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

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Abstract

**Background:** Modification of teichoic acids with D-alanine by the products of the *dlt* operon protects Gram-positive bacteria against major antimicrobial host defense molecules such as defensins, cathelicidins, myeloperoxidase or phospholipase. The *graRS* regulatory genes have recently been implicated in the control of D-alanylation in *Staphylococcus aureus*.

**Results:** To determine the impact of the GraRS regulatory system on resistance to antimicrobial host defense mechanisms and virulence of *S. aureus*, we compared inactivation of *S. aureus* wild type and its isogenic *graRS* deletion mutant by the human cathelicidin LL-37 or human neutrophil granulocytes in vitro, and the ability to cause infection in vivo. We show here that *graRS* deletion considerably alters bacterial surface charge, increases susceptibility to killing by human neutrophils or the defense peptide LL-37, and attenuates virulence of *S. aureus* in a mouse infection model.

**Conclusion:** Our results indicate that *S. aureus* can regulate its surface properties in order to overcome innate host defenses.

**Background**

*Staphylococcus aureus*, a frequent cause of human infections, is highly resistant to antimicrobial factors of the innate immune system such as cationic antimicrobial peptides (CAMPs) [1,2] which are produced by epithelial cells and neutrophils [3,4]. These peptides generally contain 10–50 amino acids and have positive net charges [5]. Due to their cationic properties, CAMPs can easily bind to the highly negatively charged bacterial cell envelope and inactivate bacteria, e.g. by forming pores in the bacterial membrane leading to bacterial lysis [6]. *S. aureus* has evolved mechanisms to alter the anionic charge of cell surface components to gain resistance to a broad variety of cationic antimicrobial factors such as CAMPs [7], phospholipase A2 [8], myeloperoxidase [9], or lysozyme [10]. One such mechanism is based upon modification of phospholipids in the cytoplasmic membrane by introducing a positively charged lysyl group into anionic phosphatidylglycerol by the MprF protein, thereby neutralizing the net charge of the membrane surface...
[11,12]. A similar reaction is mediated by products of the
dltABCD operon, which are responsible for attachment of
positively charged D-alanine residues into negatively
charged phosphate groups in the backbone of teichoic
acids [7,9]. Mechanisms involved in the regulation of
these resistance factors are not yet well understood in
Gram-positive bacteria. Herbert et al. recently found that
the S. aureus two-component regulatory system graRS pos-
itive regulates expression of the dtl operon. In a S. aureus
SA113 graRS deletion mutant, the content of D-alanine in
teichoic acids was reduced by 47% and the mutant
showed reduced resistance to various antibiotics includ-
ing polymyxin B, gallidermin, and vancomycin [10,13].
Accordingly, graRS have previously been implicated in
regulation of vancomycin intermediary resistance [14]. As
the dtl operon plays a key role in S. aureus resistance to cat-
ionic antimicrobial host molecules, the graRS system may
be important in evasion of host defense mechanisms such as
catonic antimicrobial peptides and neutrophil killing.

Results
The graRS mutant shows altered cell surface charge but
unaltered lysyl-phosphatidylglycerol (LPG) content
In order to study if reduced expression of the dtl operon
upon graRS disruption results in altered cell surface
charge, we compared binding of the red-coloured, cat-
onic protein cytochrome c to wild type S. aureus SA113
(WT), the isogenic graRS mutant, and the plasmid-com-
plemented mutant.

The graRS mutant bound significantly more cytochrome c
than the WT or the complemented mutant (Fig. 1A),
which is in accordance with the recently described
reduced content of D-alanine residues in the teichoic
acids of the mutant [10].

