureus*). Proteins encoded in the CtsR/HrcA operon are shown as diamonds; proteins encoded in the σ^B operon are shown as hexagons; numbers refer to the locus tags of *S. aureus* NCTC 8325.

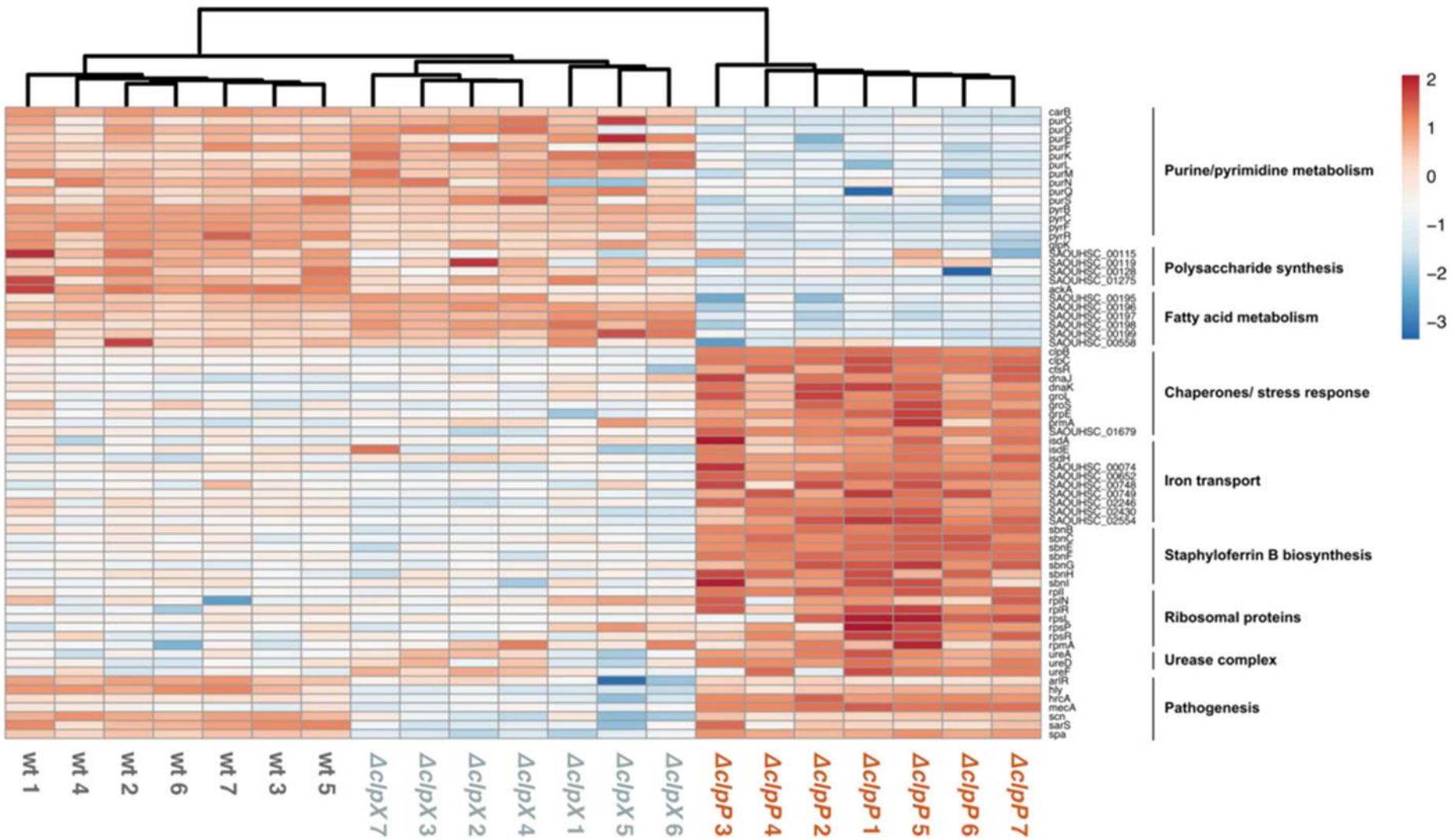

Figure 17: Heatmap of dysregulated proteins in *S. aureus* wildtype in comparison to *clpX* or *clpP* deletion strains (NCTC 8325 background) (Kirsch *et al.* 2021). Rows indicate proteins and columns indicate samples. High and low expressions are shown in red and blue, respectively.

Deletions of the *clpP* and *clpX* genes reveal an extensive dysregulation of several hundred intra- and extracellular proteins which could largely be linked to distinct pathways, e.g., virulence, purine/pyrimidine biosynthesis, iron uptake, and stress response (Kirsch *et al*., 2021). However, only *clpP* deletion leads to a significantly decreased expression of a high number of proteins involved in the purine and pyrimidine metabolism (especially, uridine monophosphate (UMP) biosynthesis; a precursor in RNA biosynthesis) which was also verified on the metabolomic level (Kirsch *et al*., 2021). Also, the now verified impaired purine production of *clpP* deletion strains was thought to be responsible for the reduced growth rate of these mutants (Frees *et al*., 2012; Kirsch *et al*., 2021) that might in parallel contribute to the vancomycin-intermediate resistant phenotype of the CmR mutants (**Chapter 4.2**, above).

