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

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It has been shown recently that modification of peptidoglycan by O-acetylation renders pathogenic staphylococci resistant to the muramidase activity of lysozyme. Here, we show that a *Staphylococcus aureus* double mutant defective in O-acetyltransferase A (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 D-alanyl groups in teichoic acids. The *oatA/dltA* double mutant showed the highest sensitivity to lysozyme; this mutant completely lacks teichoic acid-bound D-alanine esters, which are responsible for the increased susceptibility to CAMPs and peptidoglycan O-acetylation. Our results demonstrate that resistance to lysozyme can be dissected into genes mediating resistance to its muramidase activity (*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.

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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-acetylglucosamine (GlucNAc), resulting in degradation of peptidoglycan (PG), and subsequent cell lysis [3,4]. Most bacterial species are sensitive to lysozyme, but some important human pathogens, such as *Staphylococcus aureus*, *Neisseria gonorrhoeae*, and *Proteus mirabilis*, are resistant. The mechanisms behind the high resistance of *S. aureus* to lysozyme are unknown, although several studies suggest that O-acetylation at position C-6 of the MurNAc residue contributes to lysozyme resistance [5–9]. Recently, we were able to prove that indeed O-acetyltransferase A (OatA) of *S. aureus* is responsible for O-acetylation of the PG, and this leads to resistance to the muramidase activity of lysozyme [10]. We also showed that the MurNAc was O-acetylated only in pathogenic, lysozyme-resistant staphylococci (e.g., *S. aureus*, *S. epidermidis*, *S. lugdunensis*, and others). All nonpathogenic species (e.g., *S. carnosus*, *S. gallinarum*, or *S. xylosus*) were lysozyme sensitive and lacked PG-specific O-acetylation. Therefore, OatA can be regarded as a general virulence factor [11].

Although the *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

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

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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 Δ -alanine esterification (Dlt) renders the cells resistant to lysozyme's CAMPs and other CAMPs. Transcriptome analysis of the glycopeptide resistance-associated (GraRS) two-component system revealed that this global regulator controls 248 genes such as other global regulators, colonization factors, or exotoxin-encoding genes. Since GraRS also upregulates the *dlt* operon, it was not surprising that in the *graRS* mutant teichoic acid Δ -alaninylation 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

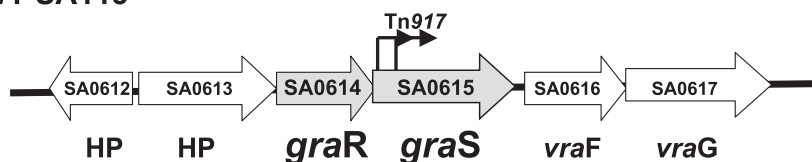
Susceptibility of *oatA* and *graRS* Single and Double Mutants to Lysozyme and CAMPs

In our search for highly susceptible lysozyme mutants in *S. aureus*, we isolated two Tn917 transposon mutants in SA113*oatA::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 (glycopeptide resistance-associated), because overexpression of GraR (response regulator) and GraS (sensor histidine kinase) increased vancomycin resistance [19]. To further study the role of TCS in lysozyme resistance, we constructed a deletion mutant by substituting *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 pTX*graRS*, a vector in which the *graRS* genes are induced into expression by xylose, confirmed the correct replacement. Whereas the *oatA/graRS* 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 *oatA/graRS* double mutant was much more lysozyme sensitive than each of the single mutants. This hypersensitivity of the double mutant can be explained by dual activities of lysozyme that act in a synergistic way. To study this phenotype in more detail, we investigated whether the *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).

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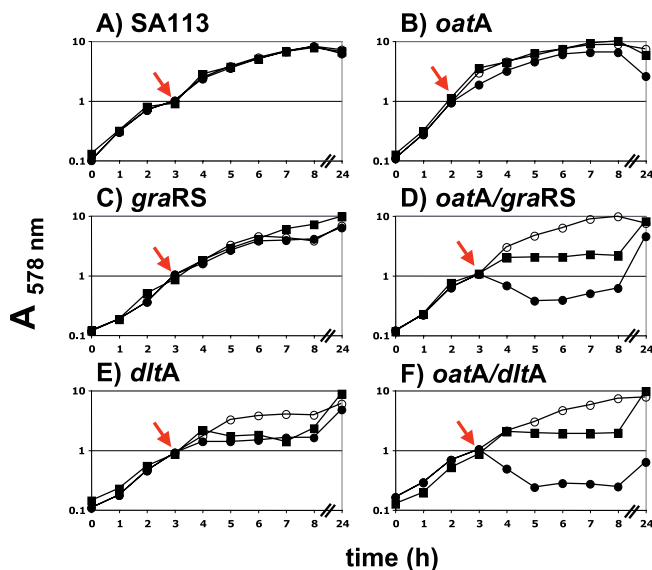


Figure 2. Susceptibility of WT SA113 and Various *S. aureus* Mutants to Lysozyme and Heat-Inactivated Lysozyme

Cells were grown in BM at 37 °C. OD_{578nm} was measured hourly for the first 8 h and after 24 h. Lys was added in the exponential growth phase at OD_{578nm} 1.0 as indicated by arrow. Catalytic inactive Lys was heated for 1 h at 100 °C.

(A) WT SA113: control (○); Lys (300 µg/ml [20.8 µM]) (●); heat-inactivated lysozyme (Lys) (300 µg/ml [20.8 µM]) (■).

(B) *oatA* mutant: control (○); Lys (300 µg/ml) (●); heat-inactivated Lys (300 µg/ml) (■).

(C) *grarS* mutant: control (○); Lys (300 µg/ml) (●); heat-inactivated Lys (300 µg/ml) (■).

(D) *oatA/grarS* mutant: control (○); Lys (50 µg/ml [3.47 µM]) (●); heat-inactivated Lys (300 µg/ml) (■).

(E) *dltA* mutant: control (○); Lys (300 µg/ml) (●); heat-inactivated Lys (300 µg/ml) (■).

(F) *oatA/dltA* mutant: control (○); Lys (20 µg/ml [1.39 µM]) (●); heat-inactivated Lys (300 µg/ml) (■).

