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

Silvia Herbert¹, Agnieszka Bera¹, Christiane Nerz¹, Dirk Kraus², Andreas Peschel², Christiane Goerke², Michael Meehl³, Ambrose Cheung³, Friedrich Götz¹*

¹ Microbial Genetics Department, University of Tübingen, Tübingen, Germany, ² Medical Microbiology and Hygiene Department, University of Tübingen, Tübingen, Germany, ³ Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire, United States of America

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 (OatA), and the glycopeptide resistance-associated two-component system, GraRS, is much more sensitive to lysozyme than S. aureus with the oatA mutation alone. The graRS 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 107R-A-W-V-A-W-R-N-R115 (LP9), polymyxin B, or gallidermin. A comparative transcriptome analysis of wild type and the graRS mutant revealed that GraRS controls 248 genes. It up-regulates global regulators (rot, sarS, or mgrA), 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 graRS mutant is also biofilm-negative. The decrease of the dlt transcript in the graRS mutant correlates with a 46.7% decrease in the content of esterified Ọ-alanyl groups in teichoic acids. The oatA/dltA double mutant showed the highest sensitivity to lysozyme; this mutant completely lacks teichoic acid–bound Ọ-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 (oatA) and genes mediating resistance to CAMPs (graRS and dlt). The two lysozyme activities act synergistically, as the oatA/dltA or oatA/graRS double mutants are much more susceptible to lysozyme than each of the single mutants.

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 β-1,4 glycosidic bonds between N-acetylmuramic acid (MurNAc) and N-acetylg glucosamine (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. canosus, 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 oatA 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

Editor: Jeffrey N. Weiser, University of Pennsylvania, United States of America

Received February 16, 2007; Accepted June 4, 2007; Published July 27, 2007

Copyright: © 2007 Herbert et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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

* To whom correspondence should be addressed. E-mail: friedrich.goetz@uni-tuebingen.de
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 glycopeptidase 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 graRS mutant teichoic acid Ω-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.

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 oatA and graRS Single and Double Mutants to Lysozyme and CAMPs

In our search for highly susceptible lysozyme mutants in S. aureus, we isolated two Tn917 transposon mutants in SA113oatA::kan that revealed higher sensitivity to lysozyme than the oatA mutation alone. Chromosomal sequencing of the flanking Tn917 insertion sites revealed that Tn917 was inserted in SA0615 [18]. SA0615 and the upstream gene SA0614 have the features of a typical TCS and were recently named GraRS (glycopeptidase 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 graRS with an erythromycin B cassette to yield SA113 graRS::erm (Figure 1). In addition, we also constructed an oatA::kan/graRS::erm double knockout. Sequencing and complementation with pTXgrraRS, a vector in which the graRS genes are induced into expression by xylose, confirmed the correct replacement. Whereas the oatAgraRS 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).

The oatAgraRS double mutant was much more lysozyme sensitive than each of the single mutants. This hyper-sensitivity 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 graRS single mutant is affected by the muramidase activity of lysozyme. Indeed, the isolated PG from the graRS single mutant was completely resistant to lysozyme hydrolysis, in contrast to the oatA mutant. As expected, PG of the oatA/graRS double mutant was also hydrolysed, although the sensitivity was less pronounced, as in the oatA 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 graRS 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 lyso-

A) WT SA113

B) SA113 graRS::ermB

Figure 1. Illustration of Construction of the graRS Deletion Mutant

(A) Gene organization in the chromosome of WT SA113; Tn917 insertions in graS gene are indicated by arrows.

(B) In the graRS deletion mutant, graRS is substituted by the erythromycin B resistance cassette. Note that ermB gene has a weak transcription terminator, and transcriptional read-through to the following vraFG genes is likely. graR, response regulator; graS, sensor histidine kinase; vraF, ABC transporter ATP-binding protein; vraG, ABC transporter permease; SA0612 and SA0613 are hypothetical proteins (HP).

doi:10.1371/journal.ppat.0030102.g001
zyme showed no activity (neither lytic nor CAMP activity) to the oatA mutant or to the isolated PG of oatA, but it was able to inhibit the growth of the oatA/graRS 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.

Comparative Transcriptome Analysis of WT and graRS Mutant

To find out which genes are responsible for the high susceptibility to CAMPs in the graRS mutant, we carried out a comparative transcriptome analysis of the WT strain and the graRS 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 graRS mutant, genes that are involved in RNA and amino acid synthesis and glycolysis shows highly gene transcription rates. In particular, the urease genes (ureA-G) all 12 pur genes were 2- to 32-fold up-regulated as compared to the WT, whereas purR (repressor) appeared not to be influenced by GraRS. Interestingly, the amount of oatA transcript increased in the graRS mutant, which could explain the slightly higher resistance of the graRS mutant to the muramidase activity of lysozyme (Figure 3). A number of genes that were down-regulated included global regulators (rot, sarS, mgrA), cell surface protein encoding genes (the Ser-Asp rich fibrinogen-binding proteins SdrC and SdrE), the major autolysin gene (atlA) and an autolysin/adesin gene (aatA) [20], exoprotein encoding genes (lbt, hlgA,B, lukM,F, and geh), transporter encoding genes (essA,essC, oppB, and norB), capsule encoding genes (capA,H,I,J,K) and PIA encoding genes (icaADBC), genes responsible for 3-alanyl esterification of teichoic acids (TAs) (dltA,B,D), and the alanine dehydrogenase gene (aldA). The pronounced decrease of expression of the ica [21–23] and dlt operons [24] and atlA [25] explains why the graRS mutant showed a biofilm-negative phenotype on microtiter plates (unpublished data). With a few genes, such as rot, uvrC, and dltA, we verified the transcriptome data by reverse transcriptase (RT)-PCR (Table 3).

Next, we asked which of the 115 less expressed genes in the graRS mutant were responsible for the increased susceptibility to CAMPs. A most likely candidate was the dlt operon (encoding enzymes involved in 3-alanylation of TAs). Its transcript was decreased 2.1-fold to 2.9-fold as compared to WT, whereas purR (repressor) appeared not to be influenced by GraRS. Interestingly, the amount of oatA transcript increased in the graRS mutant, which could explain the slightly higher resistance of the graRS mutant to the muramidase activity of lysozyme (Figure 3). A number of genes that were down-regulated included global regulators (rot, sarS, mgrA), cell surface protein encoding genes (the Ser-Asp rich fibrinogen-binding proteins SdrC and SdrE), the major autolysin gene (atlA) and an autolysin/adesin gene (aatA) [20], exoprotein encoding genes (lbt, hlgA,B, lukM,F, and geh), transporter encoding genes (essA,essC, oppB, and norB), capsule encoding genes (capA,H,I,J,K) and PIA encoding genes (icaADBC), genes responsible for 3-alanyl esterification of teichoic acids (TAs) (dltA,B,D), and the alanine dehydrogenase gene (aldA). The pronounced decrease of expression of the ica [21–23] and dlt operons [24] and atlA [25] explains why the graRS mutant showed a biofilm-negative phenotype on microtiter plates (unpublished data). With a few genes, such as rot, uvrC, and dltA, we verified the transcriptome data by reverse transcriptase (RT)-PCR (Table 3).