In summary, the omics data of cervimycin treated *S. aureus* and the cervimycin-resistant mutant CmR-02 exhibited certain similarities, in particular the de-repression of the CtsR/HrcA operon and the depletion of the purine synthesis proteins that are both extensively influenced by the ClpP protease.

A database search in the *Staphylococcus aureus* Transcriptome Meta-Database (SATMD: https://satmd.org/index.php, last access: 23.12.2022; Nagarajan and Elasri (2007), last access 23.12.2022) verified the induction of the CtsR/HrcA operon by heat stress (43 and 48 °C), but also revealed further inducers of this stress response.

Interestingly, an excess of metal ions as well as metal ion chelation induced the heat stress response in *S. aureus*. Excess copper (100 µM copper sulfate for 30 min) induced the expression of the *ctsR, clpC, clpP*, and *clpB* genes in *S. aureus*, probably due to the presence of misfolded proteins or the modulators of CtsR repressor activity may sense copper directly (Baker *et al*., 2010). Indeed, the McsA protein of *S. aureus*, which protects the CtsR repressor from degradation by ClpCP under non-stress conditions, was shown to bind different metal ions via four CXXC motifs (Krüger *et al*., 2001; Sitthisak *et al*., 2012). Consequently, metal ion binding by McsA might de-protect CtsR and lead to a de-repression of the CtsR/HrcA operon. Another de-repression mechanism involves the McsB protein, a putative kinase, which directly modifies, i.e., phosphorylates, CtsR and, thus, removes the repressor from the DNA and targets CtsR for degradation by the ClpCP protease during stress (Krüger *et al*., 2001). The heat shock proteins ClpB, ClpC, ClpP, GroEL, DnaK, and GrpE were also more abundant in *S. aureus* cells treated with the macrocyclic polyketide kendomycin (Elnakady *et al*., 2016). Kendomycin directly or indirectly affected the cell division machinery, protein stability, and programmed cell death in *S. aureus* (Elnakady *et al*., 2016), activities which were finally assigned to the iron

and copper chelation of the compound (Tranter *et al*., 2020).

Some plant extracts also strongly induced the heat shock response. Tee tree oil, orange oil, and sodium houttuyfonate, which is the easily polymerized major constituent of the volatile oil of the plant *Houttuynia cordata* THUNB., concordantly induced the heat shock response of *S. aureus*, but always in combination with the cell wall stress response and/or cell lysis (Cuaron *et al*., 2012; Liu *et al*., 2011; Muthaiyan *et al*., 2012). However, the complex composition of these extracts hardly allows the assignment of the observed effects to specific compounds within these mixtures. In addition to that, the solvent ethanol alone also induces the heat shock response in *S. aureus* which is attributed to a rapid denaturation of protein and membrane structures (Chatterjee *et al*., 2006; McDonnell & Russell, 1999).

Cell envelope-targeting agents like oxacillin also induce some genes of the protein stress response, implicating that treatment of cells with cell-wall-active antibiotics causes the accumulation of damaged, misfolded, and aggregated proteins, necessitating the production of stress-chaperone and protease proteins to deal with the accumulation of aberrant proteins (Utaida *et al*., 2003). Enduracidin, which is related to ramoplanin, and telavancin, a vancomycin derivative, both target the cell envelope of *S. aureus* by binding to intermediates along the pathway to mature peptidoglycan and strongly induce the protein and the cell wall stress response (Song *et al*., 2012). Altered gene expression due to the membrane depolarization effect of telavancin was only observed after longer incubation times (Song *et al*., 2012).

Targocil, which is a late-stage inhibitor of the wall teichoic acid (WTA) biosynthesis, also causes cell wall and protein stress in *S. aureus* (Campbell *et al*., 2012). Targocil targets TarG, which is part of the WTA transporter, leading to accumulation of WTA intermediates (Campbell *et al*., 2012). Either the toxicity of the accumulated WTA intermediates or the prevention of the bactoprenol phosphate carrier lipid recycling then results in the bacteriostatic effect (Campbell *et al*., 2012). Targocil led to a stronger protein stress response than other cell wall-active antibiotics which might indicate that misfolded and aberrant proteins may accumulate to a greater extent upon targocil treatment or the accumulated WTA polymers may be sensed as defective/misfolded proteins (Campbell *et al*., 2012). Consistently with the latter hypothesis, tunicamycin, which is an early-stage WTA biosynthesis inhibitor, causes no protein stress response (Campbell *et al*., 2011).

Under cervimycin treatment, we saw a strong induction of the protein stress response, but not of the cell wall stress response (**Chapter 4.3**, above). We used *S. aureus* SG511 Berlin as model strain for these analyses to have the same genetic background as for the CmR mutants

(**Chapter 4.2**, above). Nevertheless, the *graS* negative genetic background of *S. aureus* SG511 Berlin (**Chapters 2.2**, above; and **4.1**, above) might distort some effects of cervimycin on the cell envelope and must be taken into account. Still, disturbance of the cell envelope was also not detected in *B. subtilis* (**Chapter 4.2**, above (*liaI* and *ypuA* cell envelope stress reporters); and glucosamine incorporation test (Herold, 2005)).

