# Modifications of the *Staphylococcus aureus* cell envelope and their roles in host-microbe interactions

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# Meiner Familie

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# Summary

*Staphylococcus aureus* is a frequent constituent of human nasal flora and a major cause of severe infections. In order to successfully establish infections, *S. aureus* has evolved several mechanisms to resist and evade the human innate immune system (36).

One mechanism is the neutralization of the cell surface net charge by *dlt*-mediated incorporation of positively charged alanine residues into teichoic acids (99). In this work we could show that *dlt*-mediated alanylation is regulated by the novel two-component regulatory system GraRS and that this regulation is crucial for *S. aureus* CAMP resistance and virulence. However, the detailed mechanisms concerning activating stimuli and involved regulatory proteins remained unknown.

CAMPs have many functions in the innate immune response. The fact that they show rather weak killing activity when tested under culture conditions with physiological NaCl concentrations or serum (13) led to a debate as to the true relevance of these peptides in direct inactivation of microorganisms. Here we could show, that carbonate ions are essential to render bacteria susceptible to CAMPs. The presence of carbonate leads to dramatic alterations of bacterial transcriptional profiles and cell wall structure. However, the detailed mechanisms that lead to increased susceptibility to CAMPs in the presence of carbonate remained unknown.

Neutrophils are the first-line cell defense of the innate immune system. They inactivate pathogens by production of CAMPs and by the oxidative burst. Former publications postulated a role of BK-channels (big conductance $Ca^{2+}$-activated $K^{+}$-channels) in production of toxic, oxygen-containing molecules, and consequently, in innate antimicrobial host defense (1). In this work we could show, that BK- channels are absent in neutrophils and are not essential in combating invading pathogens.

Besides the resistance against innate antimicrobial defenses, evasion of recognition by receptors of the innate immune system is essential to establish an infection. Recent studies have shown, that modifications of muropeptides strongly impair recognition of peptidoglycan structures by receptors such as NOD1 (19,44). *S. aureus* modifies its muropeptides by D-glutamate amidation. However, we could not detect an impact of muropeptide amidation on the immune stimulating capacity of *S. aureus* in this work. The role of this modification on CAMP resistance e.g. could be an interesting question for future studies.

# Introduction

## 1.1. The innate immune system

The immune system of vertebrates is divided into two parts. Besides the adaptive immune system, the innate immune system defends the host against invasion of pathogens by both humoral and cellular mechanisms.

The first line of innate defense is formed by the epithelia, which protect the human body from invading microorganisms both physically and by production of antimicrobial molecules. These antimicrobial defense molecules are also produced by specialized cells of the innate immune system, such as granulocytes. They are produced constitutively or after contact with pathogens and can be divided into two groups, cationic antimicrobial peptides (CAMPs) and bacteriolytic enzymes. Another mechanism, besides production of antimicrobial defense molecules, is the oxidative burst. Granulocytes and other cells of the innate immune system, such as monocytes and macrophages, produce high amounts of toxic, oxygen-containing molecules that lead to lysis of microorganisms in the lysosome of immune cells.

Further important functions of monocytes, macrophages and also dendritic cells are presentation of antigens and production of cytokines that lead to enhanced immune reaction and, if immoderate, to sepsis.

Some cell types that play key roles in eliminating pathogens are shown in tab. 1.

**Tab. 1.** Cells and their functions in host defense

| Cell | Function |
|---|---|
| epithelial cell | physical barrier; production of CAMPs; production of IL-8 that leads to recruitment of macrophages and granulocytes |
| neutrophil granulocyte | phagocytosis and inactivation of pathogens by CAMPs, enzymes and oxidative burst |
| macrophage | phagocytosis and inactivation of pathogens by oxidative burst; antigen presentation; production of cytokines (e.g. TNF-α, IL-1, IL-6, IL-8, IL12) |
| dendritic cell | antigen presentation; production of cytokines (e.g. IL-6, IL-12, IL-18, INF-α, INF-γ) |

Another important, humoral factor is the complement system, which leads both to direct inactivation and opsonization of microorganisms.

### 1.1.1. Cationic antimicrobial peptides (CAMPs)

CAMPs are amphiphilic peptides, which usually contain 10-50 amino acids, and have cationic net charge (53). Due to their positive charge, they show high affinity for anionic bacterial cell surface components (tab. 2.). CAMPs also contain regions with hydrophobic amino acids that enable them to integrate into bacterial membranes. Multimerization leads to pore formation and, subsequently, to lysis of the bacterium (112). Another discussed mechanism is the interaction with intracellular targets, e.g. the highly negatively charged DNA. This could lead to abrogation of transcription and replication and finally to cell death (15).

Host cells are protected from destruction by CAMPs by incorporation of mainly unpolar or neutral lipids and by high contents of cholesterol in the cytoplasmic membrane (103,126).

**Tab. 2.** Opposing charges of antimicrobial host factors and components of the bacterial membrane. Some exemplary molecules are shown.

| **Cationic antimicrobial host factors** | **Anionic molecules of bacterial cell surface components** |
|---|---|
| defensins | peptidoglycan |
| cathelicidin | lipopolysaccharid |
| thrombocidin | lipid A |
| lysozyme | wall teichoic acids |
| lactoferrin | lipoteichoic acids |
| cathepsin G | phosphatidylglycerol |
| group IIA-phospholipase $A_2$ ($PLA_2$) | cardiolipin |
| myeloperoxidase | |

Based on their structure, CAMPs can be divided into three groups. The structures of some examples of the different groups are shown in fig. 1.

**Defensins** show β-sheet structure and are stabilized by three disulphide bridges. According to the pattern of the disulphide bridges, defensins are devided into α-defensins and β-defensins. α-defensins are mainly produced by neutrophil granulocytes (HNPs, human neutrophil peptides) and by Paneth cells in the intestinal tract (cryptdins). β-defensins are mainly produced by epithelial tissues, both keratinized, such as the skin, and non-keratinized, such as the epithelia of the respiratory, gastrointestinal and urogenital tract (hBDs, human beta-defensins) (38,53,80).

**Cathelicidins** are wide spread in mammals. However, in humans just one cathelicidin is described, the LL-37. It is α-helical and is produced by granulocytes and different epithelia (92).

**Thrombocidins** are derived from chemokines and produced by platelets (72).

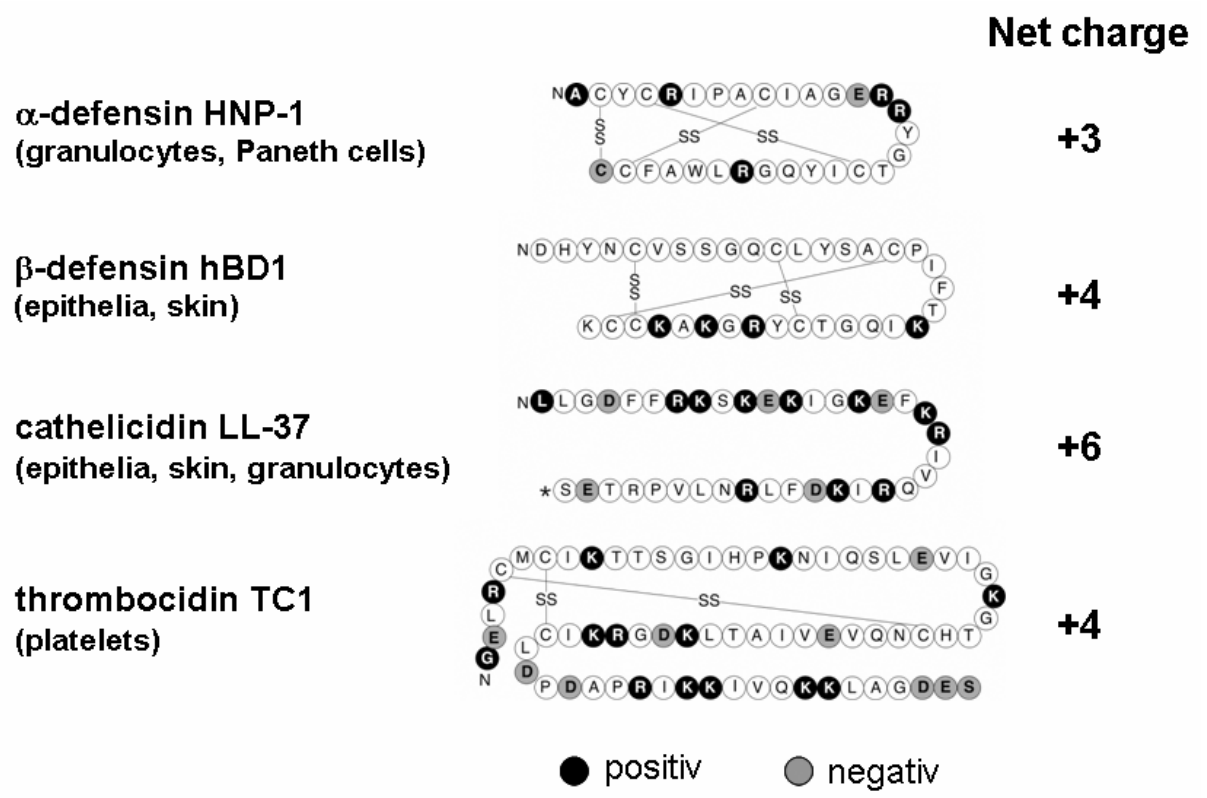


**Fig. 1.** Structure of some exemplary human cationic antimicrobial peptides and their net charge. Charged amino acid residues are highlighted.

All CAMPs are processed from propeptides and can be modified posttranslationally (e.g. the θ-defensins, which are found in Rhesus monkeys, are posttranslationally cyclized) (39,97).

CAMPs can be produced constitutively, e.g. hBD1, and induced by contact with pathogens, e.g. hBD2 and LL-37 in keratinocytes (13). Production of α-defensins in Paneth cells is also induced after contact with bacteria (97), and the release of thrombocidins by platelets is induced after contact with thrombin (129).

The fact that CAMPs seem not to be antimicrobially active when assayed *in vitro* under physiological concentrations of NaCl (13) lead to a debate as to the true relevance of these molecules in innate immunity. Bivalent cations such as $Mg^{2+}$ and $Ca^{2+}$ can also lead to abrogation of killing activity of CAMPs *in vitro* in concentrations

present in body fluids (14,81). However, this contradicts the results of several animal models in which the *in vivo* relevance of these peptides had clearly been shown. Which factors facilitate the direct antimicrobial activity of CAMPs under physiological conditions *in vivo* remained unclear.

CAMPs have, besides their direct antimicrobial activity, many other functions. They have been shown to bind LPS, thereby preventing inflammation. LL-37 is able to induce apoptosis (in epithelial cells) or inhibit apoptosis (in granulocytes). Many CAMPs can act as chemokines and induce chemokine production. Furthermore LL-37 seems to play a role in wound healing (13).

### 1.1.2. Bacteriolytic enzymes

This group of natural antibiotics contains several proteins with enzymatic properties, which are able to destroy the bacterial cell envelope. Important examples are lysozyme and group IIA-phospholipase A2, which are produced by various cell types of the innate immune system and are key components in the host´s defense against bacterial pathogens (90).

### 1.1.3. Oxidative burst

Phagocytes, such as neutrophil granulocytes and macrophages, produce high amounts of toxic, oxygen-containing molecules by which microorganisms can be killed in the phagolysosome. Some examples are hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH^{\bullet}$), hypochlorous acid (HOCl), superoxid anions ($O_2^-$), singlet oxygen ($^1O_2$) and nitric oxide (NO). Enzymes that play key roles in the synthesis of these molecules are myeloperoxidase, the NADPH-oxidase and the nitric oxide synthase. Another factor that facilitates killing in the phagolysosome is acidification.

### 1.1.4. Complement system

The complement system consists of humoral factors. It acts directly antimicrobially by forming pores in surfaces of Gram-negative bacteria by the membrane attack complex (MAC) and facilitates effective phagocytosis by opsonizing both Gram-positives and Gram-negatives. Gram-positives are resistant against the MAC.

### 1.1.5. Further mechanisms of host antimicrobial defense

Iron is an essential factor for bacterial survival. Humans produce various iron-binding proteins such as transferrin and lactoferrin. Lactoferrin acts directly antimicrobially in high concentrations, however, more important seems to be the limitation of free iron by lactoferrin, which leads to decreased bacterial growth and virulence (22). Bacteria acquire free iron in the host´s milieu by binding it to siderophores such as the enterobactin of *E. coli* (34). Immune cells are able to produce lipocalin 2, which binds the siderophore-iron complex, thereby preventing uptake into the bacterial cell (35).

An antimicrobial protein which is produced by the human skin is psoriasin. It has been shown to protect the human skin from colonisation by *E. coli* (45).

Further mechanisms that protect the host from invading pathogens are acidic conditions in the stomach, digestion enzymes and bile salts in the intestinal tract. And also the normal human bacterial flora can be regarded as a defense mechanism. Many commensal bacteria compete for ecological niches. They also produce antibacterial factors that can inactivate other bacterial species, thereby preventing colonization with pathogens. Such bacterial defense molecules are e.g. epidermin, produced by *Staphylococcus epidermidis*, and colicins, produced by *E. coli* (108).

Production of antimicrobial fatty acids, high salt concentrations, and the low pH on the surface of human skin can also be regarded as crucial factors that ensure a bacterial ecology essential for human health.

## 1.2. Receptors of the innate immunity and their bacterial ligands

The activation of the innate immunity by conserved bacterial structures is crucial for a successful defense against bacterial pathogens. These pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs). The best studied PAMP is the lipopolysaccharide (LPS) of Gram-negative bacteria. The corresponding structure in Gram-positive bacteria is, in a certain manner, the lipoteichoic acid (LTA). Further potent activators of the innate immunity are bacterial CpG-motives, peptidoglycan (PG) and peptidoglycan fragments (the muropeptides), which are common for Gram-positives and Gram-negatives. A well known muropeptide is the muramyldipeptide (MDP), which represents a strong activator of the innate immune response. Further PAMPs are flagellin, lipopeptides, porins and glyco-structures such as the lipoarabinomannan of mycobacteria. Various other anionic polymers on the bacterial surface can be recognized by receptors of immune cells.

Further very important PAMPs are formylated peptides, which are produced by bacteria but not by eukaryotic cells. They are recognized by the formyl peptide receptor (FPR), which is expressed mainly by neutrophil granulocytes (77). The activation of FPR leads to chemotactic recruitment of granulocytes to the place of infection and is thereby one of the first-line mechanisms of the innate immune response (30).

The PRRs play a crucial role in the detection of PAMPs. A well known group of PRRs is the Toll-like receptor (TLR) family. 10 TLRs have been described in humans to date. They are expressed on myeloid cells, including macrophages, granulocytes and dendritic cells, and also nonmyeloid cells such as epithelial and endothelial cells and fibroblasts.

### 1.2.1. Toll-like receptors

Most of the TLRs recognize bacterial structures that are common for Gram-positives, Gram-negatives and *Mycoplasma*. TLR5 recognizes flagellin, which is present in all flagellated bacteria (54). TLR9 is activated by unmethylated CpG-DNA-motives (6). These motives are more frequent in bacteria than in eukaryots, which facilitates the discrimination between host and microbial DNA. A member of the TLR family which solely binds Gram-negative structures is TLR4, which is activated by LPS (122).

Atypical LPS from *Porphyromonas gingivalis* is also recognized by TLR2 (5,107). TLR2 is activated by several other bacterial structures, including LTA of Gram-positives (52,65,114), porins of various Gram-negative species such as *Neisseria*, *Shigella* und *Haemophilus* (87,105,106), and the lipoarabinomannan of *Mycobacteria* (123). The role of peptidoglycan as an activator of TLR2 remains a matter of debate (115,131).

Some bacterial structures are recognized by heterodimers of TLRs. Triacylated lipopeptides of Gram-negative bacteria are recognized by a dimer of TLR1 and TLR2 (18,121). TLR2 can also form dimers with TLR6. This complex is activated by diacylated lipopeptides of *Mycoplasma* species and by LTA of group B streptococci (16,55,117).

TLR3, 7 and 8 recognize double and single stranded RNA and are therefore important in response to viral infections (25,116,125).

The TLRs and their ligands are summarized in tab. 3.

**Tab. 3.** TLRs and their microbial ligands. *The role of peptidoglycan as an activator of TLR2 remains a matter of debate.

| TLR | Microbial ligand | Species | Ref. |
|---|---|---|---|
| TLR1/TLR2 | triacylated lipopeptides | Gram-negative bacteria | (18,121) |
| TLR2 | LTA | Gram-positives, e.g. *S. aureus*, *S. pneumoniae* | (52,65,114) |
| | atypical LPS | Gram-negatives, e.g. *Porphyromonas gingivalis* | (5,107) |
| | porins | Gram-negatives, e.g. *Neisseria*, *Shigella* | (87,105,106) |
| | peptidoglycan* | Gram-positives, -negatives | (115,131) |
| | lipoarabinomannan | mycobacteria | (123) |
| TLR2/TLR6 | diacylated lipopeptides | mycoplasma | (16,117) |
| | LTA | group B-streptococci | (55) |
| TLR4 | LPS | Gram-negatives | (122) |
| TLR5 | flagellin | flagellated Gram-positives, -negatives | (54) |
| TLR9 | CpG | Gram-positives, -negatives, mycobacteria | (6) |
| TLR3 | dsRNA | viruses | (116) |
| TLR7,8 | ssRNA | viruses | (25,125) |

### 1.2.2. NODs

Further receptors that play a crucial role in recognition of bacterial structures are the intracellular NODs (NOD = nucleotide-binding oligomerization domain-like receptor) (63). For humans, two NODs have been described in detail, NOD1 and NOD2. Both

are expressed by various cell types including epithelial cells and myeloid cells such as macrophages. NOD1 and NOD2 recognize different structures of the bacterial cell wall (37). The minimal motive for activation of NOD1 is a dipeptide consisting of D-glutamate (D-Glu) and meso-diaminopimelic acid (meso-DAP) (19,42). This motive is usually found in the PG of Gram-negatives, but also exists in *Bacillus* species. In contrast, NOD2 recognizes MDP, which consists of muramic acid, L-alanine and iso-glutamine. this motive is common for both Gram-negatives and Gram-positives (43,60). It has been shown, that modifications of NOD1-activating structures can cause partial or even total abrogation of the proinflammatory capacity of these motives. Amidation of the D-Glu residue partially inhibits NOD1 stimulation (19) and amidation of meso-DAP totally abrogates stimulatory properties of the dipeptide (44). Gram-positive bacteria also modify their PG structures. *S. aureus* is known to amidate a major part of the D-Glu residues in the peptide chains of its PG, resulting in formation of iso-glutamine. This amidation has a profound impact on the physicochemical properties of the molecule, since it removes the negative charge from the D-Glu α-carboxyl group. Accordingly, the net charge of MDP at physiological pH changes from -2 to -1. If this modification has an impact on the stimulatory capacity of MDP, remains to be elucidated.

### 1.2.3. Further PRRs

Further important receptors that play a role in innate immunity are e.g. the mannose receptor and the scavenger receptors, which are mainly expressed in macrophages and recognize sugar structures and anionic polymers on bacterial surfaces. In addition, there are co-receptors that are important for the recognition of ligands by TLRs, e.g. CD14, which binds LBP-coupled LPS together with TLR4 (LBP = lipopolisaccharide binding protein), or CD36, which acts as a facilitator or co-receptor for di-acylglyceride recognition by the TLR2/6 complex (58).

## 1.3. Resistance mechanisms of Gram-positive bacteria against the innate immune response – lessons from *S. aureus*

In order to successfully establish an infection, pathogenic microorganisms such as *S. aureus* have to resist the host innate antimicrobial defense to a certain extent. Therefore, *S. aureus* has evolved several mechanisms to resist and evade these host defense mechanisms (36). In the following chapter, several known mechanisms will be described examplarily. The focus will be mainly on the resistance mechanisms against cationic antimicrobial peptides and their regulation. At the end of the chapter resistance mechanisms against further components of the innate immune system will be briefly described.

### 1.3.1. Proteolytic degradation of CAMPs

The production of peptidases and proteases that cleave the defense peptides is one mechanism to gain resistance to CAMPs in *S. aureus*. *S. aureus* secretes several of these enzymes, e.g. the metallo-protease aureolysin and the serin-protease V8, which are capable of cleaving LL-37. This correlates with staphylococcal *in vitro* resistance to CAMPs (118).

### 1.3.2. Production of extracellular CAMP-binding molecules

Another resistance mechanism against $\alpha$-defensins is the production of the fibrinolytic enzyme staphylokinase (SAK). This protein has, besides fibrinolytic activity, high affinity for human $\alpha$-defensins. By binding these molecules, SAK production leads to significant $\alpha$-defensins resistance, and production of SAK correlates with *in vitro* susceptibility of *S. aureus* isolates (61).

### 1.3.3. CAMP-specific export systems

Bacterial resistance against small, amphipathic, antimicrobial molecules is often mediated by energy-dependent export systems, which extrude the substances from the membrane. Due to their broad substrate specificity these systems are often called multi drug resistance exporter (MDR). CAMPs also show amphipathic, membrane damaging properties and can therefore be exported by MDRs. The QacA

efflux pump leads to resistance against platelet-derived CAMPs, the so called tPMPs (thrombin-induced microbicidal proteins), in *S. aureus*. (75). However, it has recently been shown, that the resistance phenotype is not related to a peptide efflux but rather results from the impact of a specific transporter on membrane structure or function (7).

### 1.3.4. CAMP-resistance by alteration of the cell surface net charge

CAMPs and most other antimicrobial molecules, including lysozyme, phopholipase A2 and RNase5, have positive net charges. In contrast to the surfaces of human cells, which normally consist of mainly uncharged or zwitterionic lipids, the bacterial surface usually has anionic properties. This is due to many anionic components such as peptidoglycan, most phospholipids, lipid A and teichoic acids. It is assumed, that antimicrobial host factors have evolved cationic properties to gain high and selective affinity for the anionic surfaces of bacteria. Since these structures are very ancient and invariable, it seems impossible for bacteria to replace them with other structures that would be less favourable for an interaction with CAMPs. However, there are some bacterial species that are able to reduce the negative charge of their cell envelope, thereby becoming resistant to inactivation by many cationic antimicrobial host factors. In *S. aureus* two mechanisms have been described in detail.

1. Modification of teichoic acids with D-alanine:

Teichoic acids of *S. aureus* and other Gram-positive bacteria consist of alternating glycerolphosphate or ribitolphosphate units, which are substituted with N-acetyl-glucosamine or D-alanine (91). These polymers are either anchored to the cytoplasmic membrane (lipoteichoic acid = LTA) or the peptidoglycan (wall teichoic acid = WTA) and show anionic properties due to the presence of negatively charged phosphate groups. By substitution of teichoic acids with D-alanine, positive amino groups are introduced, which leads to a partial neutralization of the polymer (99). The modification of the net charge leads to a decreased interaction of CAMPs with the bacterial surface and, consequently, to a reduced sensitivity to a wide range of cationic host defense molecules (99). The products of the *dlt*ABCD operon, which exists in the genomes of many Gram-positives of the low G+C branch, are responsible for the transfer of D-alanine residues to teichoic acids (99).

2. Modification of phospholipids with L-lysine

Most bacterial phospholipids, such as phosphatidylglycerol and cardiolipin, also show anionic properties (59). *S. aureus* and many other bacteria are able to modify the majority of phosphatidylglycerol with L-lysine (104). This modification leads, similar to the alanylation of teichoic acids, to neutralization of the cell envelope and accordingly to reduced susceptibility to many CAMPs (68,74,98). The lysinylation of phosphatidylglycerol is mediated by the membrane protein MprF (95,120). Homologues are found in various bacterial species. Besides human pathogens, it is also found in plant pathogens and even archaeal species (127).

3. Formation of cationic slime polymers and capsules

The production of biofilms is a further mechanism to develop resistance to CAMPs in *S. aureus*. This is most probably due to the cationic properties of the sugar polymer (PIA, polysaccharid intercellular adhesin) that is responsible for biofilm formation. These cationic properties lead to repulsion of CAMPs from the bacterial cell surface (96).

Capsules usually do not represent a major diffusion barrier for small molecules such as CAMPs. However, in some cases, the extracellular slime matrix has been shown to provide protection against certain CAMPs (17).

### 1.3.5. Further modifications of the cell envelope

Another modification of the cell envelope of *S. aureus*, implicated in CAMP resistance, is the modification of fatty acids which are introduced into lipids of the bacterial membrane. An elevated level of longer-chain, unsaturated fatty acids leads to an altered fluidity of the membrane, which results in decreased sensitivity for platelet-derived CAMPs (8). This hypothesis is supported by the decreased susceptibility of a *cspA* (the major cold shock gene) mutant for a cathepsin G-derived CAMP (64). The deletion of *cspA* leads to the loss of the membrane carotinoid staphyloxanthin, which also may result in altered membrane fluidity.

In order to integrate into lipid bilayers CAMPs need a certain membrane potential. This is, in contrast to eukaryots, present in bacterial surfaces due to the respiratory chain that is located in the cytoplasmic membrane. Yeaman *et al.* showed that mutations in the respiratory chain that lead to the phenotype of the so called small

colony variant (SCV) result in loss of the membrane potential and thereby to decreased sensitivity to CAMPs (130).

There are further modifications of the staphylococcal cell envelope that are discussed as resistance mechanisms against CAMPs. It is known, that *S. aureus* amidates the majority of D-glutamate of muropeptides. By this amidation, a usually negatively charged hydroxyl group is neutralized by an amino group, which results in reduced negative charge of the muropeptide. If this modificaton contributes to resistance against cationic host factors, has to be elucidated in more detail in future studies.

### 1.3.6. Regulation of resistance to antimicrobial peptides

The mechanisms involved in regulation of bacterial resistance to antimicrobial peptides such as environmental signals and regulatory proteins are not yet well understood. However, there are some regulatory systems described in Gram-negative bacteria which play key roles in the resistance to CAMPs. The two-component system PhoP/PhoQ e.g. plays a key role in virulence of *S. enterica*, *P. aeruginosa* und *Y. pseudotuberculosis* (47). PhoP/PhoQ recognizes changes in magnesium and calcium concentrations (40) and is activated by subinhibitory concentrations of CAMPs, as recently shown for *S. enterica* (3). The sensor kinase PhoQ directly recognizes CAMPs, thereby displacing PhoQ-bound divalet cations and leading to activation of the response regulator PhoP (4). PhoP/PhoQ regulates proteins including PagP, which is essential for the modification of lipid A, leading to CAMP resistance in *S. enterica* (50). A second system that is regulated by PhoP/PhoQ in *S. enterica* are the pmrA/pmrB genes (47). The pmrA/pmrB system recognizes extracellular iron concentrations (128) and regulates transcription of genes including *pmrE* and *pmrHFIJKLM*, which are responsible for the synthesis of a lipid A variant with 4-aminoarabinose modification (48). This modification leads to a reduction of the anionic net charge of bacterial lipid A and, subsequently, to decreased sensitivity for CAMPs. A related system in *P. aeruginosa* seems to respond directly to CAMP exposure (88).

Much less is known about the regulation and involved stimuli of CAMP-resistance in Gram-positives. The global regulatory system *agr* is involved in regulation of the *dlt* operon (29). The *dlt* operon is responsible for alanylation of teichoic acids, which leads to resistance to various cationic antimicrobial defense molecules (99). Li *et al*. recently showed that the new three component regulatory system *aps* in *S.*

*epidermidis* controls both the *dlt* operon and the *mprF* gene, which is responsible for lysinylation of phosphatidylglycerol (82). Similar results have been found for the listerial transcription factor VirR, which also regulates both loci (86).
Inactivation of further regulation systems in *S. pyogenes* (93) and *L. monocytogenes* (23) also leads to decreased sensitivity for CAMPs. However, the controlled genes remained unknown.

### 1.3.7. Resistance to bacteriolytic enzymes

Concerning resistance to bacteriolytic enzymes, some involved mechanisms have been described in *S. aureus* recently. These mechanisms are based on the modification of cell envelope structures. Koprivnjak *et al*. showed that the sensitivity of a *dlt* mutant for group IIA-phospholipase A2 is 30-100 fold increased compared to the wild type (68). Group IIA-phospholipase A2 has cationic properties and binds to highly anionic polymers such as teichoic acids, which promotes interaction of the enzyme with the bacterial membrane. By alanylation, and thereby neutralization of the net charge of teichoic acids, *S. aureus* is able to weaken the attack of the phospholipase on the membrane (68).
*S. aureus* has also evolved efficient mechanisms against inactivation by lysozyme. The backbone of peptidoglycan consists of alternating N-acetyl-muramic acid and N-acetyl-glucosamine. Lysozyme normally recognizes this structure and cleaves the polymer between the two sugars. O-acetylation at position C-6 of the muramic acid leads to complete resistance of *S. aureus* to lysozyme (10). Other factors involved in resistance to lysozyme are attachment of wall teichoic acids to the C-6 position of the muramic acid and the degree of cross linkage of the peptidoglycan (9).

### 1.3.8. Further resistance mechanisms to innate immunity

As mentioned above, granulocytes and macrophages are potent cells in combating invading pathogens. *S. aureus* has evolved various mechanisms to counter inactivation by these cells. One of them is production and secretion of leukocidin, a toxin that directly kills leukocytes such as granulocytes (41,62). The production of a polysaccharid capsule inhibits phagocytosis (94). In the lysosome the pathogen has to face antimicrobial factors including the oxidative burst and high concentrations of CAMPs. To survive the attack of CAMPs, *S. aureus* has evolved the mechanisms

described above. The production of the membrane carotinoid staphyloxanthin is an efficient mechanism to resist high concentrations of toxic, oxygen-containing molecules of the oxidative burst (21,84).

A cell-associated protein that plays a key role in resistance to phagocytosis of *S. aureus* is protein A. This protein is able to bind the Fc part of human IgG, preventing opsonization by the antibody (110). A similar effect is mediated by the staphylokinase. This enzyme activates the conversion of plasminogen to plasmin. Plasmin is able to cleave IgG. This cleavage leads to prevention of opsonization. (111).

Another antibody-binding protein is the staphylococcal superantigen-like 7 (SSL-7 ), which binds $IgA_1$ and $IgA_2$ (76). Consequently, these antibodies are not recognized by receptors of immune cells anymore.

After invading the host, the pathogen has to face not only cellular but also humoral components of the innate immunity. There are several mechanisms described that enable *S. aureus* to circumvent the effects of these components. The complement system e.g. is target of several secreted or cell associate factors which are produced by *S. aureus* to prevent an effective function of the complement cascade. CHIPS (chemotaxis inhibitory protein of *S .aureus*) e.g. binds to the C5a-receptor and the formyl-peptide-receptor, which prevents an efficient chemotactic recruitment of granulocytes to the place of infection (27,101). Another secreted protein is SCIN (staphylococcal complement inhibitor). It binds to C4b2a und C3bBb, thereby preventing successful conversion of these factors to C3, which is an essential step in the complement cascade (109). The Efb-protein (extracellular fibrinogen binding molecule) binds directly to C3, which is responsible for opsonisation and subsequent phagocytosis of the pathogen (78,79).

Protein A not only blocks opsonization by binding IgG, but also blocks complement activation by C1q (110). The C1q molecule is recognized by a specific site in the Fc fragment of IgG. This recognition is hindered by cleavage of IgG by plasmin, which was converted from plasminogen after SAK activation (111).

A further protein secreted by *S. aureus* is FLIPr (FPRL1 inhibitory protein). This protein directly binds to the FPRL1 (formyl peptide receptor like 1). FPRL1 can be activated e.g. by fMLP (formylated peptide consisting of methionine, leucin and prolin). However, a bacterial ligand for FPRL1 has not been described yet. In higher

concentrations, FLIPr can also bind to the FPR which is crucial for recognition of bacterial formylated peptides and activation of chemotaxis (102).

The above described mechanisms of the innate immune system and staphylococcal resistance mechanisms are summarized in tab. A.4.

**Tab. 4.** Defense mechanisms of the innate immunity and resistance mechanisms of *S. aureus*

| **Defense mechanism** | **Resistance mechanism of *S. aureus*** | **Reference** |
|---|---|---|
| production of CAMPs | proteolytic degradation | (118) |
| | extracellular binding | (61) |
| | specific export | (75) |
| | modification of the cell envelope net charge | (74,95,98,99,120) |
| | biofilm formation | (96) |
| | polysaccharid capsule | (17) |
| | modification of membrane fluidity | (8,64) |
| | small colony variant phenotype (SCV) | (130) |
| production of bacteriolytic enzymes | modification of the cell envelope net charge | (68) |
| | O-acetylation | (10) |
| | WTA | (9) |
| oxidative burst | staphyloxanthin | (21,84) |
| phagocytosis | leukocidin | (41,62) |
| | polysaccharid capsule | (94) |
| | CHIPS | (27,101) |
| | FLIPr | (102) |
| | protein A | (110) |
| complement system | SCIN | (109) |
| | Efb | (78,79) |
| | SSL-7 | (76) |
| | staphylokinase | (111) |

# Muropeptide Modification-Amidation of Peptidoglycan D-Glutamate Does Not Affect the Proinflammatory activity of *Staphylococcus aureus*

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## Abstract

Peptidoglycan muropeptides, potent proinflammatory components, are amidated in *Staphylococcus aureus* for unknown reasons. To study whether this modification may modulate proinflammatory capacity, cytokine induction by isogenic *S. aureus* strains with different amidation levels and by synthetic amidated/nonamidated muramyldipeptides was evaluated. However, amidation did not significantly affect cytokine induction. This finding contributes to defining peptidoglycan receptor specificities and indicates that further rationales for muropeptide amidation have to be considered.

## Text

*Staphylococcus aureus* is a frequent constituent of human nasal microflora and a major cause of severe endogenous infections (11). The staphylococcal cell wall determines several key aspects of the infection process, such as adherence (11, 28), immune recognition (13), and resistance to host defenses (21, 22). While many adhesive surface proteins have been investigated (12), the structure, function, and variability of nonproteinaceous polymers, such as peptidoglycan (PG) or teichoic acids, have remained parts of a neglected field of research. *S. aureus* is known to modify the canonical PG structure by O acetylation of the glycan strand and by amidation of the α-carboxyl group of the D-glutamate (D-Glu) residue in muropeptides, resulting in the formation of D-isoglutamine (16, 29). While O acetylation confers lysozyme resistance (3), the primary role of D-Glu amidation has remained unclear. Nevertheless, the latter modification is known to affect the level of methicillin resistance (4, 17) and to contribute to vancomycin susceptibility in *S. aureus* (8). The amino group for D-Glu amidation in *S. aureus* muropeptides is most probably derived from free glutamine (17, 23). However, the responsible transamidase enzyme has not yet been identified. Gustafson *et al*. described a *femC* mutant with 48% decreased muropeptide amidation (17, 26). The *femC* mutant has a disrupted *glnR* gene encoding the regulator of the glutamine synthetase (Fig. 1A), which results in reduced glutamine synthetase activity. As a consequence of lower amounts of the amino group donor, muropeptide amidation is reduced in this mutant (17).

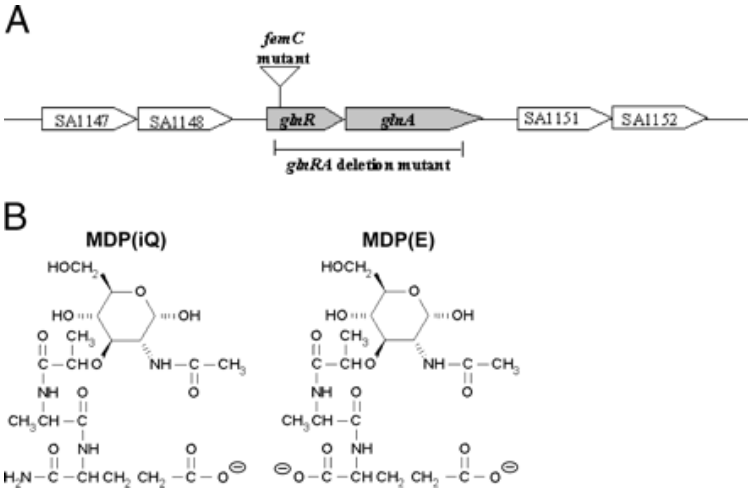


**FIG. 1.** The *S. aureus* glutamine synthetase operon, with the insertion site of Tn*551* in the *femC* mutant and the deleted region in the *glnRA* mutant (A), and structures of amidated MDP(iQ) and nonamidated MDP(E) (B).