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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

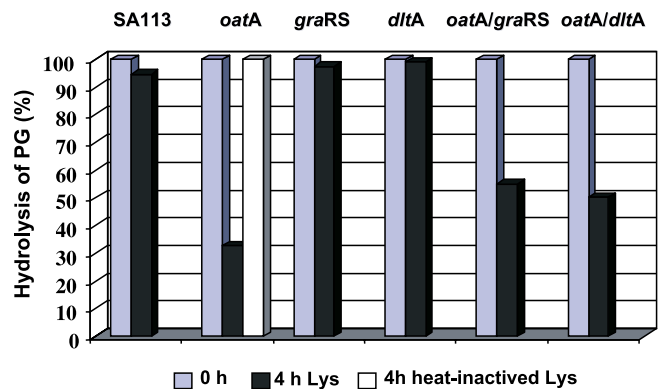


Figure 3. Susceptibility of PG Isolated from WT and Various Mutants to Hydrolysis by Lysozyme

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

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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*, *mgaA*), cell surface protein encoding genes (the Ser-Asp rich fibrinogen-binding proteins SdrC and SdrE), the major autolysin gene (*atlA*) and an autolysin/adhesin gene (*aaa*) [20], exoprotein encoding genes (*hly*, *hlyA*, *hlyB*, *hlyC*, *hlyD*, *hlyE*, *hlyF*, *hlyG*, *hlyH*, *hlyI*, *hlyJ*, *hlyK*, *hlyL*, *hlyM*, *hlyN*, *hlyO*, *hlyP*, *hlyQ*, *hlyR*, *hlyS*, *hlyT*, *hlyU*, *hlyV*, *hlyW*, *hlyX*, *hlyY*, *hlyZ*, *hlyA*, *hlyB*, *hlyC*, *hlyD*, *hlyE*, *hlyF*, *hlyG*, *hlyH*, *hlyI*, *hlyJ*, *hlyK*, *hlyL*, *hlyM*, *hlyN*, *hlyO*, *hlyP*, *hlyQ*, *hlyR*, *hlyS*, *hlyT*, *hlyU*, *hlyV*, *hlyW*, *hlyX*, *hlyY*, *hlyZ*), transporter encoding genes (*essA*, *essB*, *essC*, *essD*, *essE*, *essF*, *essG*, *essH*, *essI*, *essJ*, *essK*, *essL*, *essM*, *essN*, *essO*, *essP*, *essQ*, *essR*, *essS*, *essT*, *essU*, *essV*, *essW*, *essX*, *essY*, *essZ*), capsule encoding genes (*capA*, *capB*, *capC*, *capD*, *capE*, *capF*, *capG*, *capH*, *capI*, *capJ*, *capK*, *capL*, *capM*, *capN*, *capO*, *capP*, *capQ*, *capR*, *capS*, *capT*, *capU*, *capV*, *capW*, *capX*, *capY*, *capZ*), genes responsible for D-alanyl esterification of teichoic acids (TAs) (*dltA*, *dltB*, *dltC*, *dltD*, *dltE*, *dltF*, *dltG*, *dltH*, *dltI*, *dltJ*, *dltK*, *dltL*, *dltM*, *dltN*, *dltO*, *dltP*, *dltQ*, *dltR*, *dltS*, *dltT*, *dltU*, *dltV*, *dltW*, *dltX*, *dltY*, *dltZ*), and the alanine dehydrogenase gene (*ald1*). 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*, *ureC*, 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 D-alanylation of TAs). Its transcript was decreased 2.1-fold to 2.9-fold as compared to WT, and indeed, the D-alanylation of TAs was decreased 46.7% in the *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 *ald1* transcription by a factor of 3.5 is in line with the decreased *dlt* transcription. Ald1 is the alanine dehydrogenase, which is involved in the synthesis of L-alanine.

Comparison of *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

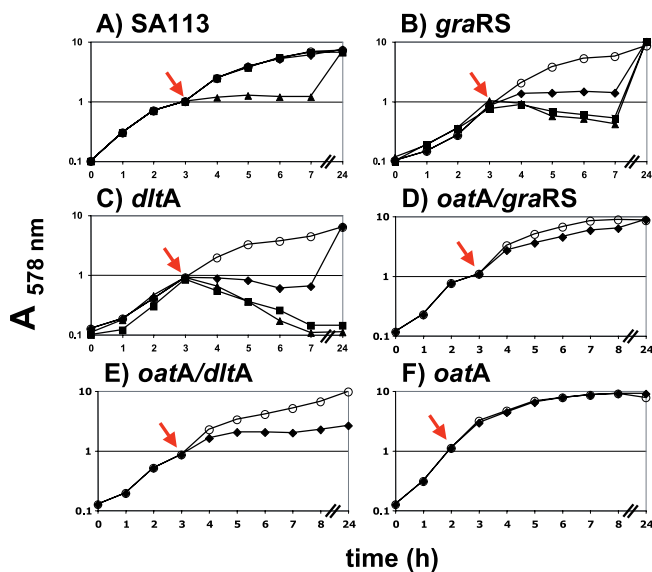


Figure 4. Susceptibility of WT and Various *S. aureus* Mutants to CAMPs (A) WT SA113: control (○); LP9 (200 μg/ml [164.9 μM]) (◆); polymyxin B (PMB) (20 μg/ml [14.4 μM]) (■); and gallidermin (Gdm) (8 μg/ml [3.64 μM]) (▲). (B) *graRS* mutant: control (○); LP9 (200 μg/ml) (◆); PMB (20 μg/ml) (■); and Gdm (8 μg/ml) (▲). (C) *dltA* mutant: control (○); LP9 (200 μg/ml) (◆); PMB (20 μg/ml) (■); and Gdm (8 μg/ml) (▲). (D) *oatA/graRS* mutant: control (○); LP9 (200 μg/ml) (◆). (E) *oatA/dltA* mutant: control (○); LP9 (200 μg/ml) (◆). (F) *oatA* mutant: control (○); LP9 (200 μg/ml) (◆). Cells were grown in BM at 37 °C. OD_{578nm} was measured hourly for the first 7–8 h and after 24 h. CAMPs were added in the exponential growth phase at OD_{578nm} 1.0 as indicated by arrow. doi:10.1371/journal.ppat.0030102.g004

CAMPs [26]. Indeed, the *dltA* mutant was more sensitive to lysozyme (Figure 2E); however, this sensitivity was not due to its muramidase activity, as the isolated PG of the *dltA* mutant was not hydrolyzed by lysozyme (Figure 3). Furthermore, growth of the *dltA* 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 *dltA* 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 *dltA* 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 *oatA/graRS* 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% D-alanyl esters, whereas the *dltA* mutant completely lacks D-alanylation 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