Next, we asked which of the 115 less expressed genes in the graRS mutant were responsible for the increased susceptibility to CAMPs. A most likely candidate was the dlt operon (encoding enzymes involved in 3-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 graRS 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 aldA transcription by a factor of 3.5 is in line with the decreased dlt transcription. AldA is the alanine dehydrogenase, which is involved in the synthesis of 3-alanine.

Comparison of graRS and dltA Mutants

Because the dlt operon is less expressed in the graRS mutant, we investigated lysozyme susceptibility with a dltA deletion mutant, which is well-known to be sensitive to
Camps [26]. Indeed, the dlt 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 mutant was not hydrolyzed by lysozyme (Figure 2A). Furthermore, growth of the dlt mutant was inhibited whether active or heat-inactivated lysozyme was applied (Figure 2E). When the susceptibility of graRS and dltA 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 mutant was more pronounced than that of the graRS mutant, and b) even in the presence of gallidermin or polymyxin B, the graRS 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 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 graRS mutant; this can possibly be explained by its proteolytic degradation. Not only the single but also the double mutants oatAGraRS and oatA/dltA were sensitive to the Camp activity of LP9, although the susceptibility was less pronounced as with the graRS and dltA single mutants. However, the oatA single mutant was completely resistant to LP9, indicating that oatA 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 graRS 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 graRS 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 graRS phenotype is unstable and that polymyxin B–resistant revertants were quickly selected. Since the dltA revealed a stable phenotype, we assume that in the selected revertants dltA expression was increased to WT levels.

Hypersensitivity of the oatA/dltA and oatA/graRS Double Mutants to Lysozyme

The highest susceptibility to lysozyme was observed with the oatA/dltA double mutant, which was more than 66-fold and 333-fold more sensitive to lysozyme than the dltA and oatA single mutants, respectively (Figure 2B, 2E, and 2F; Table 4). The oatA/graRS mutant is not quite as sensitive as the oatA/dltA mutant. Another difference is that the oatA/dltA mutant stays lysozyme sensitive even after 24 h of cultivation (Figure 2D and 2F), indicating that the dltA mutant phenotype cannot easily revert to the WT phenotype. The lower susceptibility of the oatA/graRS double mutant can possibly be explained by the fact that the TA in this mutant still contains 53.3% β-alanyl esters, whereas the dltA mutant completely lacks β-alanylmylation in its TAs (Table 3).