PG has potent proinflammatory properties (5). It is sensed by the human innate immune system via NOD1 or NOD2 proteins (15, 18). An additional role of the TLR2 receptor in PG sensing has been described previously (10) but remains a matter of debate (27). The minimal structure required for NOD1 recognition is a dipeptide consisting of D-Glu and meso-diaminopimelate (mesoDAP), which is mainly produced by gram-negative bacteria (14, 15). D-Glu amidation (leading to D-isoglutamine) strongly impairs PG recognition by NOD1 (7), and the amidation of mesoDAP completely abrogates the ability of NOD1 to detect PG (15). NOD2 has a different substrate specificity as it requires a muramyldipeptide (MDP) composed of N-acetylmuramic acid, L-alanine, and D-Glu (Fig. 1B) (19, 27). *S. aureus* PG is recognized by NOD2 but not by NOD1 since it contains L-lysine instead of meso-DAP (7, 15). However, the relevance of D-Glu amidation for the efficiency of NOD2 recognition has remained unclear. MDP amidation has a profound impact on the physicochemical properties of the molecule since it removes the negative charge from the D-Glu α-carboxyl group (Fig. 1B). Accordingly, the net charge of MDP changes from –2 to –1 at physiological pH as a result of D-Glu amidation. In an attempt to study whether this structural difference has an impact on proinflammatory activity, we evaluated the tumor necrosis factor alpha (TNF-α)-stimulating capacities of *S. aureus* strains with different levels of PG amidation and of synthetic MDPs with and without amidation.

**Proinflammatory capacities of *S. aureus* wild type and *femC* mutant.** *S. aureus* NCTC8325 wild type and the isogenic *femC* mutant (17) were grown overnight in glutamine-deficient, lipopolysaccharide-free RPMI 1640 medium (Sigma), harvested,

and washed twice in phosphate-buffered saline (PBS). The mutant grew slower than the wild type did, but both strains reached similar bacterial densities. After heat inactivation at 90°C for 15 min, bacteria were washed again with PBS and adjusted to $10^9$ bacteria/ml by using a Neubauer chamber. Endotoxin contamination of these preparations was below the detection limit of 10 pg/ml of the Limulus amoebocyte lysate test (Cambrex). A total of 360 µl whole blood from healthy volunteers was incubated with 40 µl of PBS containing increasing numbers of bacteria at 37°C for 8 h. Subsequently, samples were centrifuged and TNF-α levels in the supernatant were determined by enzyme-linked immunosorbent assay (R&D Systems). Both bacterial strains stimulated TNF-α production in a dose-dependent fashion (Fig. 2A). However, there was no significant difference in TNF-α levels induced by wild-type or *femC* mutant bacteria, suggesting that the modulation of muropeptide structure by D-Glu amidation may not affect the inflammatory activity of *S. aureus* cells.

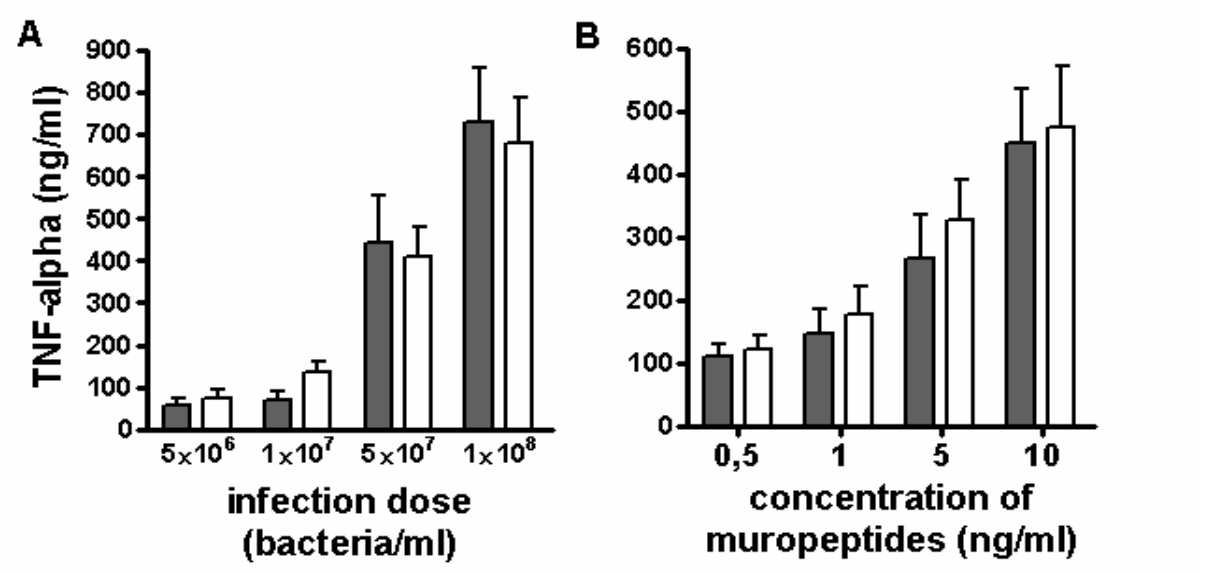


**FIG. 2.** TNF-α induction by *S. aureus* strains (A) and synthetic MDPs (B). (A) Increasing numbers of *S. aureus* wild-type (gray bars) or *femC* mutant bacteria (white bars) were incubated with whole human blood, and TNF-α levels were determined. (B) MDP(iQ) (gray bars) or MDP(E) (white bars) was incubated with whole human blood, and TNF-α levels were determined. The means and standard errors of the means (error bars) of four independent experiments, run in duplicates, are shown. The small differences between the strains in panel A are not significant as calculated by Student's *t* test.

In an attempt to obtain a mutant strain that completely lacks D-Glu amidation and might cause a stronger phenotypic difference, we deleted the entire operon composed of glutamine synthetase (*glnA*) and a corresponding regulator gene (*glnR*) (Fig. 1A). However, the resulting mutant grew only upon supplementation with significant amounts of glutamine and did not have any advantage relative to the *femC* mutant (data not shown).

**Proinflammatory capacities of synthetic MDPs with or without D-Glu amidation.** In order to study whether *S. aureus* MDPs with or without D-Glu amidation differ in their levels of proinflammatory activity when administered in the absence of other bacterial molecules, we chemically synthesized a nonamidated MDP with a D-Glu residue at position 2 of the peptide strand MDP(E), which is not commercially available. Synthesis of the acetylmuramyl-L-alanyl-D-glutamyl peptide was accomplished using Fmoc/tBu solid-phase peptide synthesis methodology (1) on acid-sensitive chlorotrityl chloride resin (2). Briefly, to 0.5 g 2-chlorotrityl chloride resin (0.6 mmol chloride/g resin) in 5 ml dichloromethane, 1 mmol of Fmoc-D-Glu(OBut)-OH and 2.5 mmol diisopropylethylamine (DIPEA) in 4 ml dichloromethane were added. After vigorous shaking for 60 min, the resin was filtered off, washed twice with dimethylformamide (DMF), and deactivated with a mixture of dichloromethane-methanol-DIPEA (80:15:5) for 15 min. After washing with DMF, the Fmoc group was deprotected with 5 ml of 20% piperidine in DMF for 5 min. After intensive washing with DMF, isopropanol, and diethyl ether, 1 mmol Fmoc-Ala-OH, 1 mmol diisoproyplcarbodiimide, and 1 mmol 1-hydroxybenzotriazole in 5 ml DMF were added and incubated for 60 min. Deprotection with piperidine and further coupling with *N*-acetylmuramic acid yielded the protected peptide. Removal of the side chain-protecting group and detachment from the resin were accomplished by treatment with trifluoroacetic acid-water-phenol (90:5:5). The peptide was precipitated by the addition of diethyl ether and lyophilized. The crude peptide was purified by preparative, reversed-phase, high-performance liquid chromatography on a Reprosil $C_8$ column (150 x 10 mm) using a flat gradient from 100% A to 40% B in 40 min (A, 0.055% trifluoroacetic acid; B, 80% acetonitrile-0.05% trifluoroacetic acid) while monitoring absorbance at 214 nm. Appropriate fractions were lyophilized, and the identity of the product was confirmed by electrospray ionization mass spectrometry

(ESI-MS). Our synthetic MDP(E) and the amidated MDP(iQ) purchased from Bachem were found to have the expected mass difference of 1 (Fig. 3).

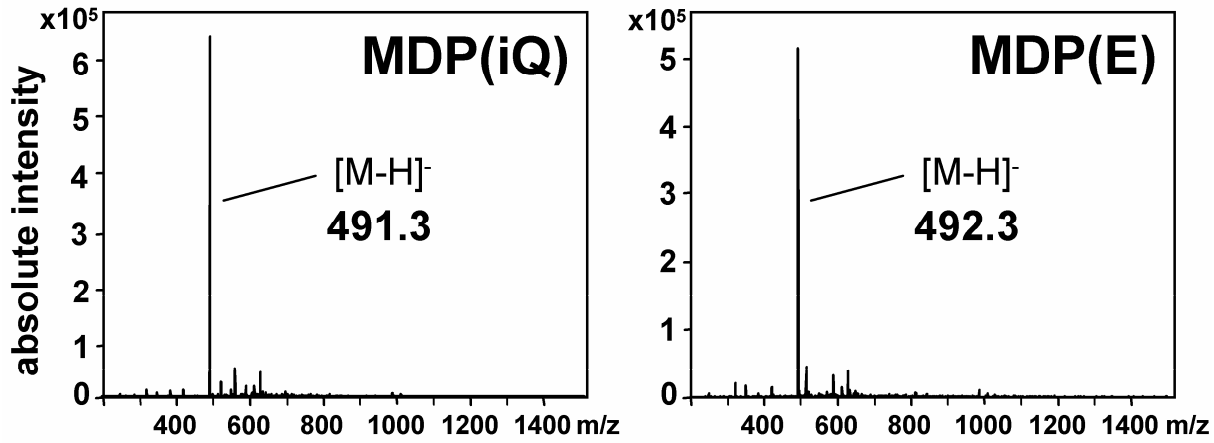


**FIG. 3.** Averaged negative ion mode ESI-MS of MDP(iQ) and MDP(E). Peaks with the expected masses for MDP(iQ) (491.3) or MDP(E) (492.3) are indicated.

MDP(E) and MDP(iQ) were dissolved in PBS. Again, the endotoxin contamination of the preparations was below the detection limit. Forty microliters of PBS containing increasing concentrations of these substances was incubated with 360 μl whole human blood at 37°C for 8 h to stimulate TNF-α production. TNF-α was quantified as described above. Both compounds stimulated TNF-α production in a dose-dependent manner. However, amidated and nonamidated MDP did not differ in their potency levels (Fig. 2B), which is in accordance with the equal proinflammatory activities of *S. aureus* wild type and the *femC* mutant.

**Concluding comments.** The inflammation-eliciting capacity of *S. aureus* plays a crucial role in the host response to infections and contributes to life-threatening complications in septicemia (5, 13). However, the diversity, relative importance, and variability of proinflammatory staphylococcal molecules, such as peptidoglycan, lipopeptides, lipoteichoic acid, formylated peptides, and others have remained uncertain. While the relative importance of lipoproteins and formylated peptides has recently been confirmed using defined mutants lacking the corresponding classes of molecules (6, 9, 25), such a strategy is impossible for PG, which is essential for bacterial viability. However, bacterial mutants with altered PG structures might help to

investigate the relative role of PG, along with the modulation of PG structure. This study demonstrates that the amidation of muropeptides does not influence the capacity of *S. aureus* or its MDP to stimulate the production of TNF-α, which contributes to our understanding of the specificity of PG-sensing host receptors. Why *S. aureus* amidates its muropeptides remains an open question. A possible reason may be the concomitant alteration of the net charge in the *S. aureus* cell envelope, which may play a role in the maintenance and turnover of the bacterial cell wall. Accordingly, muropeptide amidation has been implicated in PG cross-linking efficiency (20, 24). Identifying the muropeptide amidase gene would enable the construction of an *S. aureus* mutant lacking any muropeptide amidation without affecting the intracellular glutamine pool. Such an approach represents an important challenge for future studies.

## Acknowledgment

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# Molecular Basis of Resistance to Muramidase and Cationic Antimicrobial Peptide Activity of Lysozyme in Staphylococci

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It has been shown recently that modification of peptidoglycan by *O*-acetylation renders pathogenic staphylococci resistant to the muramidase activity of lysozyme. Here, we show that a *Staphylococcus aureus* double mutant defective in *O*-acetyltransferase A (Oat*A*), and the glycopeptide resistance-associated two-component system, GraRS, is much more sensitive to lysozyme than *S. aureus* with the *oat*A mutation alone. The *gra*RS single mutant was resistant to the muramidase activity of lysozyme, but was sensitive to cationic antimicrobial peptides (CAMPs) such as the human lysozyme-derived peptide $_{107}$R-A-W-V-A-W-R-N-R$_{115}$ (LP9), polymyxin B, or gallidermin. A comparative transcriptome analysis of wild type and the *gra*RS mutant revealed that GraRS controls 248 genes. It up-regulates global regulators (*rot, sar*S, or *mgr*A), various colonization factors, and exotoxin-encoding genes, as well as the *ica* and *dlt* operons. A pronounced decrease in the expression of the latter two operons explains why the *gra*RS mutant is also biofilm-negative. The decrease of the *dlt* transcript in the *gra*RS mutant correlates with a 46.7% decrease in the content of esterified D-alanyl groups in teichoic acids. The *oat*A*/dlt*A double mutant showed the highest sensitivity to lysozyme; this mutant completely lacks teichoic acid–bound D-alanine esters, which are responsible for the increased susceptibility to CAMPs and peptidoglycan *O*-acetylation. Our results demonstrate that resistance to lysozyme can be dissected into genes mediating resistance to its muramidase activity (*oat*A) and genes mediating resistance to CAMPs *(gra*RS and *dlt)*. The two lysozyme activities act synergistically, as the *oat*A*/dlt*A or *oat*A*/gra*RS double mutants are much more susceptible to lysozyme than each of the single mutants.

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**Abbreviations:** CAMP, cationic antimicrobial peptide; GraRS, glycopeptide resistance-associated; MIC, minimal inhibition concentration; MurNAc, *N*-acetylmuramic acid; OatA, *O*-acetyltransferase A; OD, optical density; PG, peptidoglycan; TA, teichoic acid; TCS, two-component system; WT, wild type; WTA, wall teichoic acid

## Author Summary

In humans, lysozyme plays an important role in the suppression of bacterial infections. However, some bacterial pathogens, such as *Staphylococcus aureus,* are completely resistant to lysozyme. Here we demonstrate that lysozyme acts on *S. aureus* in two ways: as a muramidase (cell wall lytic enzyme) and as a cationic antimicrobial peptide (CAMP). *S. aureus* has developed resistance mechanisms against both activities by modifying distinct cell wall structures. Modification of the peptidoglycan by *O*-acetylation (OatA) renders the cells resistant to the muramidase activity. Modification of teichoic acids by D-alanine esterification (Dlt) renders the cells resistant to lysozyme's CAMPs and other CAMPs. Transcriptome analysis of the glycopeptide resistance-associated (GraRS) two-component system revealed that this global regulator controls 248 genes such as other global regulators, colonization factors, or exotoxin-encoding genes. Since GraRS also upregulates the *dlt* operon, it was not surprising that in the *gra*RS mutant teichoic acid D-alanylation is markedly decreased, which explains its increased sensitivity to CAMPs. By comparative analysis of mutants we were able to dissect genes that were responsive to the dual activities of lysozyme. Here we show how efficiently *S. aureus* is protected from the human defense system, which enables this pathogen to cause persistent infections.

## Introduction

In humans, lysozyme is found in a wide variety of fluids, such as tears, breast milk, and respiratory and saliva secretions, as well as in cells of the innate immune system, including neutrophils, monocytes, macrophages, and epithelial cells [1,2]. Lysozyme is an important protein in the innate defense response against invading microorganisms and acts on bacteria by hydrolyzing the ß-1,4 glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlucNAc), resulting in degradation of peptidoglycan (PG), and subsequent cell lysis [3,4]. Most bacterial species are sensitive to lysozyme, but some important human pathogens, such as *Staphylococcus aureus, Neisseria gonorrhoeae,* and *Proteus mirabilis,* are resistant. The mechanisms behind the high resistance of *S. aureus* to lysozyme are unknown, although several studies suggest that *O*-acetylation at position *C*-6 of the

MurNAc residue contributes to lysozyme resistance [5–9]. Recently, we were able to prove that indeed *O*-acetyltransferase A (OatA) of *S. aureus* is responsible for *O*-acetylation of the PG, and this leads to resistance to the muramidase activity of lysozyme [10]. We also showed that the MurNAc was *O*-acetylated only in pathogenic, lysozyme-resistant staphylococci (e.g., *S. aureus, S. epidermidis, S. lugdunensis,* and others). All nonpathogenic species (e.g., *S. carnosus, S. gallinarum,* or *S. xylosus*) were lysozyme sensitive and lacked PG-specific *O*-acetylation. Therefore, OatA can be regarded as a general virulence factor [11].

Although the *oat*A mutant was less resistant to lysozyme than the wild type (WT) *S. aureus,* it still was more resistant than, for example, *Micrococcus luteus,* suggesting that other factors, such as a high degree of peptide cross-linking, may also contribute to lysozyme resistance [12]. Recently, we showed that the presence of wall teichoic acid (WTA) increased lysozyme resistance [13]. One also has to consider that lysozyme does not only comprise muramidase activity but also antimicrobial peptide activity, as demonstrated by catalytically inactivate lysozyme or peptides isolated from digested lysozyme, and by synthetic lysozyme-derived peptides [14–17].

Here, we show that the extremely high resistance of *S. aureus* to lysozyme can be genetically dissected as a) resistance to muramidase activity and b) resistance to inherent cationic antimicrobial peptide (CAMP) activity. Furthermore, we characterized via transcriptome analysis the two-component system (TCS), GraRS, which, in addition to many virulence genes, also controls the *dlt* operon to mediate resistance to lysozyme and other CAMPs.

## Results

### Susceptibility of *oat*A and *gra*RS Single and Double Mutants to Lysozyme and CAMPs

In our search for highly susceptible lysozyme mutants in *S. aureus,* we isolated two Tn*917* transposon mutants in SA113*oat*A*::kan* that revealed higher sensitivity to lysozyme than the *oat*A mutation alone. Chromosomal sequencing of the flanking Tn*917* insertion sites revealed that Tn*917* was inserted in SA0615 [18]. SA0615 and the upstream gene SA0614 have the features of a typical TCS and were recently named GraRS (glycopeptide resistance-associated), because overexpression of

GraR (response regulator) and GraS (sensor histidine kinase) increased vancomycin resistance [19]. To further study the role of TCS in lysozyme resistance, we constructed a deletion mutant by substituting *gra*RS with an erythromycin B cassette to yield SA113 *gra*RS*::erm* (Figure 1). In addition, we also constructed an *oat*A*::kan/gra*RS*::erm* double knockout. Sequencing and complementation with pTX*gra*RS, a vector in which the *gra*RS genes are induced into expression by xylose, confirmed the correct replacement. Whereas the *oat*A*/gra*RS double mutant was highly susceptible to lysozyme, both single mutants were only marginally affected, but were still more sensitive than the WT, which is completely lysozyme resistant (Figure 2A–2D).

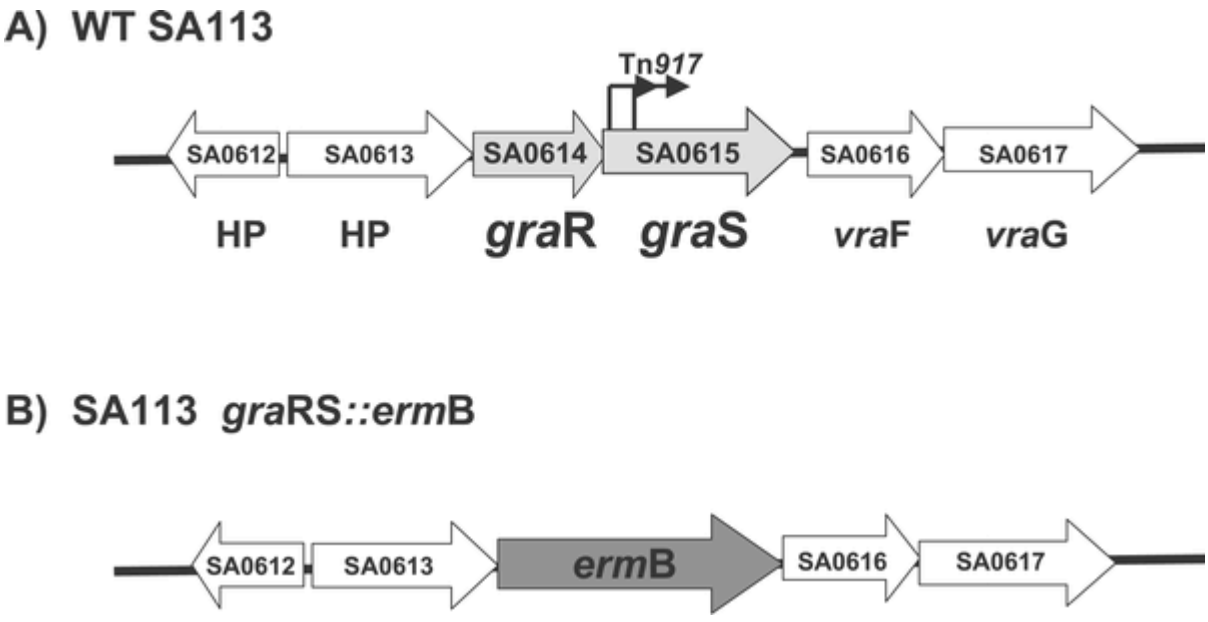



**Figure 1.** Illustration of Construction of the *gra*RS Deletion Mutant

(A) Gene organization in the chromosome of WT SA113; Tn*917* insertions in *gra*S gene are indicated by arrows.

(B) In the *gra*RS deletion mutant, *gra*RS is substituted by the erythromycin B resistance cassette. Note that *erm*B gene has a weak transcription terminator, and transcriptional read-through to the following *vra*FG genes is likely. *gra*R, response regulator; *gra*S, sensor histidine kinase; *vra*F, ABC transporter ATP-binding protein; *vra*G, ABC transporter permease; SA0612 and SA0613 are hypothetical proteins (HP).

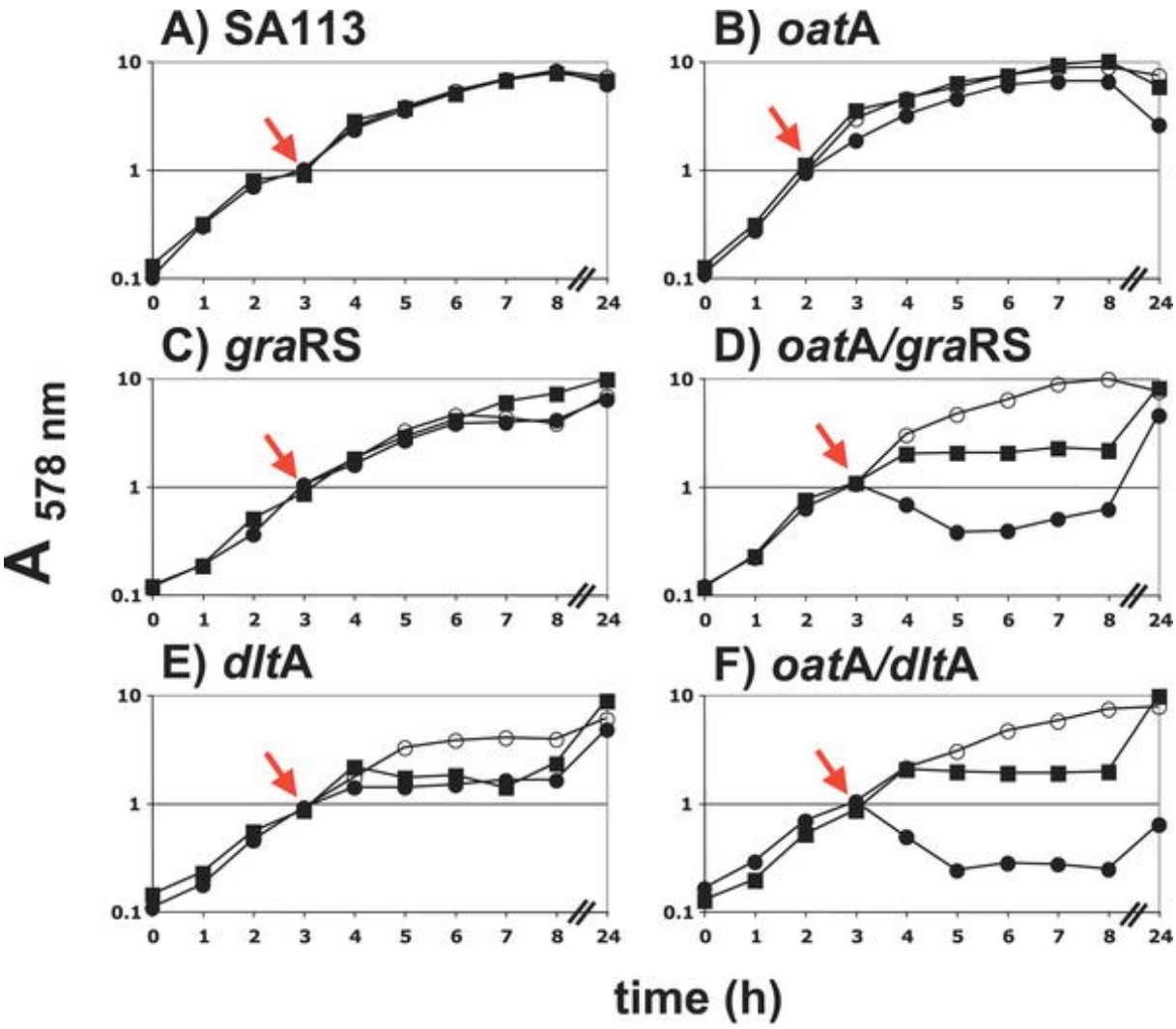
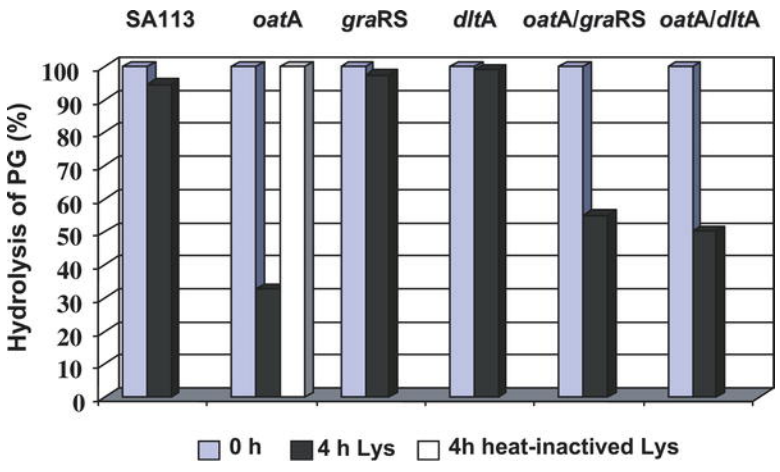



**Figure 2.** Susceptibility of WT SA113 and Various *S. aureus* Mutants to Lysozyme and Heat-Inactivated Lysozyme

Cells were grown in BM at 37 °C. $OD_{578nm}$ was measured hourly for the first 8 h and after 24 h. Lys was added in the exponential growth phase at $OD_{578nm}$ 1.0 as indicated by arrow. Catalytic inactive Lys was heated for 1 h at 100 °C. (A) WT SA113: control (○); Lys (300 µg/ml [20.8 µM]) (•); heat-inactivated lysozyme (Lys) (300 µg/ml [20.8 µM]) (▪). (B) *oat*A mutant: control (○); Lys (300 µg/ml) (•); heat-inactivated Lys (300 µg/ml) (▪). (C) *gra*RS mutant: control (○); Lys (300 µg/ml) (•); heat-inactivated Lys (300 µg/ml) (▪). (D) *oat*A/*gra*RS mutant: control (○); Lys (50 µg/ml [3.47 µM]) (•); heat-inactivated Lys (300 µg/ml) (▪). (E) *dlt*A mutant: control (○); Lys (300 µg/ml) (•); heat-inactivated Lys (300 µg/ml) (▪). (F) *oat*A/*dlt*A mutant: control (○); Lys (20 µg/ml [1.39 µM]) (•); heat-inactivated Lys (300 µg/ml) (▪).

The *oat*A/*gra*RS double mutant was much more lysozyme sensitive than each of the single mutants. This hypersensitivity of the double mutant can be explained by dual activities of lysozyme that act in a synergistic way. To study this phenotype in more detail, we investigated whether the *gra*RS single mutant is affected by the muramidase activity of lysozyme. Indeed, the isolated PG from the *gra*RS single mutant was completely resistant to lysozyme hydrolysis, in contrast to the *oat*A mutant. As expected, PG of the *oat*A/*gra*RS double mutant was also hydrolysed, although the sensitivity was less pronounced, as in the *oat*A single mutant (Figure 3). Therefore, the increased sensitivity of the double mutant likely came from its higher susceptibility to lysozyme's CAMP activity. This was confirmed by the addition of LP9, polymyxin B, or gallidermin to a growing culture, which caused immediate growth arrest in the *gra*RS mutant, whereas the WT was much less affected (Figure 4A and 4B), and only the lantibiotic gallidermin inhibited the WT. In addition, we demonstrated that heat-inactivated lysozyme exhibits CAMP activity, but no muramidase activity. Heat-inactivated lysozyme showed no activity (neither lytic nor CAMP activity) to the *oat*A mutant or to the isolated PG of *oat*A, but it was able to inhibit the growth of the *oat*A/*gra*RS double mutant (Figures 2B, 2D, and 3). This result suggests that GraRS controls genes involved in CAMP resistance. This effect was not only achieved with hen egg-white, but also with human lysozyme.

**Figure 3.** Susceptibility of PG Isolated from WT and Various Mutants to Hydrolysis by Lysozyme




PG (0.5 mg/ml) isolated from WT SA113 and mutants were incubated with catalytic active lysozyme (Lys); in addition, the *oat*A mutant was incubated with heat-inactivated Lys (300 µg/ml) in 80 mM sodium phosphate-buffered saline. Lysis of PG was measured as a decrease in $OD_{660nm}$ and calculated in percent. The diagram shows 100% of PG in the beginning (0 h) and the remaining undigested PG after 4 h of lysozyme treatment.

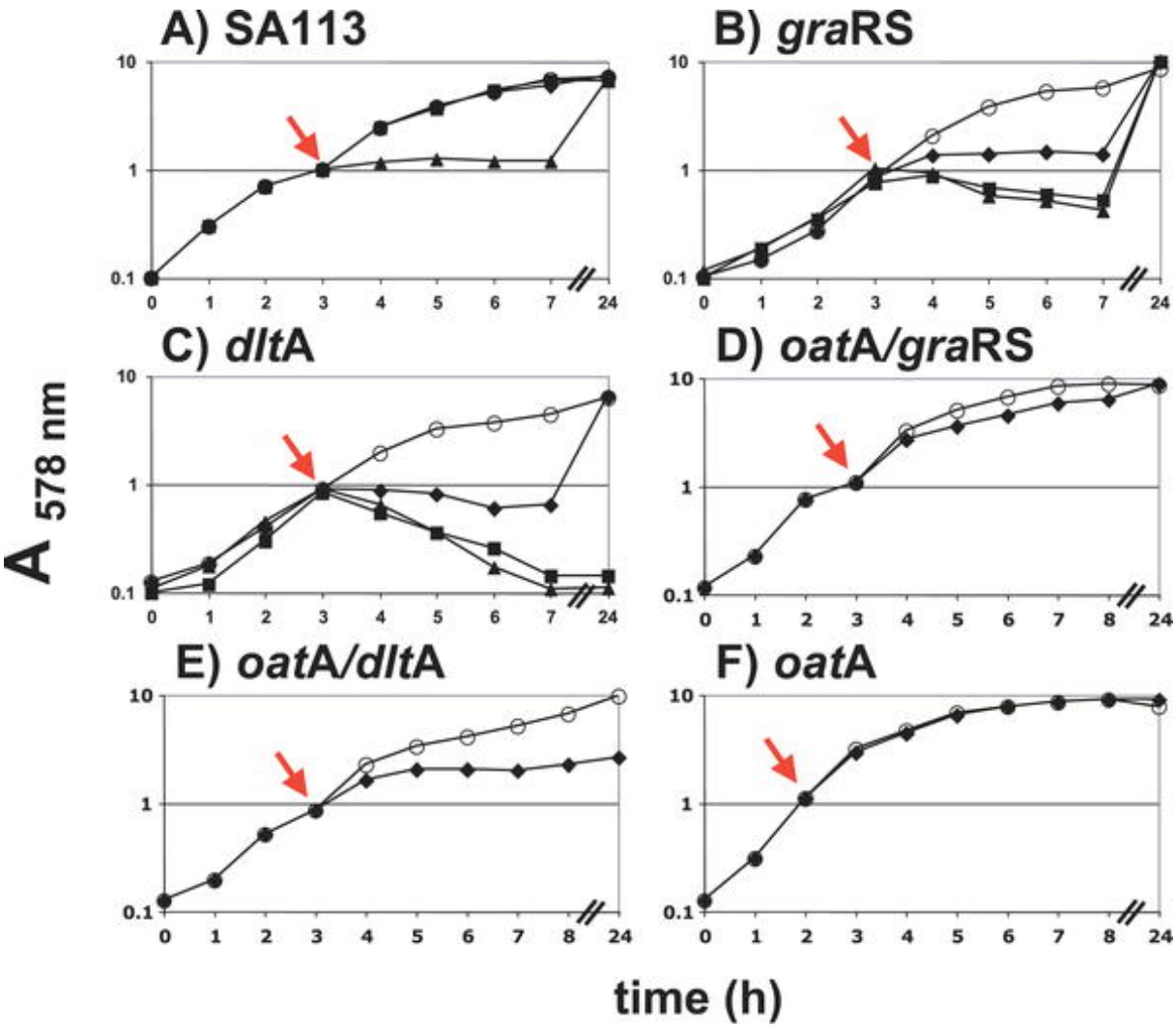



**Figure 4.** Susceptibility of WT and Various *S. aureus* Mutants to CAMPs

(A) WT SA113: control (○); LP9 (200 μg/ml [164.9 μM]) (◆); polymyxin B (PMB) (20 μg/ml [14.4 μM]) (■); and gallidermin (Gdm) (8 μg/ml [3.64 μM]) (▲). (B) *gra*RS mutant: control (○); LP9 (200 μg/ml) (◆); PMB (20 μg/ml) (■); and Gdm (8 μg/ml) (▲). (C) *dlt*A mutant: control (○); LP9 (200 μg/ml) (◆); PMB (20 μg/ml) (■); and Gdm (8 μg/ml) (▲). (D) *oat*A/*gra*RS mutant: control (○); LP9 (200 μg/ml) (◆). (E) *oat*A/*dlt*A mutant: control (○); LP9 (200 μg/ml) (◆). (F) *oat*A mutant: control (○); LP9 (200 μg/ml) (◆).

Cells were grown in BM at 37 °C. $OD_{578nm}$ was measured hourly for the first 7–8 h and after 24 h. CAMPs were added in the exponential growth phase at $OD_{578nm}$ 1.0 as indicated by arrow.

**Comparative Transcriptome Analysis of WT and *gra*RS Mutant**

To find out which genes are responsible for the high susceptibility to CAMPs in the *gra*RS mutant, we carried out a comparative transcriptome analysis of the WT strain and the *gra*RS mutant. We detected 115 genes whose mRNAs were up-regulated (Table 1) and 133 genes whose mRNAs were down-regulated by GraRS (Table 2). The complete list of up- and down-regulated genes with their National Center for Biotechnology Information PID numbers is presented in Dataset S1. In order to give an impression of which genes are controlled by GraRS, some examples are mentioned below.