However, a more obvious cause for protein stress was not mentioned yet, the disturbance of the protein production machinery, the ribosome. Ribosomes translate the genetic information encoded in messenger RNA in a multi-stage process to assemble amino acids into proteins. These large and complex biosynthetic machineries are composed of ribosomal RNA (rRNA) and proteins (r-proteins). Functional disturbance of the ribosome represents a validated mechanism of successful antibiotics, including translation inhibition (ribosome stalling), induction of mistranslation, and premature termination of translation. Truncation or mistranslation of proteins at the ribosome by puromycin or streptomycin treatment induce the protein stress response, but not stalling of the ribosome as observed after tetracycline, chloramphenicol, and erythromycin treatment (Ng *et al.*, 2003).

The cervimycin stress response resembles the mistranslation-inducing aminoglycoside gentamicin. Due to the lack of protein in the functional regions of the ribosome, the rRNA is of fundamental importance and, consequently, most ribosome inhibitors target the rRNA-rich surfaces on the 30S and 50S subunits (Poehlsgaard & Douthwaite, 2005). Thus, a large variety of natural compounds aim at the ribosome by binding to relatively few sites (Figure 18).

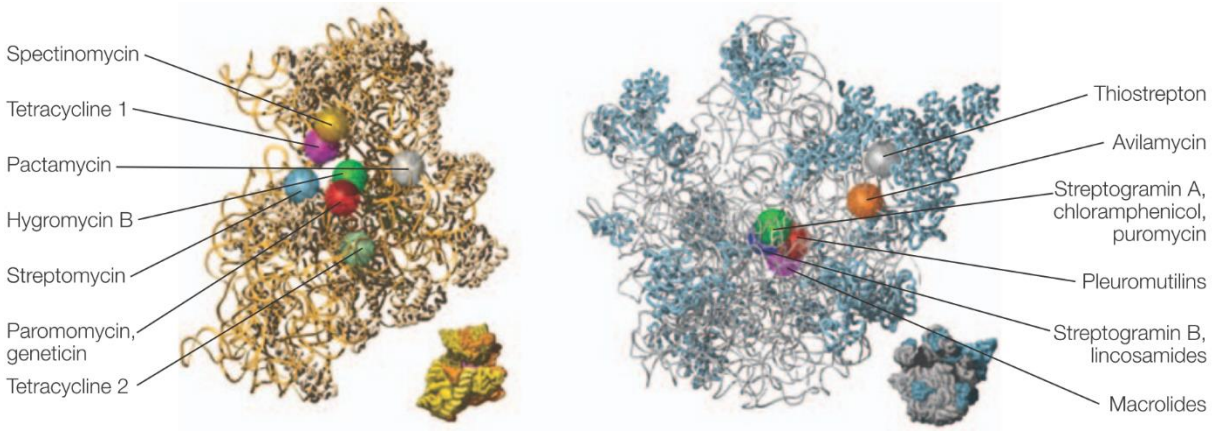

Figure 18: Binding sites of antibiotics on the bacterial ribosome; 30S ribosomal subunit on the left, 50S ribosomal subunit on the right (Poehlsgaard and Douthwaite 2005). Ribosomal RNAs are shown in yellow and grey and ribosomal proteins in bronze and blue.

Drugs that bind the 50S subunit either block the polypeptide exit tunnel by binding at overlapping sites (erythromycin and streptogramin B) or interfere with amino acyl tRNA

binding at the peptidyl transferase center (PTC) (lincomycin; streptogramin A; pleuromutilin; florphenicol; linezolid) (Foster, 2017). Tetracycline binds to the 30S subunit close to the decoding center and causes aminoacyl tRNA dissociation, while aminoglycosides, which bind in close proximity, cause misreading of the mRNA (Foster, 2017). Fusidic acid targets the elongation factor G (EF-G) and inhibits the ribosome translocation following peptide bond formation, while mupirocin blocks the isoleucine tRNA synthetase, leading to depletion of isoleucyl-tRNA and accumulation of the corresponding uncharged tRNA (Foster, 2017).

Strikingly, aminoglycosides are the only bactericidal ribosome-targeting antibiotics (Foster, 2017). Aminoglycosides greatly increase the risk that erroneous charged t-RNAs will be bound to the small subunit, increasing the error rate in translation from <1 in 1000 to around 1 in 100 so that every average sized protein has several incorrect amino acids (Walsh & Wencewicz, 2016). This so-called 'local' mistranslation leads to a change at a single codon, resulting in codon-specific local mistranslation events and aberrant protein synthesis (Mohler & Ibba, 2017). Secondly, faulty membrane proteins cause membrane damage (Davis *et al*., 1986) and, finally, a massive energy-dependent uptake of aminoglycosides will produce a 'cationic disturbance' of the membrane integrity (Baquero & Levin, 2020). But errors in protein synthesis can also occur earlier, although at a lower frequency, during tRNA aminoacylation (Kelly *et al*., 2019). Key players in this process are aminoacyl-tRNA synthetases which not only catalyze the attachment of cognate amino acids to their respective tRNAs, but also selectively hydrolyze incorrectly activated non-cognate amino acids and/or misaminoacylated tRNAs (Mohler & Ibba, 2017). In *E. coli*, errors in tRNA aminoacylation by mutation of the alanyl-tRNA synthetase AlaRS are not well tolerated, since they lead to a gross perturbation of the proteome homeostasis with potentially catastrophic effects on fitness and viability (Kelly *et al*., 2019). However, none of the T-box regulated tRNA synthetases was significantly differentially expressed in cervimycin treated *S. aureus*.