The high susceptibility of the double mutants is based on the dual activities of lysozyme: a) the oatA mutant is sensitive to the muramidase activity of lysozyme but is insensitive to Camps (Figures 2B, 3, and 4F), and b) the dltA and graRS 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 oatA/dltA 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 graRS 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 graRS mutant at a concentration of 100 μg/ml. However, when the graRS 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 graRS 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
| Function of Proteins | N315 ORF | N315 Gene | N315 Product | Protein Location | Change in Expression (n-Fold) | One-Sample t-Test-Benjamini-Hochberg (Adv) | rot, mgrA, arlRS Effect |
|----------------------|----------|-----------|--------------|-----------------|-------------------------------|------------------------------------------|------------------------|
| Virulence factors (cell surface proteins, exotoxins, colonization factors) | SA0519 | sdC | Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein | SCW | 9.6 | 0.013 | rot, arl up |
| | SA0521 | sdE | 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 | coa | Staphylocoagulase precursor | S | 2.2 | 0.164 | mgr down |
| | SA0222 | 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 | lukM | Leukocidin chainLukM | S | 2.5 | 0.042 | rot down, mgr up |
| | SA1813 | lukF | Synergolymentropic toxin precursor | S | 2.1 | 0.010 | mgr up |
| | SA2207 | hlgA | Gamma-hemolysin component A | S | 2.1 | 0.010 | mgr up |
| | SA2209 | hlgB | Gamma-hemolysin component B | S | 2.1 | 0.005 | rot, arl down |
| | SA0270 | luk | HP, similar to secretory antigen precursor SsaA | S | 2.9 | 0.012 | arl up |
| | SA0271 | essA | Virulence factor EssA | S | 4.4 | 0.012 | rot, arl up |
| | SA0620 | luk | HP, similar to secretory antigen precursor SsaA | S | 2.1 | 0.013 | rot up |
| | SA2097 | luk | HP, similar to secretory antigen precursor SsaA | S | 3.0 | 0.014 |
| | SA2431 | isaB | Immunodominant antigen B | S | 2.2 | 0.022 | mgr down |
| | SA2459 | icaA | Intercellular adhesion protein A | M | 4.8 | 0.032 |
| | SA2460 | icaD | Intercellular adhesion protein D | M | 4.9 | 0.006 |
| | SA2461 | icaB | Intercellular adhesion protein B | SCW | 3.3 | 0.063 |
| | SA2462 | icaC | Intercellular adhesion protein C | M | 2.5 | 0.068 |
| | SA0108 | sarS | Staphylocoacal accessory regulator A homolog | C | 7.2 | 0.007 | rot up, mgr down |
| | SA0614 | graR | C | 3.1 | 0.062 |
| | SA0641 | mgrA | HTH-type transcriptional regulator MgrA, MarR family | C | 3.1 | 0.007 |
| | SA0856 | spxA | Transcriptional regulator Spx | C | 5.6 | 0.024 |
| | SA1583 | rot | Repressor of toxins Rot | C | 3.8 | 0.009 | arl up |
| | SA1678 | furB | Transcriptional regulator Fur family homolog | C | 2.1 | 0.023 | mgr up |
| | SA2174 | hlg | HP, similar to transcriptional regulator | C | 2.0 | 0.046 |
| | SA0106 | lctP | Lactate transporter, LctP family | M | 3.2 | 0.021 |
| Regulators | SA0109 | sirC | Lipoprotein | M | 3.5 | 0.013 |
| | SA0111 | sirA | Lipoprotein | M | 2.0 | 0.086 |
| | SA0138 | HP, similar to alklyphosphonate ABC transporter | M | 2.1 | 0.004 | arl down |
| | SA0204 | azoR | FMN-dependent NADH-azoreductase | C | 2.2 | 0.021 |
| | SA0207 | HP, similar to maitol/maltodextrin-binding protein | M | 2.1 | 0.035 |
| | SA0208 | Maltose/maltodextrin transport permease homolog | M | 2.1 | 0.064 |
| | SA0268 | HP, similar to ABC transporter system permease protein | M | 2.3 | 0.007 |
| | SA0272 | essaA | Protein EssA | M | 4.7 | 0.022 | arl up |
| | SA0273 | essA | Protein EssA | M | 2.8 | 0.071 |
| | SA0276 | essC | Protein EssC | M | 2.6 | 0.027 | arl up |
| | SA0295 | HP, similar to outer membrane protein precursor | M | 2.3 | 0.010 |
| | SA0423 | aas | N-acetylumuramoyl-L-alanine amidase | SCW | 3.6 | 0.015 |
| | SA0518 | aoz | FMN-dependent NADPH-azoreductase | C | 2.1 | 0.019 |
| | SA0793 | dltA | d-alanine-o-alanyl carrier protein ligase | C | 2.9 | 0.066 |
| | SA0794 | dltB | DltB membrane protein | M | 2.1 | 0.010 |
| | SA0796 | dltD | Poly (glycerophosphate chain) o-alanine transfer protein | M | 2.3 | 0.008 | rot up |
| | SA0845 | oppB | 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 peptid 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 | atlA | Autolysin (N-acetylumuramoyl-L-alanine amidase/endo-beta-N-acetylglucosaminidase | SCW | 2.4 | 0.015 |
| | SA1269 | norB | 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 UFP0342 protein family | M | 2.3 | 0.072 |
| | SA1979 | HP, similar to ferrichrome ABC transporter | M | 2.1 | 0.032 |
| Function of Proteins                  | N315 ORF | N315 Gene | N315 Product                          | Protein Location | Change in Expression (n-Fold) | One-Sample t-Test-Benjamini–Hochberg (Adv) | rot, mgrA, arlRS Effect |
|--------------------------------------|----------|-----------|---------------------------------------|-----------------|-----------------------------|---------------------------------------------|------------------------|
| l-lactate permease ictP homolog      | SA2156   |           |                                       | ORF             | 3.7                         | 0.015                                       |                        |
| HP, similar to lipoprotein inner     | SA2217   |           |                                       | M               | 2.1                         | 0.022                                       |                        |
| membrane ABC transporter             |          |           |                                       |                 |                             |                                             |                        |
| HP, similar to ABC transporter       | SA2302   | stpC      |                                       | M               | 3.1                         | 0.008                                       | mgr up                 |
| HP, similar to membrane spanning     | SA2303   | smpC      |                                       | M               | 5.5                         | 0.010                                       | rot, mgr, arl up       |
| protein                               |          |           |                                       |                 |                             |                                             |                        |
| HP, belongs to ABC-transporter       | SA2475   |           |                                       | M               | 2.3                         | 0.067                                       |                        |
| HP, belongs to the UPF0397 protein   | SA2477   |           |                                       | M               | 2.3                         | 0.068                                       |                        |
| family                               | SA2480   | drp35     | Lactonase Drp35                        | C               | 2.3                         | 0.036                                       |                        |
| Capsular polysaccharide biosynthesis | SA2457   | capA      | capA                                  | M               | 2.0                         | 0.008                                       | arl down               |
| Capsular polysaccharide synthesis    | SA0152   | capH      | Capsular polysaccharide synthesis      | M               | 2.0                         | 0.033                                       | mgr up                 |
| enzyme Cap5H                         |          |           | enzyme Cap5I                          | M               | 2.0                         | 0.034                                       | mgr up                 |
| Capsular polysaccharide synthesis    | SA0153   | capJ      | Capsular polysaccharide synthesis      | M               | 2.3                         | 0.084                                       | mgr up                 |
| enzyme Cap5J                         |          |           | enzyme Cap5K                          | M               | 2.7                         | 0.025                                       | mgr up                 |
| Alkaline shock protein 23, ASP23     | SA1984   | asp23     |                                       | C               | 2.1                         | 0.082                                       |                        |
| HP, similar to deoxyxypurine kinase  | SA0515   |           |                                       |                 | 2.1                         | 0.014                                       |                        |
| HP, similar to carbamate kinase      | SA1013   |           |                                       |                 |                             |                                             |                        |
| Alcohol-acetaldehyde dehydrogenase   | SA0143   | adhE      |                                       |                 |                             |                                             | rot down               |
| Branched-chain amino acid transport  | SA0180   |           |                                       |                 |                             |                                             |                        |
| HP, similar to aspartokinase II      | SA1225   | lysC      |                                       |                 |                             |                                             |                        |
| HP, similar to threonine deaminase   | SA1271   |           |                                       |                 |                             |                                             |                        |
| HP, similar to alanine dehydrogenase | SA1272   | ald1      |                                       |                 |                             |                                             |                        |
| HP, similar to 5OS ribosomal protein | SA1502   | rpsT      |                                       |                 |                             |                                             |                        |
| HP, similar to glycerophosphodiester | SA0220   |           |                                       |                 |                             |                                             |                        |
| phosphodiesterase                   |          |           |                                       |                 |                             |                                             | rot, arl up            |
| HP, similar to replication protein   | SA0027   |           |                                       |                 |                             |                                             |                        |
| for plasmid                          | SA1709   |           |                                       |                 |                             |                                             |                        |
| HP, similar to ferritin              |          |           |                                       |                 |                             |                                             |                        |
| Holin homolog (bacteriophage phiN315)| SA1760   |           |                                       |                 |                             |                                             |                        |
| 2.4                                 | 0.104    |            |                                       |                 |                             |                                             |                        |
| HP (bacteriophage phiN315)           | SA1766   |           |                                       |                 |                             |                                             |                        |
| 6.0                                 | 0.087    |            |                                       |                 |                             |                                             |                        |
| HP (bacteriophage phiN315)           | SA1785   |           |                                       |                 |                             |                                             |                        |
| 2.5                                 | 0.028    |            |                                       |                 |                             |                                             |                        |
| HP (bacteriophage phiN315)           | SA1793   |           |                                       |                 |                             |                                             |                        |
| 23.3                                | 0.074    |            |                                       |                 |                             |                                             |                        |
| Hypothetical genes                   | SA0037   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0077   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0081   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0090   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0100   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0161   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0213   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0221   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0262   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0279   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0283   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0291   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0292   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0378   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0408   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0424   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0623   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0651   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0738   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0739   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0772   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA0890   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA1056   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA1151   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA1828   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2101   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2153   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2198   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2256   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2332   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2339   |           |                                       |                 |                             |                                             |                        |
| HP                                   | SA2373   |           |                                       |                 |                             |                                             |                        |

Lysozyme Resistance in *Staphylococcus aureus*
graRS mutant were completely resistant to lysozyme at a concentration of 50 mg/ml. However, the graRS 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 n-alanylation of the TAs. The oatA mutant was more susceptible to lysozyme than the graRS mutant, but, similar to WT, was completely insensitive to heat-inactivated lysozyme or CAMPs, indicating that oatA is only sensitive to the muramidase activity of lysozyme. The oatAggraRS double mutant was almost 17-fold more sensitive to lysozyme than the oatA 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 dltA single mutant was over 25-fold more sensitive to lysozyme than the WT and 5-fold more sensitive than the oatA single mutant, demonstrating the importance of lysozyme’s CAMP activity. Furthermore, the dltA 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 oatA/dltA double mutant revealed the highest susceptibility to lysozyme. Indeed, it has a 20-fold greater sensitivity to lysozyme than the oatAggraRS double mutant. The oatA/dltA double mutant is 333-fold and 66-fold more sensitive than the single oatA or dltA mutants, which illustrates the extremely high synergistic effect of lysozyme when it can exert both muramidase and CAMP activities. Overexpression of graRS in the graRS mutant or the WT by pTXgraRS 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 oatA 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 C6 position in MurNac as in the O-acetyl group. TagO is a specific UDP-N-acetylmuramylpentose-4-phosphate acetylglucosamine transferase, which is involved in the first step of WTA synthesis. The tagO deletion mutant completely lacks WTA [30]. Although the tagO mutant still shows high lysozyme resistance, a oatA/tagO double mutant, however, is much more susceptible to lysozyme’s muramidase activity than the oatA 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 oatA-minus background by increased lysozyme susceptibility in an oatAggraRS double mutant. The graRS mutant was more susceptible to CAMPs than the WT. We assume that the reason for the increased susceptibility of the graRS mutant was a decrease in dlt expression and, consequently, GraRS up-regulates dlt expression. The Dlt enzymes modify TAs by the incorporation of n-alanine esters rendering the cells resistant to CAMPs, very likely by repulsion [26]. We showed that the dltA mutant is even more susceptible to lysozyme-derived LP9 and other CAMPs than the graRS mutant, because in the dltA mutant, n-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 oatA or that of the graRS mutant. The latter effect is surprising, as the graRS mutant is sensitive to the other CAMPs (LP9, gallidermin, polymyxin B). However, the oatAggraRS 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 dltA mutant to heat-inactivated lysozyme can also be explained by better access to the cell envelope because of the lack of n-alanine esters in TAs.

