Investigation of the *Staphylococcus aureus* GraSR Regulon Reveals Novel Links to Virulence, Stress Response and Cell Wall Signal Transduction Pathways

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**Abstract**

The GraS/GraR two-component system has been shown to control cationic antimicrobial peptide (CAMP) resistance in the major human pathogen *Staphylococcus aureus*. We demonstrated that *graX*, also involved in CAMP resistance and cotranscribed with *graRS*, encodes a regulatory cofactor of the GraSR signaling pathway, effectively constituting a three-component system. We identified a highly conserved ten base pair palindromic sequence (5′ ACAAA TTTGT 3′) located upstream from GraR-regulated genes (*mprF* and the *dlt* and *vraFG* operons), which we show to be essential for transcriptional regulation by GraR and induction in response to CAMPs, suggesting it is the likely GraR binding site. Genome-based predictions and transcriptome analysis revealed several novel GraR target genes. We also found that the GraSR TCS is required for growth of *S. aureus* at high temperatures and resistance to oxidative stress. The GraSR system has previously been shown to play a role in *S. aureus* pathogenesis and we have uncovered previously unsuspected links with the AgrCA peptide quorum-sensing system controlling virulence gene expression. We also show that the GraSR TCS controls stress reponse and cell wall metabolism signal transduction pathways, sharing an extensive overlap with the WalKR regulon. This is the first report showing a role for the GraSR TCS in high temperature and oxidative stress survival and linking this system to stress response, cell wall and pathogenesis control pathways.

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**Introduction**

The opportunistic pathogen *Staphylococcus aureus* is both a commensal and a major Gram-positive pathogen, causing a variety of infections ranging from superficial skin abscesses to more serious diseases such as pneumonia, meningitis, endocarditis, septicemia and toxic shock syndrome [1]. The ubiquitous nature of this pathogen stems mostly from its capacity to survive a large variety of environmental conditions as well as an impressive ability to resist host innate immune defense mechanisms and produce systemic infections, often in healthy humans [2,3]. This unique adaptive potential has made *S. aureus* one of the major causes of nosocomial infections today, compounded by the rapid emergence of multiple antibiotic-resistant strains over the past few decades [4], particularly methicillin and vancomycin-intermediate resistant strains (MRSA and VISA). Until recently, vancomycin had remained the weapon of last resort, but the recent appearance of the enterococcal *vanA* vancomycin-resistance gene cluster in *S. aureus* highlights the growing threat this bacterium poses to human health and the urgent need for developing novel therapeutic approaches [5,6].

Cationic antimicrobial peptides (CAMPs) are an important component of host innate immunity and understanding the molecular mechanisms involved in resistance is a key factor in staphylococcal treatment research. CAMPs have both cationic and amphipathic properties and are classified according to their length and secondary structure [7]. They are produced by certain immune, skin and epithelial cells in all living kingdoms, as defenses against microbial proliferation, and many are known to act by forming pores in the cell membrane, through interactions with bacterial cell envelope components [8]. However, recent work has shown that several CAMPs, including indolicidin and colistin, can also kill by inhibiting intracellular processes such as protein and DNA synthesis as well as septum formation and division [9].

To counteract CAMP antimicrobial activity during infection, Gram-positive bacteria have developed several resistance mechanisms, including degradation, sequestration or electrostatic repulsion [10]. In *Bacillus subtilis* and related Gram-positive bacteria, D-alanylation of wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), mediated by the DltABCD enzymes, as well as MprF-dependent lysylation of phosphatidylglycerol, prevent CAMP-binding by increasing the bacterial surface positive charge [10,11].