Table 1. 115 *S. aureus* SA113 Genes Up-Regulated by GraRS

Function of Proteins	N315 ORF	N315 Gene	N315 Product	Protein Location	Change in Expression (n-Fold)	One-Sample t-Test-Benjamini-Hochberg (Adv)	<i>rot</i> , <i>mgrA</i> , <i>arlRS</i> Effect
Virulence factors (cell surface proteins, exotoxins, colonization factors)	SA0519	<i>sdrC</i>	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	SCW	9.6	0.013	<i>rot</i> , <i>arl</i> up
	SA0521	<i>sdrE</i>	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	SCW	2.9	0.034	<i>mgr</i> , <i>arl</i> up
	SA1003	<i>fib</i>	HP, similar to fibrinogen-binding protein	SCW	2.5	0.036	
	SA1004		HP, similar to fibrinogen-binding protein	SCW	2.2	0.164	<i>mgr</i> down
	SA0222	<i>coa</i>	Staphylocoagulase precursor	S	2.1	0.013	<i>rot</i> up
	SA0309	<i>geh</i>	Glycerol ester hydrolase, lipase 2	S	2.2	0.012	<i>rot</i> down
	SA1811	<i>hlyB</i>	Truncated beta-hemolysin	S	2.2	0.007	<i>rot</i> down
	SA1812	<i>lukM</i>	Leukocidin chainLukM	S	2.5	0.042	<i>rot</i> down, <i>mgr</i> up
	SA1813	<i>lukF</i>	Synergohymenotropic toxin precursor	S	2.1	0.010	<i>mgr</i> up
	SA2207	<i>hlgA</i>	Gamma-hemolysin component A	S	2.1	0.010	<i>mgr</i> up
	SA2209	<i>hlgB</i>	Gamma-hemolysin component B	S	2.1	0.005	<i>rot</i> , <i>arl</i> down
	SA0270		HP, similar to secretory antigen precursor SsaA	S	2.9	0.012	<i>arl</i> up
	SA0271	<i>esxA</i>	Virulence factor EsxA	S	4.4	0.012	<i>rot</i> , <i>arl</i> up
	SA0620		HP, similar to secretory antigen precursor SsaA	S	2.1	0.013	<i>rot</i> up
	SA2097		HP, similar to secretory antigen precursor SsaA	S	3.0	0.014	
	SA2431	<i>isaB</i>	Immunodominant antigen B	S	2.2	0.022	<i>mgr</i> down
	SA2459	<i>icaA</i>	Intercellular adhesion protein A	M	4.8	0.032	
	SA2460	<i>icaD</i>	Intercellular adhesion protein D	M	4.9	0.006	
	SA2461	<i>icaB</i>	Intercellular adhesion protein B	SCW	3.3	0.063	
	SA2462	<i>icaC</i>	Intercellular adhesion protein C	M	2.5	0.068	
Regulators	SA0108	<i>sarS</i>	Staphylococcal accessory regulator A homolog	C	7.2	0.007	<i>rot</i> up, <i>mgr</i> down
	SA0614	<i>graR</i>		C	3.1	0.062	
	SA0641	<i>mgrA</i>	HTH-type transcriptional regulator MgrA, MarR family	C	3.1	0.007	
	SA0856	<i>spxA</i>	Transcriptional regulator Spx	C	5.6	0.024	
	SA1583	<i>rot</i>	Repressor of toxins Rot	C	3.8	0.009	<i>arl</i> up
	SA1678	<i>furB</i>	Transcriptional regulator Fur family homolog	C	2.1	0.023	<i>mgr</i> up
	SA2174		HP, similar to transcriptional regulator	C	2.0	0.046	
Cell wall (cellular processes, transport, membrane, lipoproteins, autolysins)	SA0106	<i>lctP</i>	Lactate transporter, LctP family	M	3.2	0.021	
	SA0109	<i>sirC</i>	Lipoprotein	M	3.5	0.013	
	SA0111	<i>sirA</i>	Lipoprotein	M	2.0	0.086	
	SA0138		HP, similar to alkylphosphonate ABC transporter	M	2.1	0.004	<i>arl</i> down
	SA0204	<i>azoR</i>	FMN-dependent NADH-azoreductase	C	2.2	0.021	
	SA0207		HP, similar to maltose/maltodextrin-binding protein	M	2.1	0.035	
	SA0208		Maltose/maltodextrin transport permease homolog	M	2.1	0.064	
	SA0268		HP, similar to ABC transporter system permease protein	M	2.3	0.007	
	SA0272	<i>esaA</i>	Protein EsaA	M	4.7	0.022	<i>arl</i> up
	SA0273	<i>essA</i>	Protein EssA	M	2.8	0.071	
	SA0276	<i>essC</i>	Protein EssC	M	2.6	0.027	<i>arl</i> up
	SA0295		HP, similar to outer membraneprotein precursor	M	2.3	0.010	
	SA0423	<i>aaa</i>	N-acetylmuramoyl-L-alanine amidase	SCW	3.6	0.015	
	SA0518	<i>azo1</i>	FMN-dependent NADPH-azoreductase	C	2.1	0.019	
	SA0793	<i>dltA</i>	D-alanine-D-alanyl carrier protein ligase	C	2.9	0.066	
	SA0794	<i>dltB</i>	DltB membrane protein	M	2.1	0.010	
	SA0796	<i>dltD</i>	Poly (glycerophosphate chain) D-alanine transfer protein	M	2.3	0.008	<i>rot</i> up
	SA0845	<i>oppB</i>	Oligopeptide transport system permease protein	M	2.2	0.039	
	SA0846		HP, similar to oligopeptide transport system permease protein OppC	M	2.1	0.049	
	SA0849		HP, similar to peptide binding protein OppA	M	2.4	0.030	<i>mgr</i> down
	SA0854		HP, similar to oligopeptide transport system permease protein OppC	M	2.1	0.053	
	SA0905	<i>atlA</i>	Autolysin (N-acetylmuramoyl-L-alanine amidase/endo-beta-N-acetylglucosaminidase	SCW	2.4	0.015	
	SA1269	<i>norB</i>	Blt-like protein, efflux pump	M	3.6	0.004	<i>mgr</i> , <i>arl</i> up
	SA1270		HP, similar to acid permease	M	3.5	0.012	<i>mgr</i> , <i>arl</i> up
	SA1663		HP, belongs to the UPF0342 protein family	M	2.3	0.072	
	SA1979		HP, similar to ferrichrome ABC transporter	M	2.1	0.032	