To analyse whether increased binding of cytochrome c by
the graRS mutant is in fact due to altered cell surface
charge by decreased teichoic acid alanylation or the
altered expression of other surface-exposed molecules, we
also examined binding of cytochrome c to a dtlA deletion
mutant, which completely lacks D-alanine substitution of
teichoic acids [7], and a dtlA/graRS double deletion
mutant, which was generated by transducing the graRS
mutation into the dtlA mutant. Due to the high binding
capacities of the dtlA and the dtlA/graRS mutant we mod-
ified the conditions in order to prevent limitation of
application cytochrome c. Deletion of the graRS genes in the
dtl mutant background did not lead to further increased
binding of cytochrome c compared to the dtlA mutant
(Fig. 1B). This finding indicates that the increased binding
of cytochrome c by the graRS mutant is due to decreased
alanylation of teichoic acids and that the resulting surface
charge alteration does not result from other regulatory
effects mediated by GraRS.

Increased binding of cationic proteins may also result from reduced mprF expression and, accordingly, reduced lysylphosphatidylglycerol (LPG) content. To control for
this possibility, we compared patterns of membrane lipids
from log-phase bacteria by thin-layer chromatography.
The amounts of LPG from WT and graRS mutant were
indistinguishable (data not shown), which corroborates
recent findings that mprF is not among the graRS-regu-
lated genes in S. aureus SA113 [10].

The graRS mutant is more susceptible to killing by LL-37 and human
neutrophil granulocytes in vitro
In an attempt to test whether the increased affinity of the
graRS mutant to cationic molecules leads to higher sus-
ceptibility to human host defense peptides, we compared
inactivation of WT and graRS mutant by the human cathe-
licidin LL-37. This antimicrobial peptide is active against
S. aureus at high concentrations or long exposure [15] but
in this experiment we chose conditions under which the
LL-37 did not affect viability of the S. aureus WT. Accord-
ingly, the WT and complemented mutant strains showed
no significant decrease in CFU following LL-37 exposure,
whereas the number of graRS mutant bacteria recovered
was only 25% of the original inoculum (Fig. 2A).

Next we investigated whether the graRS mutant is killed
to a lesser extent than the parental strain by human neutrophils,
which produce high amounts of LL-37 and other CAMPs
as components of their antibacterial killing arsenal. The
genetic defects.

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

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

Significantly less bacteria were detected in the kidneys of animals, which had been infected with the graRS mutant than those infected with the WT bacteria. (Fig. 3) This finding is in coincidence with the increased susceptibility to clearance by CAMPs and neutrophils, corroborating the central importance of these host factors in innate defense.

**Discussion**

A large variety of regulatory systems has been described in *S. aureus* during the last decades [16-18]. While many systems have been shown to control adhesion and toxin production [19], much less is known about the regulation of genes involved in the resistance to antimicrobial peptides, such as the *dlt* genes. However, the critical role of this operon in infection [7,9] suggests that it may be appropriately regulated in response to environmental stimuli. *Listeria monocytogenes* expresses the VirR transcription factor, which regulates both the *dlt* operon and the *mprF* gene [20]. The *S. aureus* regulatory genes *rot* and *arl* have previously been shown to have a moderate influence on transcription of the *dlt* operon [21,22]. We have recently demonstrated that inactivation of the GraRS system leads to only 13% of the wild-type transcription level of the *dlt* operon and a decreased level of teichoic acid alanylation in *S. aureus* SA113 [10]. Other important genes regulated by graRS in *S. aureus* include the *vraF* genes involved in the resistance to vancomycin [10,13]. Similar data have recently been presented for the *Staphylococcus epidermidis* *aps* system [23]. This three component regulatory system is homologous to the graRS system of *S. aureus*. However, when initially describing the graRS system in *S. aureus*, Meehl et al. failed to recognize the three-component nature of this system [13]. Li et al. recently showed that deletion of graS in the community-associated MRSA strain MW2 leads to decreased minimal inhibitory concentrations of some cationic antimicrobial peptides such as the human beta-defensin hBD3 or the human cathelicidin LL-37 and, similar to our studies, to decreased virulence of the mutant in a mouse infection model [24]. Furthermore, they could show that several important factors in CAMP resistance including the *dlt* operon and *mprF* are activated by a diverse panel of antimicrobial peptides [24]. The fact that *mprF* is not regulated by graRS in *S. aureus* SA113 could be due to the deficiency of this strain in the global gene regulatory system *agr* [25] or to other genetic defects.
Conclusion
We could show here that, besides its role in influencing the effectiveness of pharmacologic antimicrobials, the GraRS regulatory system plays a key role in resistance to natural antimicrobials of our innate immune system against *S. aureus* and merits further attention in an era of increasing reports of virulent and drug-resistant strains of this foremost human pathogen.