Two-component systems (TCSs) play an important role in these mechanisms by coordinating the expression of resistance genes, when CAMPs are detected at the cell surface. TCSs are typically composed of a membrane histidine kinase (HK), acting as a signal sensor/transducer, through phosphorylation of its cognate response component.
regulator (RR), which acts as a transcription activator or repressor. Most S. aureus genomes have a sophisticated arsenal of sixteen sets of environmental monitoring TCS genes, with an additional one present in the staphylococcal cassette chromosome mec element of MRSA strains [12]. Among these systems, the well-studied AgrCA peptide quorum-sensing TCS controls the expression of several virulence genes [13] and VraSR was shown to be responsible for resistance to cell wall-damaging compounds, including β-lactam antibiotics and some CAMPs [14].

The main regulatory pathway controlling CAMP resistance in staphylococci, however, is the GraSR Glycopeptide Resistance Associated TCS, aka ApsSR (Antimicrobial Peptide Sensor), which has been extensively studied over the past five years [15–18]. First discovered in S. aureus as a locus whose overexpression led to increased vancomycin resistance, the GraSR TCS was shown to be required for resistance of S. aureus and S. epidermidis to several CAMPs, by controlling expression of vraFG operons [15–19]. Additionally, the first gene of the graRS operon encodes GraX, a protein of unknown function that also plays a role in CAMP resistance [16–19]. Missense mutations in the graRS locus have been linked to CAMP sensitivity of certain S. aureus strains [20], and the system also plays a role in biofilm formation [21,22]. GraS was shown to play a role in survival of S. epidermidis and S. aureus within neutrophils [23] and the GraSR system has been implicated in S. aureus virulence in several experimental models [18,24–26].

In this study we set out to further define the GraSR regulon in S. aureus. We identified a highly conserved palindromic sequence as the likely GraR binding site, and showed that the GraSR TCS is required for growth of S. aureus at high temperature and resistance to oxidative stress. Using a combination of genome-based predictions and transcriptional analysis, we revealed several novel GraR target genes as well as unsuspected links with the AgrCA and WalKR TCSs.

Results

GraX, GraS and GraR are required for Staphylococcus aureus colistin resistance

In an effort to fully define the GraSR regulon of Staphylococcus aureus, and determine the roles of GraXSR in colistin resistance, we constructed mutant strains ST1036 (ΔgraRS) and ST1070 (ΔgraXRS) in the S. aureus HG001 background [27] by removing the entire coding sequences of the genes (ΔgraRS), or by an in-frame deletion (ΔgraX; see Materials and Methods). GraX, predicted to be a cytoplasmic protein, presents weak similarities to sugar epimerases, and has been shown to be involved in CAMP resistance along with the GraSR TCS, but its specific role remains to be established.

Minimal inhibitory concentration (MIC) values for resistance to colistin, a bacterial CAMP, were determined by following growth in TSB at 37°C over a 12 h period, using a Biotek Synergy Microplate reader, with decreasing concentrations of colistin (Table 1). The ΔgraRS and ΔgraX mutants displayed acute sensitivity to colistin compared to the parental strain. However, the ΔgraX mutant appeared to be more resistant to colistin than the ΔgraRS mutant. We therefore analyzed graR expression in the ΔgraX mutant by quantitative RT-PCR (qRT-PCR), showing that graR expression is increased approximately 2-fold compared to the parental strain (data not shown). This is likely through stabilization of the graR transcript due to increased proximity with the operon promoter in the ΔgraX mutant, suggesting that CAMP sensitivity of the ΔgraX mutant may in fact be underestimated. We also observed that the ΔgraRS and ΔgraX mutants were highly sensitive to nisin (data not shown) as previously observed [17,18].

In order to complement the ΔgraRS mutant, an intact copy of the graR gene was introduced on a multicopy plasmid, resulting in strain ST1116. Complementation of the ΔgraRS mutant by constitutive expression of the graR gene fully restored colistin resistance (Table 1). Indeed, it is well known that response regulator overexpression can complement the absence of the cognate kinase, due to its phosphorylation by other phosphate donors such as acetyl phosphate or aspecific kinase activity within the cell [28,29].