Resistance mutations in ribosomal genes are rare. Resistance mechanisms towards ribosome-targeting agents can be divided into three categories (reviewed by Foster (2017)), (i) active efflux by transporters, (ii) ribosome protection by ribosome or drug modifying enzymes, and (iii) mutations in ribosomal genes. However, the latter mechanism was only described for the synthetic oxazolidinone antibiotic linezolid (Foster, 2017) (Figure 19).

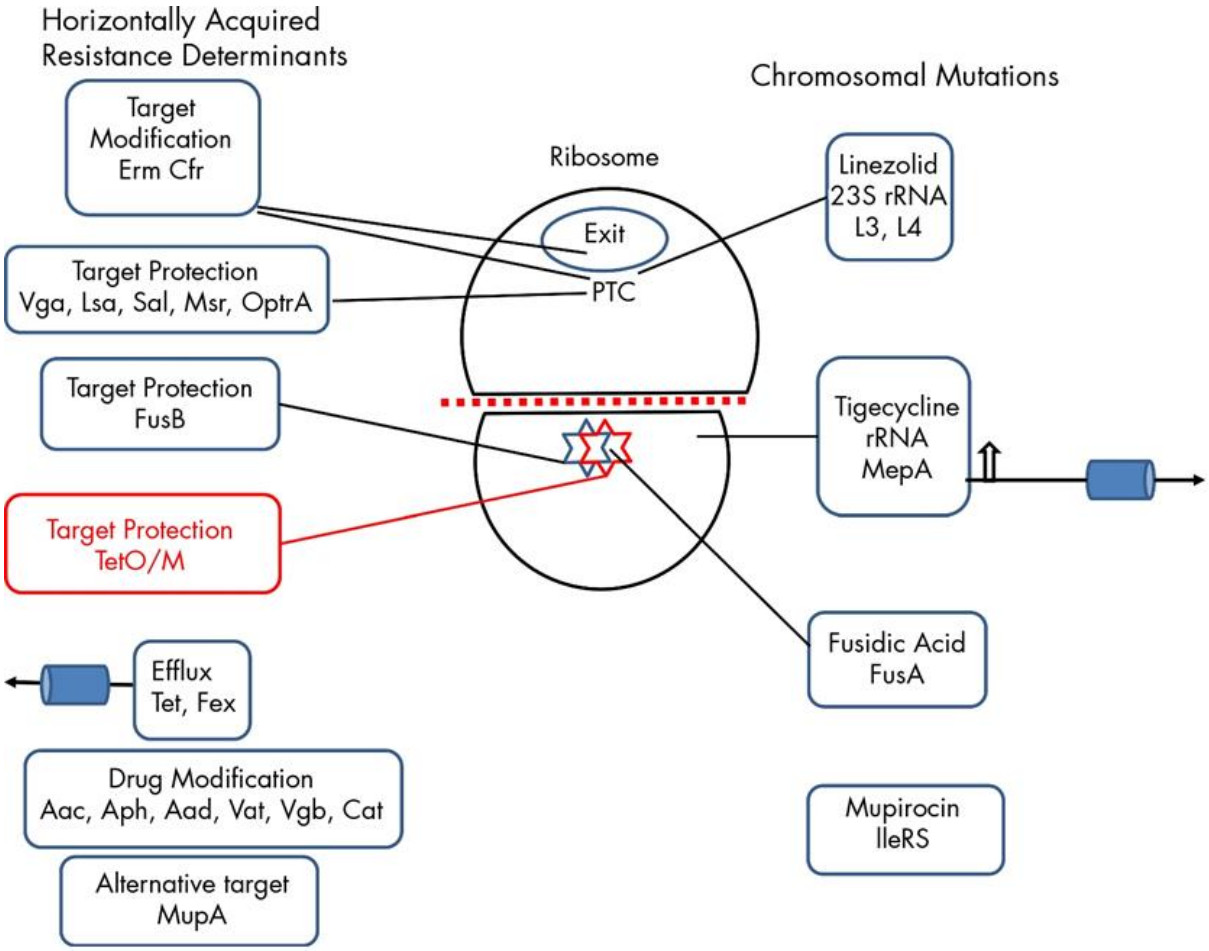

Figure 19: Resistance mechanisms to ribosome-targeting drugs acquired by horizontal gene transfer (left) or by mutations in chromosomal genes (modified after Foster (2017)).

Taking into account that the *S. aureus* genome harbors five copies of rRNA genes and the *E. coli* genome even more (seven copies), the rare occurrence of resistance mutations within these genes is not surprising. Notably, mutations in linezolid-resistant isolates occur in single copies within the V domain of the 23S rRNA and the 50S ribosomal proteins L3 and L4 (Gu *et al*., 2013). Nevertheless, mutations within multiple copies of rRNA genes or loss of copies of rRNA genes are rare due to the high fitness cost (Foster, 2017; Meka *et al*., 2004). Aminoglycoside resistance is mainly conferred by (i) transferases (acetyltransferases, phosphotransferases, nucleotidyltransferases) that modify the aminoglycoside molecule and (ii) the lack of enzymes responsible for active transport of aminoglycosides into the bacterial cell (Mlynarczyk-Bonikowska *et al*., 2022) and not by ribosomal mutations.