In the *gra*RS mutant, genes that are involved in RNA and amino acid synthesis and glycolysis shows highly gene transcription rates. In particular, the urease genes (*ure*A-G) all 12 *pur* genes were 2- to 32-fold up-regulated as compared to the WT, whereas *pur*R (repressor) appeared not to be influenced by GraRS. Interestingly, the amount of *oat*A transcript increased in the *gra*RS mutant, which could explain the slightly higher resistance of the *gra*RS mutant to the muramidase activity of lysozyme (Figure 3). A number of genes that were down-regulated included global regulators (*rot, sar*S*, mgr*A), cell surface protein encoding genes (the Ser-Asp rich fibrinogen-binding proteins SdrC and SdrE), the major autolysin gene (*atl*A) and an autolysin/adhesin gene *(aaa)* [20], exoprotein encoding genes (*hlb, hlg*A,B, *luk*M,F, and *geh*), transporter encoding genes (*ess*A/*ess*C, *opp*B, and *nor*B), capsule encoding genes (*cap*A,H,I,J,K) and PIA encoding genes (*ica*ADBC), genes responsible for D-alanyl esterification of teichoic acids (TAs) (*dlt*A,B,D), and the alanine dehydrogenase gene (*ald*1). The pronounced decrease of expression of the *ica* [21–23] and *dlt* operons [24] and *atl*A [25] explains why the *gra*RS mutant showed a biofilm-negative phenotype on microtiter plates (unpublished data). With a few genes, such as *rot, ure*C, and *dlt*A, we verified the transcriptome data by reverse transcriptase (RT)-PCR (Table 3).

**Table 1.** 115 *S. aureus* SA113 genes up-regulated by GraRS

| N315 ORF | N315 gene | N315 product | Protein-location (C) (S) (SCW) (M) | Change in expression (*n*-fold) | One Sample t-Test-Benjamini Hochberg (Adv) | *rot*, *mgr*A, *arl*RS effect |
|---|---|---|---|---|---|---|
| **Virulence factors (cell surface proteins, exotoxins, colonization factors)** | | | | | | |
| SA0519 | *sdr*C | Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein | SCW | 9.6 | 0.013 | *rot, arl* up |
| SA0521 | *sdr*E | Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein | SCW | 2.9 | 0.034 | *mgr, arl* up |
| SA1003 | *fib* | HP, similar to fibrinogen-binding protein | SCW | 2.5 | 0.036 | |
| SA1004 | | HP, similar to fibrinogen-binding protein | SCW | 2.2 | 0.164 | *mgr* down |
| SA0222 | *coa* | Staphylocoagulase precursor | S | 2.1 | 0.013 | *rot* up |
| SA0309 | *geh* | Glycerol ester hydrolase, lipase 2 | S | 2.2 | 0.012 | *rot* down |
| SA1811 | *hlb* | Truncated beta-hemolysin | S | 2.2 | 0.007 | *rot* down |
| SA1812 | *luk*M | Leukocidin chainLukM | S | 2.5 | 0.042 | *rot* down, *mgr* up |
| SA1813 | *luk*F | Synergohymentropic toxin precursor | S | 2.1 | 0.010 | *mgr* up |
| SA2207 | *hlg*A | Gamma-hemolysin component A | S | 2.1 | 0.010 | *mgr* up |
| SA2209 | *hlg*B | Gamma-hemolysin component B | S | 2.1 | 0.005 | *rot*, *arl* down |
| SA0270 | | HP, similar to secretory antigen precursor SsaA | S | 2.9 | 0.012 | *arl* up |
| SA0271 | *esx*A | Virulence factor EsxA | S | 4.4 | 0.012 | *rot*, *arl* up |
| SA0620 | | HP, similar to secretory antigen precursor SsaA | S | 2.1 | 0.013 | *rot* up |
| SA2097 | | HP, similar to secretory antigen precursor SsaA | S | 3.0 | 0.014 | |
| SA2431 | *isa*B | Immunodominant antigen B | S | 2.2 | 0.022 | *mgr* down |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA2459 | *ica*A | Intercellular adhesion protein A | M | 4.8 | 0.032 | |
| SA2460 | *ica*D | Intercellular adhesion protein D | M | 4.9 | 0.006 | |
| SA2461 | *ica*B | Intercellular adhesion protein B | SCW | 3.3 | 0.063 | |
| SA2462 | *ica*C | Intercellular adhesion protein C | M | 2.5 | 0.068 | |
| **Regulators** | | | | | | |
| SA0108 | *sar*S | Staphylococcal accessory regulator A homologue | C | 7.2 | 0.007 | *rot* up, *mgr* down |
| SA0614 | *gra*R | | C | 3.1 | 0.062 | |
| SA0641 | *mgr*A | HTH-type transcriptional regulator MgrA, MarR family | C | 3.1 | 0.007 | |
| SA0856 | *spx*A | Transcriptional regulator Spx | C | 5.6 | 0.024 | |
| SA1583 | *rot* | Repressor of toxins Rot | C | 3.8 | 0.009 | *arl* up |
| SA1678 | *fur*B | Transcriptional regulator Fur family homolog | C | 2.1 | 0.023 | *mgr* up |
| SA2174 | | HP, similar to transcriptional regulator | C | 2.0 | 0.046 | |
| **Cell wall (cellular processes, transport, membrane, lipoproteins, autolysins)** | | | | | | |
| SA0106 | *lct*P | Lactate transporter, LctP family | M | 3.2 | 0.021 | |
| SA0109 | *sir*C | Lipoprotein | M | 3.5 | 0.013 | |
| SA0111 | *sir*A | Lipoprotein | M | 2.0 | 0.086 | |
| SA0138 | | HP, similar to alkylphosphonate ABC transporter | M | 2.1 | 0.004 | *arl* down |
| SA0204 | *azo*R | FMN-dependent NADH-azoreductase | C | 2.2 | 0.021 | |
| SA0207 | | HP, similar to maltose/maltodextrin-binding protein | M | 2.1 | 0.035 | |
| SA0208 | | Maltose/maltodextrin transport permease homologue | M | 2.1 | 0.064 | |
| SA0268 | | HP, similar to ABC transporter system permease protein | M | 2.3 | 0.007 | |
| SA0272 | *esa*A | Protein EsaA | M | 4.7 | 0.022 | *arl* up |
| SA0273 | *ess*A | Protein EssA | M | 2.8 | 0.071 | |
| SA0276 | *ess*C | Protein EssC | M | 2.6 | 0.027 | *arl* up |
| SA0295 | | HP, similar to outer membraneprotein precursor | M | 2.3 | 0.010 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0423 | *aaa* | *N*-acetylmuramoyl-*L*-alanine amidase | SCW | 3.6 | 0.015 | |
| SA0518 | *azo1* | FMN-dependent NADPH-azoreductase | C | 2.1 | 0.019 | |
| SA0793 | *dlt*A | D-alanine-D-alanyl carrier protein ligase | C | 2.9 | 0.066 | |
| SA0794 | *dlt*B | DltB membrane protein | M | 2.1 | 0.010 | |
| SA0796 | *dlt*D | Poly (glycerophosphate chain) D-alanine transfer protein | M | 2.3 | 0.008 | *rot* up |
| SA0845 | *opp*B | Oligopeptide transport system permease protein | M | 2.2 | 0.039 | |
| SA0846 | | HP, similar to oligopeptide transport system permease protein OppC | M | 2.1 | 0.049 | |
| SA0849 | | HP, similar to peptide binding protein OppA | M | 2.4 | 0.030 | *mgr* down |
| SA0854 | | HP, similar to oligopeptide transport system permease protein OppC | M | 2.1 | 0.053 | |
| SA0905 | *atl*A | autolysin (*N*-acetylmuramoyl-L-alanine amidase/endo-beta-N-acetylglucosaminidase | SCW | 2.4 | 0.015 | |
| SA1269 | *nor*B | Blt-like protein, efflux pump | M | 3.6 | 0.004 | *mgr*, *arl* up |
| SA1270 | | HP, similar to acid permease | M | 3.5 | 0.012 | *mgr*, *arl* up |
| SA1663 | | HP, belongs to the UPF0342 protein family | M | 2.3 | 0.072 | |
| SA1979 | | HP, similar to ferrichrome ABC transporter | M | 2.1 | 0.032 | |
| SA2156 | | L-lactate permease lctP homolog | M | 3.7 | 0.015 | |
| SA2217 | | HP, similar to lipoprotein inner membrane ABC-transporter | M | 2.1 | 0.022 | |
| SA2302 | *stp*C | HP, similar to ABC transporter | M | 3.1 | 0.008 | *mgr* up |
| SA2303 | *smp*C | HP, similar to membrane spanning protein | M | 5.5 | 0.010 | *rot*, *mgr*, *arl* up |
| SA2475 | | HP, belongs to ABC-transporter | M | 2.3 | 0.067 | |
| SA2477 | | HP, belongs to the UPF0397 protein family | M | 2.3 | 0.068 | |
| SA2480 | *drp35* | Lactonase Drp35 | C | 2.3 | 0.036 | |
| **Adaption to stress conditions** | | | | | | |
| SA2457 | *cap*A | Capsular polysaccharide biosynthesis, capA | M | 2.0 | 0.008 | *arl* down |
| SA0151 | *cap*H | Capsular polysaccharide synthesis enzyme O-acetyltransferase Cap5H | M | 2.0 | 0.033 | *mgr* up |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0152 | *cap*I | Capsular polysaccharide synthesis enzyme Cap5I | M | 2.0 | 0.034 | *mgr* up |
| SA0153 | *cap*J | Capsular polysaccharide synthesis enzyme Cap5J | M | 2.3 | 0.084 | *mgr* up |
| SA0154 | *cap*K | Capsular polysaccharide synthesis enzyme Cap5K | M | 2.7 | 0.025 | *mgr* up |
| SA1984 | *asp23* | Alkaline shock protein 23, ASP23 | C | 2.1 | 0.082 | |
| **Others (RNA, DNA, carbohydrates, amino acids, protein, lipid synthesis)** | | | | | | |
| SA0515 | | HP, similar to deoxypurine kinase | C | 2.1 | 0.014 | |
| SA1013 | | HP, similar to carbamate kinase | C | 2.3 | 0.023 | |
| SA0143 | *adh*E | Alcohol-acetaldehyde dehydrogenase | C | 2.0 | 0.008 | *rot* down |
| SA0180 | | Branched-chain amino acid:cation transporter | M | 2.0 | 0.010 | |
| SA1225 | *lys*C | Aspartokinase II | C | 2.1 | 0.015 | |
| SA1271 | | Threonine deaminase IlvA homolog | C | 2.7 | 0.077 | *arl* up |
| SA1272 | *ald1* | Alanine dehydrogenase | C | 3.5 | 0.007 | *mgr*, *arl* up |
| SA1502 | *rpl*T | 50S ribosomal protein L20 | C | 2.1 | 0.060 | |
| SA0220 | | HP, similar to glycerophosphodiester phosphodiesterase | C | 2.1 | 0.008 | *rot*, *arl* up |
| SA0027 | | Truncated replication protein for plasmid | | 3.6 | 0.096 | |
| SA1709 | | HP, similar to ferritin | | 2.6 | 0.034 | |
| **Phage-related functions** | | | | | | |
| SA1760 | | holin homolog (Bacteriophage phiN315) | M | 2.4 | 0.104 | |
| SA1766 | | HP (Bacteriophage phiN315) | | 6.0 | 0.087 | |
| SA1785 | | HP (Bacteriophage phiN315) | | 2.5 | 0.028 | |
| SA1793 | | HP (Bacteriophage phiN315) | | 23.3 | 0.074 | |
| **Hypothetical genes** | | | | | | |
| SA0037 | | HP | | 3.4 | 0.062 | |
| SA0077 | | HP | | 3.8 | 0.073 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0081 | | HP | | 2.3 | 0.041 | |
| SA0090 | | HP | | 6.4 | 0.089 | *mgr* up |
| SA0100 | | HP | | 2.8 | 0.008 | |
| SA0161 | | HP | | 2.0 | 0.075 | |
| SA0213 | | HP | | 2.4 | 0.014 | |
| SA0221 | | HP | | 2.2 | 0.062 | *mgr* down |
| SA0262 | | HP | | 2.3 | 0.062 | |
| SA0279 | | HP | | 2.2 | 0.005 | *mgr* up |
| SA0283 | | HP | | 2.3 | 0.042 | |
| SA0291 | | HP | | 3.3 | 0.068 | *rot*, *mgr* up |
| SA0292 | | HP | | 3.9 | 0.030 | *rot* up |
| SA0378 | | HP | | 3.5 | 0.021 | |
| SA0408 | | HP | | 2.7 | 0.048 | *rot* up |
| SA0424 | | HP | | 2.4 | 0.038 | |
| SA0623 | | HP | | 2.8 | 0.059 | |
| SA0651 | | HP | | 3.1 | 0.027 | *rot* up |
| SA0738 | | HP | | 5.5 | 0.058 | |
| SA0739 | | HP | | 4.7 | 0.010 | *rot* up |
| SA0772 | | HP | | 2.1 | 0.096 | |
| SA0890 | | HP | | 3.4 | 0.012 | |
| SA1056 | | HP | M | 3.4 | 0.012 | |
| SA1151 | | HP | | 2.1 | 0.022 | |
| SA1828 | | HP | | 2.2 | 0.040 | |
| SA2101 | | HP | | 2.6 | 0.012 | |
| SA2153 | | HP | | 2.2 | 0.010 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA2198 | HP | | 2.1 | 0.062 | | |
| SA2256 | HP | | 2.2 | 0.040 | | |
| SA2332 | HP | | 2.4 | 0.019 | | |
| SA2339 | HP | M | 4.5 | 0.012 | | |
| SA2373 | HP | | 2.2 | 0.034 | *mgr* down | |
| SA2449 | HP | | 2.4 | 0.060 | | |
| SA2474 | HP | | 2.1 | 0.076 | | |

Cytoplasm (C), Secreted (S), Secreted Cellwall-bound (SCW), Membrane (M)

**Table 2.** 133 *S. aureus* SA113 genes down-regulated by GraRS

| N315 ORF | N315 gene | N315 product | Protein-location (C) (S) (SCW) (M) | Change in expression (*n*-fold) | One Sample t-Test-Benjamini Hochberg (Adv) | *rot*, *mgr*A, *arl*RS effect |
|---|---|---|---|---|---|---|
| **Regulators** | | | | | | |
| SA0017 | *vic*R | Response regulator | C | 2.0 | 0.004 | |
| SA1071 | *fab*R | Transcription factor FapR | C | 2.3 | 0.015 | |
| SA1690 | *rec*X | Regulatory protein RecX | C | 2.0 | 0.021 | |
| SA2296 | | HP, similar to transcriptional regulator, MerR family | C | 2.1 | 0.012 | |
| SA2320 | *pfo*R | Putative regulatory protein PfoR | M | 5.7 | 0.010 | *mgr*, *arl* down |
| SA2418 | | HP, similar to two-component response regulator | C | 2.1 | 0.007 | |
| **Cell wall (cellular processes, transport, membrane, lipoproteins)** | | | | | | |
| SA0252 | *lrg*A | holin-like protein LrgA | M | 2.6 | 0.017 | *mgr, arl* up |
| SA0469 | *fts*H | cell-division protein | M | 2.2 | 0.004 | |
| *SA0616 | *vra*F | ABC transporter ATP-binding protein | M | 8.6 | 0.005 | |
| *SA0617 | *vra*G | ABC transporter permease | M | 4.8 | 0.029 | |
| SA0708 | *sec*A | preprotein translocase subunit | M | 2.1 | 0.012 | |
| SA0719 | *trx*B | Thioredoxine reductase | M | 2.5 | 0.016 | |
| SA0937 | | Cytochrome D ubiquinol oxidase subunit 1 homolog | M | 2.5 | 0.010 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0997 | *mur*I | Glutamate racemase | C | 2.4 | 0.009 | |
| SA1127 | *cin*A | Competence-damage inducible protein CinA | C | 2.0 | 0.024 | |
| SA1140 | *glp*F | Gycerol uptake facilitator | M | 2.2 | 0.014 | |
| SA1206 | *fem*A | Aminoacyltransferase femA (factor essential for expression of methicillin resistance) | M | 2.0 | 0.006 | |
| SA1212 | *opp*-2D | Oligopeptide transport ATPase | M | 2.2 | 0.032 | |
| SA1214 | *opp*-2B | Oligopeptide transporter membrane permease domain | M | 2.5 | 0.036 | |
| SA1255 | *crr* | Glucose-specific phosphotransferase enzyme IIA component | M | 2.2 | 0.011 | |
| SA1519 | *aap*A | D-serine/D-alanine/glycine TRANSPORTER | M | 2.4 | 0.004 | |
| SA1653 | TRAP | Signal transduction protein TRAP | M | 2.4 | 0.005 | |
| SA1654 | | HP, similar to ABC transporter ecsB | M | 2.5 | 0.015 | |
| SA1655 | | ABC transporter ecsA homolog | M | 2.3 | 0.012 | |
| SA1916 | | HP, belongs to the UPF0340 protein family | M | 2.4 | 0.063 | |
| SA1960 | *mtl*F | PTS system, mannitol specific IIBC component | M | 2.2 | 0.040 | *mgr* down |
| SA2056 | | HP, similar to acriflavin resistance protein | M | 2.0 | 0.010 | |
| SA2234 | *oup*CD | Probable glycine betaine/carnitine/choline ABC transporter opuCD | M | 2.3 | 0.025 | |
| SA2324 | | HP, similar to thioredoxin | M | 2.2 | 0.015 | |
| SA2354 | *oat*A | *O*-acetyltransferase A | M | 2.5 | 0.012 | |
| ***** *vra*FG were down-instead of up-regulated because of transcriptional read-through into the *vra*FG genes by the very weak *erm*B transcription terminator. | | | | | | |
| **RNA (nucleotides-, nucleic acids-synthesis, regulation)** | | | | | | |
| SA0016 | *pur*A | Adenylosuccinate synthase | C | 2.2 | 0.019 | *mgr* down |
| SA1724 | *pur*B | Adenylosuccinate lyase | C | 2.6 | 0.004 | *mgr* up |
| SA0133 | *deo*C1 | Deoxyribose-phosphate aldolase | C | 2.8 | 0.005 | |
| SA0134 | *deo*B | Phosphopentomutase | C | 2.7 | 0.041 | |
| SA0915 | *fol*D | FolD bifunctional protein | C | 6.0 | 0.004 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0916 | *pur*E | Putative phosphoribosylaminoimidazole carboxylase PurE | C | 19.4 | 0.005 | |
| SA0917 | *pur*K | Phosphoribosylaminoimidazole carboxylase ATPase subunit | C | 22.2 | 0.007 | |
| SA0918 | *pur*C | Phosphoribosylaminoimidazole-succinocarboxamide synthase | C | 14.2 | 0.010 | |
| SA0920 | *pur*Q | Phosphoribosylformylglycinamidine synthase I PurQ | C | 19.9 | 0.007 | |
| SA0921 | *pur*L | Phosphoribosylformylglycinamidine synthetase PurL | C | 13.8 | 0.009 | |
| SA0922 | *pur*F | Phosphoribosylpyrophosphate amidotransferase PurF | C | 28.7 | 0.004 | |
| SA0923 | *pur*M | Phosphoribosylformylglycinamidine cyclo-ligase PurM | C | 21.6 | 0.006 | *rot* up |
| SA0924 | *pur*N | Phosphoribosylglycinamide formyltransferase | C | 25.5 | 0.004 | |
| SA0925 | *pur*H | Bifunctional purine biosynthesis protein PurH | C | 17.7 | 0.004 | |
| SA0926 | *pur*D | Phosphoribosylamine-glycine ligase PurD | C | 8.1 | 0.004 | |
| SA1172 | | HP, similar toGMP reductase | C | 4.3 | 0.008 | |
| SA1237 | *xpa*C | HP, similar to 5-bromo-4-chloroindolyl phosphate hydrolysis protein | C | 2.0 | 0.008 | |
| SA1914 | *upp* | Uracil phosphoribosyl transferase | C | 2.3 | 0.014 | |
| SA1938 | *pdp* | Pyrimidine nucleoside phosphorylase | C | 2.1 | 0.010 | |
| SA1939 | | Deoxyribose-phosphate aldolase | C | 2.5 | 0.014 | |
| SA1098 | *cod*Y | Transcription pleiotropic repressor CodY | C | 2.2 | 0.072 | |
| SA1289 | | Putative bifunctional biotin ligase/biotin operon repressor | C | 2.1 | 0.004 | |
| SA1411 | *hrc*A | Heat-inducible transcriptional repressor | C | 2.2 | 0.010 | |
| SA2410 | *nrd*D | Anaerobic ribonucleoside-triphosphate reductase | C | 4.5 | 0.004 | *rot* up |
| **Adaption to stress conditions** | | | | | | |
| SA0470 | *hsl*O | Heat-shock protein HSP33 homologue | C | 2.1 | 0.009 | |
| SA0480 | *cts*R | Transcription repressor of class III stress genes homologue | C | 2.3 | 0.010 | |
| SA0509 | *hch*A | Molecular chaperoneHchA | C | 2.5 | 0.012 | |
| SA1096 | *clp*Q | Heat shock protein HslV | C | 2.5 | 0.004 | |
| SA1238 | | HP, similar to tellurite resistance protein | C | 2.5 | 0.009 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA1408 | *dna*J | DnaJ protein (HSP40) | C | 2.9 | 0.006 | |
| SA1409 | *dna*K | DnaK protein | C | 2.4 | 0.012 | |
| SA1410 | *grp*E | GrpE protein (HSP-70 Cofactor HSP20) | C | 2.6 | 0.021 | |
| SA1535 | *tpx* | HP, similar to thioredoxin peroxidase | C | 2.5 | 0.021 | |
| **Carbohydrate mechanism** | | | | | | |
| SA0728 | *pgk* | Phosphoglycerate kinase | C | 2.2 | 0.016 | |
| SA0729 | *tpiA* | Triosephosphate isomerase | C | 2.1 | 0.065 | |
| SA0730 | *gpm*I | 2, 3-diphosphoglycerate-independentphosphoglycerate mutase | C | 2.0 | 0.029 | |
| SA0731 | *eno* | Enolase (2-phosphoglycerate dehydrogenase) | C | 2.1 | 0.010 | |
| SA0958 | | Putative myo-inositol-1 (or 4)-monophosphatase | C | 2.0 | 0.006 | |
| SA1088 | *suc*C | Succinyl-CoA synthetase, beta chain | C | 3.9 | 0.004 | |
| SA1089 | *suc*D | Succinyl-CoA synthetase, alpha chain | C | 3.2 | 0.004 | |
| SA1184 | *acn*A | Aconitate hydratase | C | 2.5 | 0.010 | |
| SA1336 | | Glucose-6-phosphate 1-dehydrogenase | C | 2.2 | 0.012 | |
| SA1517 | *cit*C | Isocitrate dehyrogenase | C | 2.2 | 0.015 | |
| SA1518 | *cit*Z | Citrate synthase II | C | 2.4 | 0.019 | |
| SA1553 | *fhs* | Formyltetrahydrofolate synthetase | C | 2.2 | 0.030 | |
| SA1996 | *lac*B | Galactose-6-phosphate isomerase LacB subunit | C | 2.4 | 0.025 | *mgr, arl* down |
| SA2001 | | HP, similar to oxidoreductase, aldo/ketoreductase family | C | 2.3 | 0.034 | *rot* up |
| SA2008 | *als*S | Alpha-acetolactate synthase | C | 2.9 | 0.004 | *mgr*, *arl* down |
| SA2304 | *fbp* | Fructose-bisphosphatase | C | 2.1 | 0.010 | |
| SA2312 | *ddh* | D-specific D-2-hydroxyacid dehydrogenase | C | 2.5 | 0.071 | *rot*, *mgr* down |
| **Amino acid synthesis** | | | | | | |
| SA0822 | *arg*G | Argininosuccinate synthase | C | 2.0 | 0.012 | |
| SA0829 | | HP, similar to 5-oxo-1,2,5-tricarboxillic-3-penten acid decarboxylase | C | 2.1 | 0.025 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0859 | | Thimet oligopeptidase homologue | C | 2.6 | 0.014 | |
| SA1347 | *bfm*BAB | Branched-chain alpha-keto acid dehydrogenase E1 | C | 2.1 | 0.010 | |
| SA1365 | *gev*PB | glycine dehydrogenase (decarboxylating) subunit 2 homolog | C | 2.7 | 0.010 | |
| SA1366 | *gev*PA | glycine dehydrogenase (decarboxylating) subunit 1 | C | 3.9 | 0.004 | |
| SA1367 | *gcv*T | Aminomethyltransferase | C | 3.6 | 0.004 | |
| SA1915 | *gly*A | Serine hydroxymethyl transferase | C | 2.3 | 0.008 | |
| SA2081 | | Urea transporter | M | 5.5 | 0.010 | *mgr*, *arl* down |
| SA2082 | *ureA* | Urease gamma subunit | C | 13.1 | 0.009 | *rot*, *mgr*, *arl* down |
| SA2083 | *ure*B | Urease beta subunit | C | 32.4 | 0.006 | *rot, mgr, arl* down |
| SA2084 | *ure*C | Urease alpha subunit | C | 24.9 | 0.007 | *rot, mgr, arl* down |
| SA2085 | *ure*E | Urease accessory protein UreE | C | 16.0 | 0.089 | *rot, mgr, arl* down |
| SA2086 | *ure*F | Urease accessory protein UreF | C | 23.7 | 0.004 | *rot, mgr, arl* down |
| SA2087 | *ure*G | Urease accessory protein UreG | C | 21.4 | 0.012 | *rot, mgr, arl* down |
| SA2088 | *ure*D | Urease accessory protein UreD | C | 10.0 | 0.007 | *rot, mgr, arl* down |
| SA2318 | *sdh*A | Putative L-serine dehydratase | C | 3.7 | 0.010 | *mgr* down |
| SA2319 | *sdh*B | Putative beta-subunit of L-serine dehydratase | C | 2.5 | 0.071 | *mgr, arl* down |
| **Others (lipid synthesis, DNA repair, coenzyme, e.g.)** | | | | | | |
| SA0842 | *fab*H | 3-oxoacyl-(acyl-carrier-protein) synthase 3 | C | 2.4 | 0.063 | |
| SA1072 | *pls*X | Fatty acid/phospholipid synthesis protein | C | 2.7 | 0.006 | |
| SA1073 | *fab*D | Malonyl CoA-acyl carrier protein transacylase | C | 2.8 | 0.011 | |
| SA1074 | *fab*G | 3-oxoacyl-reductase, (acyl-carrier protein) reductase | C | 2.7 | 0.006 | |
| SA0484 | *rad*A | DNA repair protein homologue | C | 2.0 | 0.096 | |
| SA1138 | *mut*L | DNA mismatch repair protein | C | 2.1 | 0.026 | |
| SA1512 | | HP, similar to formamidopyrimidine-DNA glycosylase | C | 2.0 | 0.012 | |
| SA0831 | *cdr* | Coenzyme A disulfide reductase | C | 2.1 | 0.009 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA0231 | | HP, similar to flavohemoprotein | C | 2.4 | 0.011 | |
| SA0998 | | HAM1 protein homolog | M | 2.4 | 0.013 | |
| SA1105 | | Putative zinc metalloprotease | | 2.1 | 0.005 | |
| SA1312 | *ebp*S | Elastin binding protein | | 2.9 | 0.038 | |
| SA1349 | | Dihydrolipoamide dehydrogenase | C | 2.1 | 0.011 | |
| SA2301 | | HP, similar to GTP-pyrophosphokinase | | 2.8 | 0.010 | |
| **Hypothetical genes** | | | | | | |
| SA0175 | | HP | | 3.6 | 0.014 | |
| SA0381 | | HP | | 2.7 | 0.019 | |
| SA0427 | | HP | | 2.2 | 0.012 | *mgr* up |
| SA0481 | | HP | C | 2.0 | 0.008 | |
| SA0558 | | HP | | 2.1 | 0.004 | |
| SA0804 | | HP | | 5.5 | 0.008 | |
| SA0805 | | HP | | 4.8 | 0.004 | |
| SA0832 | | HP | | 2.4 | 0.007 | |
| SA0833 | | HP | | 2.1 | 0.024 | |
| SA0860 | | HP | | 2.5 | 0.037 | |
| SA0903 | | HP | | 2.1 | 0.014 | |
| SA1173 | | HP | | 2.6 | 0.004 | |
| SA1280 | | HP, conserved | | 2.0 | 0.012 | |
| SA1534 | | HP | | 2.4 | 0.007 | |
| SA1723 | | HP | C | 3.2 | 0.016 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| SA1937 | | HP | | 2.2 | 0.010 | |
| SA2005 | | HP | | 2.3 | 0.023 | |
| SA2050 | | HP | | 2.2 | 0.017 | |
| SA2138 | | HP | | 2.4 | 0.045 | |
| SA2160 | | HP | | 2.3 | 0.011 | |
| SA2297 | | HP | | 2.3 | 0.016 | *rot* down |
| Cytoplasm (C), Secreted (S), Secreted Cellwall-bound (SCW), Membrane (M) | | | | | | |

**Table 3.** RT-PCR Values and D-Alanylation of TAs

| **Strains** | **RT-PCR *rot* (%)** | **RT-PCR *ure*C (%)** | **RT-PCR *dlt*A (%)** | **D-alanylation (%)** |
|---|---|---|---|---|
| SA113 | 100 | 1.4 | 100 | 100 |
| *gra*RS*::erm* | 8 | 100 | 13 | 53.3* |
| *dlt*A*::spc* | nd | nd | 0 | 0 |

Unless noted otherwise, values represent the mean of three independent RT-PCRs;
*The value represents one of three independent experiments
nd, not determined

Next, we asked which of the 115 less expressed genes in the *gra*RS mutant were responsible for the increased susceptibility to CAMPs. A most likely candidate was the *dlt* operon (encoding enzymes involved in D-alanylation of TAs). Its transcript was decreased 2.1-fold to 2.9-fold as compared to WT, and indeed, the D-alanylation of TAs was decreased 46.7% in the *gra*RS mutant compared to WT (Table 3). It has been previously shown that inactivation of the *dlt* operon in *S. aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides [26]. The observed decrease of *ald*1 transcription by a factor of 3.5 is in line with the decreased *dlt* transcription. Ald1 is the alanine dehydrogenase, which is involved in the synthesis of L-alanine.

### Comparison of *gra*RS and *dlt*A Mutants

Because the *dlt* operon is less expressed in the *gra*RS mutant, we investigated lysozyme susceptibility with a *dlt*A deletion mutant, which is well-known to be sensitive to CAMPs [26]. Indeed, the *dlt*A mutant was more sensitive to lysozyme (Figure 2E); however, this sensitivity was not due to its muramidase activity, as the isolated PG of the *dlt*A mutant was not hydrolyzed by lysozyme (Figure 3). Furthermore, growth of the *dlt*A mutant was inhibited whether active or heat-inactivated lysozyme was applied (Figure 2E). When the susceptibility of *gra*RS and *dlt*A mutants to LP9, polymyxin B, and gallidermin were compared, both mutants were similarly more susceptible to these CAMPs (Figure 4B and 4C). However, there were two distinctions: a) the susceptibility of the *dlt*A mutant was more pronounced

than that of the *gra*RS mutant, and b) even in the presence of gallidermin or polymyxin B, the *gra*RS mutant started to grow after some time and reached the same optical density (OD) values after 24 h as the control culture lacking CAMPs. In contrast, the *dlt*A mutant remained sensitive to gallidermin and polymyxin B and was unable to resume growth. In the presence of LP9, growth resumed after a similar lag period as in the *gra*RS mutant; this can possibly be explained by its proteolytic degradation. Not only the single but also the double mutants *oat*A/*gra*RS and *oat*A/*dlt*A were sensitive to the CAMP activity of LP9, although the susceptibility was less pronounced as with the *gra*RS and *dlt*A single mutants. However, the *oat*A single mutant was completely resistant to LP9, indicating that *oat*A is resistant to CAMPs (Figure 4D–4F). With respect to gallidermin- and polymyxin B–induced cell lysis, it has been observed that CAMPs such as lantibiotics induce autolysis in staphylococci by increasing PG hydrolase activity [27]. We assume that gallidermin and polymyxin B, which are also CAMPs, very likely have a similar effect.

We asked whether the increasing insensitivity of the *gra*RS mutant after prolonged growth is some short lasting CAMP-induced adaptation or whether it is based on selection of resistant mutants. To answer this question, we inoculated from a 24-h *gra*RS culture treated with polymyxin B (Figure 5B) a new culture and challenged it again with polymyxin B (Figure 5C). The subculture revealed no growth retardation, which suggests that the *gra*RS phenotype is unstable and that polymyxin B–resistant revertants were quickly selected. Since the *dlt*A revealed a stable phenotype, we assume that in the selected revertants *dlt*A expression was increased to WT levels.

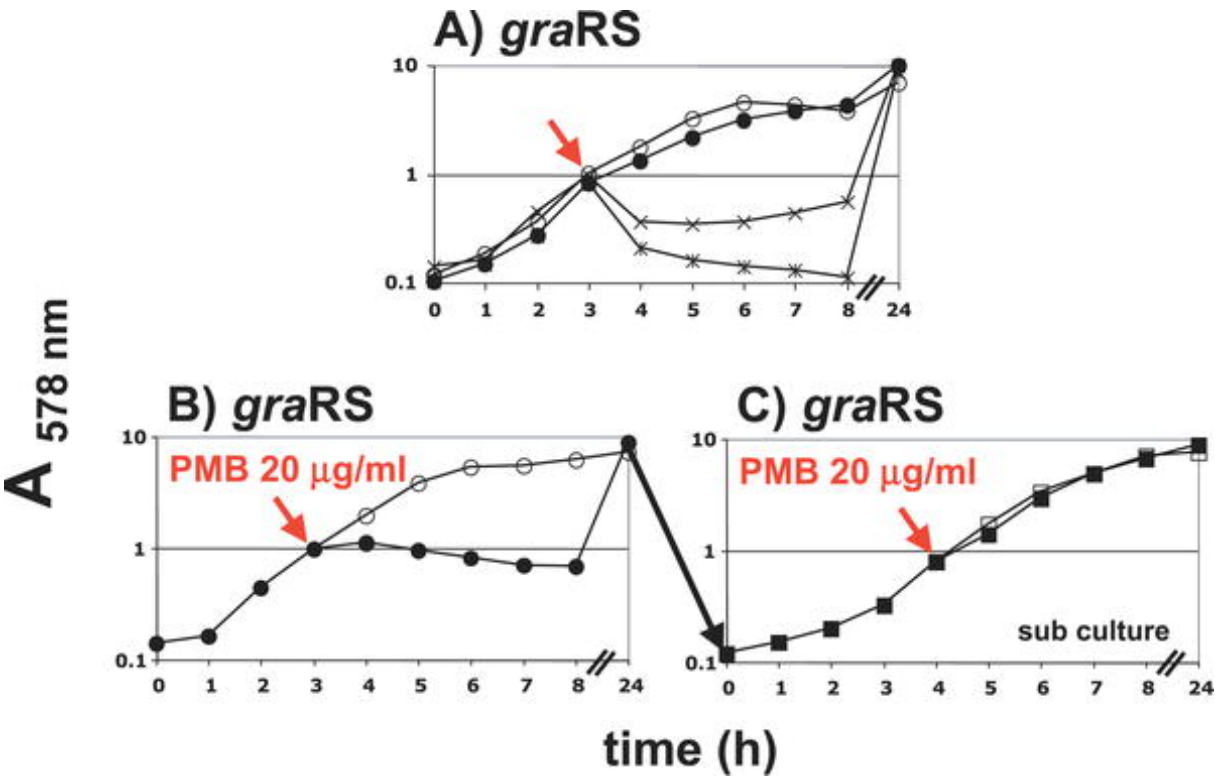



**Figure 5.** Susceptibility of S. aureus graRS Mutant to Mutanolysin, Mutanolysin and LP9 or Lysozyme, and Polymyxin B

(A) *gra*RS mutant: control (○); mutanolysin (Mut) (100 µg/ml [4.35 µM]) (•); Mut (100 µg/ml) and LP9 (200 µg/ml) (✱); Mut (50 µg/ml [2.18 µM]) and Lys (300 µg/ml) (×).

(B) *gra*RS mutant: control (○); polymyxin B (PMB) (20 µg/ml) (•).

(C) *gra*RS subculture of 5B: control (□); PMB (20 µg/ml) (▪).

Cells were grown in BM at 37 °C. $OD_{578nm}$ was measured hourly for the first 8 h and after 24 h. Cationic agents were added in the exponential growth phase at $OD_{578nm}$ 1.0 as indicated by arrow.

The highest susceptibility to lysozyme was observed with the *oat*A/*dlt*A double mutant, which was more than 66-fold and 333-fold more sensitive to lysozyme than the *dlt*A and *oat*A single mutants, respectively (Figure 2B, 2E, and 2F; Table 4). The *oat*A/*gra*RS mutant is not quite as sensitive as the *oat*A/*dlt*A mutant. Another difference is that the *oat*A/*dlt*A mutant stays lysozyme sensitive even after 24 h of cultivation (Figure 2D and 2F), indicating that the *dlt*A mutant phenotype cannot easily revert to the WT phenotype. The lower susceptibility of the *oat*A/*gra*RS double mutant can possibly be explained by the fact that the TA in this mutant still contains

53.3% D-alanyl esters, whereas the *dlt*A mutant completely lacks D-alanylation in its TAs (Table 3).