GraXSR do not autoregulate their own synthesis

As shown in Fig. 1A, the graXRS operon is located directly upstream from the vraFG operon, encoding an ABC transporter [19]. To define the graXRS operon promoter region, we analyzed its expression using primer extension experiments. Total RNA was extracted from strain HG001 during mid-exponential growth in TSB at 37°C and used for primer extension experiments. We identified a unique transcriptional start site in the graXRS operon promoter region (Fig. 1B), and the preceding nucleotide sequence revealed appropriately spaced potential −10 and −35 regions, sharing strong similarities with the consensus sequences of promoters recognized by the vegetative form of RNA polymerase holoenzyme, EσV (Fig. 1C).

Several two-component systems are known to positively autoregulate their own synthesis, such as the VraSR cell envelope stress response and AgrCA peptide quorum-sensing virulence regulatory systems of S. aureus [13,30]. In order to test whether this was also the case for the GraSR system, a transcriptional fusion was constructed between the graXRS operon upstream region and the lacZ gene of E. coli using the pSA14 plasmid (see Materials and Methods). To study graXRS operon expression, the graX-lacZ fusion was introduced into strains HG001, ST1036 (ΔgraRS) and ST1070 (ΔgraX), and β-galactosidase activity was measured during mid-exponential growth at 37°C in TSB after a 30 mn treatment with or without 200 µg ml−1 colistin (Fig. 2). No significant differences in graX-lacZ expression levels were observed between the three strains, or in the presence or absence of colistin, indicating that GraXSR do not autoregulate their own synthesis, and that their cellular levels are not induced by the presence of CAMPs such as colistin.

Identification of potential GraR-binding sites in the promoters of known GraR regulated genes

Although several genes involved in CAMP resistance are known to be controlled by GraSR (mpfF, dlt and vraFG operons), the
specific nucleotide sequence constituting the GraR operator sequence remains unknown. In order to identify potential GraR-binding sites upstream from the coding regions of these genes, we first identified their transcription initiation sites through primer extension analysis. Total RNA was extracted from strain HG001 during mid-exponential growth in TSB at 37°C after treatment with 200 μg ml⁻¹ colistin and used for primer extension experiments. We identified a unique transcriptional start site in the mprF and vraFG promoter regions (Fig. 3A) and two initiation sites for the dltXABCD operon. The first (not shown here), is located 30 bp upstream from the dltX (SAOUHSC_00868) translation initiation codon, and was previously identified in S.

Figure 1. The graXRS operon is transcribed from a σ⁵ promoter. (A) The graXRS/vraFG locus of S. aureus HG001. (B) Primer extension analysis of graXRS mRNA was carried out using total RNA extracted from S. aureus strain HG001 during mid-exponential growth in TSB at 37°C. Primer extension experiments were performed using the graX-specific oligonucleotide MF63 (lane 1). The corresponding Sanger dideoxy chain termination sequencing reactions (GATC) were carried out on a PCR-generated DNA fragment fragment corresponding to the graX upstream region (MF62/MF63). The transcriptional start site is boxed. (C) Nucleotide sequence of the graXRS operon upstream region. Potential σ⁵-type -35 and –10 sequences are boxed and the transcriptional start site is labelled +1.

doi:10.1371/journal.pone.0021323.g001

Figure 2. GraXSR do not control their own synthesis. Expression of the graXRS operon was followed using a graX-lacZ transcriptional fusion in S. aureus strains HG001, ST1036 (ΔgraRS) and ST1070 (ΔgraX). β-Galactosidase assays were performed as described in Materials and Methods and measured during mid-exponential growth at 37°C in TSB (grey bars) or after treatment with 200 μg ml⁻¹ colistin for the HG001 strain (black bar). Means and standard deviations values are presented from at least three independent experiments.

doi:10.1371/journal.pone.0021323.g002
 aureus SA113 [31] whereas the second (Fig. 3A), 110 bp further upstream, had not been reported. We identified appropriately spaced potential −10 and −35 regions upstream from all three transcription initiation sites (Fig. 3B). The mprF and dltXABCD −10 regions share strong similarities with the consensus sequence recognized by the vegetative form of RNA polymerase holoenzyme, Eσ^5. However, all three −35 regions as well as the −10 region of the vraFG operon showed only weak similarities with RNA polymerase Eσ^5 consensus promoter sequences, consistent with the existence of a positive transcriptional regulator [32].