Cervimycin treatment caused a strong protein stress response in *S. aureus*. However, the cause for this stress response is not clear yet. Notably, based on the omics data, there are some similarities to the ribosome-targeting aminoglycosides. Previous attempts to screen for ribosome interference (precursor incorporation tests and *Bacillus* bioreporters:

Chapter 4.3, above) only aimed at a ribosome stalling mode of action and mistranslation as well as premature translation might have been overseen with these tests. In addition, the lack of resistance mutations in ribosomal genes of cervimycin-resistant mutants is no proof against a ribosome-targeting mode of action, as explained above. Nevertheless, the subsequent membrane damage caused by faulty membrane proteins after aminoglycoside treatment also induces a cell wall stress response (shown for gentamicin by Jones *et al*. (2017)) which has not been observed in cervimycin treated cells.

As mentioned above, a selection for cervimycin-resistant *S. aureus* mutants revealed one mutational hotspot within the CtsR/HrcA operon, leading to the inactivation of the ClpP protease or the cognate ClpC ATPase. The inactivation of this important protein degradation system is quite counterintuitive, since a strong protein stress response occurred in cervimycin-treated *S. aureus*. Notably, the inactivation of *clpP* led to a complete de-repression of the CtsR/HrcA operon in CmR-02 and in a *clpP* deletion mutant (Michel *et al*., 2006). Since ClpC is thought to act as a chaperone for the CtsR repressor, *clpC* inactivation should have the same outcome (Frees *et al*., 2004).

However, the second mutational hotspot of the CmR mutants was the WalRK two-component system which has an essential role in the positive regulation of cell wall hydrolases in *S. aureus* (Dubrac *et al*., 2007). Consequently, inhibitors of WalK (the angucycline waldiomycin) and WalR (walrycins A and B from a chemical library) potently inhibit the growth of low G+C Gram-positive bacteria (Gotoh *et al*., 2010; Kato *et al*., 2017). Interestingly, such cell wall hydrolases were identified as ClpP substrates using a $ClpP^{TRAP}$, a proteolytically inactive ClpP variant, and accumulated in a *clpP* mutant, while transcription of the corresponding genes was not altered (Frees *et al*., 2014). And the major autolysin Atl was identified as a potential ClpCP substrate (Graham *et al*., 2013). Also, Atl processing was slowed down in a *clpP* deletion strain probably due to a stronger affinity of the Atl intermediates to the mutant cell wall or an effect on the expression of extracellular proteases (Bæk *et al*., 2014). However, the mechanism of Atl processing was so far not characterized (Schlag *et al*., 2010) and the link between the intracellular degradation of cell wall hydrolases by ClpP, which mediate their function outside the cell, is not well understood yet (Frees *et al*., 2014). But a combination of *walK* and *clpP* mutations was described as a mechanism of either vancomycin- (Shoji *et al*., 2011) or daptomycin-resistance (Song *et al*., 2013), both targeting the cell envelope of *S. aureus*.

The *walK* mutations in the CmR mutants, which were localized in different functional domains of the kinase, decreased the WalRK activity, reducing the cell wall hydrolase activity of AtlA in the zymographic analyses, increasing cell wall thickness, and leading to a vancomycin-intermediate resistant phenotype (Figure 20; **Chapter 4.3**, above).