**Table 4.** MIC Values of SA113 and Various Mutants

| **Strains** | **Lysozyme µg/ml (µM)** | | **Polymyxin B µg/ml (µM)** | | **Gallidermin µg/ml (µM)** | |
|---|---|---|---|---|---|---|
| SA113 | > 50.000 | (3470) | 350 | (252.5) | 9 | (4.1) |
| *gra*RS::*erm* | > 50.000 | (3470) | 20 | (14.4) | 2.5 | (1.14) |
| *oat*A*::kan* | 10.000 | (694) | 350 | (252.5) | 9 | (4.1) |
| *oat*A*::kan*/*gra*RS::*erm* | 600 | (41.6) | 25 | (18) | 3 | (1.36) |
| *dlt*A::*spc* | 2.000 | (138.8) | 10 | (7.2) | 1.2 | (0.55) |
| *oat*A*::kan*/*dlt*A::*spc* | 30 | (2.08) | 10 | (7.2) | 1.2 | (0.55) |
| *gra*RS::*erm* (pTX*gra*RS) | > 50.000 | (3470) | > 800 | (577.2) | 9 | (4.1) |
| SA113 (pTX*gra*RS) | > 50.000 | (3470) | > 500 | (360.8) | 9 | (4.1) |

The results represent the mean of three to five independent serial dilution experiments;
cells were grown in Basic Medium without glucose but with 0.5 % xylose as an inductor.

The high susceptibility of the double mutants is based on the dual activities of lysozyme: a) the *oat*A mutant is sensitive to the muramidase activity of lysozyme but is insensitive to CAMPs (Figures 2B, 3, and 4F), and b) the *dlt*A and *gra*RS mutants are sensitive to CAMPs, but insensitive to the muramidase activity of lysozyme (Figures 3, 4B, and 4C). The extremely high lysozyme susceptibility of the *oat*A/*dlt*A double mutant can only be explained by a synergistic effect of the two activities.

### Increased Lytic Activity of Mutanolysin by Lysozyme and LP9 in the *gra*RS Mutant

Mutanolysin is a muramidase that is able to hydrolyze *O*-acetylated PG [28] but does not normally cause cell lysis in WT *S. aureus* or its *gra*RS mutant at a concentration of 100 µg/ml. However, when the *gra*RS mutant was treated with mutanolysin in combination with lysozyme or LP9, the lytic activity (indicated by decrease in OD) was strongly increased (Figure 5A). Because the *O*-acetylated *gra*RS mutant is insensitive to the catalytic activity of lysozyme, we assume that mutanolysin acts

through its lytic activity, and LP9 and lysozyme through their CAMP properties. We have not investigated how the stimulating effect of lysozyme and LP9 on cell lysis is accomplished. However, we assume that it is caused by the concerted action of PG hydrolysis by mutanolysin and induced autolysis by lysozyme and LP9, as mentioned above.

**Minimal Inhibition Concentration Values of SA113 and Various Mutants**

The minimal inhibition concentration (MIC) values for lysozyme, polymyxin B, and gallidermin in WT and various mutants are summarized in Table 4. Both the WT and the *gra*RS mutant were completely resistant to lysozyme at a concentration of 50 mg/ml. However, the *gra*RS mutant was 17- and 4-fold more susceptible to polymyxin B or gallidermin. The sensitivity to the CAMPs is very likely due to the aforementioned decrease in expression of the *dlt* operon, which corresponds with decreased D-alanylation of the TAs. The *oat*A mutant was more susceptible to lysozyme than the *gra*RS mutant, but, similar to WT, was completely insensitive to heat-inactivated lysozyme or CAMPs, indicating that *oat*A is only sensitive to the muramidase activity of lysozyme. The *oat*A/*gra*RS double mutant was almost 17-fold more sensitive to lysozyme than the *oat*A mutant, which can be explained by the fact that this double mutant is sensitive to both the muramidase and the CAMP activities of lysozyme. The two activities exert a synergistic effect on the double mutant. The *dlt*A single mutant was over 25-fold more sensitive to lysozyme than the WT and 5-fold more sensitive than the *oat*A single mutant, demonstrating the importance of lysozyme's CAMP activity. Furthermore, the *dlt*A mutant exhibited the highest susceptibility to polymyxin B and gallidermin, but was completely insensitive to lysozyme's muramidase activity (Figure 3).

With a MIC of only 30 µg/ml, the *oat*A/*dlt*A double mutant revealed the highest susceptibility to lysozyme. Indeed, it has a 20-fold greater sensitivity to lysozyme than the *oat*A/*gra*RS double mutant. The *oat*A/*dlt*A double mutant is 333-fold and 66-fold more sensitive than the single *oat*A or *dlt*A mutants, which illustrates the extremely high synergistic effect of lysozyme when it can exert both muramidase and CAMP activities. Overexpression of *gra*RS in the *gra*RS mutant or the WT by pTX*gra*RS resulted in an approximately 2-fold increase in polymyxin B resistance, indicating that even in WT cells, CAMP resistance can be further increased.

## Discussion

One of our research aims was to identify genes involved in staphylococcal lysozyme resistance. We have already elucidated two genes and corresponding enzymes that contribute to resistance against the muramidase activity of lysozyme. Since the target of muramidase is PG, it is not surprising that the mechanism of resistance is masking PG by modification. In *S. aureus* there are two PG modifications that are involved in resistance to lysozyme's muramidase activity. One modification is *O*-acetylation catalyzed by the PG-specific *O*-acetyltransferase A, OatA, and we have shown that the *oat*A mutant is more susceptible to the muramidase activity of lysozyme than the WT [10]. The other modification is WTA [29] that is covalently linked to the same $C_6$ position in MurNAc as in the *O*-acetyl group. TagO is a specific UDP-*N*-acetylglucoseamine transferase, which is involved in the first step of WTA synthesis. The *tag*O deletion mutant completely lacks WTA [30]. Although the *tag*O mutant still shows high lysozyme resistance, a *oat*A/*tag*O double mutant, however, is much more susceptible to lysozyme's muramidase activity than the *oat*A mutation alone [13]. Here, we show that the high lysozyme resistance of *S. aureus* is not only based on resistance to the muramidase activity of lysozyme, but also to its inherent CAMP resistance.

The described global two-component regulator, GraRS, was identified in an *oat*A-minus background by increased lysozyme susceptibility in an *oat*A/*gra*RS double mutant. The *gra*RS mutant was more susceptible to CAMPs than the WT. We assume that the reason for the increased susceptibility of the *gra*RS mutant was a decrease in *dlt* expression, and consequently, GraRS up-regulates *dlt* expression. The Dlt enzymes modify TAs by the incorporation of D-alanine esters rendering the cells resistant to CAMPs, very likely by repulsion [26]. We showed that the *dlt*A mutant is even more susceptible to lysozyme-derived LP9 and other CAMPs than the *gra*RS mutant, because in the *dlt*A mutant, D-alanine esters were completely absent in TAs, the mutant was stable, and no revertants were observed. Heat-inactivated lysozyme does not affect either the growth of the *oat*A or that of the *gra*RS mutant. The latter effect is surprising, as the *gra*RS mutant is sensitive to the other CAMPs (LP9, gallidermin, polymyxin B). However, the *oat*A/*gra*RS mutant was sensitive to heat-inactivated lysozyme, suggesting that the bulky molecule has better access to

the cell envelope when the PG is de-*O*-acetylated. Likewise, sensitivity of the *dlt*A mutant to heat-inactivated lysozyme can also be explained by better access to the cell envelope because of the lack of D-alanine esters in TAs.

The next interesting question was, how do CAMPs act in the *dlt*A, *oat*A/*gra*RS, or *oat*A/*dlt*A mutants? Killing of Gram-negative bacteria could be demonstrated by lysozyme-derived peptides that were transported through the outer membrane and damaged the inner membrane by pore formation [17]. Several authors assume that lysozyme and CAMPs are not only acting as membrane permeabilization agents, but also activate autolytic wall enzymes of Gram-positive bacteria, thus causing cell lysis [31–33]. It has also been shown that lipoteichoic acids can bind and inhibit autolysins, depending on their degree of D-alanylation [34–36]. Similar results were also obtained in a *dlt* mutant of *Lactococcus lactis,* which showed increased autolysis [37]. In line with these observations, the *gra*RS and *dlt*A mutants also showed increased autolysis when treated with Triton X-100 (unpublished data), suggesting that in these mutants, too, CAMPs activate autolytic enzymes. We assume that the observed synergistic effect of lysozyme in the *oat*A/*gra*RS and *oat*A/*dlt*A double mutants is caused by the simultaneous activation of autolytic enzymes and the muramidase activity of lysozyme. A similar synergistic effect is seen by treatment with mutanolysin in combination with LP9 (inducing autolysis) or lysozyme (cannot exert its muramidase activity as the PG is *O*-acetylated) as shown in the *gra*RS single mutant (Figure 5A). For the first time (to our knowledge), we have traced and dissected genes that were responsive to the dual activities of lysozyme.

Until now, little was known about the two-component system GraRS. We became interested in the regulation of GraRS because we wanted to trace the gene(s) that caused the increased CAMP susceptibility in the *gra*RS mutant. Comparative transcriptome analysis of SA113, an 8325-derivative, and its *gra*RS mutant revealed that 115 genes were up-regulated and 133 genes were down-regulated by GraRS (Tables 1 and 2). Among the down-regulated genes was the *vra*FG operon, which immediately follows the *gra*RS operon. However, in studying intermediate level of vancomycin resistance in *S. aureus,* Ambrose Cheung and colleagues found that *vra*FG is positively controlled by GraRS [38]. This contradictory result can be explained by the genetic organization of our *gra*RS*::erm*B deletion mutant (Figure 1). In our mutant, the *erm*B cassette is in the same orientation as the *vra*FG genes. Since the *erm*B transcription terminator is very weak, we assume that there is a

transcriptional read-through into the *vra*FG genes. This explains why in our *gra*RS deletion mutant, the *vra*FG genes were up-regulated instead of down-regulated.

GraRS up-regulates transcription of global regulators such as the SarA homologs Rot, SarS, and MgrA. We compared our GraRS transcriptome results with that of the recently published transcriptome studies of Rot [39], MgrA [40], and ArlRS [41] (Tables 1 and 2; Figure 6). Rot is a repressor of exoproteins but positively regulates cell surface proteins, and SarS is a positive activator of protein A. MgrA appears to be an antagonist to Rot, as it up-regulates exoproteins and down-regulates cell surface proteins, including the regulator SarS. We found that Rot and MgrA regulate some of the GraRS-controlled genes in the same direction. For these few genes we do not know whether their up- or down-regulation is directly affected by GraRS or indirectly via up-regulation of Rot and MgrA, respectively. Moreover, there are some genes that were regulated in opposite directions (Figure 6, boxed genes). Interestingly, GraRS up-regulates both regulators, Rot 3.8- and MgrA 3.1-fold. GraRS controls many genes involved in cell wall synthesis and transport (57 genes). Among the transporters are the EssA and EssC proteins, involved in transport of the virulence factor EsxA, oligopeptide transport system (OppB), or NorB, which encodes the Blt-like protein that is an efflux pump involved in multidrug resistance, all of which are up-regulated by GraRS. Interestingly, *smp*C, which encodes a membrane-spanning protein with unknown transport functions, is the only gene that is increased by all four regulators (GraRS, Rot, MgrA, and ArlRS). The gene which had the highest (23.3-fold) up-regulation by GraRS was SA1793, which encodes a hypothetical protein with a phage-related function. Many of the down-regulated genes are involved in RNA and amino acid synthesis or glycolysis. *lrg*A, which encodes a holin-like protein with murein hydrolase activity, is also down-regulated by GraRS but up-regulated by ArlRS and MgrA. Most of the genes are exclusively regulated by GraRS, such as *ica, pur, mgr*A, *sir*A,C, *atl*A, *aaa, dna*J,K, *grp*E, and *vra*F,G. These results illustrate that there is a distinct cross-regulation between GraRS, ArlRS, Rot, MgrA, and probably some other global regulators.

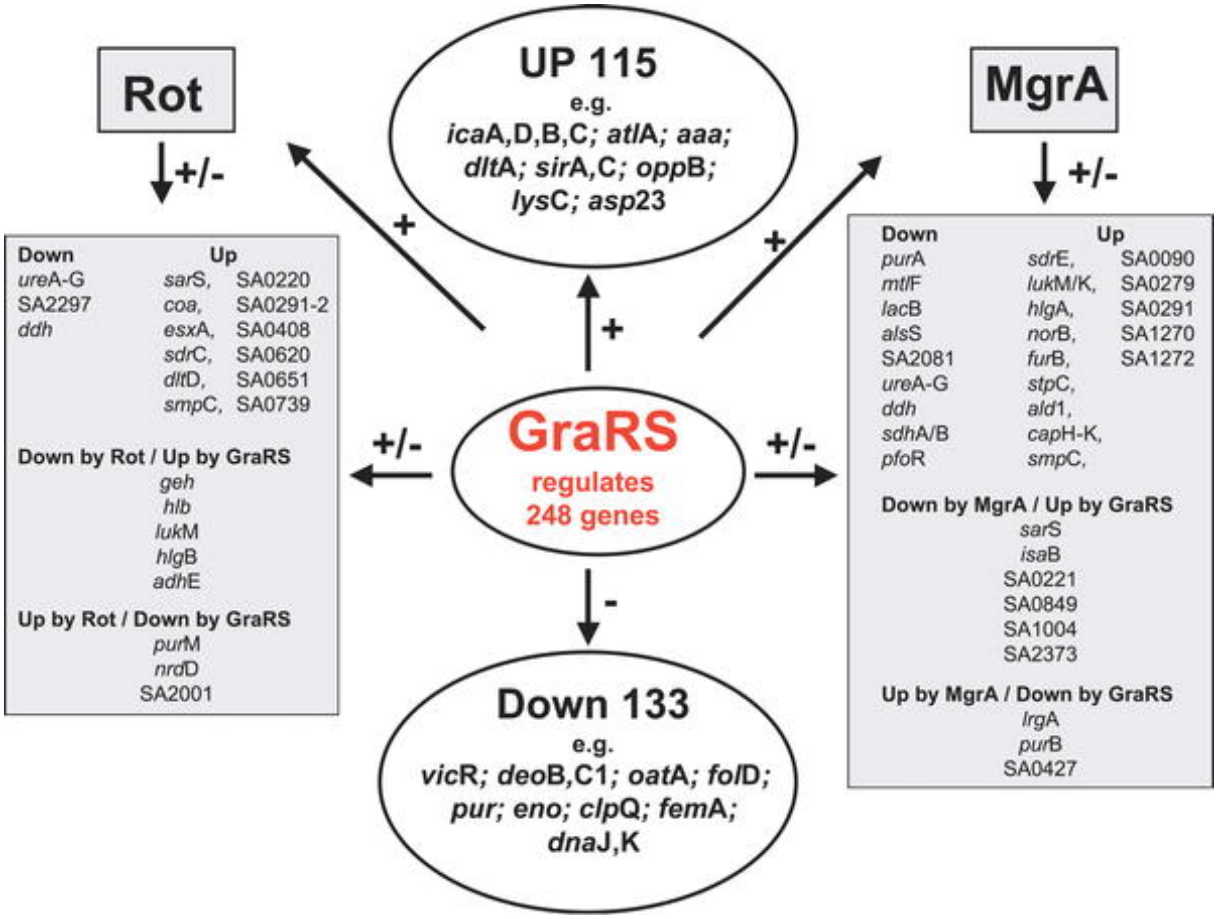



**Figure 6.** Interplay of GraRS–TCS with Other Global Regulators

Of the 248 genes regulated by GraRS, 115 genes are up-regulated and 133 genes are down-regulated. GraRS also upregulates the global regulators Rot and Mgr (both are homologs of SarA). Genes that are controlled by both GraRS and Rot or GraRS and MgrA are boxed. Example genes that are exclusively controlled by GraRS are circled.

GraRS is not only important for resistance to glycopeptides, lysozyme, and other CAMPs. Our data suggest that GraRS also has an intermediate role between other global regulators (Agr, MgrA, Rot, and SarA), as GraRS up-regulates both adhesins as well as exoproteins and toxins (e.g., *hlb, hlg*A,B, *luk*M,F, *geh*). GraRS is possibly involved in the establishment of persistent infections by the up-regulation of colonization factors (e.g., *ica, atl, aaa, fib, sir*A, *sir*C, *sdr*C, *sdr*E), factors involved in resistance to CAMPs *(dlt),* factors involved in intermediary vancomycin resistance

(*vra*F,G, as mentioned above), and factors involved in biofilm formation (e.g., *dlt, atl, ica*). It would be interesting to study the *gra*RS mutant in an animal model for chronic infection.

## Materials and Methods

### Bacterial strains and plasmids.

All of the strains and plasmids that were used are listed in Table 5. Bacteria were grown in Basic Medium (BM) (1% tryptone; Gibco BRL Life-Technologies), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, 0.1% $K_2HPO_4$, 0.1% glucose, or 0.5% xylose).

| Strain or plasmid | Comment | Reference or source |
|---|---|---|
| ***S. aureus*** | | |
| RN4220 | Mutant strain of 8325-4, accept foreign DNA | Kreiswirth |
| SA113 | Mutant strain of 8325, with an agr$^-$ background and 11 bp deletion in *rsb*U | Iordanescu |
| SA113Δ*oat*A | Mutant of SA113 (Δ*oat*A*::kan*) | Bera |
| SA113Δ*dlt*A | Mutant of SA113 (Δ*dlt*A*::spc*) | Peschel |
| SA113Δ*gra*RS | Mutant of SA113 (Δ*gra*RS*::erm*) | This study |
| SA113Δ*oatA*/Δ*gra*RS | Double mutant of SA113 (Δ*oat*A*::kan*/Δ*gra*RS*::erm*) | This study |
| SA113Δ*oat*A/Δ*dlt*A | Double mutant of SA113 (Δ*oat*A*::kan*/Δ*dlt*A*::spc*) | This study |
| SA113Δ*gra*RS + (pTX*gra*RS) | Δ*gra*RS*::erm* and pTX15 containing xylose inducible *gra*RS genes | This study |
| SA113 + (pTX*gra*RS) | SA113 and pTX15 containing xylose inducible *gra*RS genes | This study |
| ***S. carnosus*** | | |
| TM300 | Host strain for cloning vector pTX15 | Götz |
| ***E. coli*** | | |
| Dh5α | Host strain for cloning vector pBT2 | Hanahan |
| **Plasmids** | | |
| pBT2 | Temperature-sensitive *E. coli-S. aureus* shuttle vector | Brückner |
| pBTud*gra*RS | pBT2 containing up- and downstream region of *gra*RS and *erm*B cassette | This study |
| pTX15 | Xylose inducible vector for complementation | Peschel |
| pTX*gra*RS | pTX15 containing xylose inducible *gra*RS genes | This study |
| pTV1ts | Vector for transposon (Tn*917*) mutagenesis | Youngman |

**Transposon mutagenesis.**

Transposon mutagenesis was carried out in the Δ*oat*A*::kan* mutant using the temperature-sensitive plasmid pTV1ts and was performed as described by Bera et al. [10].

**Construction of plasmids, homologous recombination, and transduction.**

Was performed as described by Bera et al. [10]. The PCR products, up- and downstream of *gra*RS (SA0614/15) (U0614/15Kpn: TGATATAGGTACCTAATTGTTT ACTAGCCGACG, U0614/15Sma: ATTTGTCCCGGGTTCTAGTAGTATTTGCATCC, D0614/15Sal: GGCCGTGTCGACTTTGTCATTTTAAACATGCG, and D0614/15Nhe: ATTGCTAGCTTGGCATAACTTGCTGCAACAGG), were cloned into the polylinker of the pBT2 vector flanking the *erm*B antibiotic cassette. Complementation of the *gra*RS deletion mutant was obtained by cloning the *gra*RS genes (1,912 bp) (C0614/15Bam: AATGATGGATCCTGGCTTTGAAGTTGACTGCC, and C0614/15Eco: AGCGCGAAT TCATTTCCTTTAGGCTTTGGCAC) into the xylose-inducible vector pTX15 in *S. carnosus* TM300. The *oat*A*::kan/dlt*A*::spc* double mutant was created by bacteriophage φ11-mediated transduction of the *oat*A*::kan* knockout into the *dlt*A*::spc* deletion mutant.

**Effects of cationic agents on exponential growth.**

Overnight cultures were diluted to an $OD_{578nm}$ of 0.1 in 50 ml of BM and the cultures were incubated with shaking at 37 °C. OD was determined every hour. Ten milliliters of each culture were transferred into a new 100-ml flask when the cultures reached an $OD_{578nm}$ of nearly 1.0. Then, cationic agents, such as hen egg-white lysozyme and human lysozyme (Sigma-Aldrich), LP9 (a lysozyme-derived 9-aa peptide, $_{107}$R-A-W-V-A-W-R-N-R$_{115}$–$NH_2$) (EMC), polymyxin B (Sigma-Aldrich), gallidermin (Genmedics), or mutanolysin (Sigma), were added. Lysozyme was inactivated by heating for 1 h at 100 °C and placed on ice. The $OD_{578nm}$ of all cultures was measured hourly up to 7–8 h and after 24 h.

**MIC assay.**

The overnight cultures were diluted in BM with 0.5% xylose to a concentration of $0.5\times10^6$ CFU per ml and aliquoted in 0.5-ml samples, and cationic agents in different

concentrations were added. The cultures were incubated with shaking at 37 °C for 20–24 h and MIC was determined.

**Biofilm assay.**

An overnight culture was diluted 1:200 in fresh TSB with 0.5% glucose, and 200 µl were filled into microtiter plates and incubated for 20–24 h at 37 °C without shaking. The supernatant was removed and the plate was washed two times with PBS (pH 7.4). The plate was dried and the cells were colored with 0.1% safranine.

**Isolation of PG.**

One liter of BM was inoculated with an overnight culture of the WT SA113 or the mutants. Strains were grown for 12 h with shaking at 37 °C. Cells were centrifuged, washed two times with cold 0.9% NaCl, diluted in 0.9% NaCl, and boiled for 20 min. After the cells were chilled on ice, they were again centrifuged and washed twice with 0.9% NaCl. The cells were disrupted in a mechanical grinding device using glass beads 150–212 µm (Sigma-Aldrich) at 4 °C, centrifuged and washed two times with cold $H_2O_{bidest}$, boiled for 30 min in 2% SDS to remove noncovalently bound proteins, and washed four times with $H_2O_{bidest}$. The cell wall fragments were diluted in 0.1 M Tris/HCl (pH 6.8) and incubated with 0.5 mg/ml trypsin for 16 h at 37 °C to degrade cell-bound proteins. After centrifugation and washing with water, the PG was lyophilized.

**Turbidometric assay of PG.**

For analyzing the susceptibility of PG to lysozyme, we used a modified method turbidometric assay as described by Clarke [42]. The PG of the WT SA113 and the mutants were sonicated and diluted to 0.5 mg in 1 ml of 80 mM PBS (pH 6.4). After the addition of 300 µg lysozyme per ml, the decrease in optical density was monitored at the beginning (0 h) and after 4 h at $OD_{660nm}$ and calculated as percentages.

**Quantification of D-alanylation of TA by HPLC.**

*S. aureus* strains were grown in BM with 0.25% glucose overnight, centrifuged, washed three times, and resuspended in ammonium acetate buffer (20 mM [pH 6.0]). The $OD_{600nm}$ was adjusted to 30. Aliquots (1 ml) were heat-inactivated by incubation

at 99 °C for 10 min and centrifuged, and pellets were dried. After incubation at 37 °C for 1 h with 100 µl of 0.1 N NaOH, 100 µl of 0.1 N HCl were added for neutralization and samples were dried. For derivatization, 100 µl of triethylamine and 100 µl of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; Sigma) (10 mM) were added. After incubation at 40 °C for 1 h, samples were dried and resuspended in DMSO:$H_2O$ (1:1). Quantification of D-alanine was performed by HPLC as previously described [43].

**RNA isolation and real-time RT-PCR.**

SA113 and the *gra*RS deletion mutant were cultivated in 50 ml of BM and harvested at mid-exponential growth phase. Before RNA isolation, two volumes of RNAprotect bacteria reagent (Qiagen) were added to 10 ml of culture and centrifuged. The cells were lysed by the addition of 50 µg/ml of lysostaphin (0.5 mg/ml) (Genmedics) in TE buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Contaminating DNA was degraded with the DNase Kit (Ambion) according to the manufacturer's instructions. LightCycler RT-PCR was carried out using the LightCycler RNA amplification Kit SYBR Green I or with the LightCycler RNA amplification kit for hybridization probes (Roche Biochemicals). The internal control *gyr* was quantified using 10-fold serial dilutions ($10^4$ to $10^8$ copies/µl) of a specific RNA standard using oligonucleotides specific for *gyr* (gyr297F: TTAGTGTGGGAAATTGTCGATAAT and gyr574R: AGTCTTGTGACAATGCGTTTACA), *dlt*A (dltA1:TGGCGTTG AAAGACTAGGC and dltA2: TTACGAACTCAGACTGGCG), *rot* (rot1: TTCAGCGAGATTGAAAGCG and rot2: GTTGCTCTACTTGCAATGG) or *ure*C (ureC1: GATATCATTGCCGCTGAAGG and ureC2: AAAGCAGATGGTGTTGCACC) as described [44]. Standard curves for *dlt*A and *rot* were generated using 5-fold serial dilutions of WT SA113 RNA or for *ure*C of the *gra*RS mutant RNA. Differences between WT and the *gra*RS mutant were determined by *n*-fold change and calculated as a percentage of the mRNA product. The specificity of the PCR was verified by size determination of the amplicons by agarose gel electrophoresis. To check for DNA contamination, each sample was subjected to PCR by using the LightCycler DNA amplification kit SYBR Green I (Roche Biochemicals). In none of the cases an amplification product was detectable.

**Transcriptome analysis.**

Transcriptome analysis was carried out as described by the microarray manufacturer Scienion and Resch et al. [45]. cDNA was synthesized from isolated RNA (1 µg) during mid-exponential growth (4 h) derived from WT SA113 (labeled in green with Cy3 [532 nm]) or from the *gra*RS mutant (labeled in red with Cy5 [635 nm]). cDNAs from WT and the *gra*RS mutant were pooled and hybridized on four DNA microarrays. Scienion performed DNA transcriptome analysis by comparing the intensity of each Cy3-labeled gene of the WT with the intensity of each Cy5-labeled gene of the *gra*RS mutant as a ratio of the medians (532/635). The threshold was set at a 2-fold difference in gene expression. Genes whose RNA level was higher in WT (2.0 and more) were categorized as being positively regulated by GraRS. In contrast, genes that had higher RNA levels (2.0 and more) in the *gra*RS mutant were described as being negatively regulated by GraRS. The significance of differences (*n*-fold) in gene expression was calculated by One-Sample *t*-Test-Benjamini–Hochberg (Adv); results <0.051 are significant, and some genes from Tables 1 and 2 are higher than 0.05.

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# The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defences

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## Abstract

Modification of teichoic acids with D-alanine protects Gram-positive bacteria against major antimicrobial host defence molecules. The *graRS* regulatory genes have recently been implicated in the control of D-alanylation in *Staphylococcus aureus*. We demonstrate here that *graRS* deletion considerably alters bacterial surface charge, increases susceptibility to killing by human neutrophils or the defence peptide LL-37, and attenuates virulence of *S. aureus*. These data indicate that *S. aureus* can regulate its surface properties in order to overcome innate host defences.

## 1. Introduction

*Staphylococcus aureus*, a frequent cause of human infections, is highly resistant to antimicrobial factors of the innate immune system such as cationic antimicrobial peptides (CAMPs) (Foster, 2005;Peschel and Sahl, 2006), which are produced by epithelial cells and neutrophils (Lehrer and Ganz, 2002;Jenssen *et al.*, 2006). These peptides generally contain 10-50 amino acids and have positive net charges (Hancock and Sahl, 2006). Due to their cationic properties, CAMPs can easily bind to the highly negatively charged bacterial cell envelope and inactivate bacteria, e.g. by forming pores in the bacterial membrane leading to bacterial lysis (Sahl *et al.*, 2005). *S. aureus* has evolved mechanisms to alter the anionic charge of cell surface components to gain resistance to a broad variety of cationic antimicrobial factors such as CAMPs (Peschel *et al.*, 1999), phospholipase A2 (Koprivnjak *et al.*, 2002), myeloperoxidase (Collins *et al.*, 2002), or lysozyme (Herbert *et al.*, 2007). One such mechanism is based upon modification of phospholipids in the cytoplasmic membrane by introducing a positively charged lysyl group into anionic phosphatidylglycerol by the MprF protein, thereby neutralising the net charge of the membrane surface (Peschel *et al.*, 2001;Staubitz *et al.*, 2004). A similar reaction is mediated by products of the *dltABCD* operon, which are responsible for attachment of positively charged D-alanine residues into negatively charged phosphate groups in the backbone of teichoic acids (Peschel *et al.*, 1999;Collins *et al.*, 2002). Mechanisms involved in the regulation of these resistance factors are not yet well understood in Gram-positive bacteria. Herbert *et al.* recently found that the *S. aureus* two-component regulatory system *graRS* positively regulates expression of the *dlt*

operon. In a *S. aureus* SA113 *graRS* deletion mutant, the content of D-alanine in teichoic acids was reduced by 44% and the mutant showed reduced resistance to various antibiotics including polymyxin B, gallidermin, and vancomycin (Meehl *et al.*, 2007;Herbert *et al.*, 2007). Accordingly, *graRS* have previously been implicated in regulation of vancomycin intermediary resistance (Cui *et al.*, 2005). As the *dlt* operon plays a key role in *S. aureus* resistance to cationic host antimicrobial molecules, the *graRS* system may be important in evasion of host defence mechanisms such as cationic antimicrobial peptides and neutrophil killing.

## 2. Results

### *2.1. The graRS mutant shows altered cell surface charge but unaltered LPG content*

In order to study if reduced expression of the *dlt* operon upon *graRS* disruption results in altered cell surface charge, we compared binding of the red-coloured, cationic protein cytochrome c to wild type *S. aureus* SA113 (WT), the isogenic *graRS* mutant, and the plasmid-complemented mutant.

The *graRS* mutant bound significantly more cytochrome c than the WT or the complemented mutant (Fig. 1A), which is in accordance with the recently described reduced content of D-alanine residues in the teichoic acids of the mutant (Herbert *et al.*, 2007).

Increased binding of cationic proteins may also result from reduced *mprF* expression and, accordingly, reduced LPG content. To control for this possibility, we compared patterns of membrane lipids from log-phase bacteria by thin-layer chromatography. The amounts of LPG from WT and *graRS* mutant were indistinguishable (Fig. 1B), which corroborates recent findings that *mprF* is not among the *graRS*-regulated genes in *S. aureus* SA113 (Herbert *et al.*, 2007).

### *2.2. The graRS mutant is more susceptible to killing by LL-37 and human neutrophil granulocytes in vitro*

In order to study if the increased affinity of the *graRS* mutant to cationic molecules leads to higher susceptibility to human host defence peptides, we compared inactivation of WT and *graRS* mutant by the human cathelicidin LL-37. The WT and complemented mutant strains showed no significant decrease in CFU following LL-37

exposure, whereas the number of *graRS* mutant bacteria recovered was only 25% of the original inoculum (Fig. 2A).

Next we investigated whether the *graRS* mutant is killed faster than the parental strain by human neutrophils, which produce high amounts of LL-37 and other CAMPs as components of their antibacterial killing arsenal. The *graRS* mutant was killed by neutrophils considerably faster than the WT strain. After 15 and 30 min, the recovered CFU of the *graRS* mutant were significantly lower than those of the WT (Fig 2B). Taken together, these data indicate that *graRS*-mediated control of CAMP resistance mechanisms is of importance for *S. aureus* evasion from neutrophil killing.

*2.3. Deletion of graRS leads to attenuated virulence in a mouse infection model*

In order to study whether reduced resistance of the *graRS* mutant to neutrophil and CAMP-mediated killing influences the ability of the bacteria to cause infections *in vivo*, we compared the virulence of WT and mutant bacteria in a mouse challenge model. Therefore female BALB/c mice (12 to 15 weeks old) were infected with *S. aureus* WT or *graRS* mutant bacteria. 72 h after infection numbers of CFU/kidney were determined.

Significantly less bacteria were detected in the kidneys of animals, which had been infected with the *graRS* mutant than those infected with the WT bacteria. (Fig. 3) This finding suggests that increased susceptibility to clearance by CAMPs and neutrophils correlates to reduced virulence *in vivo*, corroborating the central importance of these host factors in innate defence.

## 3. Discussion

A large variety of regulatory systems has been described in *S. aureus* during the last decades (Novick, 2003;Bronner *et al.*, 2004;Cheung *et al.*, 2004). While many systems have been shown to control adhesion and toxin production (Ziebandt *et al.*, 2004), much less is known about the regulation of genes involved in the resistance to antimicrobial peptides, such as the *dlt* genes. However, the critical role of this operon in infection (Peschel *et al.*, 1999;Collins *et al.*, 2002) suggests that it may be appropriately regulated in response to environmental stimuli. *Listeria monocytogenes* expresses the VirR transcription factor, which regulates both the *dlt* operon and the *mprF* gene (Mandin *et al.*, 2005). Similar data have recently been presented for the

*Staphylococcus epidermidis aps* system (Li *et al.*, 2007). The *S. aureus* regulatory genes *rot* and *arl* have previously been shown to have a moderate influence on transcription of the *dlt* operon (Said-Salim *et al.*, 2003;Koprivnjak *et al.*, 2006). We have recently demonstrated that inactivation of the GraRS system leads to only 13% of the wild-type transcription level of the *dlt* operon in *S. aureus* SA113 (Herbert *et al.*, 2007). Other important genes regulated by *graRS* in *S. aureus* include the *vraFG* genes involved in the resistance to vancomycin (Meehl *et al.*, 2007;Herbert *et al.*, 2007). Thus this regulatory system plays a key role in influencing the effectiveness of both pharmacologic antimicrobials and natural antimicrobials of our innate immune system against *S. aureus*, and merits further attention in an era of increasing reports of virulent and drug-resistant strains of this foremost human pathogen.

## 4. Materials and Methods

### *4.1. Strains and growth conditions*

*Staphylococcus aureus* SA113 (WT), the isogenic *graRS* mutant (Herbert *et al.*, 2007), and the plasmid-complemented mutant (Herbert *et al.*, 2007) were inoculated in basic medium (BM; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 0.1% $K_2HPO_4$) with aliquots of overnight cultures and incubated at 37°C until logarithmic phase was reached. In the case of the complemented mutant, BM was modified by replacing glucose with 0.5% xylose to allow for expression of the plasmid-encoded *graRS* genes (Herbert *et al.*, 2007). To prepare bacteria for the mouse infection model, precultures of the staphylococcal strains which were grown for 8 h in tryptic soy broth (TSB) were diluted 1:100 into fresh TSB and incubated for 18 h without shaking.

### *4.2. Cytochrome c binding assay*

Log-phase bacteria were harvested, washed twice with potassium phosphate buffer containing 0.01% human serum albumin (KPi buffer) and bacterial density adjusted to an $OD_{600}$ of 3. Bacteria from 1.5-ml aliquots were resuspended in 500 µl cytochrome c (Sigma) solution (0.5 mg/ml) and incubated at 37°C. In order to prevent bacterial sedimentation, samples were vigorously shaken. After 15 min, samples were centrifuged and the supernatant was assayed photometrically at 410 nm.

*4.3. Lipid analyses*

Comparison of membrane lipid patterns by extraction of polar lipids and subsequent thin-layer chromatography was performed as recently described (Peschel *et al.*, 2001).

*4.4. Inactivation assay with human defence peptide LL-37*

Log-phase bacteria were harvested, washed twice with potassium phosphate buffer containing 0.01% human serum albumin (KPi buffer) and bacterial density adjusted to an $OD_{600}$ of 1.5. Samples (40 µl) with a final concentration of 20 µg/ml LL-37 were shaken at 37°C. After 20 min, 160 µl ice-cold KPi buffer was added to block further antimicrobial action and appropriate aliquots were plated on BM agar plates. After 24 h incubation at 37°C, CFU were enumerated.