GraR is a member of the OmpR subfamily of response regulators, with a typical winged helix-turn-helix domain [33] extending from residues 173 to 203. Although response regulators belonging to the OmpR family are known to bind to short direct repeats [34], orthologs of GraR such as VirR of Bacillus subtilis were in fact shown to bind to inverted repeat sequences [35,36]. We failed to identify any significant direct repeat sequences in the upstream regions of mprF and the vraFG and dlt operons. However, a global study aimed at identifying response regulator binding sites in low G+C Gram-positive bacteria [37] reported the presence of an imperfect palindromic sequence (5′ AAGTGACA-N4-TGTCATTT 3′) within the end of the graS coding region, upstream from the vraFG operon which is known to be controlled by the GraSR system [16,17,18,19]. We were able to identify this palindromic sequence as also being present upstream from the −35 sequences of mprF and the upstream dlt operon promoter. The three inverted repeats are highly conserved, allowing us to align them in order to produce a potential GraR-binding site consensus sequence (Fig. 3B). In agreement with our results showing that GraXSR do not autoregulate their own synthesis, the potential GraR operator sequence is not present in the graXRS operon upstream promoter region.

**Figure 3. Identification of potential GraR-binding sites in the promoters of known GraR-regulated genes.** (A) Primer extension analysis of mprF, dltXABCD and vraFG transcripts was carried out using total RNA extracted from S. aureus strain HG001 treated with 200 µg ml⁻¹ colistin during mid-exponential growth at 37°C in TSB, using specific oligonucleotides for mprF, dltX and vraF (lanes 1 to 3 respectively). The corresponding Sanger dideoxy chain termination sequencing reactions (GATC) were carried out on PCR-generated DNA fragments corresponding to the respective upstream regions (see Table 5). The transcriptional start sites are boxed. (B) Alignment of the potential GraR DNA-binding sites in the mprF, dltXABCD and vraFG promoter regions. The potential GraR-binding site is shaded and conserved nucleotides are shown in white. Potential −35 and −10 sequences are underlined and the transcriptional start sites are indicated in bold.

doi:10.1371/journal.pone.0021323.g003

GraSR-dependent gene expression requires GraX, CAMPs and the consensus binding site

To determine the roles of GraX and this potential GraR-binding site in CAMP resistance, we constructed transcriptional lacZ fusions with the vraFG operon and mprF gene promoters, using the pSA14 vector, with or without the potential GraR operator sequence (vraF-lacZ and ΔvraF-lacZ or mprF-lacZ and ΔmprF-lacZ respectively). The fusions were introduced into strains HG001, ST1036 (ΔgraRS) and ST1070 (ΔgraX) and β-galactosidase activity was measured during mid-exponential growth at 37°C in TSB with or without a 30 min treatment with 50 µg ml⁻¹ colistin (Fig. 4A). In the absence of GraX, GraSR or the potential GraR-binding site (strains ST1052 ΔgraX vraF-lacZ, ST1041 ΔgraRS vraF-lacZ and ST1040 ΔvraF-lacZ, expression of vraF-lacZ was strongly lowered, even in the presence of colistin (Fig. 4A). Comparable results were observed using the mprF-lacZ and ΔmprF-lacZ fusions, although mprF clearly displays a higher level of expression in the absence of GraSR and GraX (Fig. 4B). We also note that there is a significant level of GraSR-dependent expression from both the vraF and mprF promoters in the absence of colistin, indicating that the GraSR system is at least partially active in the absence of inducer, or that it is responding to some other signal under these conditions.