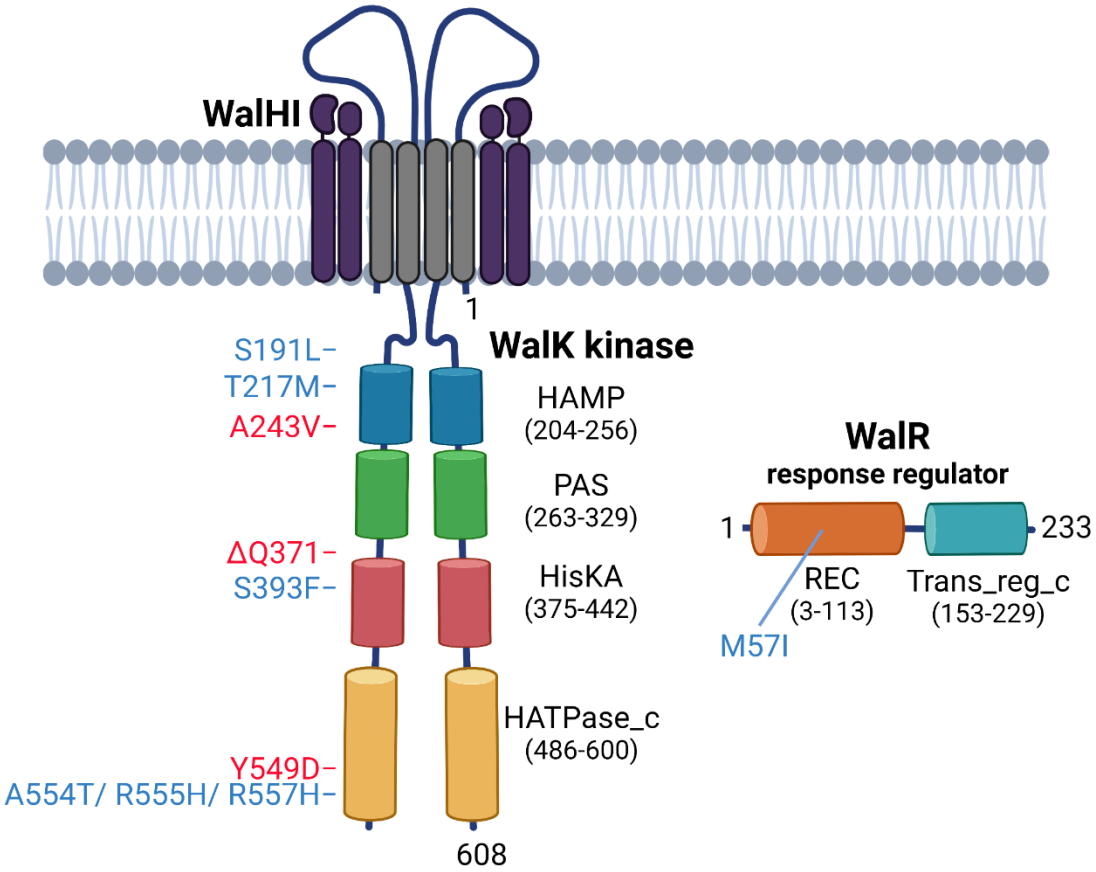

Figure 20: Domain architecture of the *S. aureus* WalK histidine kinase and its cognate WalR regulator (created with Biorender.com, last access: 23.12.2022). The domain architecture and the coordinates were derived from the SMART database (https://smart.embl.de/, last access: 23.12.2022). A predicted extracellular PAS domain (Szurmant *et al.*, 2008) is not shown. The accessory proteins WalH and WalI (purple) are positive regulators of WalRK in *S. aureus* (Cameron *et al.*, 2016; Gajdiss *et al.*, 2020). Mutation sites identified in cervimycin-resistant mutants are marked in red, mutations identified in cervimycin-susceptible revertants are shown in blue.

Although the additional *walK* (and in one case *walR*) mutations in the cervimycin-susceptible revertants only had a modest effect on the *in vitro* WalK autophosphorylation activity, major differences in the autolysin transcription and cell lysis were observed, indicating that they at least partially restore WalK activity *in vivo*. Notably, four revertant strains harbored additional mutations in the ATP-lid (amino acids 531 to 561 in the *S. aureus* WalK protein) within the ATP-binding and hydrolyzing HATPase_c domain of WalK which was recently described as activating mechanism (Tan *et al.*, 2022), possibly by controlling the affinity or

hydrolysis of ATP. Nevertheless, the position of the mutation within the ATP-lid seems to be highly decisive, since mutations altering amino acids R555 and R557 activated WalK (Tan *et al*., 2022), while the I544 mutation of the laboratory-derived vancomycin-intermediate resistant *S. aureus* VC40 abolished *in vitro* WalK activity (Kienemund, 2016). Nevertheless, the *walK* mutations in the CmR mutant did not cluster around a potential binding site of cervimycin towards WalK (Figure 20) and decreased the WalK activity, indicating an indirect effect of these mutations rather than direct binding and inhibition of the kinase.

The balance between peptidoglycan biosynthesis and cell wall hydrolysis is crucial during the growth of *S. aureus*. Salamaga *et al*. (2021) demonstrated that stasis occurs, when detrimental WalRK depletion is counteracted by the addition of the otherwise bactericidal cell envelope-targeting antibiotics vancomycin and oxacillin. In CmR-02, the WalR regulon was inconsistently dysregulated, since the major autolysin Atl and Sle1 were down-regulated and the minor autolysins were up-regulated. Interestingly, in an *atl* deletion mutant, secondary peptidoglycan hydrolases are also increased in the secretome and the corresponding genes are transcriptionally up-regulated, suggesting a compensatory mechanism for the *atl* mutation (Pasztor *et al*., 2010) which might also be the case in the CmR mutants.

Link between heat shock response and autolysin activity. Autolysin expression and the heat shock response are interlinked processes in *S. aureus*. Heat shocked (40 °C for 10 min) *S. aureus* cells exhibited decreased Triton X-100 induced lysis in comparison to cells grown at 30 °C (Qoronfleh & Wilkinson, 1986). The missing autolysin activity under thermal stress was thought to rely on the thermolability of autolysins or the loss of magnesium cations in the cell envelope that are important for autolysin targeting (Qoronfleh & Wilkinson, 1986). However, since other agents known to induce heat shock proteins, like $CdCl_2$ or ethanol, also reduced the autolysin activity, complexing with heat shock proteins was thought to inhibit the autolysin activity (Qoronfleh & Wilkinson, 1986). In line with this, autolysins were highly expressed in cervimycin treated *S. aureus*, probably to compensate for the binding of autolysin by the highly expressed chaperones.

Another option was the reduced secretion of autolysins in cervimycin treated cells due to incorrect signal peptides originating from protein mistranslation, secretion of inactive autolysins, or a general secretion inhibition mechanism. The former mechanism would explain the similarities to the mistranslation inducing aminoglycosides, although the missing cell wall stress response represents a major difference between the substances. For the latter mechanism, the cervimycin stress response was compared with the stress response of the signal peptidase I

(SPase) inhibitor arylomycin (Craney & Romesberg, 2015), since secretion stress of *S. aureus* is not well characterized. Indeed, expression of the GroES chaperone is induced by SPase inhibition, but the primary stress response includes the activation of the cell wall stress response and an alternative protein release mechanism by de-repression of the *aryRABC* operon (Craney & Romesberg, 2015, 2017) that was not observed in cervimycin treated *S. aureus*.