The next interesting question was, how do CAMPs act in the dltA, oatAggraRS, or oatA/dltA 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 n-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
Table 2. 133 S. aureus SA113 Genes Down-Regulated by GraRS

| Function of Proteins | N31S ORF | N31S Gene | N31S Product | Protein Location | Change in Expression (n-Fold) | One-Sample t-Test-Benjamini-Hochberg (Adv) | rot, mgrA, arlRS Effect |
|----------------------|----------|-----------|--------------|------------------|------------------------------|---------------------------------------------|-------------------------|
| Regulators           | SA0017   | vicR      | Response regulator | C               | 2.0                          | 0.004                                       |                         |
|                      | SA1071   | fabR      | Transcription factor FapR | C               | 2.3                          | 0.015                                       |                         |
|                      | SA1690   | recX      | Regulatory protein RecX | C               | 2.0                          | 0.021                                       |                         |
|                      | SA2296   |           | HP, similar to transcriptional regulator, MerR family | C               | 2.1                          | 0.012                                       |                         |
|                      | SA2320   | pfoR      | 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   | lrgA      | Holin-like protein LrgA | M               | 2.6                          | 0.017                                       | mgr, arl up             |
|                      | SA0469   | ftsH      | Cell-division protein | M               | 2.2                          | 0.004                                       |                         |
|                      | SA0616   | vroF      | ABC transporter ATP-binding protein | M               | 8.6                          | 0.005                                       |                         |
|                      | SA0617   | vroG      | ABC transporter permease | M               | 4.8                          | 0.029                                       |                         |
|                      | SA0708   | secA      | Preprotein translocase subunit | M               | 2.1                          | 0.012                                       |                         |
|                      | SA0719   | trxB      | Thioredoxin reductase | M               | 2.5                          | 0.016                                       |                         |
|                      | SA0937   |           | Cytochrome D ubiquinol oxidase subunit 1 homolog | M               | 2.5                          | 0.010                                       |                         |
|                      | SA0997   | muI       | Glutamate racemase | C               | 2.4                          | 0.009                                       |                         |
|                      | SA1127   | cinA      | Competence-damage inducible protein CinA | C               | 2.0                          | 0.024                                       |                         |
|                      | SA1140   | gpf       | Glycerol uptake facilitator | M               | 2.2                          | 0.014                                       |                         |
|                      | SA1206   | femA      | 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   | cr        | Glucose-specific phosphotransferase enzyme IIA component | M               | 2.2                          | 0.011                                       |                         |
|                      | SA1519   | aapA      | α-serine/α-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 ecbB | M               | 2.5                          | 0.015                                       |                         |
|                      | SA1655   | ecbA      | ABC transporter ecbA homolog | M               | 2.3                          | 0.012                                       |                         |
|                      | SA1916   |           | HP, belongs to the UPF0340 protein family | M               | 2.4                          | 0.063                                       |                         |
|                      | SA1960   | mtiF      | PTS system, mannitol specific IBC component | M               | 2.2                          | 0.040                                       | mgr down               |
|                      | SA2056   |           | HP, similar to acriflavine resistance protein | M               | 2.0                          | 0.010                                       |                         |
|                      | SA2234   | oupCD     | Probable glycine betaine/carnitine/choline ABC transporter opuCD | M               | 2.3                          | 0.025                                       |                         |
|                      | SA2324   |           | HP, similar to thioredoxin | M               | 2.2                          | 0.015                                       |                         |
|                      | SA2354   | oaaA      | O-acetyltransferase A | M               | 2.5                          | 0.012                                       |                         |
| RNA (nucleotides, nucleic acid synthesis, regulation) | SA0016   | purA      | Adenylosuccinate synthase | C               | 2.2                          | 0.019                                       | mgr down               |
|                      | SA1724   | purB      | Adenylosuccinate lyase | C               | 2.6                          | 0.004                                       | mgr up                  |
|                      | SA0133   | deoC1     | Deoxyribose-phosphate aldolase | C               | 2.8                          | 0.005                                       |                         |
|                      | SA0134   | deoB      | Phosphopentomutase | C               | 2.7                          | 0.041                                       |                         |
|                      | SA0915   | folD      | FolD bifunctional protein | C               | 6.0                          | 0.004                                       |                         |
|                      | SA0916   | purE      | Putative phosphoribosylaminomimidazole carboxylase PurE | C               | 19.4                          | 0.005                                       |                         |
|                      | SA0917   | purK      | Phosphoribosylaminomimidazole carboxylase APase subunit | C               | 22.2                          | 0.007                                       |                         |
|                      | SA0918   | purC      | Phosphoribosylaminomimidazole-succinocarboxamide synthase | C               | 14.2                          | 0.010                                       |                         |
|                      | SA0920   | purQ      | Phosphoribosylformylglycinamidine synthase PurQ | C               | 19.9                          | 0.007                                       |                         |
|                      | SA0921   | purL      | Phosphoribosylformylglycinamidine synthetase PurL | C               | 13.8                          | 0.009                                       |                         |
|                      | SA0922   | purF      | Phosphoribosylpyrophosphate amidotransferase PurF | C               | 28.7                          | 0.004                                       |                         |
|                      | SA0923   | purM      | Phosphoribosylformylglycinamidine cyclo-ligase PurM | C               | 21.6                          | 0.006                                       | rot up                 |
|                      | SA0924   | purN      | Phosphoribosylglycinamidine formyltransferase | C               | 25.5                          | 0.004                                       |                         |
|                      | SA0925   | purH      | Bifunctional purine biosynthesis protein PurH | C               | 17.7                          | 0.004                                       |                         |
|                      | SA0926   | purD      | Phosphoribosylamine-glycine ligase PurD | C               | 8.1                           | 0.004                                       |                         |
|                      | SA1172   |           | HP, similar toGMP reductase | C               | 4.3                           | 0.008                                       |                         |
|                      | SA1237   | xpoC      | HP, similar to 5-bromo-4-chloroindolyl phosphate hydrolysis protein | C               | 2.0                           | 0.008                                       |                         |
|                      | SA1914   | upp       | Uricid phosphoribosyl transferase | C               | 2.3                           | 0.014                                       |                         |
|                      | SA1938   | pdc       | Pyrimidine nucleoside phosphorylase | C               | 2.1                           | 0.010                                       |                         |
|                      | SA1939   | deoxyribose-phosphate aldolase | C               | 2.5                           | 0.014                                       |                         |
|                      | SA1098   | codY      | Transcription pleiotropic repressor CodY | C               | 2.2                           | 0.072                                       |                         |
Table 2. Continued.