*4.5. Inactivation assay with human neutrophil granulocytes*

Bacteria were grown to logarithmic phase, washed, and adjusted in KPi buffer as described above. Neutrophils were isolated from peripheral blood of healthy volunteers by ficoll/histopaque gradient centrifugation as described previously (Schmitz *et al.*, 1997) and resuspended in HBSS-HSA (HBSS containing 0.05% human serum albumin). Bacterial and neutrophil suspensions were mixed to final concentrations of $5x10^6$/ml bacteria and $2.5x10^6$/ml neutrophils. Bacteria were opsonized by addition of pooled human serum (Sigma) to a final concentration of 10%. Samples (500 µl) were shaken at 37°C. After 15, 30 and 60 min, aliquots were diluted in ice-cold water and vortexed vigorously to disrupt the neutrophils and halt bacterial killing. Appropriate dilutions were plated on BM agar plates and incubated at 37°C for 24 h for enumeration of CFU.

*4.6. Mouse infection model*

Female BALB/c mice (12 to 15 weeks old) were infected intraperitoneally with *S. aureus* WT or the *graRS* mutant. Briefly, precultures of the staphylococcal strains which were grown for 8 h in tryptic soy broth (TSB) were diluted 1:100 into fresh TSB, incubated for 18 h without shaking, washed twice in PBS, adjusted to $3x10^8$ CFU/ml in PBS and 400 µl of these suspensions were injected intraperitoneally. 72 h after infection, mice were sacrificed, one kidney was aseptically removed, weighed,

homogenized, and serially diluted in PBS for plating on Todd Hewitt agar plates. After 24 h incubation at 37°C, the numbers of CFU/kidney were determined.

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**Figures:**

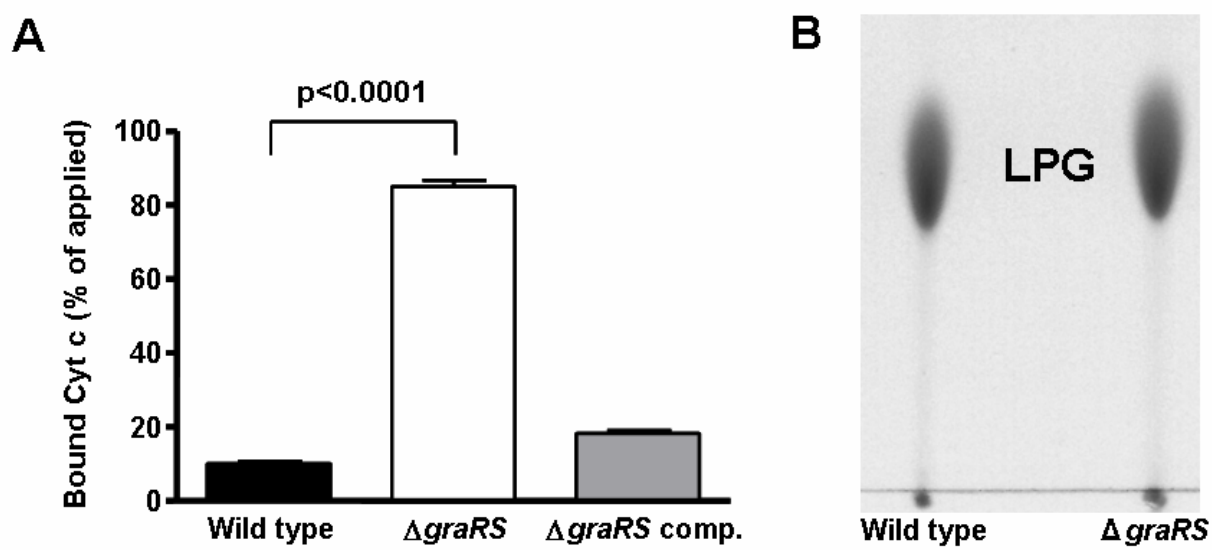


**Fig. 1.** (A) Binding of cationic cytochrome c (Cyt c) by *S. aureus* Sa113 wild type (black bar), *graRS* mutant (white bar) and complemented mutant (grey bar). The means and SEM of three independent experiments are shown. *P* values < 0.05 as calculated by Student's *t* test were regarded statistically significant. (B) TLC analysis of polar lipids. Amino group-containing lipids were visualized by ninhydrin treatment. The lipid spots corresponding to lysylphosphatidylglycerol (LPG) are indicated.

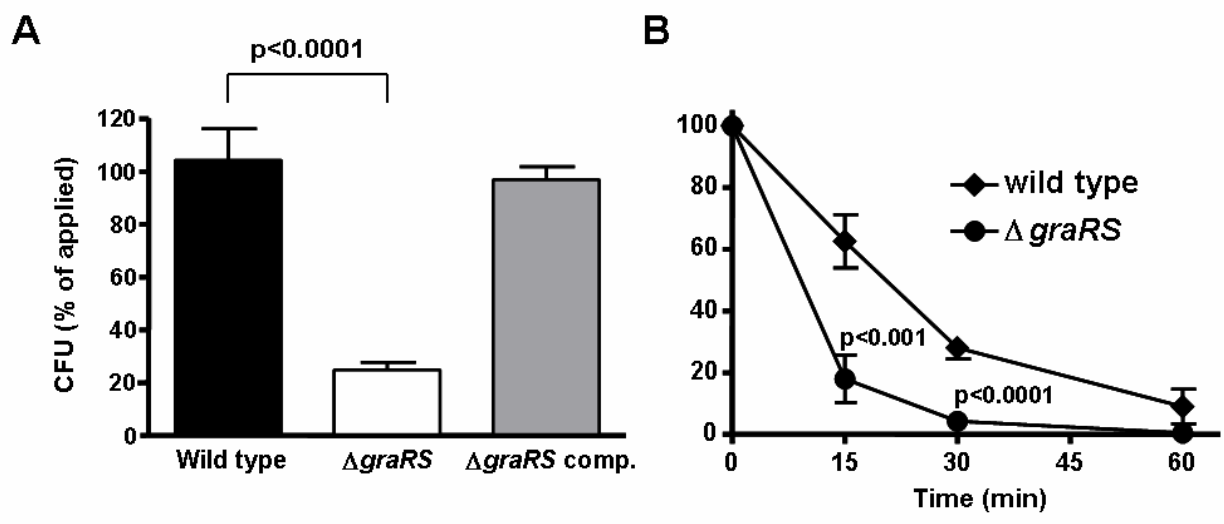


**Fig. 2.** (A) Inactivation of wild type (black bar), *graRS* mutant (white bar) and complemented mutant (grey bar) by LL-37 after 20-min incubation with 20 µg/ml LL-37. (B) Inactivation of wild-type (squares) and mutant bacteria (circles) by human neutrophils after 15, 30 and 60 minutes. The means and SEM of three independent experiments run in duplicate (A) and the means and SD of two independent experiments run in duplicate (B) are shown. *P* values < 0.05 as calculated by Student's *t* test were regarded statistically significant.

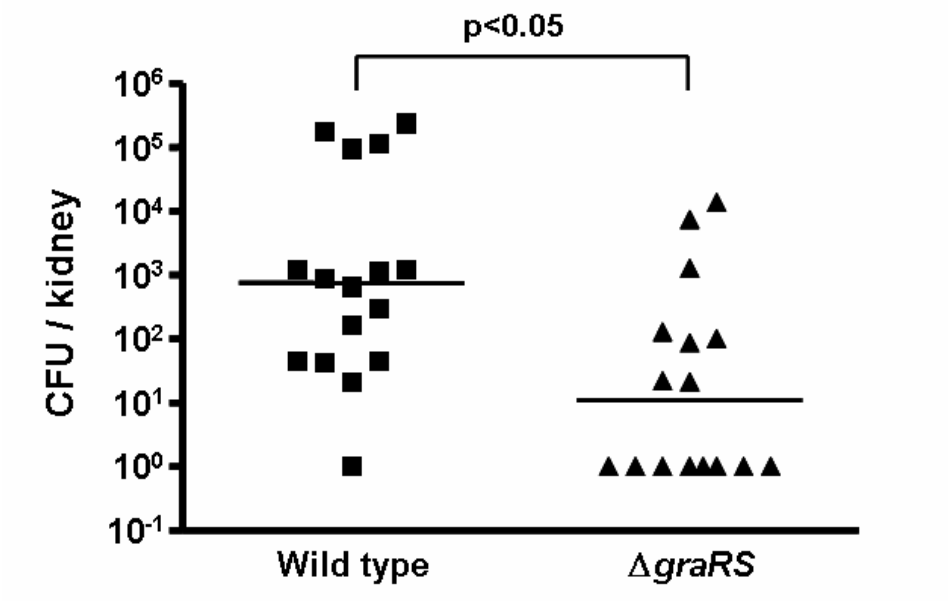


**Fig. 3.** CFU of wild-type and mutant bacteria in kidneys of mice 72 h after intraperitoneal infection with $1.2x10^8$ bacteria. For each strain 16 animals were tested. Medians are given as horizontal lines. A *P* value < 0.05 as calculated by Student's t test was regarded statistically significant.

# The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides

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## Abstract

Antimicrobial peptides (AMPs) have been shown in animal and human systems to be effective natural antibiotics. However, it is unclear how they convey protection; they often appear inactive when assayed under culture conditions applied to synthetic antibiotics. This inactivation has been associated with loss of function in physiological concentrations of NaCl or serum. In this study we show that the balance of host ionic conditions dictate microbial sensitivity to AMPs. Carbonate is identified as the critical ionic factor present in mammalian tissues that imparts the ability of AMPs such as cathelicidins and defensins to kill at physiological NaCl concentrations. After adapting to carbonate-containing solutions, global changes occur in *Staphylococcus aureus* and *Escherichia coli* structure and gene expression despite no change in growth rate. Our findings show that changes in cell wall thickness and Sigma factor B expression correspond to the increased susceptibility to the AMP LL-37. These observations provide new insight into the factors involved in enabling function of innate immune effector molecules, and suggest that discovery of new antimicrobials should specifically target pathogens as they exist in the host and not the distinctly different phenotype of bacteria grown in culture broth.

**Key Words:** leukocyte recruitment • cathelicidin • AMP activity • dermatitis

## Introduction

Antimicrobial Peptides (AMPs) are present throughout the animal and plant kingdoms, an observation that suggests these molecules have played an important role in the evolution of defense by multicellular organisms (1). The persistence of AMP activity stands in marked contrast to the human experience with design of effective pharmacologic antibiotics. In common clinical usage, the duration of effectiveness for synthetic antibiotics is very short due to the rapid evolution of various microbial strategies for resistance. Studies in animal models, however, have demonstrated that evolutionarily conserved gene-encoded antibiotics such as cathelicidins and defensins provide protection against microbial infection, while their

absence results in increased risk of infectious disease (2,3,4,5). Clinical correlations suggest this immune function extends to humans, as patients with impaired epithelial AMP production (e.g., atopic dermatitis) are highly susceptible to secondary infection, while those with increased AMP production (e.g., psoriasis) are relatively protected (6).

It has been suggested that the study of AMPs may provide insight into design of more effective antibiotic therapies (1). To date this hope has yet to translate into reality. AMPs, such as the human cathelicidin LL-37, are highly membrane active. Their ability to kill bacteria correlates with charge and reflects an ability to disrupt the organized membrane structure of the target organism. A dependence on ionic interaction is confirmed when antimicrobial activity is evaluated in solutions containing salt. The addition of NaCl to microbial culture broth at concentrations as low as 50 mM suppresses the activity of LL-37 and many other AMPs (7,8). These results led many investigators to conclude that AMPs can only kill microbes in solutions of low ionic strength or when the peptide is expressed at very high local concentrations, observations that contradict experience in several animal models.

An alternative explanation for the function of AMPs is that these peptides have evolved from simple antibiotics to act as signals of injury. Several examples now exist that show mammalian cells respond to AMPs with specific changes in gene expression. These responses include extracellular matrix synthesis (9), angiogenesis (10), leukocyte recruitment, and cytokine release (11). Surface receptors such as FPRL-1 (12) and CCR6 (13) may mediate the host cell response to AMPs. Thus, it is possible that the association between AMP expression and infection seen in animal models and human disease may be the result of their ability to augment a cellular immune defense program rather than direct microbicidal action.

This study sought to address the apparent contradictions regarding how AMPs can function as natural antibiotics. The direct bactericidal activity of AMPs was evaluated *in vitro* in an animal cell culture environment. Our findings show that when select bacteria are grown in mammalian ionic conditions, they respond with an altered gene expression profile that renders them susceptible to AMPs. These observations provide insight into the true parameters of AMP activity, and suggest a novel paradigm for evaluating the function of these peptides in innate immune defense.

## Materials and Methods

### Bacterial strains

Bacterial strains used were *Staphylococcus aureus* Rosenbach ATCC 25923, *S. aureus* N315, *S. aureus* NKSB (SigB knockout in the N315 strain), methicillin-resistant *S. aureus* (MRSA) strains 81056 and 81025, *Salmonella enterica* serovar Dublin Lane, and *Escherichia coli* O29.

### Antimicrobial assays

Bacteria were grown at 37°C in tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA) to stationary phase, diluted 1:100 into assay medium, and grown to early log phase. Assay medium contained 20% TSB, 10% fetal bovine serum (FBS), and 150 mM NaCl, unless otherwise noted, together with 70% of additional media or media components. The additional 70% volume used to complete the assay medium included minimal essential media (MEM) (Gibco, Grand Isle, NY, USA); the buffers from MEM, which are 27 mM $NaHCO_3$ and 1.1 mM $NaH_2PO_4$; 1 mM $NaH_2PO_4$ ± 50 mM $NaHCO_3$. A 50 mM glycine-HCl buffer (Fisher, Tustin, CA, USA) was also used in place of 50 mM $NaHCO_3$ in Fig. 1c due to its similar buffering capacity. All solutions were adjusted to pH 7.4, unless otherwise noted in the figure legend. Antimicrobial peptides were added to 0.1 mL bacterial suspension containing ~1 x $10^6$ CFU/mL in a 96-well flat bottom plate (Costar, Corning, NY, USA). Plates were incubated at 37°C for the indicated times. Inhibition of bacterial growth was determined by comparing the change in turbidity at $OD_{600}$ in the presence of peptide to that in the absence of peptide. Minimal inhibitory concentration (MIC) was defined as the concentration of peptide that resulted in no detectable bacterial growth. Bactericidal and bacteristatic activity was determined by plating serial dilutions of bacteria on tryptic soy agar and enumerating colony forming units after overnight incubation at 37°C. The minimal bactericidal concentration was determined as the minimum peptide concentration resulting in no surviving colonies. Statistical significance was determined using GraphPad Prism software (v. 21., GraphPad Software, San Diego, CA, USA). Means were calculated with standard deviation, then a 2-way ANOVA was applied with a Bonferroni post-test. *P* values <0.001 were considered significant.

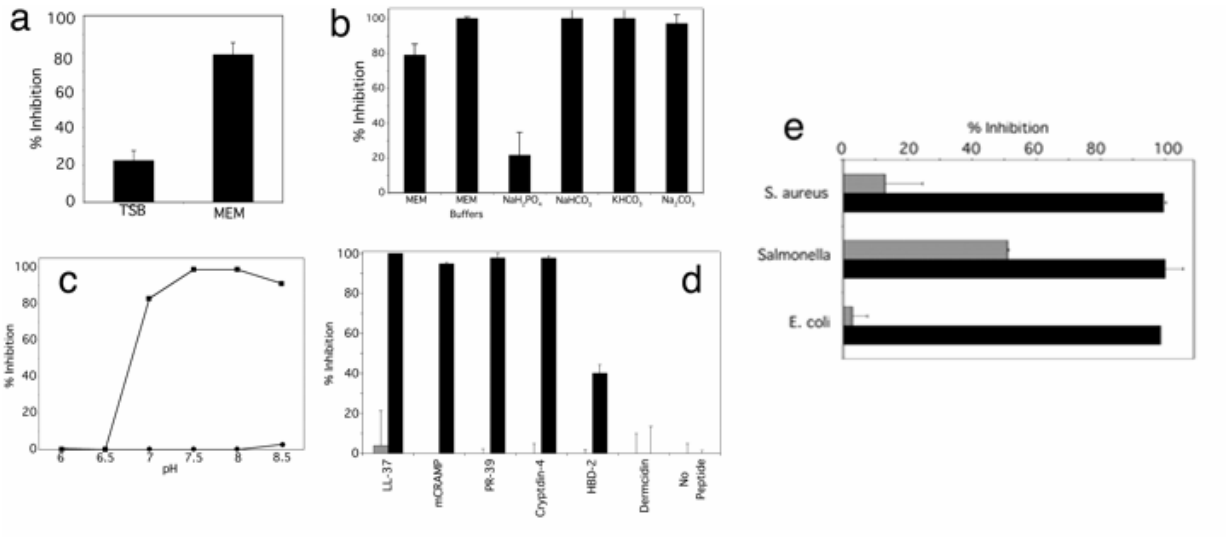


**Figure 1.** Carbonate-containing media increase the antimicrobial activity of LL-37. a) 32 μM LL-37 inhibited overnight growth of *S. aureus* Rosenbach poorly in the bacterial growth media TSB-containing 125 mM NaCl and 10% FBS, yet was effective in MEM with the same salt and serum concentrations. b) Serial deletion of MEM components revealed that $NaHCO_3$ was responsible for the enhancement of antimicrobial activity in the presence of salt and serum in MEM. The importance of carbonate was confirmed with other carbonate-containing compounds, including 50 mM $KHCO_3$ and $Na_2CO_3$, which similarly increased antimicrobial activity. pH in all media was 7.4. c) Carbonate itself, and not pH, was shown to be responsible for antimicrobial enhancement. Inhibition of *S. aureus* by 32 μM LL-37 overnight was measured at various pH in media containing 20% TSB, 10% FBS 1 mM $NaH_2PO_4$, and 150 mM NaCl, with either 50 mM $NaHCO_3$ (squares) or 50 mM Glycine-HCl buffer (circles). Only bicarbonate-containing media was able to confer increased antimicrobial activity to LL-37. d) Inhibition of *S. aureus* growth overnight was measured in the presence of 32 μM of an array of structurally diverse antimicrobial peptides. Antimicrobial activity was measured in 20% TSB, 1 mM $NaH_2PO_4$, with (black bars), or without (gray bars) 50 mM $NaHCO_3$ at pH 7.4. Antimicrobial activity of human (LL-37) and murine (mCRAMP) cathelicidins, the linear porcine (PR-39) cathelicidin, and the ß sheet murine (Cryptdin-4) and human (HBD-2) defensins, but not dermcidin, was enhanced by $NaHCO_3$. e) $NaHCO_3$ enhances antimicrobial activity against Gram negative and positive bacteria. *S. aureus* and *E. coli* were resistant to 16 μM mCRAMP without 50 mM $NaHCO_3$ (gray bars), but became susceptible in its presence (black bars) in 20% TSB, 1 mM $NaH_2PO_4$ at pH 7.4. *Salmonella* dublin's susceptibility was also increased in the presence of $NaHCO_3$.

**Reverse-transcriptase PCR and quantitative real-time PCR**

Approximately 1 x $10^8$ CFU of log phase bacteria were homogenized in 1 mL of TRIzol Reagent (Gibco, Grand Island, NY, USA) in a mini BeadBeater (Biospec Products, Bartlesville, OK, USA) with 0.1 mm glass beads on max power for 5 min and RNA was extracted per TRIzol manufacturer's directions. RT-PCR was performed on 1.5 µg RNA with Retroscript kit (Ambion, Austin, TX, USA). Briefly, first strand synthesis combined RNA with 0.4 mM dNTPs and 4 µM decamers in 16 µL at 80°C for 3 min. Reaction was iced and 2 µL 10x RT-PCR buffer, 1 µL RNase inhibitor, and 1 µL mMLV RT added followed by incubation at 42°C for 1 h, then denatured at 94°C for 10 min. Real-time (RT) quantitative PCR was performed using an Applied Biosystems 7000 Sequence Detection System (Foster City, CA, USA). Primers were designed for the target genes *sigB* and *fliA*, as well as 16 s rRNA as an endogenous control. The sequences of the *sigB* primers are: F-AAAGATGGTTCAACTGTTACGCTATTAG, R-GGTCATCTTGTTGCCCCATAA. The sequences of the *fliA* primers are: F-GCGGCATTGGGTTACTTAATG, R-CGTTCCTTGTAGGGCGTCATA. The sequences of the 16 s primers are: F-GTTATCCGGAATTATTGGGCG, R-CCGGGCTTTCACATCAGACT. 5 µL RT reaction was used in SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with 100 nM primers. Thermal profile: 50°C 2 min, 95°C 10 min, 40 x (94°C 15 s, 60°C 1 min). Results were analyzed using the Comparative Ct Method, as described (14). Product specificity was confirmed by generating dissociation curves for the reactions.

**Membrane permeability**

*E. coli* strain ML-35p, which constitutively expresses a plasmid-encoded periplasmic ß-lactamase and cytoplasmic ß-galactosidase but lacks lactose permease, was used to measure inner and outer membrane permeability. After an overnight incubation at 37°C, the culture was adjusted to 2.5–5 x $10^7$ CFU/mL ($A_{600}$=0.03) in buffer A (1 mM $NaH_2PO_4$, 20% TSB) or B (1 mM $NaH_2PO_4$, 20% TSB and 25 mM $NaHCO_3$).

To test permeabilization of the inner membrane, formation *o*-nitrophenol (ONP) as the hydrolytic product of ß-galactosidase on the colorless substrate *o*-nitrophenyl-ß-d-galactosidase (ONPG) was determined by an increase in absorbance at 420 nm ($A_{420}$). ONPG was prepared at a stock concentration of 3 mg/mL in 10 mM sodium phosphate buffer, pH 7.0 (NaPB). To study permeabilization of the outer membrane,

we followed ß-lactamase activity by measuring the decrease in $A_{390}$ due to the substrate nitrocefin and increase of $A_{486}$ due to the red product. Nitrocefin was prepared at a stock concentration of 500 µg/mL in NaPB with 0.05 % DMSO (DMSO). The assay mixture contained 43 µL of bacteria from stock cultured in buffer A or B, with final concentrations of 4 µM LL-37, 0.3 mg/mL ONPG or 25 µg/mL nitrocefin. Samples were incubated at 37°C in sterile 96-well microtiter plates (Corning Inc., Corning, NY, USA) and the kinetics of ß-galactosidase or ß-lactamase activity calculated by measurement of the $A_{420}$ or $A_{390}/A_{486}$, respectively, with a Spectra max PLUS 384 (Molecular Devices, Sunnyvale, CA, USA). To test bacterial growth, the $A_{600}$ was also monitored.

As a positive control, 2.5–5 x $10^7$ CFU/mL of *E. coli* ML-35p was permeabilized with 1 µg/mL of polymyxin B (Calbiochem, La Jolla, CA, USA), 1% Triton X-100 in buffer A or B.

Nuclear membrane permeabilization in *S. aureus* was also examined by fluorometric measurement. 74 µL of *S. aureus* cultured as above were dispensed onto each well of a sterile 96-well microtiter plate (Nunc, Roskilde, Denmark). 2 µL of 640 µM LL-37 or $H_2O$ and 4 µL of 4 µM Sytox Green (Molecular Probes, Eugene, OR, USA) in 0.08% DMSO were added to each sample and the plates were incubated at 37°C in the dark. Fluorescence emission was measured with a microplate reader (Fluoroskan Ascent FL, Labsystems, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. 1 µg/mL polymyxin B, 1% Triton X-100 was used as a positive control.

**Isolation and analysis of *S. aureus* membrane lipids**

Bacteria were grown as described above for the antibacterial assay in the presence or absence of carbonate and disrupted using glass beads and a Disintegrator S (Biomatic GmbH, Rodgau, Germany). Lipids were isolated and analyzed as described recently (15). Briefly, polar lipids were extracted by the Bligh-Dyer procedure, vacuum dried, and dissolved in chloroform/methanol (2:1, by vol). Equal amounts of lipid extracts were spotted onto silica 60 F_254 HPTLC plates (Merck, Darmstadt, Germany) and developed with chloroform/methanol/water (65:25:4, by vol) in the first direction and chloroform/acetic acid/methanol/water (80:15:12:4, by vol) in the second direction. All lipids were visualized with molybdatophosporic acid spray (Merck) followed by charring at 120°C and treatment with ammonia vapor to

improve the contrast. Phospholipids or amino group-containing lipids were selectively stained with Molybdenum Blue (Sigma) or ninhydrin spray (Merck), respectively. The lipid fatty acid composition was analyzed by gas chromatography as described recently (15).

**Electron microscopy**

Electron microscopy was performed on *S. aureus* Rosenbach grown to log phase in 20% TSB, 1 mM $NaH_2PO_4$, with or without addition of 25 mM $NaHCO_3$. 32 μM LL37 or an equal volume of water was added and the bacteria were cultured 4 h. Bacteria were pelleted at 1000 x *g* for 5 min at 4°C, fixed in Karnovsky's Solution 24 h at 4°C, and processed for EM on a Zeiss EM 10B Transmission Electron Microscope by the VA San Diego Microscopy Core Facility.

**Microarray analysis**

*E. coli* was grown, as described earlier, in the presence or absence of $NaHCO_3$ (buffer A or B) for 2 h. The samples for microarray analysis were prepared as described in Affymetrix Gene Chip Expression Analysis Procedure (Affymetrix, Santa Clara, CA, USA). Briefly, after centrifugation of bacteria, RNA extraction was carried out using RNeasy Mini kit (Qiagen, Germantown, MD, USA). Total RNA from four separate experiments was pooled for cDNA synthesis, RNA removed by hot-alkali treatment and cDNA was purified using a Qiaquick PCR Purification kit (Qiagen). cDNA was fragmented by DNase I treatment and terminally labeled using Enzo® BioArray™ Terminal Labeling Kit with Biotin-ddUTP (Affymetrix, Santa Clara, CA, USA). The target was hybridized onto probe arrays. Arrays were scanned at the UCSD-VA Gene Chip Core using an Affymetrix Genechip 3000 High Resolution Scanner.

Subsequent analysis was performed using GeneSpring software (Silicon Genetics, Redwood City, CA, USA). The raw data were normalized by housekeeping genes, spiked in controls, Total Intensity, Mean Centering, Lowess correction, Chen's Ratio Statistics, and filtered based on genes being present in at least one condition and having at least a 2-fold change.

## Results

### The presence of carbonate increases peptide antimicrobial activity

Several human AMPs, such as cathelicidins and members of the defensin family of defense peptides, have been observed to lose antimicrobial activity *in vitro* with the addition of physiological NaCl concentrations or plasma proteins (7,8,16). However, in animal model systems the expression of these gene products correlates with increased microbial resistance (4,17,18) (Lee *et al*., in press). To resolve the apparent contradiction between observations in animal systems and those measured *in vitro*, the antimicrobial activity of the human cathelicidin peptide, LL-37, against the pathogen *S. aureus* was evaluated in animal tissue culture media containing 10% serum and a physiological NaCl concentration. In contrast to the bacteriologic culture media TSB, LL-37 showed significant growth inhibition of *S. aureus* in MEM media with 10% serum (Fig. 1a).

To identify the factor in animal cell culture media responsible for increasing antimicrobial activity, the components of MEM were systematically tested. All media tested contained 20% TSB, 150 mM NaCl, and 10% FBS at pH 7.4. 20% TSB was chosen because it allowed robust bacterial growth in the absence of AMPs. The concentration of TSB did not affect the ability of LL-37 to kill *S. aureus*, as no difference in killing was observed when the assay was performed in MEM containing 0, 5, 10, or 20% TSB (data not shown). The presence of only the salts from MEM, $CaCl_2$, KCl, and $MgSO_4$ was not sufficient to enhance antimicrobial activity, nor were phenol red, amino acids or vitamins present in this medium (data not shown). Addition of the buffering components of MEM (27 mM $NaHCO_3$ and 1.1 mM $NaH_2PO_4$,) was the only treatment that showed effects similar to that of complete MEM (Fig. 1b). Further testing identified the carbonate anion as the active molecule (Fig. 1b). As a control, similar testing was performed using solutions buffered with either $NaHCO_3$ or glycine-HCl. No increase in antimicrobial activity was seen at any pH in glycine-based buffers (Fig. 1c), indicating the unique carbonate effect was not simply a reflection of pH stabilization. We also examined whether environmental $CO_2$ could diffuse into the media to potentiate killing. In the absence of carbonate, overnight growth and assay in a 5% $CO_2$ environment did not result in significantly enhanced antimicrobial activity.

**Carbonate enhances the antimicrobial activity of a structurally diverse panel of peptides and renders diverse bacteria susceptible**

To determine whether the observed carbonate-induced increase in antimicrobial activity applied to peptides other than LL-37, a structurally diverse panel of AMPs was tested (Fig. 1d). The human and murine cathelicidins LL-37 and mouse cathelin-related antimicrobial peptide (mCRAMP) are both α-helical, the porcine cathelicidin PR-39 is a proline/arginine-rich linear peptide, and murine α-defensin cryptdin-4 (19) and human ß defensin 2 (hBD-2) (20) have ß sheet structures. None of the peptides tested had significant activity in the presence of NaCl and serum without carbonate (MIC>64 μM), but in the presence of $NaHCO_3$ all showed significant inhibition of bacterial growth (i.e., LL-37 MIC=4 μM). Of the panel tested here, only the anionic human defense peptide dermcidin did not show increased antimicrobial activity in the presence of carbonate.

Next, to compare the influence of carbonate on *S. aureus* with enhanced resistance to pharmacologic antibiotics, we tested two MRSA isolates, ATCC 81025 and 81056, and found both demonstrated an increase in susceptibility to LL-37 (MIC>32 μM in the absence of carbonate, and >98% inhibition of 81056 and 100% inhibition of 81025 at 8 μM with carbonate). A similar AMP-enhancing effect was seen with two important Gram-negative human pathogens. Enteroinvasive O29 *E. coli* were completely inhibited by 16 μM mCRAMP in the presence of carbonate but were resistant to cathelicidin without it. Similarly, *Salmonella dublin* Lane was inhibited ~ 50% without carbonate, while totally inhibited in its presence (Fig. 1e).

**Carbonate acts on bacteria to render them susceptible**

It has been reported that carbonate can affect the α-helical structure of LL-37 (21). This opens the possibility that the potentiating effects of carbonate could reflect structural changes in AMPs to somehow increase their potency, although the diversity of peptides tested seemed to diminish this possibility. To test whether the effects observed were due to action on the peptide or on the bacteria, we studied the ability of LL-37 to kill *S. aureus* as a function of the time of exposure to $NaHCO_3$ (Fig. 2). Bacteria were grown to log phase in the presence or absence of $NaHCO_3$, then washed free of media and resuspended in fresh media with or without $NaHCO_3$ and with or without LL-37. *S. aureus* grown initially without $NaHCO_3$ were not inhibited by LL-37 even if $NaHCO_3$ was added at the time of LL-37 exposure. In contrast, bacteria

grown initially in the presence of $NaHCO_3$ were killed by LL-37 even if carbonate was removed prior to LL-37 addition. Separate experiments demonstrated that bacteria must grow for at least 2 h in carbonate-containing media to show increased susceptibility to LL-37 (data not shown).

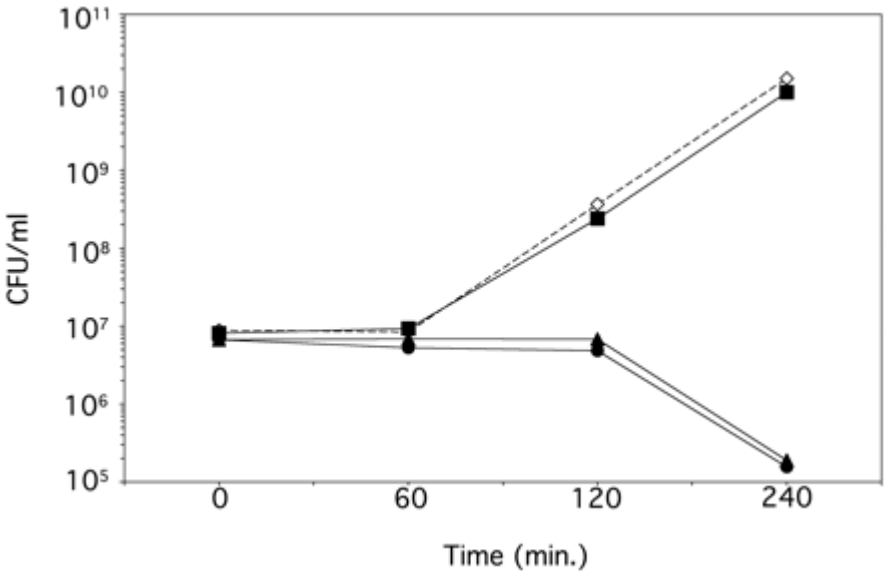


**Figure 2.** Preincubation of bacteria with $NaHCO_3$ is required for enhanced antimicrobial activity. *S. aureus* was grown to log phase in 20% TSB, 10% FBS, 1 mM $NaH_2PO_4$, 150 mM NaCl with or without 50 mM $NaHCO_3$, washed to remove $NaHCO_3$, and exposed to 12.8 µM LL-37 at time 0 h in media with or without $NaHCO_3$. Surviving bacteria were enumerated at various time points for 4 conditions. Open diamonds on a dashed line represent bacteria grown in $NaHCO_3$ before and after washing, but not exposed to LL-37. Squares represent bacteria cultured without $NaHCO_3$ both before and after washing and exposed to LL-37. Triangles represent bacteria grown in $NaHCO_3$ before and after washing and exposed to LL-37. Circles represent bacteria grown in $NaHCO_3$ before washing and assayed after washing without $NaHCO_3$ with LL-37. Exposure of the bacteria to $NaHCO_3$ is required for enhanced susceptibility, but this susceptibility persists when the $NaHCO_3$ is removed before exposure to the peptide

To determine whether altered susceptibility to AMPs correlates with changes in membrane permeability, *S. aureus* and *E. coli* membrane permeability was examined directly. *S. aureus* cultured in carbonate exhibited an increase in permeability to Sytox green in response to LL-37 (Fig. 3a). This effect was also observed in *E. coli* by measuring ß-galactosidase release in response to Polymyxin B (Fig. 3b) or LL-37

(Fig. 3c). These data suggested that differences in the bacterial cell membrane or in membrane repair and synthesis mechanisms may make them more susceptible to permeabilization by cationic compounds. One important mechanism of bacterial AMP resistance reflects modification of cell wall lipids with cationic substitutions to decrease surface negative charge (15). Examination of membrane charge by binding of cytochrome *c* (22) and direct analysis of fatty acid and lipid composition of *S. aureus* showed no significant changes in response to growth in carbonate-containing media (data not shown). However, transmission electron microscopy of *S. aureus* treated with $NaHCO_3$ did show thinning of the cell wall (Fig. 4a, c). Subsequent addition of LL-37 produced severe membrane blebbing, disintegration of the cell wall and widespread bacterial lysis (Fig. 4d).

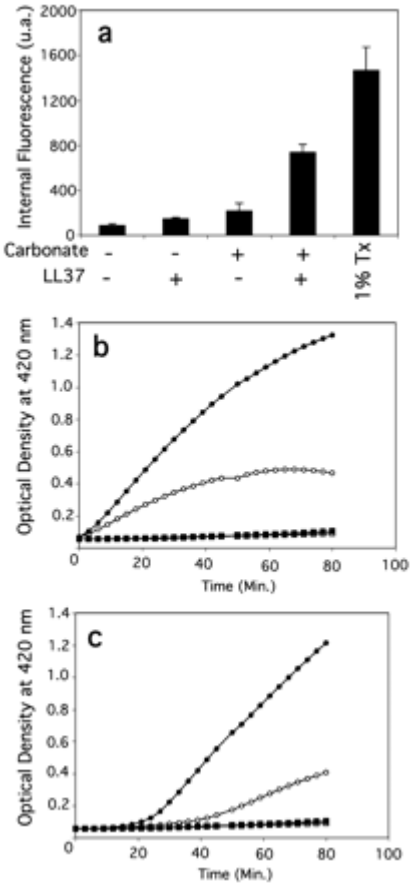


**Figure 3.** $NaHCO_3$ causes *S. aureus* and *E. coli* to become more susceptible to permeabilization. *S. aureus* or *E. coli* were grown to log phase with or without 25 mM $NaHCO_3$ and exposed to 4 μM LL-37 (a, c) or 1 μg/mL polymyxin B (b). Increases in *S. aureus* membrane permeability in response to carbonate and LL-37 was measured in panel a by uptake of the nuclear stain, Sytox Green. 1% Triton-X 100 (1%Tx) was used as a positive control. *E. coli* permeability was determined by measuring the release of ß-galactosidase activity into the media in panels b, c. The membranes of *E. coli* cultured in $NaHCO_3$ (filled symbols, b, c) were more permeable after polymyxin B or LL-37 treatment (circles, b, c). Without peptides (squares, b, c), $NaHCO_3$ did not make the *E. coli* significantly more permeable.