Table 1. 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
	SA2156		L-lactate permease lctP homolog	M	3.7	0.015	
	SA2217		HP, similar to lipoprotein inner membrane ABC transporter	M	2.1	0.022	
	SA2302	<i>stpC</i>	HP, similar to ABC transporter	M	3.1	0.008	<i>mgr</i> up
	SA2303	<i>smpC</i>	HP, similar to membrane spanning protein	M	5.5	0.010	<i>rot, mgr, arl</i> up
	SA2475		HP, belongs to ABC-transporter	M	2.3	0.067	
	SA2477		HP, belongs to the UPF0397 protein family	M	2.3	0.068	
	SA2480	<i>drp35</i>	Lactonase Drp35	C	2.3	0.036	
Adaptation to stress conditions	SA2457	<i>capA</i>	Capsular polysaccharide biosynthesis, capA	M	2.0	0.008	<i>arl</i> down
	SA0151	<i>capH</i>	Capsular polysaccharide synthesis enzyme O-acetyltransferase Cap5H	M	2.0	0.033	<i>mgr</i> up
	SA0152	<i>capI</i>	Capsular polysaccharide synthesis enzyme Cap5I	M	2.0	0.034	<i>mgr</i> up
	SA0153	<i>capJ</i>	Capsular polysaccharide synthesis enzyme Cap5J	M	2.3	0.084	<i>mgr</i> up
	SA0154	<i>capK</i>	Capsular polysaccharide synthesis enzyme Cap5K	M	2.7	0.025	<i>mgr</i> up
	SA1984	<i>asp23</i>	Alkaline shock protein 23, ASP23	C	2.1	0.082	
Others (RNA, DNA, carbohydrates, amino acids, protein, lipid synthesis)	SA0515		HP, similar to deoxypurine kinase	C	2.1	0.014	
	SA1013		HP, similar to carbamate kinase	C	2.3	0.023	
	SA0143	<i>adhE</i>	Alcohol-acetaldehyde dehydrogenase	C	2.0	0.008	<i>rot</i> down
	SA0180		Branched-chain amino acid:cation transporter	M	2.0	0.010	
	SA1225	<i>lysC</i>	Aspartokinase II	C	2.1	0.015	
	SA1271		Threonine deaminase IlvA homolog	C	2.7	0.077	<i>arl</i> up
	SA1272	<i>ald1</i>	Alanine dehydrogenase	C	3.5	0.007	<i>mgr, arl</i> up
	SA1502	<i>rplT</i>	50S ribosomal protein L20	C	2.1	0.060	
	SA0220		HP, similar to glycerophosphodiester phosphodiesterase	C	2.1	0.008	<i>rot, arl</i> up
	SA0027		Truncated replication protein for plasmid		3.6	0.096	
	SA1709		HP, similar to ferritin		2.6	0.034	
Phage-related functions	SA1760		Holin homolog (bacteriophage phiN315)	M	2.4	0.104	
	SA1766		HP (bacteriophage phiN315)		6.0	0.087	
	SA1785		HP (bacteriophage phiN315)		2.5	0.028	
	SA1793		HP (bacteriophage phiN315)		23.3	0.074	
Hypothetical genes	SA0037		HP		3.4	0.062	
	SA0077		HP		3.8	0.073	
	SA0081		HP		2.3	0.041	
	SA0090		HP		6.4	0.089	<i>mgr</i> up
	SA0100		HP		2.8	0.008	
	SA0161		HP		2.0	0.075	
	SA0213		HP		2.4	0.014	
	SA0221		HP		2.2	0.062	<i>mgr</i> down
	SA0262		HP		2.3	0.062	
	SA0279		HP		2.2	0.005	<i>mgr</i> up
	SA0283		HP		2.3	0.042	
	SA0291		HP		3.3	0.068	<i>rot, mgr</i> up
	SA0292		HP		3.9	0.030	<i>rot</i> up
	SA0378		HP		3.5	0.021	
	SA0408		HP		2.7	0.048	<i>rot</i> up
	SA0424		HP		2.4	0.038	
	SA0623		HP		2.8	0.059	
	SA0651		HP		3.1	0.027	<i>rot</i> up
	SA0738		HP		5.5	0.058	
	SA0739		HP		4.7	0.010	<i>rot</i> up
	SA0772		HP		2.1	0.096	
	SA0890		HP		3.4	0.012	
	SA1056		HP	M	3.4	0.012	
	SA1151		HP		2.1	0.022	
	SA1828		HP		2.2	0.040	
	SA2101		HP		2.6	0.012	
	SA2153		HP		2.2	0.010	
	SA2198		HP		2.1	0.062	
	SA2256		HP		2.2	0.040	
	SA2332		HP		2.4	0.019	
	SA2339		HP	M	4.5	0.012	
	SA2373		HP		2.2	0.034	<i>mgr</i> down

Table 1. Continued.

Function of Proteins	N315 ORF	N315 Gene	N315 Product	Protein Location	Change in Expression (n-Fold)	One-Sample t-Test-Benjamini-Hochberg (Adv)	<i>rot</i> , <i>mgrA</i> , <i>arlRS</i> Effect
	SA2449		HP		2.4	0.060	
	SA2474		HP		2.1	0.076	

C, cytoplasm; HP, hypothetical protein; M, membrane; S, secreted; SCW, secreted cell wall-bound.
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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 D-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 *oatA/graRS* 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 *oatA/graRS* 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 pTX*graRS* 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-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 *oatA/graRS* 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 D-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, D-alanine esters were completely absent in TAs, the mutant was stable, and no revertants were observed. Heat-inactivated lysozyme does not affect either the growth of the *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 *oatA/graRS* 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 D-alanine esters in TAs.

The next interesting question was, how do CAMPs act in the *dltA*, *oatA/graRS*, 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 D-alanylation [34–36]. Similar results were also obtained in a *dlt* mutant of *Lactococcus lactis*, which showed increased autolysis [37]. In line with these observations, the