| Function of Proteins | N315 ORF | N315 Gene | N315 Product | Protein Location | Change in Expression (n-Fold) | One-Sample t-Test Benjamini-Hochberg (Adv) | rot, mgrA, arlRS Effect |
|----------------------|---------|----------|--------------|-----------------|------------------------------|-------------------------------------------|-------------------------|
| Amino acid synthesis |         |          |              |                 |                              |                                           |                         |
| SA0829               | Putative bifunctional biotin ligase/biotin operon repressor | C | 2.1 | 0.004 | | |
| SA1411               | hrcA    | Heat-inducible transcriptional repressor | C | 2.2 | 0.010 | | |
| SA2410               | ndrD    | Anaerobic ribonucleoside-triphosphate reductase | C | 4.5 | 0.004 | rot up | |
| Adaptation to stress conditions |         |          |              |                 |                              |                                           |                         |
| SA0470               | hslO    | Heat-shock protein HSP33 homolog | C | 2.1 | 0.009 | | |
| SA0480               | ctsR    | Transcription repressor of class III stress genes homolog | C | 2.3 | 0.010 | | |
| SA0509               | hchA    | Molecular chaperone HchA | C | 2.5 | 0.012 | | |
| SA1096               | clpQ    | Heat shock protein HslV | C | 2.5 | 0.004 | | |
| SA1238               | HP, similar to tellurite resistance protein | C | 2.5 | 0.009 | | |
| SA1408               | dnaJ    | DnaJ protein (HSP40) | C | 2.9 | 0.006 | | |
| SA1409               | dnaK    | DnaK protein | C | 2.4 | 0.012 | | |
| SA1410               | gprE    | GrpE protein (HSP-70 cofactor HSP20) | C | 2.6 | 0.021 | | |
| SA1535               | ipx     | HP, similar to thioredoxin peroxidase | C | 2.5 | 0.021 | | |
| SA0728               | pgk     | Phosphoglycerate kinase | C | 2.2 | 0.016 | | |
| Carbohydrate mechanism |         |          |              |                 |                              |                                           |                         |
| SA0729               | tpIA    | Triosephosphate isomerase | C | 2.1 | 0.065 | | |
| SA0730               | gpmI    | 2,3-diphosphoglycerate-independent phosphoglycose 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               | sucC    | Succinyl-CoA synthetase, beta chain | C | 3.9 | 0.004 | | |
| SA1089               | sucD    | Succinyl-CoA synthetase, alpha chain | C | 3.2 | 0.004 | | |
| SA1184               | acnA    | Aconitase hydratase | C | 2.5 | 0.010 | | |
| SA1336               | Glucose-6-phosphate 1-dehydrogenase | C | 2.2 | 0.012 | | |
| SA1517               | citC    | Isocitrate dehydrogenase | C | 2.2 | 0.015 | | |
| SA1518               | citZ    | Citrate synthase II | C | 2.4 | 0.019 | | |
| SA1553               | rhs     | Formyltetrahydrofolate synthetase | C | 2.2 | 0.030 | | |
| SA1996               | lacB    | Galactose-6-phosphate isomerase LacB subunit | C | 2.4 | 0.025 | mgr, arl down | |
| SA2001               | HP, similar to oxidoreductase, ald/o ketoreductase family | C | 2.3 | 0.034 | rot up | | |
| SA2008               | aliS    | Alpha-acetolactate synthase | C | 2.9 | 0.04 | mgr, arl down | |
| SA2304               | Rp      | Fructose-bisphosphatase | C | 2.1 | 0.010 | | |
| SA2312               | dfh     | D-3-hydroxy-3-methylglutaryl-CoA reductase | C | 2.5 | 0.071 | rot, mgr down | |
| SA0822               | argG    | Arginosuccinate synthase | C | 2.0 | 0.012 | | |
| SA0829               | Putative HP, similar to 5-oxo-1,2,5-tricarbocyclic-3-penten acid decarboxylase | C | 2.1 | 0.025 | | |
| SA0859               | Thimet oligopeptidase homolog | C | 2.6 | 0.014 | | |
| SA1347               | bfmBAB  | Branched-chain alpha-keto acid dehydrogenase E1 | C | 2.1 | 0.010 | | |
| SA1365               | gevPB   | Glycine dehydrogenase (decarboxylating) subunit 2 homolog | C | 2.7 | 0.010 | | |
| SA1366               | gevPA   | Glycine dehydrogenase (decarboxylating) subunit 1 | C | 3.9 | 0.004 | | |
| SA1367               | gcvT    | Aminomethyltransferase | C | 3.6 | 0.004 | | |
| SA1915               | glyA    | Serine hydroxymethyltransferase | C | 2.3 | 0.008 | | |
| SA2081               | Urea transporter | M | 5.5 | 0.010 | mgr, arl down | | |
| SA2082               | ureA    | Urea gamma subunit | C | 13.1 | 0.009 | rot, mgr, arl down | |
| SA2083               | ureB    | Urease beta subunit | C | 32.4 | 0.006 | rot, mgr, arl down | |
| SA2084               | ureC    | Urease alpha subunit | C | 24.9 | 0.007 | rot, mgr, arl down | |
| SA2085               | ureE    | Urease accessory protein UreE | C | 16.0 | 0.089 | rot, mgr, arl down | |
| SA2086               | ureF    | Urease accessory protein UreF | C | 23.7 | 0.004 | rot, mgr, arl down | |
| SA2087               | ureG    | Urease accessory protein UreG | C | 21.4 | 0.012 | rot, mgr, arl down | |
| SA2088               | ureD    | Urease accessory protein UreD | C | 10.0 | 0.007 | rot, mgr, arl down | |
| SA2318               | sdiA    | Putative l-serine dehydratase | C | 3.7 | 0.010 | mgd down | |
| SA2319               | sdiB    | Putative beta-subunit of l-serine dehydratase | C | 2.5 | 0.071 | mgd, arl down | |
| Others (lipid synthesis, DNA repair, coenzyme) |         |          |              |                 |                              |                                           |                         |
| SA0842               | fobH    | 3-oxoacyl-(acyl-carrier protein) synthase 3 | C | 2.4 | 0.063 | | |
| SA1072               | plkX    | Fatty acid/phospholipid synthesis protein | C | 2.7 | 0.006 | | |
| SA1073               | fabD    | Malonyl CoA-acyl carrier protein transacylase | C | 2.8 | 0.011 | | |
| SA1074               | fabG    | 3-oxoacyl-reductase, (acyl-carrier protein) reductase | C | 2.7 | 0.006 | | |
| SA0484               | radA    | DNA repair protein homolog | C | 2.0 | 0.096 | | |
| SA1138               | mutL    | 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 | | |


graRS and dltA 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 oatA/graRS and oatA/dltA 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 graRS 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 graRS mutant. Comparative transcriptome analysis of SA113, an 8325-derivative, and its graRS 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 vraFG operon, which immediately follows the graRS operon. However, in studying intermediate level of vancomycin resistance in S. aureus, Ambrose Cheung and colleagues found that vraFG is positively controlled by GraRS [38]. This contradictory result can be explained by the genetic organization of our graRS::ermB deletion mutant (Figure 1). In our mutant, the ermB cassette is in the same orientation as the vraFG genes. Since the ermB transcription terminator is very weak, we assume that there is a transcriptional read-through into the vraFG genes. This explains why in our graRS deletion mutant, the vraFG 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