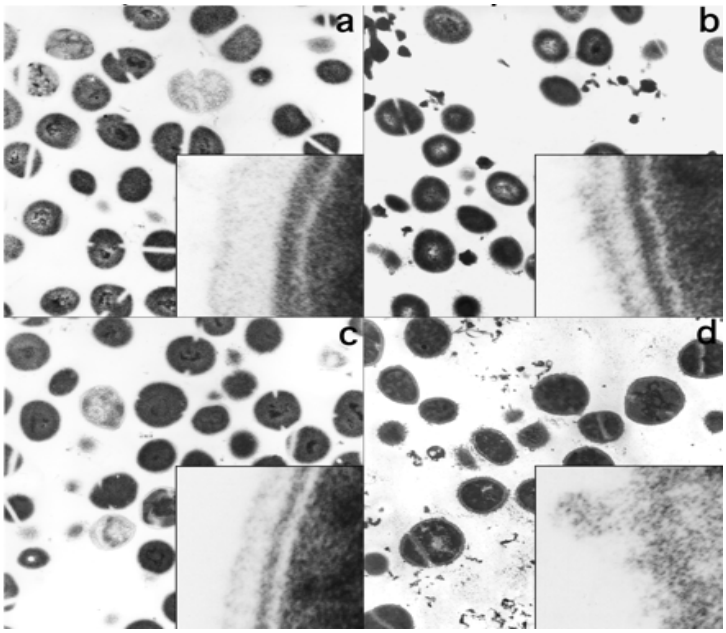


**Figure 4.** Electron microscopy reveals bacterial cell wall lysis by LL-37 and carbonate. *S. aureus* was grown for 4 h in 20% TSB, 1 mM $NaH_2PO_4$ with or without 25 mM $NaHCO_3$ or 32 μM LL-37. The presence of 25 mM $NaHCO_3$ alone (c) resulted in thinner but intact bacterial cell walls compared with untreated bacteria (a). 32 μM LL-37 caused a small amount of damage to bacterial cell walls in the absence of $NaHCO_3$ (b), but totally ablated them in its presence (d). Low magnification images are provided to verify similar cell numbers and demonstrate the general condition of the bacteria. High magnification images in the inset allow closer examination of the cell wall. Panel magnification, 33,000x. Inset magnification, 137,500x.

**Carbonate alters gene expression of key regulatory factors**

Our data demonstrated that carbonate enhancement of bacterial AMP susceptibility occurred without alterations in membrane charge, but with potential structural alterations in the cell wall. The potential effects of carbonate on bacterial genes not previously associated with AMP sensitivity was explored by global transcriptional profiling. DNA microarray analysis of *E. coli* O29 cultured in the presence or absence of $NaHCO_3$ identified changes in the expression of several candidate genes (Table 1). Notably, > 30 flagellar genes, which have been implicated in virulence in *Salmonella* and stress response (23), were decreased >2-fold by culture in carbonate in *E. coli* O29. In fact, of the 10 genes with the largest decrease in expression shown in Table 1, five were flagellar genes. A large decrease in expression was also observed for *bar*A (24), which encodes a kinase that controls various virulence factors through the global regulator OmpR (25).

**Table 1.** Culture in the presence of carbonate alters *E. coli* gene expression[a]

| Gene | Protein | Fold decrease |
|---|---|---|
| proM | tRNA-Pro | 14.8 |
| serT | tRNA-Ser | 12.7 |
| hdeA | Defense against acid stress | 11.8 |
| barA | Histidine kinase | 7.4 |
| fliD | Flagellar filament cap protein HAP2 | 7.4 |
| fliZ | Structural component | 7.3 |
| flgL | Second hook-filament junction zone | 6.6 |
| cysC | Adenylsulfate kinase | 6.2 |
| fliA | $\sigma^{28}$ | 6.1 |
| yhjH | New member of flagellar regulon | 6.0 |

| Gene | Protein | Fold increase |
|---|---|---|
| LAMCG_mRNA | | 39.5 |
| yagL | DNA binding protein | 15.7 |
| yhhH | Hypothetical protein | 9.5 |
| elaA | Acyltransferase (predicted) | 8.0 |
| b2361 | Hypothetical protein | 5.4 |
| nadB | L-aspartate oxidase | 4.5 |
| aceB | Malate synthase A | 4.4 |
| rfaJ | Lipopolysaccharide 1,2-glucosyltransferase | 4.1 |
| spy | Spheraplast protein Y (Cpx regulon member) | 3.8 |
| b3913 | Hypothetical protein | 3.7 |
| betB | Betaine aldehyde dehydrogenase | 3.6 |
| nadA | Quinolinate synthetase | 3.6 |

[a] Alterations in *E. coli* gene expression were determined in response to culture in 25 mM $NaHCO_3$ as described in Materials and Methods. These data represent the 10 genes with the largest fold increase or decrease in response to carbonate.

For confirmatory analysis we chose the *fli*A gene, which encodes a sigma factor that regulates many flagellar genes and can influence bacterial virulence and AMP sensitivity (23). Comparison of *fli*A to sigma factors in *S. aureus* revealed sequence similarities to the alternative sigma factor, *sig*B. SigB contributes to *S. aureus* virulence and is involved in a broad range of stress responses, including control of cell wall thickness and integrity (26,27). Using quantitative RT-PCR, we verified a 5-fold decrease in *E. coli fli*A expression and a 10-fold decrease in *S. aureus sig*B upon growth in carbonate-containing media (Fig. 5a, b). Thus, one important effect of carbonate upon the bacteria appears to be suppression of global regulatory genes involved in coordinating potential resistance phenotypes.

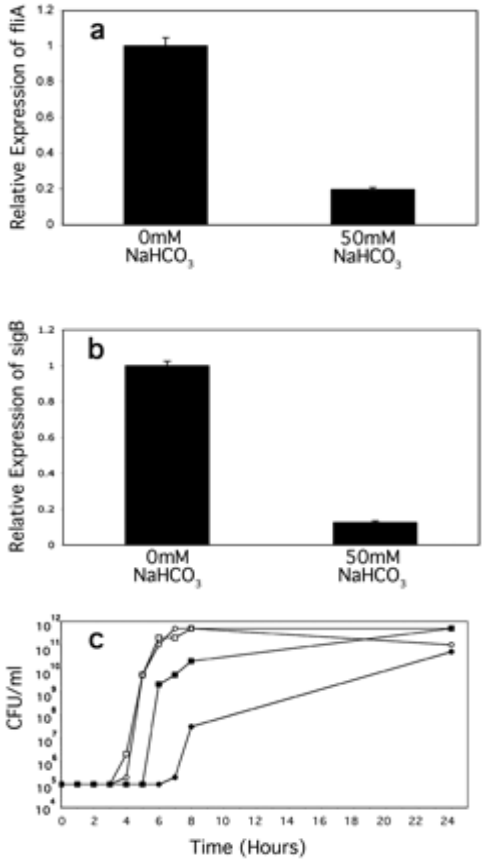


**Figure 5.** $NaHCO_3$ suppresses expression of the alternate sigma factors FliA in *E. coli* and SigB in *S. aureus*, which increases their susceptibility to AMPs. RNA was extracted from log phase *E. coli* (*a*) and *S. aureus* Rosenbach (*b*) cultured with or without 25 mM $NaHCO_3$ and expression levels determined via quantitative RT-PCR. Expression was normalized first to 16 s rRNA as an endogenous control, then to the bacteria grown without $NaHCO_3$ to determine a relative level of expression. *c) S. aureus* N315 deficient in SigB, termed NKSB (circles), was inhibited by LL-37 to a greater degree than the parental strain (squares). Addition of 16 μM LL-37 (filled symbols) to NKSB resulted in a longer and larger inhibition of growth than it did on Two-way ANOVA statistical analysis confirmed that the difference between both N315 and NKSB treated with LL-37 and their untreated controls was significant at $P < 0.001$ at 6 to 8 h. The difference between NKSB and N315 treated with LL-37 was not significant by 2-way ANOVA.

To directly test the hypothesis that a decrease in the expression of *sig*B in *S. aureus* could contribute to the increase sensitivity to LL-37 observed when cultured in carbonate, we evaluated *S. aureus* ΔsigB mutant (NKSB) for growth inhibition in response to LL-37. We found the ΔsigB mutant NKSB to be more susceptible to LL-37 during early growth periods from 2 to 8 h post-inoculum (Fig. 5c). Calculation of MIC in this period showed MIC of 16 μM for NKSB compared with wild-type parent strain MIC of >64 μM. However, unlike *S. aureus* grown in carbonate-containing buffers, the ΔsigB mutant increase in sensitivity was transient.

## Discussion

The last decade has seen important advances in the discovery of antimicrobial peptides and proteins with lytic activity against pathogens including bacteria, viruses, fungi, and even cancer cells (28). Many of these molecules are thought to be integral mediators of host barrier defense, including the cathelicidins and defensins, whose family members have been described in most human epithelia (29). AMPs are either constitutively expressed at locations where initial interaction with potential invading microbes occurs or induced upon recognition of injury. The *in vivo* significance of several of these peptides has been demonstrated in animal models, yet many of these molecules appear to be inactive, or minimally active, when antimicrobial activity is directly evaluated *in vitro* using physiologic salt concentrations or serum (7,21). This has lead to a debate as to the true relevance of these so-called "natural antibiotics" to immune defense. Here we demonstrate that bacterial susceptibility is significantly enhanced in the mammalian ionic environment in which AMPs have evolved their defense role.

Carbonate is a ubiquitous molecule in many microenvironments of the body, including blood, sweat, and gastrointestinal, urogenital, and respiratory tracts. Secretions containing carbonate at the concentrations evaluated in this study are generated from or bathe AMP-expressing tissues such as skin, gut, airway epithelia, and neutrophils (30,31,32). Our findings show that when grown in the presence of carbonate, both Gram-positive and Gram-negative bacteria may show dramatically increased AMP sensitivity. For example, the major human pathogen *S. aureus* has been described as resistant to mammalian AMPs, with an MIC to many cathelicidins and defensins of >32 μM (8). In mouse studies, the concentration of cathelicidin in wounds has been

estimated as only between 3 and 10 μM (33). Our data now show that in the presence of carbonate at physiologic concentrations *S. aureus* is suppressed by the murine cathelicidin mCRAMP at 8 μM, within the effective concentration range found *in vivo*.

The AMP-enhancing effect of carbonate is due to alterations in bacterial susceptibility. Carbonate enhances activity of a broad range of antimicrobials including polymyxin and peptides with α-helical, ß-sheet, and linear structures. Carbonate does not need to be present in the media at the same time as the AMP to produce increased bacterial susceptibility, suggesting its effects are independent of any potential changes in the structural conformation of AMPs as previously reported. Culture of *S. aureus* and *E. coli* in the presence of carbonate increased the ability of LL-37 and polymyxin B to produce membrane permeabilization, indicating the anion has a deleterious effect on the bacteria's ability to resist external stresses that threaten its membrane integrity.

Bacterial growth in the presence of carbonate appears to produce significant changes in gene expression that correspond to the enhanced AMP sensitivity phenotype. In the Gram-negative *E. coli*, no difference in growth rate was observed upon carbonate treatment, but a global alteration in gene expression was seen with more than 300 gene transcripts altered >2-fold. Among the genes with significantly decreased expression was the global regulator *fli*A, as confirmed by quantitative RT-PCR analysis. Analysis of the Gram-positive *S. aureus* response to carbonate also showed marked changes in transcript abundance, including genes involved in virulence, stress response, and cell wall maintenance. Sharing homology to *fli*A, the *S. aureus* global regulatory gene *sig*B was also significantly suppressed when the Gram-positive pathogen was grown in the presence of carbonate.

In earlier investigations, deletion of *sig*B restored methicillin susceptibility in resistant strains of *S. aureus* (34), while overexpression of *sig*B increased cell wall thickness as well as resistance to cell wall antibiotics (27). Ultrastructural analysis of *S. aureus* grown in the presence of carbonate shows a decrease in cell wall thickness that corresponded to the enhanced susceptibility to AMP mediated lysis. Since SigB mutants of *S. aureus* are known to have decreased cell wall thickness, this suggests that functional phenotypic changes observed in our study in response to carbonate may be partly linked to *sig*B. However, the deletion of *sig*B did not confer the same level of susceptibility as culture of the parental strain in carbonate-containing media.

Moreover, carbonate further enhanced the susceptibility of *sig*B-deficient bacteria to LL-37 (data not shown). This indicates that additional effects beyond suppression of *sig*B expression are involved in the AMP-enhancing properties of carbonate. Our data indicate that investigation of families of genes that are involved in the control of cell wall synthesis and repair may be fruitful in understanding microbial resistance systems and how native antimicrobial defense systems can function effectively.

The discovery that carbonate enhances microbial susceptibility to AMPs is important from many perspectives. First, it reconciles the apparent paradox described between the demonstrated *in vivo* innate immune function of AMPs and their lack of *in vitro* antimicrobial activity. The presence of carbonate in mammalian tissues may dictate alterations in bacterial gene expression and cell wall integrity that render them more susceptible to innate immune defense molecules. This phenomenon may also represent a mechanism to maintain susceptible microbial populations. AMPs are evolutionarily conserved, predicting their presence in the environment for millennia, yet bacteria have only developed incomplete resistance. The low level of carbonate in external reservoirs for microbial growth ensures that in these settings no survival advantage exists for the further development of AMP resistance.

These observations also provide an alternate paradigm for antimicrobial discovery and suggest that analysis of microbial susceptibility to compounds should be performed with organisms grown in an environment corresponding to the mammalian host for which they represent a potential pathogen. Potential new classes of effective antimicrobials may be discovered by this approach. Compounds that may have otherwise been deemed ineffective during *in vitro* screening may be effective in the animal. This approach would represent a major change in the continuing struggle for development of effective antibiotics in the setting of rapidly evolving antibiotic resistance, and would exploit the immune defense strategy evolved by natural antibiotic peptides.

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# Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity

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## Abstract

Large-conductance $Ca^{2+}$-activated $K^+$ (BK) channels are reported to be essential for NADPH oxidase-dependent microbial killing and innate immunity in leukocytes. Using human peripheral blood and mouse bone marrow neutrophils, pharmacological targeting, and BK channel gene-deficient ($BK^{-/-}$) mice, we stimulated NADPH oxidase activity with 12-*O*-tetradecanoylphorbol-13-acetate (PMA) and performed patch-clamp recordings on isolated neutrophils. Although PMA stimulated NADPH oxidase activity as assessed by $O_2^-$ and $H_2O_2$ production, our patch-clamp experiments failed to show PMA-activated BK channel currents in neutrophils. In our studies, PMA induced slowly activating currents, which were insensitive to the BK channel inhibitor iberiotoxin. Instead, the currents were blocked by $Zn^{2+}$, which indicates activation of proton channel currents. BK channels are gated by elevated intracellular $Ca^{2+}$ and membrane depolarization. We did not observe BK channel currents, even during extreme depolarization to +140 mV and after elevation of intracellular $Ca^{2+}$ by *N*-formyl-L-methionyl-L-leucyl-phenylalanine. As a control, we examined BK channel currents in cerebral and tibial artery smooth muscle cells, which showed characteristic BK channel current pharmacology. Iberiotoxin did not block killing of *Staphylococcus aureus* or *Candida albicans*. Moreover, we addressed the role of BK channels in a systemic *S. aureus* and *Yersinia enterocolitica* mouse infection model. After 3 and 5 days of infection, we found no differences in the number of bacteria in spleen and kidney between $BK^{-/-}$ and $BK^{+/+}$ mice. In conclusion, our experiments failed to identify functional BK channels in neutrophils. We therefore conclude that BK channels are not essential for innate immunity.

killing assay; reactive oxygen species; BK-deficient mice; mice infection

## Introduction

Neutrophils are the first-line cell defense of the innate immune system. Neutrophils kill microorganisms by ingesting them into phagocytic vacuoles and bombarding them with reactive oxygen species (ROS; e.g., $O_2^-$ and $H_2O_2$) and enzymatic contents of cellular granules (23). ROS generation is mediated through the activation of NADPH oxidase in the plasma membrane. NADPH oxidase transfers electrons out of the cell into the phagocytic vacuole to reduce $O_2$ to $O_2^-$. The resulting negative charge movement (44) should be compensated. Two currents have been suggested to compensate electron flux into the vacuole: an $H^+$ current through voltage-gated proton channels (16, 25, 34) and a $K^+$ current (38). $K^+$ flux activates neutrophil granule proteases, which are necessary to resist staphylococcal and candidal infections (38, 45). Ahluwalia *et al.* (1) recently proposed that the $K^+$ flux is caused by opening of big-conductance $K^+$ (BK) channels in neutrophils.

BK channels, also known as slo and maxi-$K^+$ channels, are broadly distributed among different cell types (for recent review see Refs. 21 and 32). BK channels are activated by membrane depolarization and by an increase in cytosolic $Ca^{2+}$ and can be suppressed by potent blockers: iberiotoxin (19), charybdotoxin (22), and tetraethylammonium (TEA) (48). The BK channel consists of four pore-forming α-subunits and tissue-specific modulatory β-subunits. BK channel α-subunit-knockout ($BK^{-/-}$) mice suffer from cerebral ataxia and Purkinje cell dysfunction (42), elevated blood pressure (41), progressive hearing loss (40), and erectile dysfunction (49). Immune disorders in these mice have not been reported.

Ahluwalia *et al*. (1) found that the phorbol ester phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC)-dependent NADPH oxidase activator (8), stimulated a rise in intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and BK channel activity in isolated neutrophils. PMA-activated currents were blocked by iberiotoxin, but not by $Zn^{2+}$ (1), an effective inhibitor of proton current (13). In the study of Ahluwalia *et al*., iberiotoxin did not affect ROS production but completely inhibited the killing of *Staphylococcus aureus, Serratia marcescens*, and *Candida albicans* by human neutrophils. The authors concluded that the BK channel is essential for innate immunity.

$K^+$ channels in neutrophils are not as well studied as those in neuronal or muscle cells (26) and even in other leukocytes such as T and B cells (2, 20). The most

comprehensive study of $K^+$ channels in neutrophils was published in 1990 by Krause and Welsh (29). They did not find BK channels in human neutrophils but found two separate $K^+$ currents: a voltage-dependent current and a $Ca^{2+}$-activated current. Neither current was sensitive to charybdotoxin. Subsequently, $Ca^{2+}$-activated intermediate-conductance $K^+$ channels were identified in cultured HL-60-derived neutrophils (47). ATP-sensitive $K^+$ channels, which may play a role in neutrophil migration and chemotaxis in the inflammatory response, may also be present in neutrophils (10). BK channels had been detected in macrophages but had not been described in neutrophils (15, 18).

Given the proposed importance of BK channels for innate immunity (1), we used BK channel gene-deleted ($BK^{-/-}$) mice to study the role of the BK channel in the immune system. We failed to find that PMA stimulated BK channel activity in mouse and human neutrophils. Iberiotoxin, a specific blocker of the BK channel, did not decrease ROS production and microorganism killing in human neutrophils. Survival of bacteria was not increased in mice after genetic depletion of the BK channel. Thus our results are in agreement with the data obtained by Femling *et al*. (17), who also could not confirm an essential role of BK channels in neutrophil function.

## Methods

*Mice.* All the experiments were performed with 2-to 3-mo-old male and female wild-type ($BK^{+/+}$) and $BK^{-/-}$ mice. $BK^{-/-}$ mice were generated as described elsewhere (42). $BK^{+/+}$ and $BK^{-/-}$ mice were of the hybrid SV129/C57BL6 background. Litter- or age-matched animals were randomly assigned to the experimental procedures. All animal experimental protocols were approved by the local animal care committees (Regierungspräsidium, Tübingen and LaGetSi, Berlin, Germany).

*Purification of cells.* Heparinized venous blood was obtained from healthy volunteers after written informed consent was obtained. Human neutrophils were isolated from blood as previously described (28, 43). Smooth muscle cells were enzymatically isolated from cerebral arteries from mice as previously described (41). Tibial smooth muscle cells were isolated using the same protocol. However, incubation with papain was prolonged to 45 min and isolation with collagenases to 10 min. Mouse neutrophils were isolated from bone marrow by a Ficoll-Histopaque gradient. Mice

were killed, the femur and the tibia from both hindlegs were removed and freed of soft tissue attachments, and the extreme distal tip of each extremity was removed. $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS; GIBCO) was forced through the bone with a syringe. The resulting cell suspension was passed over a 70-µm sterile nylon filter (BD Falcon) for removal of cell aggregates and other debris. The filtrate was layered over a double Ficoll-Histopaque gradient (1:1 Histopaque 1119 and 1083, Sigma) and centrifuged at 700 *g* for 30 min. The intermediate layer was collected and washed three times with HBSS. Purity of isolated bone marrow neutrophils was 70% as assessed by staining with Hemacolor (Merck). Neutrophils for further experiments were selected on the basis of their morphology.

*Mice infection.* Freshly thawed, plasmid-harboring *Yersinia enterocolitica* strain WA-314 serotype *O:8* organisms suspended in 0.1 ml of sterile PBS, pH 7.4, were used for intravenous infection as described previously (5, 7). To determine the actual number of bacteria administered, we plated serial dilutions of the inoculum on Mueller-Hinton agar and counted colony-forming units (CFUs) after 36 h of incubation at 26°C. $BK^{+/+}$ and $BK^{-/-}$ mice were killed by carbon dioxide asphyxiation at 5 days after infection with 5 x $10^3$ bacteria. The spleens were aseptically removed, and a single-cell suspension was prepared using 5 ml of PBS containing 0.1% BSA. Duplicates of 0.1 ml of serial dilutions of these preparations were plated on Mueller-Hinton agar. The limit of detectable CFUs was 25 ($\log_{10}$ = 1.4). A total of nine mice were infected per group.

$BK^{+/+}$ and $BK^{-/-}$ mice were also infected intravenously with $10^7$ *S. aureus* SA113 and killed 3 days later. The number of bacteria in the spleen was determined by serial dilution and plating on Luria broth plates.

*Electrophysiology.* Patch-clamp studies were performed on freshly isolated neutrophils and smooth muscle cells. Membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments). Data were acquired and analyzed with a CED1401 interface and CED Patch and Voltage Clamp Software (version 6.08, Cambridge Electronic Design). Cells were voltage clamped at –30, –40, or 0 mV and pulsed for 300 ms from –100 to +140 or +100 mV in 20-mV increments every 2 s. Currents were measured at the end of the pulse at +140 or +100 mV. In some

experiments, currents were recorded from a holding potential of –40 mV during linear voltage ramps at 0.5 V/s from –100 to +100 mV applied every 10 s. In experiments designed to measure proton currents, cells were voltage clamped at –60 mV and pulsed for 8 s in 20-mV increments every 20 s.

If not otherwise indicated, the experiments were performed in the perforated-patch configuration. A stock solution of 100 mg/ml amphotericin B in DMSO was prepared and diluted in the pipette solution to give a final concentration of 200 µg/ml. Stable access was obtained after 10–20 min. Cell capacitance was 3.1 ± 0.2 ($n$ = 36, range 1.7–5 pF) for human neutrophils and 2.0 ± 0.1 ($n$ = 54, range 1.5–2.5 pF) for mouse neutrophils. We did not routinely correct for series resistance. Agents were applied to the bath with a gravity-driven perfusion system. Several extracellular and pipette solutions were used. In first set of experiments, we used solutions identical to a high-$Na^+$ extracellular solution containing (in mM) 140 NaCl, 2.5 KCl, 0.5 $MgCl_2$, 1.2 $CaCl_2$, 10 HEPES, and 5 glucose (with pH adjusted to 7.4 with NaOH) and a high-$K^+$ pipette solution containing (in mM) 140 KCl, 10 NaCl, 2 $MgCl_2$, 0.7 $CaCl_2$, 1 EGTA, and 10 HEPES (with pH adjusted to pH 7.3 with KOH) to record currents. In another set of experiments, we used a high-$Na^+$ extracellular solution containing (in mM) 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (with pH adjusted to 7.4 with NaOH) and a high-$K^+$ pipette solution containing (in mM) 110 $K^+$-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (with pH adjusted to 7.2 with KOH), which is routinely used in our laboratory to record BK channel currents (37, 41).

We performed some experiments using symmetrical high-$K^+$ aspartate solutions. The bath solution contained (in mM) 110 K-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 20 glucose (with pH adjusted to 7.2 with KOH), and the pipette solution contained (in mM) 110 $K^+$-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (with pH adjusted to 7.2 with KOH). In another set of experiments, the symmetrical high-$K^+$ aspartate solutions were supplemented with $NH_4$ (14) to facilitate the measurements of proton currents. The extracellular solution contained (in mM) 80 $K^+$-aspartate, 25 $(NH_4)_2SO_4$, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 1 EGTA (with pH adjusted to 7.0 with KOH), and the pipette solution contained (in mM) 80 $K^+$-aspartate, 25 $(NH_4)_2SO_4$, 2 $MgCl_2$, 5 HEPES, and 1 EGTA (with pH adjusted to 7.0 with KOH).

Values are means ± SE. Statistical analysis was performed by one-way analysis of variance and paired *t*-test.

*Confocal imaging.* For $Ca^{2+}$ measurements, cells were loaded with 5 µM fluo 4-AM (Molecular Probes) and 0.01% pluronic acid (Calbiochem) for 30 min at 5°C in HEPES-PSS (in mM: 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with NaOH). After they were loaded, the cells were washed with HEPES-PSS three times for removal of extracellular fluo 4-AM and plated at low density on 22-mm glass coverslips, which were mounted on the stage of an inverted Nikon microscope equipped with an UltraVIEW spinning-disk confocal system (Perkin Elmer). Fluo 4-AM was excited at the 488-nm line of an argon laser, and the fluorescence was measured at >510-nm emission. $Ca^{2+}$ signals were monitored in individual cells before and after drug application. Drugs were added to the chamber as concentrated stock solutions to reach the desired final concentration. Two-dimensional fluorescence images were recorded at a rate of 2 frames/s and analyzed with the temporal mode of the UltraVIEW software (Perkin Elmer).

*Measurement of $O_2^-$ generation by ferricytochrome c reduction.* $O_2^-$ was measured using the assay of SOD-inhibitable reduction of ferricytochrome *c*, as described elsewhere (36). Neutrophils ($0.75 \times 10^6$) were preincubated with 100 nM iberiotoxin (Sigma) or 10 µM diphenylene iodonium for 15 min at 37°C and then activated with 0.025 or 1 µg/ml PMA or buffer control. Experiments were done in duplicate. Samples were incubated in 96-well plates at 37°C for up to 60 min, and the absorption of samples with and without 300 U/ml SOD was scanned repetitively at 550 nm using a Microplate Autoreader (Molecular Devices, Munich, Germany). The final ferricytochrome *c* concentration was 250 µM, and the final cell concentration was $3.75 \times 10^6$/ml.

*Measurement of cellular oxidant stress by dihydrorhodamine oxidation.* The generation of reactive oxygen radicals was additionally assessed using dihydrorhodamine-1,2,3 (DHR), as described previously (28). Briefly, prewarmed neutrophils [$1 \times 10^7$/ml HBSS with $Ca^{2+}$ and $Mg^{2+}$ ($HBSS^{++}$, Biochrom)] were loaded with 1 µM DHR for 10 min at 37°C and then incubated with 100 nM iberiotoxin for 15

min at 37°C. Cells were activated with 0.025 or 1 µg/ml PMA or buffer control at 37°C. After 45 min, the reactions were stopped by addition of ice-cold 1% BSA-PBS. Samples were analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany). Data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined, and the mean fluorescence intensity (representing the amount of generated rhodamine 123) is reported.

*Killing of C. albicans by human neutrophils.* Killing of *C. albicans* was assessed as previously described (12, 35). *C. albicans* were selected from single colonies grown on Sabouraud-agar plates, inoculated into Sabouraud broth, and grown overnight at 30°C. The microorganisms were washed twice in $HBSS^{++}$-HSA and adjusted to a density of 5 x $10^7$ cells/ml. Pooled human serum (Sigma) was added to a final concentration of 10%, and microorganisms were opsonized for 10 min at 37°C. Neutrophils were isolated from peripheral blood as described above and resuspended in $HBSS^{++}$ containing 0.05% human serum albumin (HSA) and 10% pooled human serum. Neutrophils were preincubated for 15 min with 100 nM iberiotoxin at 37°C. Opsonized microorganisms were added at a microorganism-to-neutrophil ratio of 2:1. A sample without neutrophils served as a control. The samples were shaken for 90 min at 37°C, and incubation was stopped by addition of 2 ml of ice-cold distilled water to disrupt the neutrophils. Aliquots (25 µl) were spread on Sabouraud agar plates, and colonies were counted after 24 h of incubation at 30°C. The percent killing was calculated as follows: [CFU sample (microorganisms) – CFU sample (neutrophils + microorganisms)]/CFU sample (microorganism) x 100.

*Killing of S. aureus by human neutrophils.* Neutrophils were isolated from peripheral blood of healthy volunteers as described previously (43) and resuspended in HBSS containing 0.05% HSA. To prepare bacteria, basic medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 0.1% $K_2HPO_4$) was inoculated with 1:100 dilution of an overnight culture and shaken at 37°C until midlogarithmic phase. The bacteria were washed twice in 10 mM potassium phosphate ($KP_i$) buffer (pH 7.0) containing 0.01% HSA and adjusted to 5 x $10^7$ bacteria/ml. Bacterial and neutrophil suspensions were mixed to final concentrations of 5 x $10^6$/ml and 2.5 x $10^6$/ml, respectively. Bacteria were opsonized by addition of pooled human serum (Sigma) to a final concentration of 10%. Samples (500 µl) with 100 nM iberiotoxin and without

iberiotoxin were shaken at 37°C. Incubation was stopped by dilution of aliquots in ice-cold, distilled water. The neutrophils were disrupted by vigorous vortexing. Appropriate sample volumes were plated on basic medium agar plates, and colonies were counted after 24 h of incubation at 37°C.

## Results

*Membrane currents in human and mouse neutrophils are not inhibited by iberiotoxin.* Iberiotoxin is a potent and specific blocker of the BK channel (19). We did not observe iberiotoxin-sensitive currents in human blood and mouse bone marrow neutrophils (Fig. 1*A*), even after stimulation with PMA. We used the amphotericin B perforated-patch mode of the patch-clamp technique to measure currents in isolated neutrophils. We used the same solutions and protocols routinely used for BK channel current measurements (37, 41) and the solutions and protocols used by Ahluwalia *et al*. (1) but failed to find iberiotoxin-sensitive currents in neutrophils. Representative currents measured in cells clamped at –30 mV and depolarized for 300 ms from –100 to +140 mV in 20-mV increments in the absence and presence of 1 μg/ml PMA, in the presence of 1 μg/ml PMA +100 nM iberiotoxin, and in the presence of 1 μg/ml PMA +3 mM $ZnCl_2$ are shown in Fig. 1*A*. Depolarization-evoked current amplitudes increased on stimulation with PMA and were not inhibited by iberiotoxin (Fig. 1*B*) but were blocked by $Zn^{2+}$, which abolishes proton currents (17). The BK channel can be also blocked by 1 mM TEA (30, 48). TEA, at 1 mM, had no effect on depolarization-evoked currents in human neutrophils ($n$ = 6 cells; data not shown).

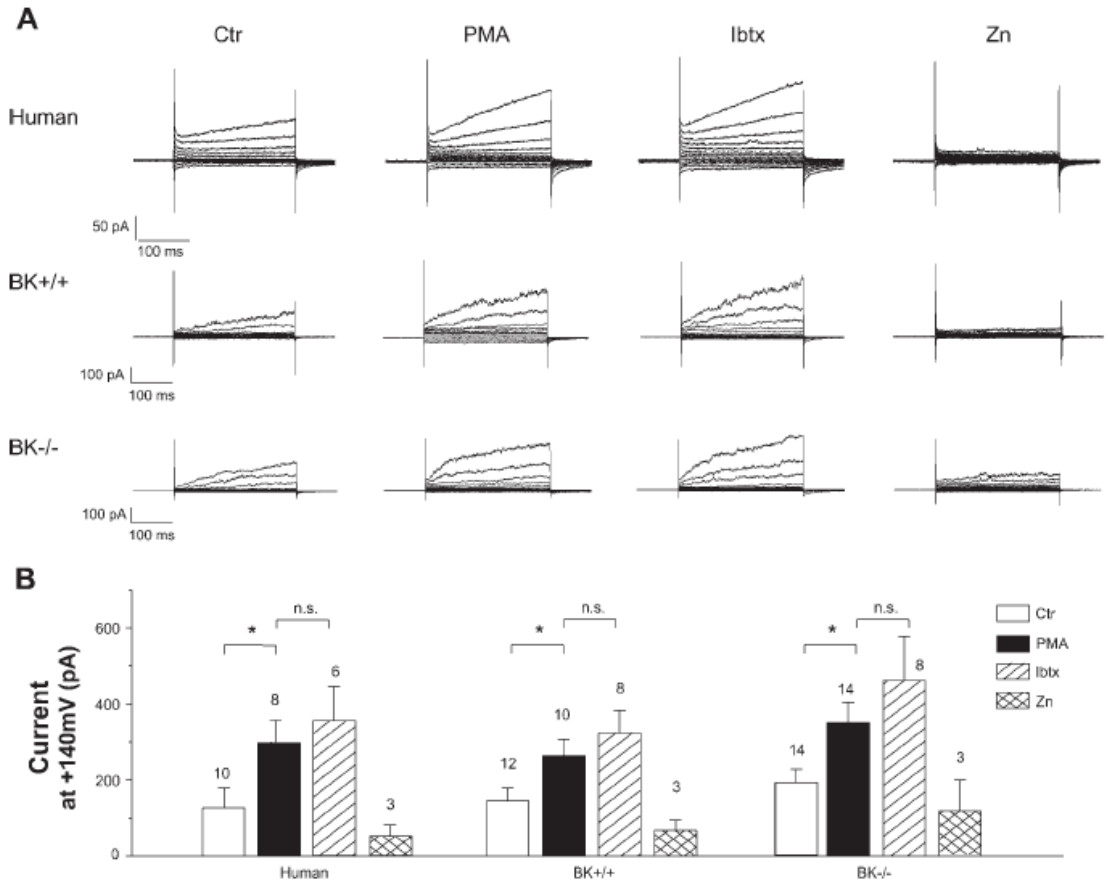


**Fig. 1.** Big-conductance $Ca^{2+}$-activated $K^+$ (BK) channel activity is absent in human and mouse neutrophils. *A*: representative currents in human and mouse $BK^{+/+}$ and $BK^{-/-}$ neutrophils recorded in the absence (Ctr) and presence of 1 μg/ml phorbol 12-myristate 13-acetate (PMA, 5–7 min after application), in the presence of 1 μg/ml PMA +100 nM iberiotoxin (Ibtx, 10–15 min after application), and in the presence of 1 μg/ml PMA +3 mM $ZnCl_2$ (Zn, 1–3 min after application). Iberiotoxin did not inhibit PMA-induced currents, indicating that BK channels are not present. Effects of PMA and $Zn^{2+}$ were significant, since only small currents were induced by "sham bath change" within 25 min. Amphotericin-perforated cells were voltage clamped at a holding potential of –30 mV and pulsed for 300 ms from –100 to +140 mV in 20-mV increments every 2 s. High-$Na^+$ external solution and high-$K^+$ internal solution were used (1). *B*: mean current amplitudes recorded in human and $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils in response to step depolarization to +140 mV in the absence and presence of 1 μg/ml PMA, in the presence of 1 μg/ml PMA +100 nM iberiotoxin, and in the presence of 1 μg/ml PMA +3 mM $ZnCl_2$. Numbers of cells are indicated above bars. *$P < 0.05$. ns, Not significant.

Next, using symmetrical high-$K^+$ aspartate solutions, we attempted to find BK channel currents. Representative currents measured in mouse neutrophils clamped at 0 mV and depolarized for 300 ms from –100 to +100 mV in 20-mV increments in the absence and presence of 1 μg/ml PMA (5–6 min) and in the presence of 1 μg/ml PMA +100 nM iberiotoxin (10–12 min) are shown in Fig. 2*A*. Depolarization-evoked current amplitudes tended to increase on stimulation with PMA and were not inhibited by iberiotoxin (Fig. 2*B*). External application of 1 mM $Ba^{2+}$ blocked the amplitude of the inward component of the current significantly but did not affect the outward component ($n$ = 3 cells each for $BK^{+/+}$ and $BK^{-/-}$ mice; data not shown), indicating the presence of inwardly rectifying $K^+$ currents previously reported in newt blood neutrophils (27).