Table 2. 133 *S. aureus* SA113 Genes Down-Regulated by GraRS

Function of Proteins	N315 ORF	N315 Gene	N315 Product	Protein Location	Change in Expression (n-Fold)	One-Sample t-Test-Benjamini-Hochberg (Adv)	<i>rot</i> , <i>mgrA</i> , <i>arlRS</i> Effect
Regulators	SA0017	<i>vicR</i>	Response regulator	C	2.0	0.004	
	SA1071	<i>fabR</i>	Transcription factor FapR	C	2.3	0.015	
	SA1690	<i>recX</i>	Regulatory protein RecX	C	2.0	0.021	
	SA2296		HP, similar to transcriptional regulator, MerR family	C	2.1	0.012	
	SA2320	<i>pfoR</i>	Putative regulatory protein PfoR	M	5.7	0.010	<i>mgr</i> , <i>arl</i> down
	SA2418		HP, similar to two-component response regulator	C	2.1	0.007	
Cell wall (cellular processes, transport, membrane, lipoproteins)	SA0252	<i>lrgA</i>	Holin-like protein LrgA	M	2.6	0.017	<i>mgr</i> , <i>arl</i> up
	SA0469	<i>ftsH</i>	Cell-division protein	M	2.2	0.004	
	SA0616 ^a	<i>vraF</i>	ABC transporter ATP-binding protein	M	8.6	0.005	
	SA0617 ^a	<i>vraG</i>	ABC transporter permease	M	4.8	0.029	
	SA0708	<i>secA</i>	Preprotein translocase subunit	M	2.1	0.012	
	SA0719	<i>trxB</i>	Thioredoxine reductase	M	2.5	0.016	
	SA0937		Cytochrome D ubiquinol oxidase subunit 1 homolog	M	2.5	0.010	
	SA0997	<i>murl</i>	Glutamate racemase	C	2.4	0.009	
	SA1127	<i>cinA</i>	Competence-damage inducible protein CinA	C	2.0	0.024	
	SA1140	<i>glpF</i>	Glycerol uptake facilitator	M	2.2	0.014	
	SA1206	<i>femA</i>	Aminoacyltransferase femA (factor essential for expression of methicillin resistance)	M	2.0	0.006	
	SA1212	<i>opp-2D</i>	Oligopeptide transport ATPase	M	2.2	0.032	
	SA1214	<i>opp-2B</i>	Oligopeptide transporter membrane permease domain	M	2.5	0.036	
	SA1255	<i>crr</i>	Glucose-specific phosphotransferase enzyme IIA component	M	2.2	0.011	
	SA1519	<i>aapA</i>	D-serine/ D-alanine/glycine TRANSPORTER	M	2.4	0.004	
	SA1653	TRAP	Signal transduction protein TRAP	M	2.4	0.005	
	SA1654		HP, similar to ABC transporter ecsB	M	2.5	0.015	
	SA1655		ABC transporter ecsA homolog	M	2.3	0.012	
	SA1916		HP, belongs to the UPF0340 protein family	M	2.4	0.063	
	SA1960	<i>mtlF</i>	PTS system, mannitol specific IIBC component	M	2.2	0.040	<i>mgr</i> down
	SA2056		HP, similar to acriflavin resistance protein	M	2.0	0.010	
	SA2234	<i>oupCD</i>	Probable glycine betaine/carnitine/choline ABC transporter opuCD	M	2.3	0.025	
	SA2324		HP, similar to thioredoxin	M	2.2	0.015	
	SA2354	<i>oatA</i>	O-acetyltransferase A	M	2.5	0.012	
RNA (nucleotides, nucleic acid synthesis, regulation)	SA0016	<i>purA</i>	Adenylosuccinate synthase	C	2.2	0.019	<i>mgr</i> down
	SA1724	<i>purB</i>	Adenylosuccinate lyase	C	2.6	0.004	<i>mgr</i> up
	SA0133	<i>deoC1</i>	Deoxyribose-phosphate aldolase	C	2.8	0.005	
	SA0134	<i>deoB</i>	Phosphopentomutase	C	2.7	0.041	
	SA0915	<i>folD</i>	FoID bifunctional protein	C	6.0	0.004	
	SA0916	<i>purE</i>	Putative phosphoribosylaminoimidazole carboxylase PurE	C	19.4	0.005	
	SA0917	<i>purK</i>	Phosphoribosylaminoimidazole carboxylase ATPase subunit	C	22.2	0.007	
	SA0918	<i>purC</i>	Phosphoribosylaminoimidazole-succinocarboxamide synthase	C	14.2	0.010	
	SA0920	<i>purQ</i>	Phosphoribosylformylglycinamide synthase I PurQ	C	19.9	0.007	
	SA0921	<i>purL</i>	Phosphoribosylformylglycinamide synthetase PurL	C	13.8	0.009	
	SA0922	<i>purF</i>	Phosphoribosylpyrophosphate amidotransferase PurF	C	28.7	0.004	
	SA0923	<i>purM</i>	Phosphoribosylformylglycinamide cyclo-ligase PurM	C	21.6	0.006	<i>rot</i> up
	SA0924	<i>purN</i>	Phosphoribosylglycinamide formyltransferase	C	25.5	0.004	
	SA0925	<i>purH</i>	Bifunctional purine biosynthesis protein PurH	C	17.7	0.004	
	SA0926	<i>purD</i>	Phosphoribosylamine-glycine ligase PurD	C	8.1	0.004	
	SA1172		HP, similar to GMP reductase	C	4.3	0.008	
	SA1237	<i>xpaC</i>	HP, similar to 5-bromo-4-chloroindolyl phosphate hydrolysis protein	C	2.0	0.008	
	SA1914	<i>upp</i>	Uracil phosphoribosyl transferase	C	2.3	0.014	
	SA1938	<i>pdp</i>	Pyrimidine nucleoside phosphorylase	C	2.1	0.010	
	SA1939		Deoxyribose-phosphate aldolase	C	2.5	0.014	
	SA1098	<i>codY</i>	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)	<i>rot</i> , <i>mgrA</i> , <i>arlRS</i> Effect
Adaptation to stress conditions	SA1289		Putative bifunctional biotin ligase/biotin operon repressor	C	2.1	0.004	
	SA1411	<i>hrcA</i>	Heat-inducible transcriptional repressor	C	2.2	0.010	
	SA2410	<i>nrdD</i>	Anaerobic ribonucleoside-triphosphate reductase	C	4.5	0.004	<i>rot</i> up
	SA0470	<i>hslO</i>	Heat-shock protein HSP33 homolog	C	2.1	0.009	
	SA0480	<i>ctsR</i>	Transcription repressor of class III stress genes homolog	C	2.3	0.010	
	SA0509	<i>hchA</i>	Molecular chaperone HchA	C	2.5	0.012	
	SA1096	<i>clpQ</i>	Heat shock protein HslV	C	2.5	0.004	
	SA1238		HP, similar to tellurite resistance protein	C	2.5	0.009	
	SA1408	<i>dnaJ</i>	DnaJ protein (HSP40)	C	2.9	0.006	
	SA1409	<i>dnaK</i>	DnaK protein	C	2.4	0.012	
Carbohydrate mechanism	SA1410	<i>grpE</i>	GrpE protein (HSP-70 cofactor HSP20)	C	2.6	0.021	
	SA1535	<i>tpx</i>	HP, similar to thioredoxin peroxidase	C	2.5	0.021	
	SA0728	<i>pgk</i>	Phosphoglycerate kinase	C	2.2	0.016	
	SA0729	<i>tpiA</i>	Triosephosphate isomerase	C	2.1	0.065	
	SA0730	<i>gpml</i>	2,3-diphosphoglycerate-independent phosphoglycerate mutase	C	2.0	0.029	
	SA0731	<i>eno</i>	Enolase (2-phosphoglycerate dehydrogenase)	C	2.1	0.010	
	SA0958		Putative myo-inositol-1 (or 4)-monophosphatase	C	2.0	0.006	
	SA1088	<i>sucC</i>	Succinyl-CoA synthetase, beta chain	C	3.9	0.004	
	SA1089	<i>sucD</i>	Succinyl-CoA synthetase, alpha chain	C	3.2	0.004	
	SA1184	<i>acnA</i>	Aconitate hydratase	C	2.5	0.010	
Amino acid synthesis	SA1336		Glucose-6-phosphate 1-dehydrogenase	C	2.2	0.012	
	SA1517	<i>citC</i>	Isocitrate dehydrogenase	C	2.2	0.015	
	SA1518	<i>citZ</i>	Citrate synthase II	C	2.4	0.019	
	SA1553	<i>fhs</i>	Formyltetrahydrofolate synthetase	C	2.2	0.030	
	SA1996	<i>lacB</i>	Galactose-6-phosphate isomerase LacB subunit	C	2.4	0.025	<i>mgr</i> , <i>arl</i> down
	SA2001		HP, similar to oxidoreductase, aldo/ketoreductase family	C	2.3	0.034	<i>rot</i> up
	SA2008	<i>alsS</i>	Alpha-acetolactate synthase	C	2.9	0.004	<i>mgr</i> , <i>arl</i> down
	SA2304	<i>fbp</i>	Fructose-bisphosphatase	C	2.1	0.010	
	SA2312	<i>ddh</i>	D-specific D-2-hydroxyacid dehydrogenase	C	2.5	0.071	<i>rot</i> , <i>mgr</i> down
	SA0822	<i>argG</i>	Argininosuccinate synthase	C	2.0	0.012	
Others (lipid synthesis, DNA repair, coenzyme)	SA0829		HP, similar to 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase	C	2.1	0.025	
	SA0859		Thimet oligopeptidase homolog	C	2.6	0.014	
	SA1347	<i>bfbBAB</i>	Branched-chain alpha-keto acid dehydrogenase E1	C	2.1	0.010	
	SA1365	<i>gevPB</i>	glycine dehydrogenase (decarboxylating) subunit 2 homolog	C	2.7	0.010	
	SA1366	<i>gevPA</i>	glycine dehydrogenase (decarboxylating) subunit 1	C	3.9	0.004	
	SA1367	<i>gcvT</i>	Aminomethyltransferase	C	3.6	0.004	
	SA1915	<i>glyA</i>	Serine hydroxymethyl transferase	C	2.3	0.008	
	SA2081		Urea transporter	M	5.5	0.010	<i>mgr</i> , <i>arl</i> down
	SA2082	<i>ureA</i>	Urease gamma subunit	C	13.1	0.009	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2083	<i>ureB</i>	Urease beta subunit	C	32.4	0.006	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
Others (lipid synthesis, DNA repair, coenzyme)	SA2084	<i>ureC</i>	Urease alpha subunit	C	24.9	0.007	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2085	<i>ureE</i>	Urease accessory protein UreE	C	16.0	0.089	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2086	<i>ureF</i>	Urease accessory protein UreF	C	23.7	0.004	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2087	<i>ureG</i>	Urease accessory protein UreG	C	21.4	0.012	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2088	<i>ureD</i>	Urease accessory protein UreD	C	10.0	0.007	<i>rot</i> , <i>mgr</i> , <i>arl</i> down
	SA2318	<i>sdhA</i>	Putative L-serine dehydratase	C	3.7	0.010	<i>mgr</i> down
	SA2319	<i>sdhB</i>	Putative beta-subunit of L-serine dehydratase	C	2.5	0.071	<i>mgr</i> , <i>arl</i> down
	SA0842	<i>fabH</i>	3-oxoacyl-(acyl-carrier protein) synthase 3	C	2.4	0.063	
	SA1072	<i>plsX</i>	Fatty acid/phospholipid synthesis protein	C	2.7	0.006	
	SA1073	<i>fabD</i>	Malonyl CoA-acyl carrier protein transacylase	C	2.8	0.011	
Others (lipid synthesis, DNA repair, coenzyme)	SA1074	<i>fabG</i>	3-oxoacyl-reductase, (acyl-carrier protein) reductase	C	2.7	0.006	
	SA0484	<i>radA</i>	DNA repair protein homolog	C	2.0	0.096	
	SA1138	<i>mutL</i>	DNA mismatch repair protein	C	2.1	0.026	
	SA1512		HP, similar to formamidopyrimidine-DNA glycosylase	C	2.0	0.012	
	SA0831	<i>cdr</i>	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	