---

**Table 2. Continued.**

| Function of Proteins | N315 ORF | N315 Gene | N315 Product | Protein Location | Change in Expression (n-Fold) | One-Sample t-Test Benjamini–Hochberg (Adv) | rot, mgrA, arlRS Effect |
|---------------------|----------|-----------|-------------|-----------------|-------------------------------|---------------------------------|----------------------|
| SA1105              | Putative zinc metalloprotease                  | 2.1 | 0.005       |                 |                               |                                 |                      |
| SA1312              | ebpS     | Elastin binding protein                       | 2.9 | 0.038       |                               |                                 |                      |
| SA1349              | Dihydropyrimidase 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                     |                                 |                      |
| SA0481              | HP       |            |              | C               | 2.0 | 0.008       |                                 |                      |
| SA0558              | HP       |            |              | 2.1             | 0.004                     |                                 |                      |
| SA0804              | HP       |            |              | 4.8             | 0.004                     |                                 |                      |
| SA0805              | HP       |            |              | 2.4             | 0.007                     |                                 |                      |
| SA0832              | 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                     |                                 |                      |

^vraFG were down-regulated instead of up-regulated because of transcriptional read-through into the vraFG genes by the very weak ermB transcription terminator.

C, cytoplasm; HP, hypothetical protein; M, membrane; S, secreted; SCW, secreted cell wall-bound.

---

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

| Strains | RT-PCR rot (%) | RT-PCR ureC (%) | RT-PCR dltA (%) | α-Alanylation (%) |
|---------|----------------|-----------------|-----------------|------------------|
| SA113   | 100            | 1.4             | 100             | 100              |
| graRS::ermB | 8               | 100             | 13              | 53.3*            |
| dltA::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.

10.1371/journal.ppat.0030102.t003

10.1371/journal.ppat.0030102.t002

doi:10.1371/journal.ppat.0030102.t002

doi:10.1371/journal.ppat.0030102.t003

doi:10.1371/journal.ppat.0030102.t003

doi:10.1371/journal.ppat.0030102.t003

doi:10.1371/journal.ppat.0030102.t003

---

PLoS Pathogens | www.plospathogens.org July 2007 | Volume 3 | Issue 7 | e1020990

Lysozyme Resistance in Staphylococcus aureus
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, smpC, 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

Table 4. MIC Values of SA113 and Various Mutants

| Strains            | Lysozyme | Polymyxin B | Gallidermin |
|--------------------|----------|-------------|-------------|
| SA113              | >50,000  | 3,470       | 350         | 252.5     | 9 | 4.1 |
| graRS::erm         | >50,000  | 3,470       | 20          | 14.4      | 2.5 | 1.14 |
| oatA-kan           | 10,000   | 694         | 350         | 252.5     | 9 | 4.1 |
| oatA-kan::graRS::erm | 600     | 41.6        | 25          | 18        | 3 | 1.36 |
| dttA::spc          | 2,000    | 138.8       | 10          | 7.2       | 1.2 | 0.55 |
| oatA-kan::dttA::spc | 30      | 2.08        | 10          | 7.2       | 1.2 | 0.55 |
| graRS::erm (pTXgraRS::erm) | >50,000 | 3,470       | >800        | 577.2     | 9 | 4.1 |
| SA113 (pTXgraRS)   | >50,000  | 3,470       | >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 inducer. doi:10.1371/journal.ppat.0030102.t004

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

(A) graRS 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) graRS mutant: control (○); polymyxin B (PMB) (20 μg/ml) (●).

(C) graRS subculture of 5B: control (○); PMB (20 μg/ml) (●).

Cells were grown in BM at 37 °C. OD578nm was measured hourly for the first 8 h and after 24 h. Cationic agents were added in the exponential growth phase at OD578nm 1.0 as indicated by arrow. doi:10.1371/journal.ppat.0030102.g005

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. doi:10.1371/journal.ppat.0030102.g006
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. lrQA, which encodes a holin-like protein with murein hydrolyase activity, is also down-regulated by GraRS but up-regulated by ArcRS and MgrA. Most of the genes are exclusively regulated by GraRS, such as ica, pur, mga, sirA,C, atlA, aaa, dnaJ/K, rpfE, and vreF.G. These results illustrate that there is a distinct cross-regulation between GraRS, ArcRS, Rot, MgrA, and probably some other global regulators.

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, hlgAB, lukMF, geh). GraRS is possibly involved in the establishment of persistent infections by the up-regulation of colonization factors (e.g., ica, atl, aaa, fih, sirA, sirC, sdrC, sdrE), factors involved in resistance to CAMPs (dlt), factors involved in intermediary vancomycin resistance (vreF.G, as mentioned above), and factors involved in biofilm formation (e.g., dlt, atl, ica). It would be interesting to study the graRS 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, http://www.invitrogen.com/), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, 0.1% K2HPO4, 0.1% glucose, or 0.5% xylose. Bacteria were grown in BM supplemented with 0.5% yeast extract (Gibco BRL), 0.5% NaCl, 0.1% K2HPO4, 0.1% glucose, or 0.5% xylose. The cultures were incubated at 37°C, and 100-ml flask when the cultures reached an OD578nm of nearly 1.0. The MIC assay. The overnight cultures were diluted in BM with 0.5% xylose to a concentration of 0.5 × 108 CFU/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 with 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% safranin.

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 twice 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. The supernatant was removed and the plate was washed two times with PBS (pH 6.8) and 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 Pshigolized.

Turbidometric assay of PG. For analyzing the susceptibility of PG to lysozyme, we used a modified method turbidometric assay as described by Clarke [32]. The MIC 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 OD600nm and calculated as percentages.

Table 1. List of Strains and Plasmids

| Strain or Plasmid | Comment | Reference or Source |
|------------------|---------|---------------------|
| S. aureus RN4220 | Mutant strain of B325-4, accept foreign DNA | Kreiswirth [46] |
| S. aureus SA113 | Mutant strain of B325, with an agr background and 11-bp deletion in rsB | Bera [10] |
| S. aureus SA113AaTA | Mutant of SA113 (AaTA-kan) | Bera [10] |
| S. aureus SA113AdIA | Mutant of SA113 (AdIA-spcl) | Peschel [26] |
| S. aureus SA113AgraRS | Mutant of SA113 (AgraRS:erm) | This study |
| S. aureus SA113AgraRS | otaA and AgraRS double mutant of SA113 (AotaA-kan/AgraRS:erm) | This study |
| S. aureus SA113AdIA | AgraRS:erm and pTX15 containing lysose inducible AgraRS genes | This study |
| S. aureus SA113AdIA | SA113 and pTX15 containing lysose inducible AgraRS genes | This study |
| S. carnosus TM300 | Host strain for cloning vector pTX15 | Gotz [48] |
| Escherichia coli DH5α | Host strain for cloning vector pBT2 | Hanahan [49] |
| pBT2 | Temperature-sensitive E. coli–S. aureus shuttle vector | Brückner [50] |
| pBTugraRS | pBT2 containing up- and downstream region of AgraRS and ernB cassette | This study |
| pTX15 | Xylose-inducing vector for complementation | Peschel [51] |
| pTugraRS | pTX15 containing xylose-inducible AgraRS genes | This study |
| pTVtis | Vector for transposon (Tn917) transposition | Youngman [52] |

Lysozyme Resistance in Staphylococcus aureus

The presence of GraRS genes in the strain 8325 indicated that GraRS is possibly involved in the regulation of the expression of colonization factors (e.g., ica, atl, aaa, fib, sirA, sirC, sdrC, sdrE), factors involved in resistance to CAMPs (dlt), factors involved in intermediary vancomycin resistance (vreF.G, as mentioned above), and factors involved in biofilm formation (e.g., dlt, atl, ica). It would be interesting to study the graRS mutant in an animal model for chronic infection.