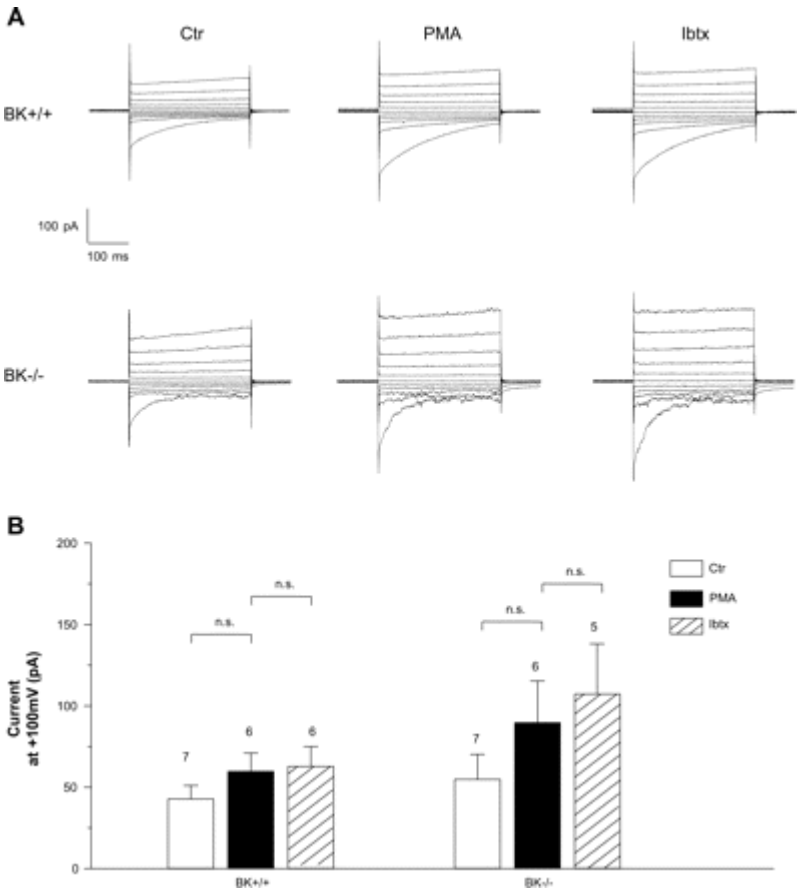


**Fig. 2.** Currents in symmetrical high-$K^+$ aspartate solutions were not sensitive to iberiotoxin. *A*: representative currents in $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils recorded in the absence and presence of 1 μg/ml PMA (5–7 min after stimulation) and in the presence of 1 μg/ml PMA +100 nM iberiotoxin (10–15 min after application). Amphotericin-perforated cells were voltage clamped at a holding potential of 0 mV and pulsed for 300 ms from –100 to +100 mV in 20-mV increments every 2 s. Symmetrical high-$K^+$ aspartate solutions were used in bath and pipette. *B*: mean current amplitudes recorded in $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils in response to step depolarization to +100 mV in the absence and presence of 1 μg/ml PMA and in the presence of 1 μg/ml PMA +100 nM iberiotoxin. Numbers of cells are indicated above bars.

The effects of PMA, a well-known PKC activator (39), on BK channel activity are not clear: inhibition (46) and activation have been reported (6). Ahluwalia *et al*. (1) found that PMA increased $[Ca^{2+}]_i$ in human neutrophils and, therefore, activated BK channels that are $Ca^{2+}$ sensitive. We also found that PMA induced a $Ca^{2+}$ increase in human neutrophils (Fig. 3*A*). Changes in $[Ca^{2+}]_i$ were measured in fluo 4-AM-loaded neutrophils using Nipkow disk confocal microscopy. The time course of changes in fluo-4 fluorescence intensity evoked by 1 μM PMA averaged from five cells in a representative experiment is shown in Fig. 3*A*. The relative fluorescence ($F/F_0$) increase in the presence of PMA was <20% but was, nonetheless, statistically significant. To achieve a more significant increase in $[Ca^{2+}]_i$, we used *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP). $[Ca^{2+}]_i$ in neutrophils was increased much more effectively by 1 μM fMLP than by PMA at the same concentration. Despite a significant increase in $[Ca^{2+}]_i$, fMLP failed to induce appropriate BK channel activity in human neutrophils (Fig. 3*B*).

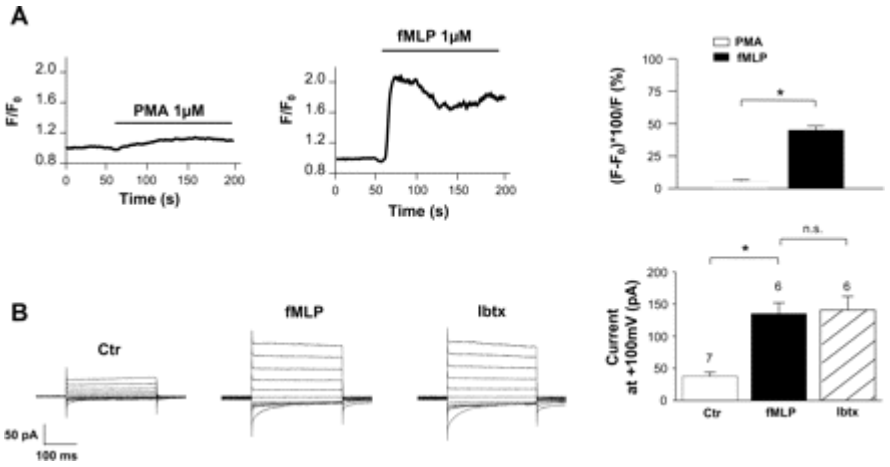


**Fig. 3.** *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) elevates cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) but fails to stimulate BK channels in human neutrophils. *A*: $[Ca^{2+}]_i$ changes induced by 1 µM PMA and 1 µM fMLP measured using Nipkow spinning-disk confocal microscopy in human neutrophils loaded with the $Ca^{2+}$ indicator dye fluo 4-AM. Time course of mean fluorescence intensity changes was averaged for 5 cells each. Increases in global $[Ca^{2+}]_i$ in human neutrophils induced by 1 µM PMA and 1 µM fMLP are expressed as percentage of fluorescence intensity changes: $(F - F_0) * 100/F_0$, where F is the average intensity over the whole area of the cell in the presence of substance and $F_0$ is fluorescence before substance application. Values are means ± SE of ≥100 cells for each substance. *B*: representative currents recorded in symmetrical high-$K^+$ aspartate solutions in a human neutrophil in the absence of fMLP, in the presence of 1 µM fMLP (5–7 min after stimulation), and in the presence of 1 µM fMLP +100 nM iberiotoxin (10–15 min after application). Amphotericin-perforated cell was voltage clamped at a holding potential of 0 mV and pulsed for 300 ms from –100 to +100 mV in 20-mV increments. Mean current amplitudes were recorded in human neutrophils in response to step depolarization to +100 mV in the absence and presence of 1 µM fMLP and in the presence of 1 µM fMLP +100 nM iberiotoxin. Numbers of cells in each experimental condition are indicated above bars. *$P < 0.05$.

As a control, we measured BK currents in smooth muscle cells isolated from mouse tibial and cerebral arteries (Fig. 4). In contrast to the neutrophils, in arterial smooth muscle cells, depolarization-evoked currents were effectively blocked by 100 nM iberiotoxin. BK channel activity in $BK^{+/+}$ cerebral smooth muscle cells was abolished by iberiotoxin (Fig. 4*A*). In contrast, BK channel currents were absent in $BK^{-/-}$ cells.

BK channel currents in $BK^{+/+}$ tibial smooth muscle cells were effectively blocked by iberiotoxin (Fig. 4*B*). In tibial and cerebral smooth muscle cells, iberiotoxin also blocked spontaneous transient outward currents, which represent coordinated openings of a cluster of BK channels caused by $Ca^{2+}$ sparks (37, 41).

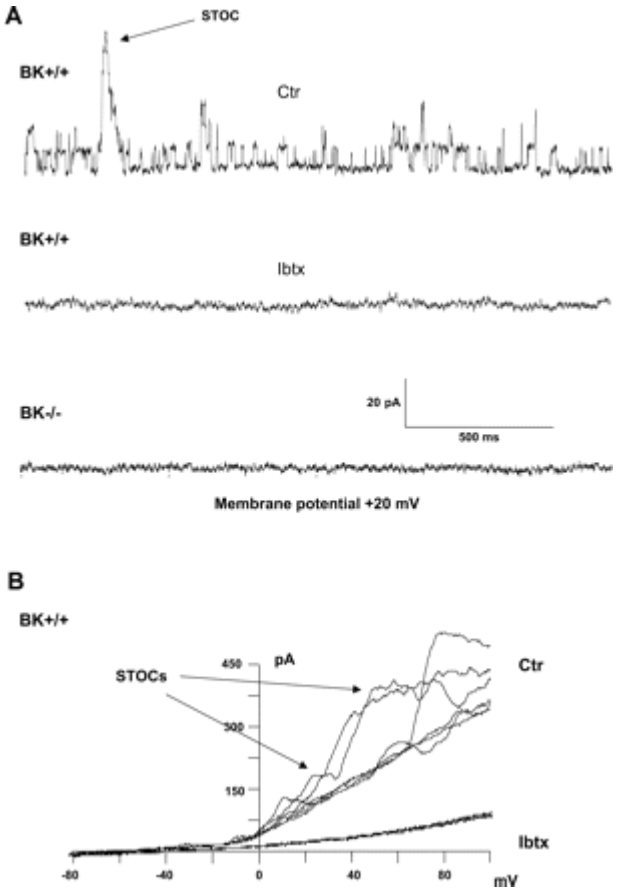


**Fig. 4.** BK channel activity in arterial smooth muscle cells. *A*: BK channel activity in a smooth muscle cell isolated from a cerebral artery of a $BK^{+/+}$ mouse (*top trace*) was blocked by 100 nM iberiotoxin (10 min after application, *middle trace*). BK channel activity was absent in a smooth muscle cell isolated from a cerebral artery of a $BK^{-/-}$ mouse (*bottom trace*). Amphotericin-perforated cells were voltage clamped at a holding potential of +20 mV. High-$Na^+$ external and high-$K^+$ internal pipette solutions were used (41). Data were filtered at 1 kHz and sampled at 2 kHz.

Arrow indicates coordinated openings of several BK channels, which are known as spontaneous transient outward current (STOC) (37). Similar results were obtained in 3 other $BK^{+/+}$ and 3 other $BK^{-/-}$ cerebral smooth muscle cells (see Ref. 41 for our previous recordings). *B*: 100 nM iberiotoxin blocked STOCs and whole cell $K^+$ current in a smooth muscle cell isolated from the tibial artery of a $BK^{+/+}$ mouse. Amphotericin-perforated cell was voltage clamped at –40 mV, and linear voltage ramps at 0.5 V/s from –100 to +100 mV were applied every 10 s. High-$Na^+$ external and high-$K^+$ internal pipette solutions were used (41). The 6 superimposed records are shown in the absence and presence of 100 nM iberiotoxin (10 min after application). Similar results were obtained in 3 other cells.

As another control, proton-like currents were recorded in human and mouse neutrophils. Symmetrical high-$K^+$ aspartate solutions were supplemented with $NH_4$ (14). Representative current traces are shown in Fig. 5. Amphotericin-perforated cells were voltage clamped at a holding potential of –60 mV and pulsed for 8 s from –60 to +60 mV in 20-mV increments every 20 s. PMA-stimulated currents were not sensitive to iberiotoxin. However, the currents were almost completely blocked by $Zn^{2+}$, which is consistent with previously reported results (17).

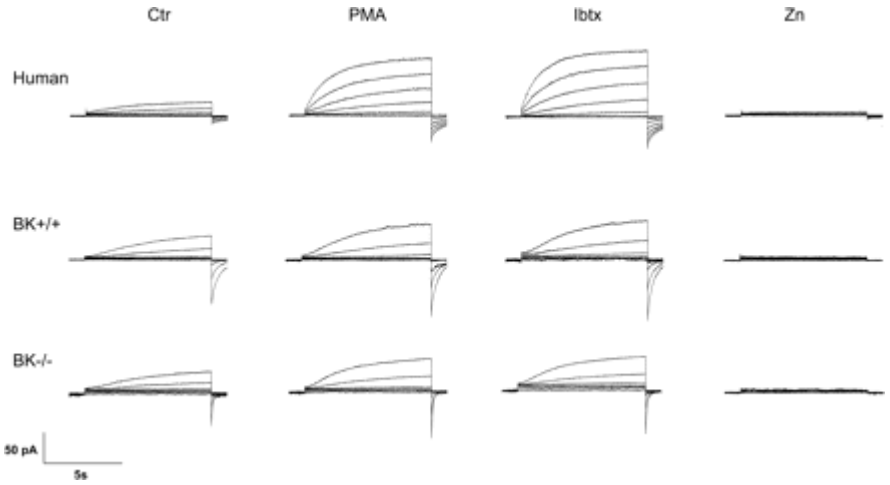


**Fig. 5.** Proton-like currents in human and mouse neutrophils were not sensitive to iberiotoxin but were blocked by $Zn^{2+}$. Currents in human and $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils were recorded in the absence and presence of 1 μg/ml PMA (5–7 min after stimulation), in the presence of 1 μg/ml PMA +100 nM iberiotoxin (10–15 min after application), and in the presence of 1 μg/ml PMA +3 mM $ZnCl_2$ (1–3 min after application). Amphotericin-perforated cells were voltage clamped at a holding potential of –60 mV and pulsed for 8 s from –60 to +60 mV in 20-mV increments every 20 s. Recordings were performed in symmetrical high-$K^+$ aspartate solutions supplemented with $NH_4$ (14). PMA-stimulated currents were not sensitive to iberiotoxin. However, they were blocked by $Zn^{2+}$. Similar results were obtained in 4 other human and 3 other $BK^{+/+}$ and 3 other $BK^{-/-}$ mouse neutrophils.

*Iberiotoxin does not inhibit killing of human neutrophils.* Ahluwalia *et al*. (1) reported that iberiotoxin is able to inhibit neutrophil killing. We tested the effect of iberiotoxin on the ability of human neutrophils to kill *S. aureus* and *C. albicans* (Fig. 6, *A* and *B*). Survival of *S. aureus* at the end of 15, 30, and 60 min of incubation with neutrophils at 37°C compared with the initial number of bacteria is shown in Fig. 6*A*. The ratio of bacteria to neutrophils was taken as 2:1. Almost all bacteria were killed at 60 min. Bacterial survival was not significantly changed by 100 nM iberiotoxin at 15, 30, or 60 min. The ability of neutrophils to kill *C. albicans* at the end of 90 min of incubation with neutrophils at 37°C is shown in Fig. 6*B*. Opsonized microorganisms were added at a microorganism-to-neutrophil ratio of 2:1. The ~40% of *C. albicans* killed in control was not decreased by 100 nM iberiotoxin. Therefore, our data indicate that iberiotoxin does not inhibit killing of *S. aureus* and *C. albicans* by neutrophils, in contrast to the data presented by Ahluwalia *et al*. (1) but in agreement with other reports (11, 17).

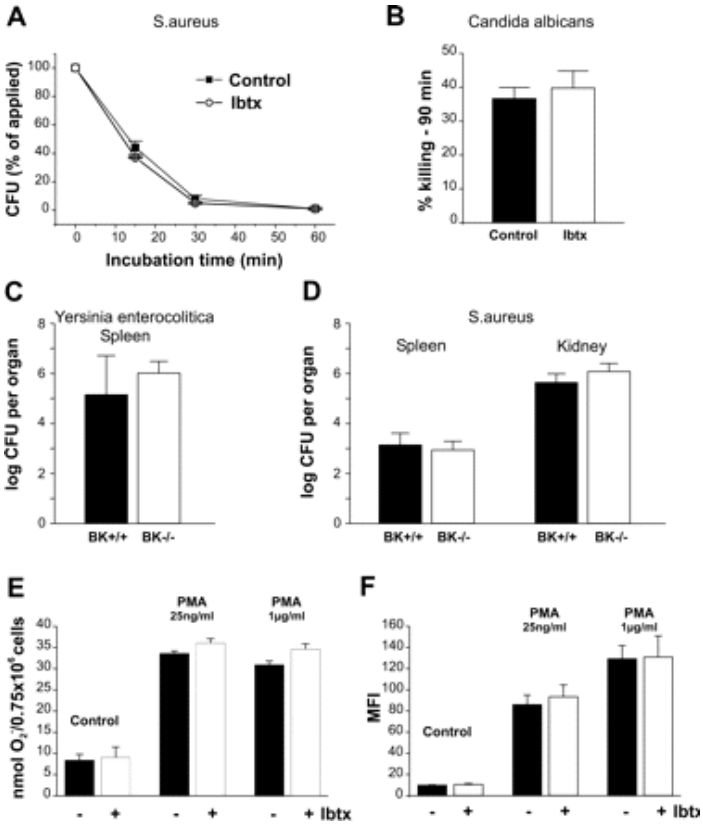


**Fig. 6.** BK channel inhibition does not influence killing activity of neutrophils and reactive oxygen species production. *A*: effect of BK channel inhibition on killing of opsonized *Staphylococcus aureus* by human neutrophils. Number of viable *S. aureus* colony-forming units (CFUs) after incubation with human neutrophils is expressed as percentage of initial count. Samples were incubated in the absence and presence of 100 nM iberiotoxin. Values are means ± SE of 2 independent experiments run in duplicate. BK channel inhibitor iberiotoxin had no effect on killing of *S. aureus* by human neutrophils. *B*: effect of BK channel inhibition on the killing of *C. albicans* by human neutrophils. BK channel inhibitor iberiotoxin had no effect on killing of *C. albicans* by human neutrophils. *C*: BK channel knockout does not reduce resistance to *Yersinia* infection in mice. Number of bacteria were counted in spleens isolated 5 days after $BK^{+/+}$ and $BK^{-/-}$ mice were infected with *Yersinia*. Values are means ± SE for 9 mice. BK channel is not essential for *Yersinia* killing. *D*: BK channel knockout does not reduce resistance to *S. aureus* infection in mice. Number of bacteria were counted in spleens and kidneys isolated 3 days after $BK^{+/+}$ and $BK^{-/-}$ mice were infected with *S. aureus*. Values are means ± SE from 10 mice. BK channel is not essential for *S. aureus* killing. *E*: effect of BK channel inhibition on $O_2^-$ generation. Data are from samples incubated for 45 min. Values are means ± SE ($n$ = 7). BK channel inhibitor iberiotoxin had no effect on respiratory burst activity of PMA-treated neutrophils. *F*: effect of BK channel inhibition on oxidant generation of PMA-treated neutrophils. Oxidants were assessed using dihydrorhodamine (DHR) oxidation assay. Values are means ± SE ($n$ = 6). Independent assay to estimate respiratory burst activity indicates that BK channel inhibitor iberiotoxin had no effect on respiratory burst of PMA-stimulated neutrophils. MFI, mean fluorescence units (i.e., amount of rhodamine-1,2,3 generated).

*Iberiotoxin does not inhibit ROS production in human neutrophils.* Effective killing of *S. aureus* and *C. albicans* requires NADPH oxidase activity and generation of ROS (4, 24). We tested the effect of iberiotoxin on generation of ROS in human neutrophils with two independent assays. $O_2^-$ was measured using the assay of SOD-inhibitable reduction of ferricytochrome *c* (Fig. 6*E*). At 25 ng/ml–1 μg/ml, PMA, a known NADPH oxidase activator (8), stimulated $O_2^-$ production. Iberiotoxin (100 nM) did not block $O_2^-$ production in neutrophils in the absence or presence of PMA. Similar results were obtained with the DHR oxidation assay. The generation of reactive oxidants was stimulated by 25 ng/ml and 1 μg/ml PMA and was not inhibited by 100 nM iberiotoxin (Fig. 6*F*). In contrast to iberiotoxin, diphenylene iodonium, a classical inhibitor of NADPH-dependent ROS production (9), strongly reduced the oxidant generation in PMA-stimulated cells (*n* = 6; data not shown). Therefore, our data show that ROS production was not inhibited by iberiotoxin. The data are in agreement with the absence of an inhibitory effect of iberiotoxin on killing activity of human neutrophils and confirm data obtained in previous studies (1, 17).

*BK channel knockout does not reduce resistance to S. aureus and Yersinia infection in mice.* If BK channels are essential for innate immunity, a reasonable expectation would be that $BK^{-/-}$ mice are less resistant to infections than $BK^{+/+}$ mice. We performed experiments with *S. aureus*- and *Yersinia*-infected mice to explore this possibility (Fig. 6*C*). $BK^{+/+}$ and $BK^{-/-}$ mice were intravenously infected with *S. aureus* or *Y. enterocolitica*. Three days after infection with *S. aureus* and 5 days after infection with *Yersinia*, the mice were killed, and the number of viable bacteria recovered from spleen and kidney was determined. The number of viable *Yersinia* and *S. aureus* was not increased by the absence of BK channels in mice (Fig. 6, *C* and *D*). Thus the data do not support the idea that BK channels are essential for innate immunity and, thus, for protection against bacterial infections.

## Discussion

We did not find that PMA stimulated BK channel activity in human or mouse neutrophils. For a positive control, we recorded iberiotoxin-sensitive BK channel currents in arterial smooth muscle cells, where their existence is well established (for recent review see Ref. 31). Electrophysiological measurements were done using the protocol and solutions routinely used in our laboratory (37, 41) and the solutions and protocol used by Ahluwalia *et al*. (1). They found that PMA significantly increases intracellular $Ca^{2+}$ in neutrophils and activates $Ca^{2+}$-dependent $K^+$ currents blocked by iberiotoxin. In contrast to iberiotoxin, PMA is not a classical pharmacological tool to study BK channels. The action of PMA is variable, ranging from channel activation (6) to inhibition (46), depending on the cell type. The reported effects were not associated with a rise in $[Ca^{2+}]_i$ but, rather, with PKC-dependent protein phosphorylation. For example, in pulmonary arterial smooth muscle cells, PMA stimulated PKC and, thereby, activated BK channels via cGMP-dependent protein kinase (6), which directly phosphorylates the pore-forming channel $\alpha$-subunits (3). Also, the increase in $[Ca^{2+}]_i$ in neutrophils is not the commonly observed effect of PMA (33). We detected the PMA-induced increase in neutrophil $[Ca^{2+}]_i$; however, the response we observed was much more modest than that reported by Ahluwalia *et al*. On the other hand, a similar concentration of fMLP increased $[Ca^{2+}]_i$ significantly but failed to stimulate BK channel activity.

Although BK channel activity was not detected in neutrophils in our study and in the report by Femling *et al*. (17), these cells successfully kill *S. aureus* and *C. albicans* (17) (Fig. 6, *A* and *B*). Iberiotoxin, a specific blocker of the BK channel, did not decrease the ability of neutrophils to eliminate microorganisms. The killing assays were performed in two independent laboratories (Berlin and Tübingen) using different experimental methods and conditions. Although Femling *et al*. (17) also obtained their results in two separate laboratories, four independent sources report no essential role for BK channels in the neutrophil killing function. Our experiments with $BK^{-/-}$ mice also do not support the idea that BK channels are essential for innate immunity. $BK^{-/-}$ mice were not less resistant to *S. aureus* and *Yersinia* infection than their $BK^{+/+}$ littermates. The notion that neutrophils function via BK channel activity should be revised.

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# Discussion

*Staphylococcus aureus* is a leading cause of nosocomial infections. According to the German national nosocomial infections surveillance system (KISS), 18% of pneumonia, 18% of catheter-associated sepses and 31% of wound infections in intensive care units in German hospitals are due to infection by *S. aureus*.
Therapy of these infections has become more and more difficult, and in several cases almost impossible due to the high resistance of many strains to most available antibiotics. According to a study by the Paul Ehrlich Gesellschaft in 2004, 22.6% of isolated staphylococci were resistant to oxacillin, which is an indicator for the detection of MRSA (methicillin resistant *S. aureus*).
*S. aureus* colonizes the anterior nares of 10-20% of the population permanently, 30-50% are colonized intermittently and the rest of the population never gets colonized (20,85). Carrier status is a big problem in health care institutions, in which colonized health care workers can transmit the pathogen to immunosuppressed patients (57,83). But not only immunosuppressed people can develop an infection by *S. aureus*. During the last years, several cases of community-acquired MRSA infections have been reported, describing severe and even lethal infections by highly virulent strains of *S. aureus* in immunocompetent people (31).
The progressive development of resistance to antibiotics and the occurrence of community-acquired MRSA has attracted much interest to *S. aureus* and a better understanding of the underlying mechanisms that enable this pathogen to cause infections and overcome a broad spectrum of human defense mechanisms.

The cell envelope of *S. aureus* plays a key role in virulence and resistance to antimicrobial host defenses (36). Cell envelope components such as the peptidoglycan (PG) and parts of it, the muropeptides, are recognized by receptors of the innate immune system including toll-like receptors (TLRs) and intracellular NODs (2,12,100). These pathogen-associated molecular patterns (PAMPs) are highly conserved and to a great extent invariable. However, there are some modifications described in bacteria that lead to a decreased stimulation of the host´s immune response to the pathogen.

The minimal structure recognized by NOD1 is a dipeptide consisting of D-glutamate (D-Glu) and meso-diaminopimelic acid (meso-DAP) (19,42). This motive is usually found in the PG of Gram-negatives but is also present in *Bacillus* species. It has been shown that modifications of NOD1 activating structures can lead to decreased proinflammatory capacity. Amidation of D-Glu partially inhibits NOD1 stimulation (19), and amidation of meso-DAP totally abrogates stimulatory properties (44).

An essential component by which *S. aureus* is recognized by the immune system is the muramyldipeptide (MDP), which consists of muramic acid, L-alanine and D-glutamate. This motive is common for both Gram-negatives and Gram-positives. It is recognized by NOD2 (43,60). *S. aureus* is known to modify most of its muropeptides by amidation of D-Glu leading to the formation of iso-glutamine (iso-Gln) (46). This amidation has a profound impact on the physicochemical properties of the molecule, since it removes the negative charge from the D-Glu α-carboxyl group. Accordingly, the net charge of MDP changes from -2 to -1 at physiological pH.

In this work we investigated the impact of muropeptide amidation on the proinflammatory capacity of *S. aureus*.

The *femC* mutant shows 48% reduced muropeptide amidation due to an insertion in the glutamine synthetase regulator. This insertion leads to decreased availability of glutamine which serves as a donor of amino groups for the amidation of muropeptides. Consequently, the level of amidation is decreased in this mutant (51).

Stimulation with *femC* mutant bacteria or the corresponding wild type led to TNF-α production in a dose dependent manner in human whole blood. However, there was no significant difference in the produced TNF-α levels after stimulation with wild-type or *femC* mutant bacteria (70).

The fact that glutamine serves as a donor of amino groups for D-Glu amidation led to the idea that deletion of the entire operon composed of glutamine synthetase (*glnA*) and the corresponding regulator (*glnR*) might lead to a total loss of muropeptide amidation and a stronger phenotype than in the *femC* mutant. However, the resulting mutant grew only upon supplementation with significant amounts of glutamine and did not have any advantage compared to the *femC* mutant (70).

In order to study if MDPs with or without amidation differ in their proinflammatory capacity when administered in the absence of other bacterial molecules, we incubated whole human blood with chemically synthesized amidated or non-amidated MDPs.

Stimulation with chemically synthesized MDPs led to production of TNF-α in a dose dependent fashion. However, we could not detect significant differences in produced TNF-α levels induced by amidated or non-amidated MDPs. This is in accordance to the equal proinflammatory capacity of wild-type and *femC* mutant bacteria and lead to the conclusion that amidation of MDPs does not influence the proinflammatory capacity of *S. aureus* (70).

Some of the modifications of PG structures leading to resistance to antimicrobial host defenses and evasion of the innate immune system have been investigated in detail during the last years. It has been shown that O-acetylation and attachment of wall teichoic acids at the C-6 position of the muramic acid leads to complete resistance of *S. aureus* to lysozyme (9,10). As mentioned above, modifications of amino acids in the stem peptide of various bacterial species lead to decreased recognition by NOD1 (19,44). Unfortunately, we could not elucidate the true relevance of muropeptide amidation in *S. aureus* in this work. One possible reason for this energy-consuming modification could be the reduction of the negative charge of D-Glu in the muropeptide. Reduction of negative charges of cell envelope components such as teichoic acids or phospholipids has been shown to be an efficient mechanism for gaining resistance to cationic antimicrobial peptides (CAMPs) in *S. aureus* (71,98,99). Muropeptide amidation, which leads to neutralization of a negatively charge carboxyl group, might play a further role in becoming resistant to CAMPs. Another factor, which is influenced by muropeptide amidation is the PG cross linking efficiency (66,119). Furthermore, D-Glu amidation has been shown to influence methicillin resistance levels (11) and vancomycin susceptibility in *S. aureus* (26). Identifying and deleting the gene coding for the putative muropeptide amidase could represent an important challenge for future studies. Unfortunately, we were not able to find a promising candidate gene in the genome of *S. aureus* yet.

As mentioned above, alteration of the net charge of cell envelope components is an efficient mechanism in *S. aureus* to gain resistance to CAMPs (68,71,98,99). MprF, a trans-membrane protein, is responsible for lysinylation of negatively charged phosphatidylglycerol (PG) in the membrane of *S. aureus* (98,120) and other gram-positive human pathogens such as *L. monocytogenes* (124). The neutralization of the membrane net charge by lysinylation of phospholipids leads to resistance to a broad range of CAMPs including defensins and cathelicidins (74,98,120). Another

modification which leads to neutralization of the cell surface net charge in *S. aureus* is the alanylation of teichoic acids. By the products of the *dltABCD* operon a positively charge alanine is attached to the sugar moieties in the backbone of teichoic acids, which is normally negatively charged due to the presence of negative phosphate groups (99). This modification has been show to mediate resistance to various CAMPs including antimicrobial peptides and bacteriolytic enzymes such as group IIA phospholipase A2 (68,99). Alanylation of teichoic acids has also been discribed in other human pathogenic species such as *S. pneumoniae*, *S. pyogenes* and *B. subtilis* in which alanylation of teichoic acids also leads to CAMP resistance (24,69,73).

To date, little is known about the regulation of MprF and Dlt-mediated CAMP resistance mechanisms in Gram-positive bacteria. In this work we could show that the novel two-component regulatory system GraRS positively regulates the *dlt* operon. The genes *dltA*, *dltB* and *dltD* were upregulated 2.1 to 2.9 fold as shown by micro-array analyses and transcription of *dltA* was reduced to 13% of the wild-type level in a *graRS* deletion mutant as shown by RT-PCR (56). Furthermore we could show, that the degree of D-alanylation of teichoic acids is decreased to 53.3% in case of the mutant compared to the wild type (56).

We further investigated the role of the *graRS* system in regulation of *S. aureus* resistance to CAMPs *in vitro* and *in vivo*.

Reduced expression of the *dlt* operon upon *graRS* disruption results in altered cell surface charge. The *graRS* mutant bound significantly more cytochrome c than the wild type or the complemented mutant, which is in accordance with the reduced content of D-alanine residues in teichoic acids of the mutant.

Increased binding of cationic proteins may also result from reduced *mprF* expression and, accordingly, reduced LPG content. We could exclude this possibility by comparing patterns of membrane lipids from log-phase bacteria by thin-layer chromatography. The amounts of LPG from wild type and *graRS* mutant were indistinguishable, which corroborates the findings that *mprF* is not among the *graRS*-regulated genes in *S. aureus* SA113 (56).

In this work we could further show, that increased affinity of the *graRS* mutant to cationic molecules leads to higher susceptibility to human host defense peptides. This was shown by comparing inactivation of wild type and *graRS* mutant by the human cathelicidin LL-37. The wild-type and complemented mutant strain showed no

significant decrease in CFU following LL-37 exposure, whereas the number of *graRS* mutant bacteria recovered was only 25% of the original inoculum.

Neutrophil granulocytes produce high amounts of LL-37 and other CAMPs as components of their antibacterial killing arsenal. We found the *graRS* mutant killed considerably faster by neutrophils than the wild-type strain. After 15 and 30 min, the recovered CFU of the *graRS* mutant was significantly lower than those of the wild type. Taken together, these data indicate that *graRS*-mediated control of CAMP resistance mechanisms is of importance for *S. aureus* evasion from neutrophil killing.

In order to study whether reduced resistance of the *graRS* mutant to neutrophil and CAMP-mediated killing influences the ability of the bacteria to cause infections *in vivo*, we compared virulence of wild-type and mutant bacteria in a mouse challenge model. Significantly less bacteria were detected in the kidneys of animals which had been infected with the *graRS* mutant than those infected with the wild-type bacteria. This finding suggests that increased susceptibility to clearance by CAMPs and neutrophils correlates to reduced virulence *in vivo*.

As mentioned above, little is known about the regulation of CAMP resistance in Gram-positives and especially in *S. aureus*. During the last years, some regulation mechanisms have been reported mainly in Gram-negative species such as *S. enterica*, *P. aeruginosa* und *Y. pseudotuberculosis* (47). In these pathogens the two-component regulatory system PhoP/PhoQ regulates proteins essential for the modification of lipid A with aminoarabinose (49). This modification leads to an altered net charge of the molecule and subsequently to resistance to many CAMPs (50). Several stimuli have been identified, which lead to activation of PhoP/PhoQ in *S. enterica* including changes in magnesium- and calcium concentrations and the direct presence of CAMPs (3,4,40).

Proteins that are known to be involved in regulation of CAMP resistance mechanisms in Gram-positives are e.g. the transcription factor VirR in *L. monocytogenes*, which has been shown to be involved in the regulation of both *mprF* and the *dlt* operon (86). The three-component regulatory system *Aps* in *S. epidermidis* also regulates *mprF* and *dlt* (82). Further systems have been shown to play roles in regulation of CAMP resistance in *S. pyogenes* (93) and *L. monocytogenes* (23). However, the regulated genes have remained unknown.

In this work we could show, that the two-component regulatory system GraRS plays an important role in regulation of the *dlt* operon (56) and thereby in resistance of *S.*

*aureus* to CAMPs both *in vitro* and *in vivo*. Further important loci regulated by GraRS are other regulatory proteins such as Rot, Mgr and Arl along with many other genes with important roles in the virulence of *S. aureus* such as the *ica* operon, which is responsible for biofilm formation, and several genes encoding toxins including leukocidin and hemolysin (56). Recently, the *vraFG* genes, which play a key role in the resistance of *S. aureus* to vancomycin have been shown to be under control of GraRS (89). Other regulatory systems which have been shown to be involved in regulation of the *dlt* genes are the global regulators Rot and Arl (67,113). However, it is still unknown which regulators bind to the *dltA* promoter or have an indirect effect on regulation of the operon.

We have shown, that the transcriptional level of *dltA* is reduced to 13 % of the wild-type level in a GraRS deletion mutant (56). However, this low level is still sufficient for an alanylation degree in teichoic acids of about 53% compared to the wild type. If the *dlt* operon is expressed constitutively to a certain degree or if other yet unknown regulators are involved in its regulation remains to be elucidated.

The exact stimuli involved in activation of regulatory systems controlling CAMP resistance in Gram-positives have also remained incompletely understood. There is some evidence that high concentrations of cations such as $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ influence the transcription of the *dlt* operon in *S. aureus* (67). However, the detailed mechanisms remained unknown. Further investigations could show, if involved regulatory systems are activated directly by sensing antimicrobial molecules, by changes in concentrations of certain molecules in the environment, changes in cell wall or membrane integrity, or by other unknown factors.

In *S. epidermidis*, another important factor in staphylococcal resistance to CAMPs, the MprF protein (74,98,120), has been shown to be regulated by the novel three-component regulatory system Aps (82). This system has been shown to be directly activated by the presence of human defensins (82). In *S. aureus*, much less is known about the regulation of MprF-mediated lysinylation of phosphatidylglycerol. However, it is assumed that this process has to be tightly regulated in order to maintain a stable membrane composition.