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)	<i>rot</i> , <i>mgrA</i> , <i>arlRS</i> Effect
Hypothetical genes	SA1105		Putative zinc metalloprotease		2.1	0.005	
	SA1312	<i>ebpS</i>	Elastin binding protein		2.9	0.038	
	SA1349		Dihydrolipoamide dehydrogenase	C	2.1	0.011	
	SA2301		HP, similar to GTP-pyrophosphokinase		2.8	0.010	
	SA0175		HP		3.6	0.014	
	SA0381		HP		2.7	0.019	
	SA0427		HP		2.2	0.012	<i>mgr</i> up
	SA0481		HP	C	2.0	0.008	
	SA0558		HP		2.1	0.004	
	SA0804		HP		5.5	0.008	
	SA0805		HP		4.8	0.004	
	SA0832		HP		2.4	0.007	
	SA0833		HP		2.1	0.024	
	SA0860		HP		2.5	0.037	
	SA0903		HP		2.1	0.014	
	SA1173		HP		2.6	0.004	
	SA1280		HP, conserved		2.0	0.012	
	SA1534		HP		2.4	0.007	
	SA1723		HP	C	3.2	0.016	
	SA1937		HP		2.2	0.010	
	SA2005		HP		2.3	0.023	
	SA2050		HP		2.2	0.017	
	SA2138		HP		2.4	0.045	
	SA2160		HP		2.3	0.011	
	SA2297		HP		2.3	0.016	<i>rot</i> down

^a*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.

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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.

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

Strains	RT-PCR <i>rot</i> (%)	RT-PCR <i>ureC</i> (%)	RT-PCR <i>dltA</i> (%)	D-Alanylation (%)
SA113	100	1.4	100	100
<i>graRS::erm</i>	8	100	13	53.3 ^a
<i>dltA::spc</i>	nd	nd	0	0

Unless noted otherwise, values represent the mean of three independent RT-PCRs.

^aThe value represents one of three independent experiments.

nd, not determined.

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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.