Electron microscopy of cervimycin treated *S. aureus* cells revealed multiple defects, including a cell division defect, cell wall thickening, a rough cell surface, and disturbed septum formation, causing septa of irregular length, thickened septa, and thinning in the upper part of septa (**Chapter 4.3**, above). Strikingly, tetracycline and aminoglycoside treatment also significantly thicken the cell wall of *S. aureus* (Figure 21).

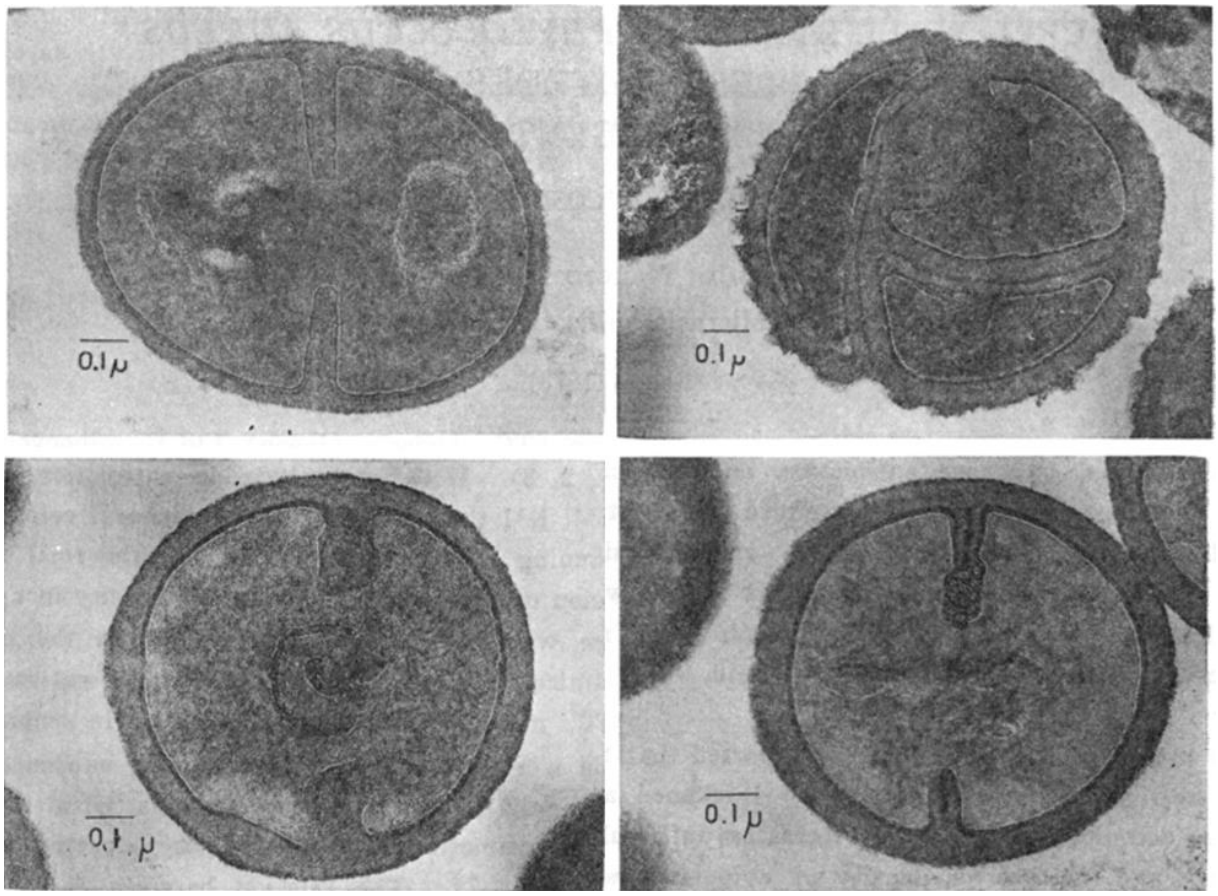

Figure 21: Tetracycline (top right), kanamycin (bottom left) and streptomycin (bottom right) treated *S. aureus* cells exhibit thickened cell walls and abnormal cell division in comparison to an untreated control (top left) (modified after Nakao and Nakazawa (1974)); electron microscopy of agar slides of *S. aureus* 209-P JC, treated with 10 µg/mL of the indicated antibiotics for 24 h.

Interestingly, similar to cervimycin, tetracycline treatment causes cell wall thickening and a rough cell surface in *S. aureus*, while the cell wall of streptomycin and kanamycin treated cells is smoother. However, tetracycline also disrupts the binary division of *S. aureus* which

was not seen with cervimycin, but might also rely on different treatment times. In regard to septum defects, streptomycin causes a similar phenotype like cervimycin in *S. aureus*, altering especially the upper part of the septum, while kanamycin also significantly thickens the cross wall.