Cationic antimicrobial peptides (CAMPs) are potent, natural antibiotics with multiple functions. Besides their direct antimicrobial properties they can bind and thereby neutralize LPS, some can induce or inhibit apoptosis, and take part in regulation of

the immune response (13). However, it has remained unclear which of these functions is most important in mediating protection of the host from invading pathogens. CAMPs seem to have rather weak direct antimicrobial killing activity when tested under culture conditions applied to synthetic antibiotics. This is due to the loss of funtion of CAMPs in physiological concentrations of NaCl, which leads to abrogation of bacterial killing, e.g. by LL-37, at NaCl-concentrations of at least 50 mM (13). Therefore, CAMPs were thought to be active only under low ionic strength or in high concentrations. However, this notion is in contrast to many animal models which have clearly shown the *in vivo* relevance of these peptides.

In this work we could show that carbonate ions are an essential factor for the antimicrobial activity of CAMPs (28). Carbonate is present in most compartments of the human body including blood, sweat, the urogenital, gastrointestinal and respiratory tract. We could show that both Gram-positive and Gram-negative bacteria are much more sensitive for killing by CAMPs when grown under physiological concentrations of carbonate. The MIC (minimal inhibitory concentration) of the murine cathelicidin mCRAMP is 32 µM for *S. aureus* grown in cell culture medium without carbonate. After growth of the bacteria in medium with physiological concentrations of carbonate, the MIC is decreased to 8 µM, which represents the concentration of mCRAMP in body fluids of mice (28).

Furthermore, we could show that this effect is due to increased sensitivity of the bacteria and not due to interaction of the CAMPs with carbonate, as growth with carbonate is sufficient to render the bacteria susceptible. The presence of carbonate during incubation with CAMPs is dispensable (28).

Which alterations in the bacterial cell lead to increased sensitivity for AMPs by carbonate could not be completely elucidated in the present work. Alterations of the membrane composition, e.g. a decreased content of positively charged lysyl-phosphatidylglycerol (LPG), which leads to resistance of *S. aureus* to AMPs, could be excluded by comparing the patterns of polar lipids of *S. aureus* after growth with or without carbonate (28).

The presence of carbonate led to drastic changes in transcritional profiles of *E. coli* und *S. aureus*. Transcription of more than 300 genes was altered by two-fold in *E. coli*. Among them the global regulator FliA. In *S. aureus* the global regulator *sigB*, which shows homology to *fliA*, was strongly repressed in the presence of carbonate.

Further genes with altered transcription levels were genes important for virulence, stress response and cell wall maintainance (28).
Ultra structure analyses of *S. aureus* cells, which were grown in the presence of carbonate, showed a reduced thickness of the cell wall. This phenotype is also known from *sigB*-deletion mutants. This finding led to the idea that the repression of *sigB* and subsequently the reduced thickness of the cell wall may lead to increased sensitivity for AMPs. However, a *sigB* mutant was not as sensitive for AMPs as wild-type bacteria grown with carbonate and cultivation of the *sigB* mutant with carbonate further enhanced sensitivity to LL-37 (28).
These results show that further, yet unknown, bacterial factors play a crucial role in rendering bacteria sensitive to AMPs in the presence of physiological concentrations of carbonate. Further investigations of these factors could lead to a better understanding of the mechanisms by which AMPs kill bacteria and the mechanisms that enable some pathogenic bacteria to resist these host defense molecules.
The discovery that carbonate enhances susceptibility of bacteria to AMPs is important for several reasons. First, it reconciles the paradox between the observed relevance of AMPs in innate immune function and the lack of antimicrobial activity when assayed *in vitro*. Second, it can be regarded as a mechanism to maintain AMP-susceptible microbial populations. In the external environment, the levels of carbonate are very low. Thus, there is no further advantage for bacteria to develop resistance mechanisms against host defense molecules. This could explain why bacteria did not succeed in getting completely resistant against these very ancient and highly conserved innate immunity components. Third, when testing new antimicrobial substances, one should better use bacteria grown in an environment corresponding to that of the host for which they represent a potential pathogen.

Leukocytes such as neutrophil granulocytes are in the first line of defense against invading pathogens. They are recruited to the place of infection and can kill microorganisms after phagocytosis by several mechanisms. Besides the production of antimicrobial peptides and bacteriolytic enzymes, they produce high amounts of toxic oxygen-containing molecules including superoxid anions ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which are synthesized by the NADPH oxidase. This mechanism was reported to be dependent on the large-conductance $Ca^{2+}$-activated $K^+$ (BK)

channel (1). However, there were conflictive results about the existence and role of these channels in neutrophils (33).

In this work, we contributed to solving the question, whether BK channels are really present in neutrophils and if they play a role in combating invading pathogens.

Patch-clamp analyses with human and mouse neutrophils after stimulation with PMA, (12-*O*-tetradecanoylphorbol-13-acetate) which is an activator of NADPH oxidase, failed to detect the presence of BK-channels. Although PMA stimulated NADPH oxidase activity, there were no BK-channel currents detectable. Furthermore, the currents were not inhibited by iberiotoxin, a well known inhibitor of BK channels. Iberiotoxin also did not lead to impaired killing activity of human neutrophils *ex vivo* against *S. aureus* and *C. albicans.* This notion corroborates the finding that iberiotoxin is not able to inhibit NADPH oxidase-dependent mechanisms *in vitro* (32).

BK channel knock-out mice did not show a significant difference in bacterial loads in spleen and liver after infection with *S. aureus* or *Y. enterocolitica* compared to wild-type mice. This finding shows that BK-channels are not important in combating invading pathogens *in vivo* (32).

Altogether our findings show, that functional BK channels are not present in neutrophils and are not essential for innate immunity.

## Concluding remarks

Several mechanisms have described by which *S. aureus* resists and evades the innate immune system in order to sucessfully establish an infection. In this work we could show that one of these mechanisms, the alanylation of teichoic acids by the products of the *dlt* operon, is regulated by the GraRS two-component regulatory system. However, it is assumed that other systems are involved in regulation, since deletion of the GraRS system leads to partial but not total loss of alanylation. A number of important questions remain unanswered, which will be challenges for future studies: Which other regulatory proteins may be involved in the control of teichoic acid alanylation and what may be the factors which lead to activation of these regulatory proteins? Are the *dlt* genes expressed constitutively to a certain degree, and which factor binds to their promoter in order to control expression?
Former studies have shown that modifications of peptidoglycan structures can lead to bacterial evasion of recognition by the immune system e.g. in Gram-negatives and *Bacillus* species. *S. aureus* modifies its peptidoglycan by amidation of muropeptide glutamate. Concerning this modification, we have to consider other rationales than evasion of the innate immune system. In this work we could show that muropeptide amidation has no impact on proinflammatory capacities of *S. aureus* muropeptides. However, the *in vivo* relevance of this energy-consuming modification remains unknown. One possible reason could be the neutralization of cell envelope components in order to gain resistance to cationic antimicrobial peptides. To test for this hypothesis, identifying and deleting the responsible gene(s) would be a great step forward.

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# Contribution to the publications

**"Muropeptide modification-amidation of peptidoglycan D-glutamate does not affect the proinflammatory activity of *Staphylococcus aureus*."**
For this work I performed stimulation assays of whole human blood, and determination of TNF-α levels by ELISA.

**"Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci"**
For this publication I determined D-alanylation degrees of teichoic acids of different staphylococcal strains.

**"The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses"**
All experiments were performed by myself, except the mouse infection model, which was done by Sascha Kristian in San Diego, California.

**"The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides."**
I contributed to this publication by performing analysis of staphylococcal lipids.

**"Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity."**
For this work I determined inactivation of *S. aureus* by neutrophil granulocytes.

**Review**

# Molecular mechanisms of bacterial resistance to antimicrobial peptides

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# Molecular Mechanisms of Bacterial Resistance to Antimicrobial Peptides

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**Abstract** Cationic antimicrobial peptides (CAMPs) are integral compounds of the antimicrobial arsenals in virtually all kinds of organisms, with important roles in microbial ecology and higher organisms' host defense. Many bacteria have developed countermeasures to limit the efficacy of CAMPs such as defensins, cathelicidins, kinocidins, or bacteriocins. The best-studied bacterial CAMP resistance mechanisms involve electrostatic repulsion of CAMPs by modification of cell envelope molecules, proteolytic cleavage of CAMPs, production of CAMP-trapping proteins, or extrusion of CAMPs by energy-dependent efflux pumps. The repertoire of CAMPs produced by a given host organism and the efficiency of microbial CAMP resistance mechanisms appear to be crucial in host–pathogen interactions, governing the composition of commensal microbial communities and the virulence of bacterial pathogens. However,

all CAMP resistance mechanisms have limitations and bacteria have never succeeded in becoming fully insensitive to a broad range of CAMPs. CAMPs or conserved CAMP resistance factors are discussed as new mediators and targets, respectively, of novel and sustainable anti-infective strategies.

## 1 Introduction

One of nature's most ancient strategies for combating unwelcome bacteria is the production of membrane-damaging antimicrobial peptides (Zasloff 2002; Hancock and Chapple 1999). Such molecules are produced by certain bacterial or archaeal strains (bacteriocins) (Riley and Wertz 2002), by plants (plant defensins) (Lay and Anderson 2005), by protozoons (Leippe and Herbst 2004), and by virtually all classes of animals (Zasloff 2002). In order to equip these molecules with a high affinity for bacterial membranes most of them have cationic properties and are referred to as CAMPs (cationic antimicrobial peptides). The antimicrobial activity of CAMPs depends on an ionic milieu comparable to the conditions found in mammalian body fluids (Dorschner et al. 2006). CAMPs include linear, usually α-helical peptides such as the amphibian magainin, the murine CRAMP, and the human LL-37 (Nizet and Gallo 2003), disulfide bridge-stabilized peptides with β-sheet structures such as the α-, β-, and θ-defensins (Ganz 2003; Lehrer 2004), and large chemokines or chemokine-derived molecules with antimicrobial activity named kinocidins (Yang et al. 2003; Dürr and Peschel 2002), to mention only a few typical classes of vertebrate CAMPs. Bacteriocins often contain unusual modifications such as thioether bridges (Guder et al. 2000).

CAMPs have been shown to play crucial roles in microbial ecology and in higher organisms' host defense. However, microorganisms have also found many ways to limit the efficacy of CAMPs (Groisman 1994; Ernst et al. 2001; Peschel 2002; Nizet 2005). Bacteriocin-producing bacteria are resistant to the produced peptides, which enable them to survive while competing microorganisms are inhibited (Riley and Wertz 2002). Bacterial commensals and pathogens of higher organisms, on the other hand, use CAMP resistance mechanisms as a prerequisite to invade and colonize host tissues (Peschel 2002). Unlike antibiotic resistance genes, most CAMP resistance genes are usually not found on plasmids, transposons, or other laterally transferable genetic elements but on the bacterial chromosome in the vicinity of housekeeping genes. At least some of them are considered to have appeared rather early in evolution and seem to be integral parts of the genomes of bacteria whose habitats involve the frequent exposure to CAMPs. As another conse-

quence of their long presence in bacteria, some of the cell wall modifications leading to CAMP resistance affect other bacterial functions such as the attachment and activity of cell wall proteins (Peschel et al. 2000), biofilm formation (Gross et al. 2001), or interaction with epithelial cells (Weidenmaier et al. 2004). Extensive research activities have led to a very large number of studies on bacterial CAMP resistance (Table 1; Fig. 1). This review focuses on established molecular principles of CAMP resistance rather than giving a complete overview on all publications concerning this topic. The ecological aspects of CAMP resistance along with their relevance in microbial biofilm formation and biofilm-associated infections are discussed elsewhere (Otto 2005).

As one would expect, CAMPs seem to be subjected to a very rapid and active evolution (Maxwell et al. 2003), probably as a means to react to the equally fast evolving bacterial resistance mechanisms (Patil et al. 2004). Accordingly, the various mammalian genera are highly variable in the sequences and structures of produced antimicrobial peptides. It can be assumed that the pattern of antimicrobial molecules of a given species is one of the factors that govern the spectrum of its commensal and pathogenic microorganisms. For instance, the production of antiretroviral θ-defensins is discussed as a crucial factor determining resistance (in monkeys) or susceptibility (humans) to HIV (Nguyen et al. 2003). The human gut is particularly rich in bacterial colonizers, which is probably the reason why specialized cells in the crypts of Liberkühn produce an extraordinarily large spectrum of antimicrobial peptides ranging from CRS peptides (mice) (Hornef et al. 2004) to various α-defensins (most mammalian species including humans) (Lehrer 2004; Ganz 2003). Elucidating the basis of microbial CAMP resistance mechanisms will be crucial for understanding, monitoring, and interfering with bacterial colonization and infection.

## 2 How Widespread Is CAMP Resistance?

Considering the fact that probably each bacterial species encounters CAMP-producing competing microorganisms or host cells, one would expect that most bacteria have evolved at least some strategies to evade CAMP-mediated killing. In fact, increasing research activities have clearly demonstrated that this is true for many microbial habitats. Skin bacteria such as staphylococci, oral bacteria such as streptococci, and intestinal bacteria such as salmonellae have been described to resist high concentrations of locally produced CAMPs (Peschel 2002; Ernst et al. 2001; Nizet 2005) (Table 1). Soil bacteria such as *Bacillus subtilis* also have CAMP resistance mechanisms, probably as a means

**Table 1** Mechanisms and prevalence of bacterial CAMP resistance

| Resistance mechanism | Species | Reference |
|---|---|---|
| **Proteolytic cleavage** | | |
| PgtE | *Salmonella enterica* | (Guina et al. 2000) |
| OmpT | *Escherichia coli* | (Stumpe et al. 1998) |
| Aureolysin, serin protease V8 | *Staphylococcus aureus* | (Sieprawska-Lupa et al. 2004) |
| Unidentified proteases | *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *Porphyromonas gingivalis*, *Prevotella* spp. | (Schmidtchen et al. 2002) |
| **Production of external CAMP-binding molecules** | | |
| SIC protein, M1 protein | *Streptococcus pyogenes* | (Frick et al. 2003; Nizet 2005) |
| Staphylokinase | *Staphylococcus aureus* | (Jin et al. 2004) |
| **CAMP-specific drug exporters** | | |
| MtrCDE | *Neisseria gonorrhoeae* | (Shafer et al. 1998) |
| EpiFEG | *Staphylococcus epidermidis*; many antibiotic producers | (Peschel and Götz 1996; Jack et al. 1998) |
| RosA/B | *Yersinia* spp. | (Bengoechea and Skurnik 2000) |
| **Alteration of the electrostatic properties of the bacterial cell surface** | | |
| Modification of lipid A with aminoarabinose | *Salmonella enterica*, many Gram-negative spp. | (Ernst et al. 2001b; Miller et al. 2005) |
| Alanylation of teichoic acids | *Staphylococcus aureus*; many Gram-positive bacteria | (Peschel et al. 1999; Abachin et al. 2002; Poyart et al. 2003; Perego et al. 1995) |
| Lysinylation of phospholipids | *Staphylococcus aureus*; many Gram-positive and Gram-negative bacteria | (Peschel et al. 2001; Staubitz and Peschel 2002; Ratledge and Wilkinson 1988) |
| **Further mechanisms** | | |
| Additional fatty acid in lipid A | *Salmonella enterica*, many Gram-negative spp. | (Guo et al. 1998; Miller et al. 2005) |
| Modification of mycolic acid | *Mycobacterium tuberculosis* | (Gao et al. 2003) |
| Reduced cytoplasmic membrane potential | *Staphylococcus aureus* | (Yeaman et al. 1998) |

**Table 1** (continued)

| Resistance mechanism | Species | Reference |
|---|---|---|
| Slime and capsule polymers, biofilm formation | *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, many other bacteria | (Campos et al. 2004; Otto 2005) |
| Inhibition of CAMP production | *Shigella* spp. | (Islam et al. 2001) |

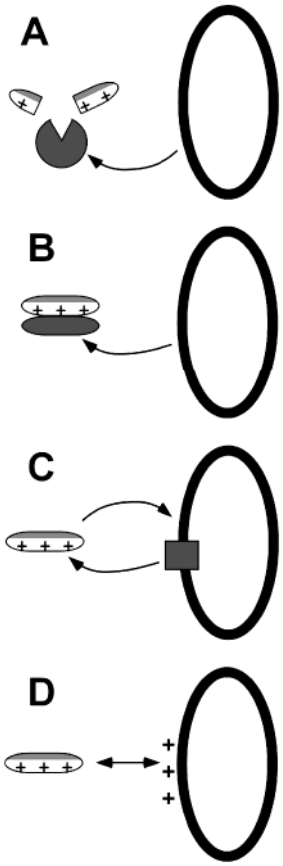


**Fig. 1A–D** Mechanisms of bacterial CAMP resistance by proteolytic cleavage of CAMPs (**A**), CAMP-trapping molecules (**B**), CAMP extruding transport proteins (**C**), or electrostatic repulsion of CAMPs (**D**)

to achieve protection against bacteriocins frequently produced by other soil microorganisms and fungi (Staubitz and Peschel 2002; Cao and Helmann 2004). The available bacterial genome sequences reveal the presence of CAMP resistance genes in the majority of microbial species, indicating that CAMP resistance is in fact a very widespread bacterial trait (Weidenmaier et al. 2003; Miller et al. 2005). Extensive investigations in some prototype species such as *Salmonella enterica* and *Staphylococcus aureus* have revealed the presence of several resistance mechanisms in one bacterial species, which seem to complement each other in order to achieve high-level resistance to a broad spectrum of CAMPs (Ernst et al. 2001; Peschel 2002). However, different isolates of one particular bacterial species may vary widely in their susceptibility to CAMPs (Midorikawa et al. 2003; Joly et al. 2004) indicating that the various mechanisms may be differently expressed or functional in different clones.

## 3
## Proteolytic Cleavage of CAMPs

The most straightforward way for a bacterial species to inactivate antimicrobial peptides is the production of peptidases and proteases that cleave CAMPs (Fig. 1A). Such enzymes have been described in Gram-negative and Gram-positive bacteria. *S. enterica* produces the outer membrane protease PgtE, which is capable of cleaving the cathelicidin LL-37 and other alpha helical CAMPs (Guina et al. 2000). *S. aureus* expresses several proteases; the metalloprotease aureolysin and the serine protease V8 can cleave LL-37 and the in vitro resistance to LL-37 has been associated with aureolysin production (Sieprawska-Lupa et al. 2004). Many other bacterial species including *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *Porphyromonas gingivalis*, and *Prevotella* spp. also produce proteases that cleave linear CAMPs (Schmidtchen et al. 2002).

## 4
## Production of External CAMP-Binding Molecules

Some bacterial species express secreted or surface-anchored proteins that bind certain CAMPs with a very high affinity and thereby prevent their access to the cytoplasmic membrane (Fig. 1B). *Streptococcus pyogenes* secretes the SIC protein, which binds and thereby inactivates LL-37 (Frick et al. 2003). Its production has been correlated with the invasiveness of *S. pyogenes* strains.

A similar approach is used by *S. aureus* in order to achieve resistance to α-defensins. The fibrinolytic exoprotein staphylokinase does not only bind to plasminogen, but also has a high affinity for human α-defensins (Jin et al. 2004). Staphylokinase thereby contributes significantly to α-defensin resistance and staphylokinase production correlates with the in vitro resistance of *S. aureus* isolates to defensins.

The *S. pyogenes* M proteins are covalently attached to the peptidoglycan. Several functions have been assigned to the various M protein domains, which are highly variable in structure and size (Bisno et al. 2003). One of the M protein serotypes, the globally disseminated M1 clone, seems to play a critical role in resistance to LL-37 by binding this peptide with the hypervariable M1 C-terminus (Nizet 2005). Inactivation of the M1 gene or its heterologous expression leads to reduced susceptibility and increased resistance to LL-37 respectively.

## 5 CAMP-Specific Drug Exporters

Protection against small amphiphatic drugs is often mediated by extrusion of the molecules by energy-dependent export proteins in the cytoplasmic membrane. Many of these resistance factors have a broad substrate spectrum and are referred to as multiple drug resistance exporters (van Veen and Konings 1997). As CAMPs also have amphiphatic, membrane-damaging properties, it is not surprising that some of the known bacterial MDRs confer resistance to certain CAMPs (Fig. 1C). The *Neisseria gonorrhoeae* MtrCDE MDR, a member of the resistance/nodulation/division (RND) class of microbial efflux pumps, contributes to resistance to the small porcine β-sheet CAMP protegrin 1 and to the α-helical human peptide LL-37 (Shafer et al. 1998). Attenuated virulence of *mtr*-mutated *N. gonorrhoeae* suggests a considerable role of the MtrCDE system in evasion of CAMP-mediated killing (Jerse et al. 2003). The QacA efflux pump contributes to *S. aureus* resistance to platelet-derived CAMPs (tPMPs, thrombin-induced microbicidal proteins) (Kupferwasser et al. 1999). However, this mechanism appears to be independent of the transport function of QacA and to result from another activity of this membrane protein.

Bacterial producers of lanthionine-containing bacteriocins usually bear ABC transporters that provide resistance against the produced antimicrobial peptide. These systems always seem to be very specific for the produced peptide and do not protect against a larger spectrum of CAMPs (Riley and Wertz 2002; Peschel and Götz 1996).

## 6
## CAMP Resistance by Altering the Electrostatic Properties of the Bacterial Cell Surface

CAMPs share positive net charges with most antimicrobial molecules and enzymes such as lysozyme, secretory group IIA phospholipase $A_2$ (PLA2), RNase 7, and myeloperoxidase. These cationic properties are in a striking contrast to the generally anionic net charge of the molecules forming the bacterial cell envelopes such as peptidoglycan, most phospholipids, lipid A (Gram-negatives) and teichoic acids (Gram-positives) (Weidenmaier et al. 2003). In contrast, the outer leaflets of human cell membranes are usually composed of uncharged or zwitterionic lipids such as phosphatidylcholine and sphingolipids (Devaux and Morris 2004), which are unfavorable for binding and integration of CAMPs. It is assumed that host defense factors have evolved cationic properties in order to impart a high and selective affinity for bacterial cell surface molecules (Weidenmaier et al. 2003). Most of the anionic bacterial cell envelope molecules are very ancient and invariable, and it seems to be impossible for microorganisms to replace these molecules with different structures that would be less favorable for interactions with CAMPs. However, many bacteria are able to modify their cell surfaces in order to reduce their negative net charge and thus acquire protection against inactivation by CAMPs (Fig. 1D). Detailed studies of this phenomenon are again available for *S. aureus* and *S. enterica*.

The teichoic acids of staphylococci and other Gram-positive bacteria are composed of alternating glycerolphosphate or ribitolphosphate groups and are substituted with N-acetylglucosamine or D-alanine (Neuhaus and Baddiley 2003). These polymers are anchored to the cytoplasmic membrane (lipoteichoic acids) or connected to the peptidoglycan (wall teichoic acids). The great number of phosphate groups impart polyanionic properties on teichoic acids. D-alanine incorporation introduces positively charged amino groups into teichoic acids, leading to a partial neutralization of the polymers (Peschel et al. 1999). This modification limits the interactions of CAMPs with the staphylococcal cell wall and decreases the susceptibility to a broad variety of cationic host factors ranging from defensins (Peschel et al. 1999) and PLA2 (Koprivnjak et al. 2002) to myeloperoxidase (Collins et al. 2002). In addition to staphylococci, this resistance mechanism has also been described in *Listeria monocytogenes* (Abachin et al. 2002), *Streptococcus agalactiae* (Poyart et al. 2003), *S. pyogenes* (Kristian et al. 2005), and *B. subtilis* (Wecke et al. 1997). The *dltABCD* operon responsible for D-alanine transfer into teichoic acids occurs in the genomes of most bacteria of the low G+C branch of Gram-positive bacteria, indicating that teichoic acid alanylation repre-

sents a very widespread CAMP resistance mechanism (Weidenmaier et al. 2003).

Most of the bacterial phospholipids such as phosphatidylglycerol, cardiolipin, and others share anionic properties with cell wall polymers (Huijbregts et al. 2000). Many bacterial species, however, including staphylococci, enterococci, listeriae and *P. aeruginosa*, are able to modify a considerable amount of phosphatidylglycerol with L-lysine (Ratledge and Wilkinson 1988), which leads again to neutralization of the cell surface net charge and, consequently, to reduced binding of CAMPs and other cationic host defense molecules. MprF, a novel membrane enzyme, is responsible for the synthesis of lysylphosphatidylglycerol (Staubitz et al. 2004; Oku et al. 2004), and its inactivation leads to a considerably increased susceptibility of *S. aureus* to a large variety of CAMPs (Peschel et al. 2001; Kristian et al. 2003a; Koprivnjak et al. 2002; Weidenmaier et al. 2005). *mprF* homologs are found in the genomes of many Gram-positive and Gram-negative bacteria, among them many human, animal, and plant pathogens, and even in some archaeal species, suggesting that these bacteria employ very similar mechanisms to achieve protection against CAMPs (Weidenmaier et al. 2003).

Many Gram-negative bacteria have similar CAMP resistance strategies. Modifications of lipid A, the conserved integral membrane part of the lipopolysaccharide, are responsible for CAMP resistance in *S. enterica* and *P. aeruginosa* (Ernst et al. 1999, 2001). The anionic character of lipid A can be reduced, for instance, by incorporation of cationic aminoarabinose (Nummila et al. 1995; Gunn et al. 1998). Many Gram-negative species bear the *pmr* genes responsible for amioarabinose transfer into lipid A in their genomes (Miller et al. 2005), suggesting that this modification is a widespread trait in Gram-negative bacteria.

Other cell wall modifications such as synthesis of the neutral phospholipid phosphatidylethanolamine (Cao and Helmann 2004), the neutralization of peptidoglycan muropeptides by iso-D-glutamate amidation (Gustafson et al. 1994), and the transfer of positively charged ethanolamine into lipopolysaccharide (Nummila et al. 1995) may also have the purpose of reducing the efficacy of CAMPs. Obviously, many of the bacterial mechanisms of CAMP resistance reflect the same molecular strategy, even though the modified target molecules and the involved genes are unrelated.

## 7
## Further Bacterial Mechanisms of CAMP Resistance

In order to kill bacteria, CAMPs need to integrate into bacterial membranes, diffuse laterally, and form complexes with other CAMP molecules, which

leads to pore formation and efflux of protons and small molecules (Sahl et al. 2004). Membrane fluidity is thus a critical aspect in CAMP-mediated killing and, in some cases, changes in the composition of lipid fatty acids have been implicated in CAMP resistance. Introduction of an additional fatty acid into the lipid A of *S. enterica* mediated by the PagA protein reduces the susceptibility to LL-37 and protegrin PG1 (Guo et al. 1998). Related genes that may play similar roles are found in several Gram-negative pathogens' genomes (Miller et al. 2005) and increased acylation of lipid A has been implicated in adaptation of *P. aeruginosa* during persistent lung infection in cystic fibrosis patients (Ernst et al. 1999). The occurrence of shorter acyl chains in mycolic acids of a *Mycobacterium tuberculosis kasB* mutant leads to increased susceptibility to defensins and lysozyme (Gao et al. 2003). Mycolic acids form an outer membrane-like shield on the mycobacterial surface and the altered acyl chains of the mutant increase the permeability for several antibiotics and CAMPs. *S. aureus* resistance to platelet microbicidal proteins has also been associated with changes in the composition of lipid fatty acids and concomitantly altered membrane fluidity (Bayer et al. 2000). Inactivation of the major cold shock gene *cspA* leads to susceptibility of *S. aureus* to a cathepsin G-derived CAMP for unclear reasons (Katzif et al. 2003). Since mutation of *cspA* also led to deficiency in the yellow membrane carotinoid staphyloxanthine, altered composition and fluidity of the cytoplasmic membrane may be the reason for CAMP susceptibility in this mutant.

CAMPs need a certain threshold membrane potential to integrate into lipid bilayers. Bacterial cytoplasmic membranes usually have a strong potential since they contain the respiratory chain generating a proton-motive force. Eukaryotic cytoplasmic membranes, in contrast, are much less energized, which is one of the factors for the relative insensitivity of eukaryotic cells for CAMPs. Spontaneous mutations in genes encoding bacterial respiratory chain components often lead to small colony phenotypes since these mutants show a strongly attenuated growth behavior. *S. aureus* small colony variants (SCVs), however, have a better capacity to persist in human cells and they are often responsible for recurrent infections (Proctor et al. 1998). SCVs have a lower membrane potential and they are less susceptible to many CAMPs (Yeaman et al. 1998). Accordingly, the SCV phenotype can be regarded as a CAMP resistance mechanism and CAMP resistance may be one of the reasons for the increased ability of SCVs to persist in host tissues.

In some instances, capsular polymers have been shown to contribute to CAMP resistance (Campos et al. 2004; Vuong et al. 2004). Bacterial capsules are usually considered as an antiopsonic and antiphagocytotic virulence factor. They do usually not represent a major diffusion barrier for small molecules such as CAMPs. In some cases, however, the extracellular slime matrix of

capsules and biofilms have been shown to provide protection against certain CAMPs (Campos et al. 2004; Vuong et al. 2004). This phenomenon may depend on the net charge of capsule polymers, as the exopolymer PIA involved in CAMP resistance in *Staphylococcus epidermidis* has a positive net charge and may thus contribute to repulsion of cationic antimicrobial molecules. Slime polymers and the special metabolic adaptations of bacteria in biofilms seem to play important roles in evasion of CAMP-mediated killing. Their relevance in CAMP resistance is reviewed in detail elsewhere in this book (Otto 2005).

Another elegant method of CAMP resistance is used by *Shigella* species, which inhibit expression of LL-37 and β-defensin 1 in human colonic epithelia cells. This event involves *Shigella* plasmid DNA (Islam et al. 2001). The underlying mechanisms are not yet understood.

## 8 Regulation of CAMP Resistance Mechanisms

Most of the regulatory mechanisms involved in resistance of bacteria against CAMPs are not well understood yet, both in terms of regulating signals and of regulatory proteins. However, there are some well-characterized regulatory pathways in Gram-negative bacteria, which have been shown to play crucial roles in CAMP resistance. The PhoP/PhoQ two-component system plays a key role in the virulence of *S. enterica*, *P. aeruginosa*, and *Yersinia pseudotuberculosis* (Groisman 2001). PhoP/PhoQ-controlled genes such as PagP are necessary for lipid A modification leading to CAMP resistance, as shown in *S. enterica* (Guo et al. 1998). PhoP/PhoQ responds to changes in the magnesium and calcium ion concentrations (García Véscovi et al. 1996), and it has been shown to be activated by the presence of subinhibitory concentrations of CAMPs in *S. enterica* (Bader et al. 2003). The sensor kinase PhoQ directly recognizes CAMPs, thereby displacing PhoQ-bound divalent cations and leading to activation of the response regulator PhoP (Bader et al. 2005). A second two-component regulatory system, PmrA/PmrB, responds to extracellular iron (Wosten et al. 2000), and it is also controlled by PhoP/PhoQ in *S. enterica* (Groisman 2001). It confers resistance to several CAMPs by transcriptional activation of two loci, *pmrE* and *pmrHFIJKLM*, which are required for the biosynthesis of a lipid A variant with 4-aminoarabinose modification (Gunn et al. 2000). This modification leads to a reduction of the anionic character of the bacterial lipid A and, consequently, to CAMP resistance, as discussed above. A related system seems to respond directly to CAMP exposure in *P. aeruginosa* (McPhee et al. 2003).

Much less is known about the regulation mechanisms and stimuli involved in CAMP resistance of Gram-positive bacteria. The global virulence regulatory system *agr* of *S. aureus* is involved in the regulation of the *dlt*-operon, responsible for the alanylation of techoic acids (Dunman et al. 2001). Another two-component regulation system, DltRS, controls expression of the *dlt* operon in *S. agalactiae* (Poyart et al. 2003). Inactivation of regulatory genes has led to increased CAMP susceptibility in *S. pyogenes* (Nizet et al. 2001) and *L. monocytogenes* (Cotter et al. 2002), but the genes controlled by these regulators have remained unknown.

## 9 Role of CAMP Resistance in Host Colonization and Infection

In addition to obligate pathogens, several bacterial commensals or opportunistic pathogens have been shown to resist high concentrations of CAMPs (Sahly et al. 2003; Shelburne et al. 2005; Brissette et al. 2004; Nishimura et al. 2004). This ability is generally considered as a prerequisite for the colonization of human epithelia whose secretions in the airway as well as in the gastrointestinal and genitourinary tracts contain high amounts of CAMPs such as β-defensins and LL-37. Only a few animal studies have addressed the role of CAMP resistance in bacterial colonization. *S. aureus* colonizes the anterior nares in 30%–40% of the human population (Peacock et al. 2001), which is one of the crucial risk factors for developing severe wound and skin infections or life-threatening systemic infections such as endocarditis and sepsis (von Eiff et al. 2001; Wertheim et al. 2004). A CAMP-susceptible *S. aureus dlt*A mutant has recently been shown to have a strongly reduced capacity to colonize the nares of cotton rats, which represent a good model of human nasal colonization (Weidenmaier et al. 2004). However, since the *dlt*A mutation leads to altered teichoic acids and since teichoic acid structure is critical in *S. aureus* binding to nasal epithelial cells, it is not yet clear whether the abrogated capacity of this mutant to colonize cotton rat nares is a result of reduced binding to epithelial cells, increased killing by nasal CAMPs, or both. Further in vivo studies will be necessary to elucidate the relevance of CAMP resistance in colonization.

The importance of CAMP resistance in localized infections of various organ systems has been demonstrated for many different pathogens and in many animal models. Skin infections caused by *S. pyogenes* (Nizet et al. 2001), *S. aureus* abscess-like tissue cage infections (Kristian et al. 2003b), *Legionella pneumophila* lung infections (Edelstein et al. 2003), *S. enterica* gastrointestinal infections (Gunn et al. 2000), and *N. gonorrhoeae* genital tract

infections (Jerse et al. 2003), to name but a few examples, are strongly affected if CAMP susceptible mutants are used. Increased bacterial killing by CAMPs produced by epithelial cells of infected organs or released by phagocytes upon contact with bacteria is most probably the reason for the observed virulence attenuation. In line with this notion, CAMP-susceptible bacterial mutants are inactivated faster and more efficiently by CAMP-producing phagocytes (Collins et al. 2002; Kristian et al. 2003a, 2005).

CAMP-susceptible *S. agalactiae* and *S. aureus* mutants are also less virulent in blood stream infections studied in mouse sepsis or rabbit endocarditis models (Poyart et al. 2003; Collins et al. 2002; Weidenmaier et al. 2005). Depending on the particular pathogen and the animal model used, alleviated killing by blood phagocytes, inactivation by microbicidal proteins released by activated platelets, or both seems to be the reason for the reduced virulence of CAMP-susceptible mutants under these conditions.

## 10 Perspectives

The production of CAMPs is a very ancient and still successful strategy of to inhibit microorganisms. Considering the short half-life of the effectiveness of modern antibiotics it seems to be a mystery how CAMPs remained so efficient during evolution. Even the great variety of bacterial CAMP resistance mechanisms has not led to microorganisms with complete resistance to all kinds of CAMPs. It seems that evolution has always found new ways to circumvent the microbial CAMP resistance mechanisms, for instance by rendering CAMPs protease-resistant or by combining two or more antimicrobial mechanisms in one molecule, as shown for the highly versatile bacteriocin nisin (Pag and Sahl 2002). The extraordinary success of CAMPs may be based on the fact that bacteria cannot completely change the composition and properties of their cytoplasmic membrane. The high metabolic costs of becoming resistant to CAMPs, for instance by the energy-consuming, extensive modifications of the cell envelope, may be another reason why it is so difficult for bacteria to develop totally efficient CAMP resistance mechanisms. Nevertheless, some CAMP resistance mechanisms seem to date back to a very early origin, as *mprF*-related genes, for instance, are found in both bacterial and some archaeal genomes (Staubitz and Peschel 2002).

The amazing effectiveness of CAMPs suggest a use of such molecules in antimicrobial therapy. In fact, several CAMPs have yielded promising results in clinical trials (Andres and Dimarcq 2004). The lactococcal bacteriocin nisin has been used as a food preservative for decades (Pag and Sahl 2002) and

daptomycin, a noncationic membrane-damaging antimicrobial lipopeptide with activity against multidrug-resistant staphylococci and enterococci has recently been approved for the use in human infections (Steenbergen et al. 2005), underscoring the therapeutic potential of membrane-active antimicrobial compounds such as CAMPs. On the other hand, highly conserved bacterial CAMP resistance proteins such as MprF or DltABCD may represent interesting new targets for novel anti-infective compounds that would not kill the bacteria but render them susceptible to innate antimicrobial host molecules (Weidenmaier et al. 2003). A deeper understanding of CAMPs and CAMP resistance mechanisms will help to exploit both innate human host defenses and bacterial evasion strategies.

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