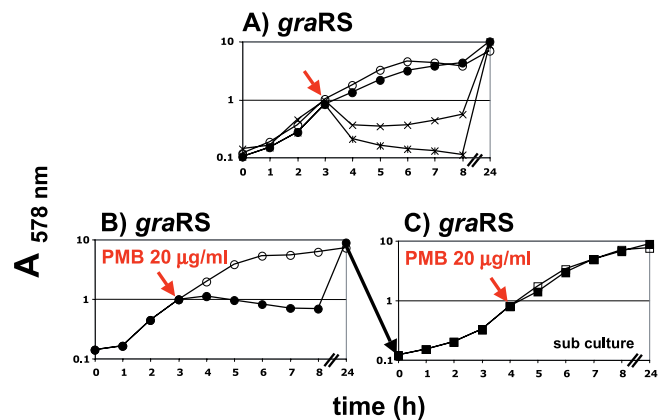
GraRS up-regulates transcription of global regulators such as the SarA homologs Rot, SarS, and MgrA. We compared our GraRS transcriptome results with that of the recently published transcriptome studies of Rot [39], MgrA [40], and ArlRS [41] (Tables 1 and 2; Figure 6). Rot is a repressor of exoproteins but positively regulates cell surface proteins, and SarS is a positive activator of protein A. MgrA appears to be

Table 4. MIC Values of SA113 and Various Mutants

Strains	Lysozyme		Polymyxin B		Gallidermin	
	μg/ml	μM	μg/ml	μM	μg/ml	μM
SA113	>50,000	3,470	350	252.5	9	4.1
<i>graRS::erm</i>	>50,000	3,470	20	14.4	2.5	1.14
<i>oatA::kan</i>	10,000	694	350	252.5	9	4.1
<i>oatA::kan/graRS::erm</i>	600	41.6	25	18	3	1.36
<i>dltA::spc</i>	2,000	138.8	10	7.2	1.2	0.55
<i>oatA::kan/dltA::spc</i>	30	2.08	10	7.2	1.2	0.55
<i>graRS::erm</i> (pTX <i>graRS</i>)	>50,000	3,470	>800	577.2	9	4.1
SA113 (pTX <i>graRS</i>)	>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

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

**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) (×).

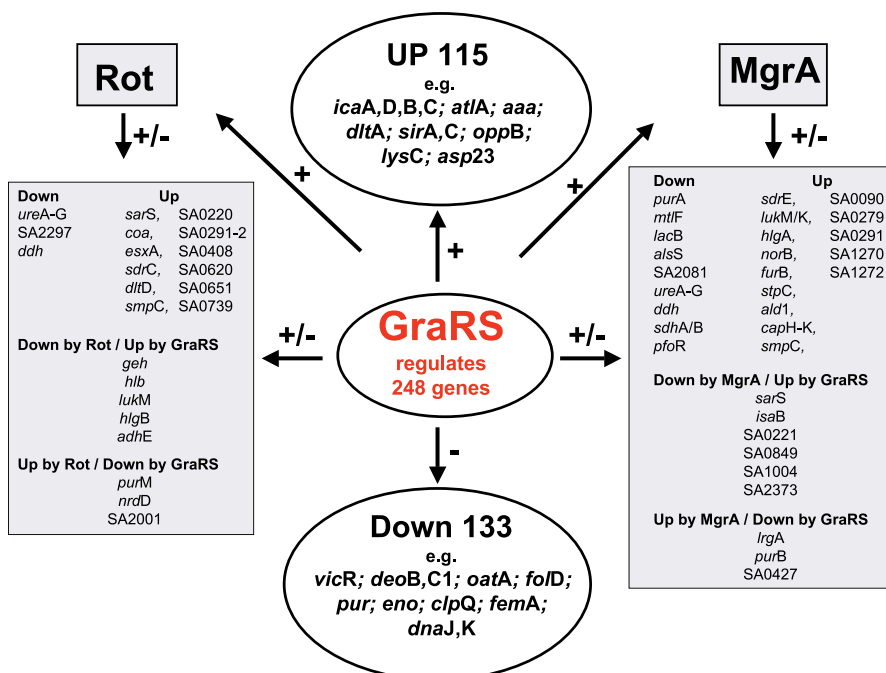
(B) *graRS* mutant: control (○); polymyxin B (PMB) (20 μg/ml) (●).

(C) *graRS* subculture of 5B: control (□); PMB (20 μg/ml) (■).

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

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that is an efflux pump involved in multidrug resistance, all of which are up-regulated by GraRS. Interestingly, *smgC*, 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

**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.

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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. *lrgA*, which encodes a holin-like protein with murein hydrolase activity, is also down-regulated by GraRS but up-regulated by ArlRS and MgrA. Most of the genes are exclusively regulated by GraRS, such as *ica*, *pur*, *mgrA*, *sirA*, *C*, *atlA*, *aaa*, *dnaJ*, *K*, *grpE*, and *vraF*, *G*. These results illustrate that there is a distinct cross-regulation between GraRS, ArlRS, 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., *hly*, *hlgA*, *B*, *lukM*, *F*, *geh*). GraRS is possibly involved in the establishment of persistent infections by the up-regulation of colonization factors (e.g., *ica*, *atl*, *aaa*, *fib*, *sirA*, *sirC*, *sdrC*, *sdrE*), factors involved in resistance to CAMPs (*dlt*), factors involved in intermediary vancomycin resistance (*vraF*, *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% K_2HPO_4 , 0.1% glucose, or 0.5% xylose).

Transposon mutagenesis. Transposon mutagenesis was carried out in the $\Delta oatA::kan$ mutant using the temperature-sensitive plasmid pTV1ts and was performed as described by Bera et al. [10].

Construction of plasmids, homologous recombination, and transduction. Was performed as described by Bera et al. [10]. The PCR products, up- and downstream of *graRS* (SA0614/15) (U0614/15Kpn: TGATATAGGTACCTAATTGTTTACTAGCCGACG, U0614/15Sma: ATTTGTCCCGGGTCTAGTAGTATTTGCATCC, D0614/15Sal: GGCCGTGTCGACTTTGTCAATTTAAACATGCG, and D0614/15Nhe: ATTGCTAGCTTGGCATAACTTGCTGCAACAGG), were cloned into the polylinker of the pBT2 vector flanking the *ermB* antibiotic cassette. Complementation of the *graRS* deletion mutant

was obtained by cloning the *graRS* genes (1,912 bp) (C0614/15Bam: AATGATGGATCCTGGCTTTGAAGTTGACTGCC, and C0614/15Eco: AGCGCGAATTCATTTCCTTTAGGCTTTGGCAC) into the xylose-inducible vector pTX15 in *S. carnosus* TM300. The *oatA::kan/dltA::spc* double mutant was created by bacteriophage ϕ 11-mediated transduction of the *oatA::kan* knockout into the *dltA::spc* deletion mutant.

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

MIC assay. The overnight cultures were diluted in BM with 0.5% xylose to a concentration of 0.5×10^6 CFU per ml and aliquoted in 0.5-ml samples, and cationic agents in different concentrations were added. The cultures were incubated with shaking at 37 °C for 20–24 h and MIC was determined.