All in all, the balance between cell wall synthesis and cell wall hydrolysis is greatly disturbed by ribosome-targeting agents, a characteristic which was shared with cervimycin treated *S. aureus*. This imbalance was further underpinned by the occurrence of D shaped cells due to cervimycin treatment (**Chapter 4.3**, above). When *S. aureus* undergoes cytokinesis, it builds a septum, generating two hemispherical daughter cells whose cell walls are only connected via a narrow peripheral ring (Zhou *et al*., 2015), described as D shape in a simplified manner. During daughter cell splitting, this ring is then resolved within milliseconds and "popping" leads to a mature spherical morphology (Wheeler *et al*., 2015; Zhou *et al*., 2015). Notably, an *atl* deletion mutant "pops" with a similar millisecond time scale as that of the wild type, but remains associated longer after "popping", leading to the characteristic clustering phenotype (Zhou *et al*., 2015). Hemispherical (D shaped) cells occur in a *sagB* deletion mutant and, even at a higher prevalence, when multiple glucosaminidases (SagB, Atl, SagA, ScaH) are depleted (Wheeler *et al*., 2015). Cells impaired in glucosaminidase activity are also impaired in their ability to increase in size after division and adopt the correct mature shape, mainly due to the dominant nonredundant glucosaminidase activity of SagB that is responsible for the archetypical short glycan chain length of *S. aureus* and has the most profound role in cell wall stiffness determination (Wheeler *et al*., 2015). Such a lack of glucosaminidase activity also occurs in amikacin treated *S. aureus* (Santhana Raj *et al*., 2007).

In conclusion, cervimycins are polyketide glycosides in which sugar residues act as a decisive factor in their architectural complexity and bioactivity, while the malonyl side chain is not crucial for the antibacterial mode of action. Probably due to the mere size of these molecules (up to 1,250 Da), the antibacterial activity is restricted to Gram-positive bacteria. Since multiple defects were observed in cervimycin treated Gram-positive bacteria, including cell division and chromosome segregation defects, cell wall thickening, and a rough cell surface, various cellular processes seemed to be disturbed, indicating a complex mode of action of cervimycin. High cervimycin concentrations disturb the incorporation of thymidine into the DNA and the *in vitro* activity of the DNA gyrase was inhibited at high cervimycin concentrations, similar to the un-glycosylated polyketide actinorhodin. This effect might be attributed to the quinone motif in the aglycone of both compounds. However, since the cervimycin stress response included a

dual de-repression of the heat shock/protein stress response and autolysin expression, but not the induction of the DNA stress response or *gyrAB* expression, the primary interaction site was expected to be somewhere else. Interestingly, this dual de-repression of the heat shock response and autolysin expression except for *atl* and *sle1*, as well as a shutdown of the purine metabolism, was shared between the cervimycin treated cells and the cervimycin-resistant mutant CmR-02. In contrast to CmR-02, where the increased expression of the CtsR/HrcA operon was attributed to the loss-of-function mutation in the *clpP* gene (resulting in $ClpP^{I29F}$), the cause of the protein stress response in cervimycin treated *S. aureus* remains to be elucidated, since numerous circumstances induce this stress response, such as an imbalance in metal ion homeostasis, treatment with ethanol or SDS, or antibiotics with different modes of action like cell envelope and ribosome-targeting antibiotics. The probably most remarkable finding was the induction of a strong protein stress response by cervimycin without concomitant induction of the cell wall stress response, a capacity which cannot be fully assigned to the GraS deficiency of our model strain *S. aureus* SG511 Berlin, since the cell wall stress response was also absent in *Bacillus*. Whether or not increased autolysin expression causes high chaperone expression or *vice versa* also needs to be clarified. Nevertheless, the D shape of *S. aureus* cells observed under cervimycin treatment indicates missing glucosaminidase activity. Since a direct interaction of cervimycin was neither proven with the essential WalRK two-component system nor with the non-essential Clp protein degradation system, a compensatory effect of the resistance mutations in the CmR strains appeared to be most likely. Although some glycosylated polyketides, such as doxorubicin, are in clinical use for a long time, their mechanism of action is partially but still not fully understood, underlining the complexity of such studies, but also the untapped potential of this class of natural products, including cervimycin.

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List of Figures

List of Tables

6. List of Publications

- Gajdiss, M., Monk, I. R., Bertsche, U., Kienemund, J., Funk, T., **Dietrich, A**., Hort, M., Sib, E., Stinear, T. P., Bierbaum, G. (2020). YycH and YycI Regulate Expression of *Staphylococcus aureus* Autolysins by Activation of WalRK Phosphorylation. Microorganisms, 8(6), 870. doi: 10.3390/microorganisms8060870

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- **Dietrich, A.**, Steffens, U., Gajdiss, M., Boschert, A. L., Dröge, J. K., Szekat, C., Sass, P., Malik, I. T., Bornikoel, J., Reinke, L., Maček, B., Franz-Wachtel, M., Nieselt, K., Harbig, T. A., Scherlach, K., Brötz-Oesterhelt, H., Hertweck, C., Sahl, H.-G., Bierbaum, G. (2022). Cervimycin-Resistant *Staphylococcus aureus* Strains Display Vancomycin-Intermediate Resistant Phenotypes. Microbiology spectrum, 10(5), e02567-22. doi: 10.1128/spectrum.02567-22

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