Table 5. List of Strains and Plasmids

| Strain or Plasmid | Comment | Reference or Source |
|------------------|---------|---------------------|
| S. aureus RN4220 | Mutant strain of B325-4, accept foreign DNA | Kreiswirth [46] |
| S. aureus SA113 | Mutant strain of B325, with an agr background and 11-bp deletion in rsB | Bera [10] |
| S. aureus SA113AaTA | Mutant of SA113 (AaTA-kan) | Bera [10] |
| S. aureus SA113AdIA | Mutant of SA113 (AdIA-spcl) | Peschel [26] |
| S. aureus SA113AgraRS | Mutant of SA113 (AgraRS:erm) | This study |
| S. aureus SA113AgraRS | otaA and AgraRS double mutant of SA113 (AotaA-kan/AgraRS:erm) | This study |
| S. aureus SA113AdIA | AgraRS:erm and pTX15 containing lysose inducible AgraRS genes | This study |
| S. aureus SA113AdIA | SA113 and pTX15 containing lysose inducible AgraRS genes | This study |
| S. carnosus TM300 | Host strain for cloning vector pTX15 | Gotz [48] |
| Escherichia coli DH5α | Host strain for cloning vector pBT2 | Hanahan [49] |
| pBT2 | Temperature-sensitive E. coli–S. aureus shuttle vector | Brückner [50] |
| pBTugraRS | pBT2 containing up- and downstream region of AgraRS and ernB cassette | This study |
| pTX15 | Xylose-inducing vector for complementation | Peschel [51] |
| pTugraRS | pTX15 containing xylose-inducible AgraRS genes | This study |
| pTVtis | Vector for transposon (Tn917) transposition | Youngman [52] |
Quantification of α-analalytic activity 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 OD600nm 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 Marfrey’s reagent (1-fluoro-2,4-dinitrophenyl-5-carboxylamide; Sigma) (10 mM) were added. After incubation at 40 °C for 1 h, samples were dried and resuspended in DMSO:H2O (1:1). Quantification of α-analalytic activity was performed by HPLC as previously described [43].

RNA isolation and real-time RT-PCR. SA113 and the graRS deletion mutant were cultured in 50 ml of BM and harvested at mid-exponential phase. Before RNA isolation, two volumes of RNAprotect bacteria reagent (Qiagen, http://www.qiagen.com/) were added to 10 ml of culture and centrifuged. The cells were lysed by the addition of 50 µg/ml of lysozymin (0.5 mg/ml) (Gennicmedics) in TE buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Contaminating DNA was degraded with the DNase Kit (Ambion, http://www.ambion.com) according to the manufacturer’s instructions. LightCycler RNA-PCR was carried out using the LightCycler RNA amplification Kit SYBR Green I (Roche Biochemicals). In none of the cases an additional control for DNA contamination, each sample was subjected to PCR by using the LightCycler DNA amplification kit for hybridization probes (Roche Biochemicals, Kit SYBR Green I or with the LightCycler Cycler RNA amplification Kit SYBR Green I). Contaminating DNA was degraded with the DNase Kit (Qiagen). Samples were then dried and resuspended in DMSO:H2O (1:1). Quantification of α-analalytic activity was performed by HPLC as previously described [43].

RNA isolation and real-time RT-PCR. SA113 and the graRS deletion mutant were cultured in 50 ml of BM and harvested at mid-exponential phase. Before RNA isolation, two volumes of RNAprotect bacteria reagent (Qiagen, http://www.qiagen.com/) were added to 10 ml of culture and centrifuged. The cells were lysed by the addition of 50 µg/ml of lysozymin (0.5 mg/ml) (Gennicmedics) in TE buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Contaminating DNA was degraded with the DNase Kit (Ambion, http://www.ambion.com) according to the manufacturer’s instructions. LightCycler RNA-PCR was carried out using the LightCycler RNA amplification Kit SYBR Green I (Roche Biochemicals). In none of the cases an additional control for DNA contamination, each sample was subjected to PCR by using the LightCycler DNA amplification kit for hybridization probes (Roche Biochemicals, Kit SYBR Green I or with the LightCycler Cycler RNA amplification Kit SYBR Green I). Contaminating DNA was degraded with the DNase Kit (Qiagen). Samples were then dried and resuspended in DMSO:H2O (1:1). Quantification of α-analalytic activity was performed by HPLC as previously described [43].

**References**

1. Jolles P, Jolles J (1984) What’s new in lysozyme research? Always a model system, today as yesterday. Mol Cell Biochem 63: 165–189.
2. Levy O (2000) Antimicrobial proteins and peptides of blood: Templates for novel antimicrobial agents. Blood 96: 2654–2672.
3. Phillips D (1966) The three-dimensional structure of an enzyme molecule. Sci Am 215: 78–90.
4. Schindler M, Assaf Y, Sharon N, Chipman DM (1977) Mechanism of lysozyme catalysis: Role of ground-state strain in subsite D in hen egg-white lysozyme catalysis: Role of ground-state strain in subsite D in hen egg-white lysozyme catalysis: Role of ground-state strain in subsite D in hen egg-white lysozyme catalysis: Role of ground-state strain in subsite D in hen egg-white lysozyme. Biochemistry 16: 423–431.
5. Blake CC, Johnson LN, Mair GA, North AC, Phillips DC, et al. (1967) Crystallographic studies of the activity of hen egg-white lysozyme. Proc R Soc London B Biol Sci 167: 378–388.
6. Blundell J, Smith GJ, Perkins HR (1980) The peptidoglycan of the cell wall of Proteus mirabilis. Eur J Biochem 95: 487–495.
7. Chen AJ, Dupont C (1991) O-acetylated peptidoglycan: Its occurrence, pathobiological significance, and biosynthesis. Can J Microbiol 38: 85–91.
8. Bera A, Herbert S, Jakob A, Vollmer W, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltansferase OaTα is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778–787.
9. Bera A, Biswas R, Herbert S, Götz F (2006) The presence of peptidoglycan O-acetyltansferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. Infect Immun 74: 4598–4604.
10. Bera A, Götz F (1997) Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis. Inhibition by chitin oligosaccharides. Infect Immun 48: 720–728.
11. Bera A, Herbert S, Götz F (2006) The presence of peptidoglycan O-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. Infect Immun 74: 4598–4604.
12. Bera A, Herbert S, Jakob A, Vollmer W, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltansferase OaTα is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778–787.
13. Bera A, Biswas R, Herbert S, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltansferase OaTα is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778–787.
14. Bera A, Herbert S, Jakob A, Vollmer W, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltansferase OaTα is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778–787.
15. Bera A, Herbert S, Jakob A, Vollmer W, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltansferase OaTα is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778–787.
25. Heilmann C, Hussain M, Peters G, Götz F (1997) Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. Mol Microbiol 24: 1013–1024.

26. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, et al. (1999) Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274: 8405–8410.

27. Bierbaum G, Sahl HG (1987) Autolytic system of Staphylococcus simulans 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. J Bacteriol 169: 5452–5458.

28. Kawata S, Takemura T, Yokogawa K (1983) Characterization of two N-acetylmuramidases from Streptomyces globisporus 1829. Agric Biol Chem 48: 261–269.

29. Endl J, Seidl HP, Fiedler F, Schleifer KH (1983) Chemical composition and structure of cell wall teichoic acids of staphylococci. Arch Microbiol 135: 215–223.

30. Weidenmaier C, Kokai-Kun JF, Kristian SA, Chanturiya T, Kalbacher H, et al. (2004) Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections. Nat Med 10: 243–245.

31. Ginsburg I (2001) Bacterial cationic peptides can also function as bacteriolysis-inducing agents mimicking beta-lactam antibiotics? It is enigmatic why this concept is consistently disregarded. Med Hypotheses 62: 367–374.

32. Ginsburg I (2001) Cationic peptides from leukocytes might kill bacteria by activating their autolytic enzymes causing bacteriolysis: Why are publications proposing this concept never acknowledged? Blood 97: 2530–2531.

33. Wecke J, Lahav M, Ginsburg I, Giesbrecht P (1982) Cell wall degradation of Strepomyces globisporus amidase. J Bacteriol 169: 5452–5458.

34. Clarke AJ (1993) Extent of peptidoglycan substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of Staphylococcus aureus, Staphylococcus carnosus and Staphylococcus simulans. J Bacteriol 175: 4550–4555.

35. Cleveland RF, Wicken AJ, Daneo-Moore L, Shockman GD (1976) Inhibition of wall autolysis in Streptococcus faecalis by lysozyme. Arch Microbiol 131: 116–123.

36. Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: Structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67: 686–723.

37. Steen A, Palumbo E, Dегhorain M, Cocconcelli PS, Delcour J, et al. (2005) Autolysis of Lactococcus lactis is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids. J Bacteriol 187: 114–124.

38. Meehl M, Herbert S, Götz F, Cheung A (2007) Interaction of the GraRS two-component system with the VraFG ABC-transporter to support vancomycin-intermediate resistance in Staphylococcus aureus. Antimicrob Agents Chemother 51: E-pub 14 May 2007.

39. Said-Salim B, Dunman PM, McAleese FM, Macapagal D, Murphy E, et al. (2005) Global regulation of Staphylococcus aureus genes by Rot. J Bacteriol 187: 610–619.

40. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY (2006) Transcription profiling of the mrgA regulon in Staphylococcus aureus. J Bacteriol 188: 1899–1910.

41. Liang X, Zheng L, Landwehr C, Lundford D, Holmes D, et al. (2005) Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of Staphylococcus aureus. J Bacteriol 187: 5486–5492.

42. Clarke AJ (1993) Extent of peptidoglycan O acetylation in the tribe Proteaceae. J Bacteriol 175: 4550–4555.

43. Kovacs M, Halfmann A, Fredike I, Heintz M, Peschel A, et al. (2006) A functional dlt operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in Streptococcus pneumoniae. J Bacteriol 188: 5797–5805.

44. Goerke C, Fluckiger U, Steinhuber A, Bisanzio V, Ulrich M, et al. (2005) Role of Staphylococcus aureus global regulators sae and sigmaB in virulence gene expression during device-related infection. Infect Immun 73: 3415–3421.

45. Resch A, Fehrenbacher B, Eisele K, Schaller M, Götz F (2005) Phage release from biofilm and planktonic Staphylococcus aureus cells. FEMS Microbiol Lett 252: 89–96.

46. Kreiswirth BN, Lofdahl S, Betley MJ, O’Reilly M, Schlievert PM, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305: 709–712.

47. Jorandlesu S, Sundeau M (1976) Two restriction and modification systems in Staphylococcus aureus NCTC8325. J Gen Microbiol 96: 277–281.

48. Götz F (1990) Staphylococcus carnosus: A new host organism for gene cloning and protein production. J Appl Bacteriol Supp. 69: 49–53.

49. Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166: 557–580.

50. Brückner R (1997) Gene replacement in Staphylococcus carnosus and Staphylococcus xylosus. FEMS Microbiol Lett 151: 1–8.

51. Peschel A, Ottenwaelder B, Götz F (1996) Inducible production and cellular location of the epidermin biosynthetic enzyme EpiB using an improved staphylococcal expression system. FEMS Microbiol Lett 137: 225–230.

52. Said-Salim B, Dunman PM, McAleese FM, Macapagal D, Murphy E, et al. (2005) Global regulation of Staphylococcus aureus genes by Rot. J Bacteriol 187: 610–619.

53. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY (2006) Transcription profiling of the mrgA regulon in Staphylococcus aureus. J Bacteriol 188: 1899–1910.

54. Liang X, Zheng L, Landwehr C, Lundford D, Holmes D, et al. (2005) Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of Staphylococcus aureus. J Bacteriol 187: 5486–5492.

55. Clarke AJ (1993) Extent of peptidoglycan O acetylation in the tribe Proteaceae. J Bacteriol 175: 4550–4555.

56. Kovacs M, Halfmann A, Fredike I, Heintz M, Peschel A, et al. (2006) A functional dlt operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in Streptococcus pneumoniae. J Bacteriol 188: 5797–5805.

57. Goerke C, Fluckiger U, Steinhuber A, Bisanzio V, Ulrich M, et al. (2005) Role of Staphylococcus aureus global regulators sae and sigmaB in virulence gene expression during device-related infection. Infect Immun 73: 3415–3421.

58. Resch A, Fehrenbacher B, Eisele K, Schaller M, Götz F (2005) Phage release from biofilm and planktonic Staphylococcus aureus cells. FEMS Microbiol Lett 252: 89–96.

59. Kreiswirth BN, Lofdahl S, Betley MJ, O’Reilly M, Schlievert PM, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305: 709–712.

60. Jorandlesu S, Sundeau M (1976) Two restriction and modification systems in Staphylococcus aureus NCTC8325. J Gen Microbiol 96: 277–281.

61. Götz F (1990) Staphylococcus carnosus: A new host organism for gene cloning and protein production. J Appl Bacteriol Supp. 69: 49–53.

62. Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166: 557–580.

63. Brückner R (1997) Gene replacement in Staphylococcus carnosus and Staphylococcus xylosus. FEMS Microbiol Lett 151: 1–8.

64. Peschel A, Ottenwaelder B, Götz F (1996) Inducible production and cellular location of the epidermin biosynthetic enzyme EpiB using an improved staphylococcal expression system. FEMS Microbiol Lett 137: 225–230.

65. Youngman P, Poth H, Green B, York K, Olmedo G, et al. (2009) Methods for genetic manipulation, cloning and functional analysis of sporulation genes in Bacillus subtilis. In: Smith I, Stepecky RA, Setlow P, editors. Regulation of procaryotic development. Washington (D.C.): American Society for Microbiology. pp. 65–69.