Biofilm assay. An overnight culture was diluted 1:200 in fresh TSB with 0.5% glucose, and 200 μ l were filled into microtiter plates and incubated for 20–24 h at 37 °C without shaking. The supernatant was removed and the plate was washed two times with PBS (pH 7.4). The plate was dried and the cells were colored with 0.1% 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 two times with cold 0.9% NaCl, diluted in 0.9% NaCl, and boiled for 20 min. After the cells were chilled on ice, they were again centrifuged and washed twice with 0.9% NaCl. The cells were disrupted in a mechanical grinding device using glass beads 150–212 μ m (Sigma-Aldrich) at 4 °C, centrifuged and washed two times with cold H_2O_{bidest} , boiled for 30 min in 2% SDS to remove noncovalently bound proteins, and washed four times with H_2O_{bidest} . The cell wall fragments were diluted in 0.1 M Tris/HCl (pH 6.8) and incubated with 0.5 mg/ml trypsin for 16 h at 37 °C to degrade cell-bound proteins. After centrifugation and washing with water, the PG was lyophilized.

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

Table 5. List of Strains and Plasmids

Strain or Plasmid	Comment	Reference or Source
<i>S. aureus</i> RN4220	Mutant strain of 8325–4, accept foreign DNA	Kreiswirth [46]
<i>S. aureus</i> SA113	Mutant strain of 8325, with an <i>agr</i> [−] background and 11-bp deletion in <i>rsbU</i>	Iordanescu [47]
<i>S. aureus</i> SA113 $\Delta oatA$	Mutant of SA113 ($\Delta oatA::kan$)	Bera [10]
<i>S. aureus</i> SA113 $\Delta dltA$	Mutant of SA113 ($\Delta dltA::spc$)	Peschel [26]
<i>S. aureus</i> SA113 $\Delta graRS$	Mutant of SA113 ($\Delta graRS::erm$)	This study
<i>S. aureus</i> SA113 $\Delta oatA/\Delta graRS$	<i>oatA</i> and <i>graRS</i> double mutant of SA113 ($\Delta oatA::kan/\Delta graRS::erm$)	This study
<i>S. aureus</i> SA113 $\Delta oatA/\Delta dltA$	<i>oatA</i> and <i>dltA</i> double mutant of SA113 ($\Delta oatA::kan/\Delta dltA::spc$)	This study
<i>S. aureus</i> SA113 $\Delta graRS$ + (pTX $graRS$)	$\Delta graRS::erm$ and pTX15 containing xylose inducible <i>graRS</i> genes	This study
<i>S. aureus</i> SA113 + (pTX $graRS$)	SA113 and pTX15 containing xylose inducible <i>graRS</i> genes	This study
<i>S. carnosus</i> TM300	Host strain for cloning vector pTX15	Götz [48]
<i>Escherichia coli</i> Dh5 α	Host strain for cloning vector pBT2	Hanahan [49]
pBT2	Temperature-sensitive <i>E. coli</i> – <i>S. aureus</i> shuttle vector	Brückner [50]
pBT $udgraRS$	pBT2 containing up- and downstream region of <i>graRS</i> and <i>ermB</i> cassette	This study
pTX15	Xylose-inducing vector for complementation	Peschel [51]
pTX $graRS$	pTX15 containing xylose-inducible <i>graRS</i> genes	This study
pTV1ts	Vector for transposon (Tn917) mutagenesis	Youngman [52]

Quantification of D-alanylation of TA by HPLC. *S. aureus* strains were grown in BM with 0.25% glucose overnight, centrifuged, washed three times, and resuspended in ammonium acetate buffer (20 mM [pH 6.0]). The OD_{600nm} was adjusted to 30. Aliquots (1 ml) were heat-inactivated by incubation at 99 °C for 10 min and centrifuged, and pellets were dried. After incubation at 37 °C for 1 h with 100 µl of 0.1 N NaOH, 100 µl of 0.1 N HCl were added for neutralization and samples were dried. For derivatization, 100 µl of triethylamine and 100 µl of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; Sigma) (10 mM) were added. After incubation at 40 °C for 1 h, samples were dried and resuspended in DMSO:H₂O (1:1). Quantification of D-alanine was performed by HPLC as previously described [43].

RNA isolation and real-time RT-PCR. SA113 and the *graRS* deletion mutant were cultivated in 50 ml of BM and harvested at mid-exponential growth phase. Before RNA isolation, two volumes of RNeasy Protect 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 lysostaphin (0.5 mg/ml) (Genmedics) in TE buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Contaminating DNA was degraded with the DNase Kit (Ambion, <http://www.ambion.com/>) according to the manufacturer's instructions. LightCycler RT-PCR was carried out using the LightCycler RNA amplification kit SYBR Green I or with the LightCycler RNA amplification kit for hybridization probes (Roche Biochemicals, <http://www.roche.com/>). The internal control *gyr* was quantified using 10-fold serial dilutions (10⁴ to 10⁸ copies/µl) of a specific RNA standard using oligonucleotides specific for *gyr* (gyr297F: TTAGTGTGGGAATTGTCGATAAT and gyr574R: AGTCTTGTCACATGCGTT TACA), *dltA* (*dltA*1: TGGCGTTGAAAGACTAGGC and *dltA*2: TTACGAACCTCAGACTGGCG), *rot* (*rot*1: TTCAGCGAGATTGAAAGCG and *rot*2: GTTGCTCTACTTGCAATGG) or *ureC* (*ureC*1: GATATCATTGCCGCTGAAGG and *ureC*2: AAAGCA-GATGGTGTGCACC) as described [44]. Standard curves for *dltA* and *rot* were generated using 5-fold serial dilutions of WT SA113 RNA or for *ureC* of the *graRS* mutant RNA. Differences between WT and the *graRS* mutant were determined by *n*-fold change and calculated as a percentage of the mRNA product. The specificity of the PCR was verified by size determination of the amplicons by agarose gel electrophoresis. To check for DNA contamination, each sample was subjected to PCR by using the LightCycler DNA amplification kit SYBR Green I (Roche Biochemicals). In none of the cases an amplification product was detectable.

Transcriptome analysis. Transcriptome analysis was carried out as

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

Supporting Information

Dataset S1. GraRS-Regulated Genes

Found at doi:10.1371/journal.ppat.0030102.sd001 (36 KB XLS).

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Author contributions. SH and FG conceived and designed the experiments and wrote the paper. SH and DK performed the experiments. SH, CN, and FG analyzed the data. SH, AB, AP, CG, MM, AC, and FG contributed reagents/materials/analysis tools.

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