Methods

Strains and growth conditions

*Staphylococcus aureus* SA113 (WT), the isogenic graRS deletion mutant [10], the plasmid-complemented graRS deletion mutant [10], the isogenic dltA deletion mutant [7] and the isogenic dltA::graRS double deletion mutant were inoculated in basic medium (BM; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 0.1% K2HPO4) with aliquots of overnight cultures and incubated at 37°C until logarithmic phase was reached. In case of the complemented mutant, BM was modified by replacing glucose with 0.5% xylose to allow for expression of the plasmid-encoded graRS genes [10].

The graRS::erm/dltA::spc double mutant was created by bacteriophage φ 11-mediated transduction of the graRS::erm mutation into the dltA::spc deletion mutant.

To prepare bacteria for the mouse infection model, precultures of the staphylococcal strains which were grown for 8 h in tryptic soy broth (TSB) were diluted 1:100 into fresh TSB and incubated for 18 h without shaking.

Cytochrome c binding assay

Log-phase bacteria were harvested, washed twice with potassium phosphate buffer containing 0.01% human serum albumin (KPi buffer) and bacterial density was adjusted to an OD600 of 3. Bacteria from 1.5 ml aliquots of overnight cultures and incubated at 37°C, CFU were enumerated. After 20 min, 160 μl ice-cold KPi buffer was added to block further antimicrobial action and appropriate aliquots were plated on BM agar plates. After 24 h incubation at 37°C, CFU were enumerated.

Inactivation assay with human neutrophil granulocytes

Bacteria were grown to logarithmic phase, washed, and adjusted in KPi buffer as described above. Neutrophils were isolated from peripheral blood of healthy volunteers by ficoll/histopaque gradient centrifugation as described previously [26] and resuspended in HBSS-HSA (HBSS containing 0.05% human serum albumin). Bacterial and neutrophil suspensions were mixed to final concentrations of 5 × 10⁹/ml bacteria and 2.5 × 10⁶/ml neutrophils. Bacteria were opsonized by addition of pooled human serum (Sigma) to a final concentration of 10%. Samples (500 μl) were shaken at 37°C. After 15, 30 and 60 min, aliquots were diluted in ice-cold water and vortexed vigorously to disrupt the neutrophils and halt bacterial killing. Appropriate dilutions were plated on BM agar plates and incubated at 37°C for 24 h for enumeration of CFU.

Mouse infection model

All procedures involving animals were approved by the UCSD Animal Care Committee, which serves to ensure that all federal guidelines concerning animal experimentation are met.

Female BALB/c mice (12 to 15 weeks old) were infected intraperitoneally with *S. aureus* WT or the graRS mutant. Briefly, precultures of the staphylococcal strains which were grown for 8 h in tryptic soy broth (TSB) were diluted 1:100 into fresh TSB, incubated for 18 h without shaking, washed twice in PBS, adjusted to 3 × 10⁸ CFU/ml in PBS and 400 μl of these suspensions were injected intraperitoneally. 72 h after infection, mice were sacrificed, one kidney was aseptically removed, weighed, homogenized, and serially diluted in PBS for plating on Todd Hewitt agar plates. After 24 h incubation at 37°C, the numbers of CFU/kidney were determined.

Authors’ contributions

DK did all experiments except for the mouse infection model, which was done by SAK and AK. AP supervised research and wrote the paper. SH generated the graRS/dltA double mutant. AP, FG and VN conceived the study and analyzed results. All authors read and approved the final manuscript.

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