As a control, we introduced a transcriptional fusion with the constitutively expressed promoter of the TU elongation factor tufA gene into S. aureus strain HG001, and no difference in tufA-lacZ expression was observed after a 30 min treatment with or without 200 µg ml⁻¹ colistin (see Supplementary Material Fig. S1A). In order to rule out a potential colistin effect independent of its function as a CAMP, we measured β-galactosidase activities of the two fusions [vraF-lacZ and mprF-lacZ] in strain HG001 following 30 min incubation in the presence or absence of 5 µg ml⁻¹ colistin.
indolicidin and found increased expression for both fusions upon addition of indolicidin (see Supplementary Material Fig. S1B).

To further investigate the role of the GraR-binding site we have shown to be required for GraR-dependent regulation, we constructed two fusions of the same length between the vraFG operon promoter sequence and the lacZ gene in the pSA14 vector. The two fusions only extend nineteen base pairs upstream from the graRS transcriptional initiation site of each gene. We identified potential GraR-binding sites on either strand upstream from 29 genes or operons (Table 2). Among these, 13 had already been described in S. aureus and another 10 genes encode putative proteins whose potential function can be deduced from sequence similarities and are suggested to be involved in different cellular pathways, whereas the remaining genes are of unknown function (Table 2).

Interestingly, 15 genetic loci encode proteins that can be classified in major functional groups. The first includes known and putative antimicrobial resistance-associated proteins: MpfR, DhaBCD, VraF, and a β-lactam antibiotic modifying enzyme named PblA [39]. The second group corresponds to transport proteins: the oligopeptide ATP-binding transporter OppF, a putative gluic acid transporter (SAOUHSC_02815) and two genes located directly downstream from the vraFG operon, encoding a putative inorganic phosphate transporter (SAOUHSC_00669 and SAOUHSC_00670) known as PtaAB in E. coli [40]. In the third group, involved in cell envelope modification, we found the tsm operon involved in teichoic acid glycosylation [41] and genes encoding the cell wall amidase SsaA, a probable autolysin regulator (AtlR-like), and the SpsB signal peptidase. The fourth class of potentially GraR-regulated genes is linked to oxidoreduction processes, including the vraABC quinol oxodase operon, ald, an alanine dehydrogenase gene, and genes encoding the YrkE-like protein containing multi-redox domains and the YibE-like protein probably involved in 2,5-didehydroglyconate reduction.

Given the functional coherence of the identified loci, we investigated the relevance of this newly defined GraSR regulon by alignment of the 29 identified potential GraR-binding sites using the WebLogo website (http://weblogo.berkeley.edu/), generating a perfect 10 bp inverted repeat consensus sequence with a high degree of conservation, constituting the likely GraR operator: 5’-ACAAA WKTGT-3’ (Fig. 6).

Expression profiling of the GraSR regulon

Having defined a consensus GraR operator sequence and several new potential members of the GraSR regulon, we wished to validate these predictions in vivo. Using the BaSysBio Sau T1 chip, a NimbleGen 385K feature tiling array designed to cover both strands of the entire Staphylococcus aureus NCTC 8325 genome (see Materials and Methods), we examined global expression changes between the parental HG001 strain and the ΔgraRS mutant (ST1036), grown to mid-exponential phase in TSB with

Figure 4. GraSR-dependent gene expression requires GraX, colistin and the consensus binding site. vraFG (A) and mprF (B) expression was followed using transcriptional lacZ fusions, with or without the upstream GraR operator sequence (vraF-lacZ, mprF-lacZ and ΔmprF-lacZ, ΔAmpF-lacZ, respectively). The fusions were introduced in S. aureus strains HG001, ST1036 (ΔgraRS) and ST1070 (ΔgraX). Expression was measured during mid-exponential growth in TSB at 37°C (grey bars) or after treatment with 50 μg ml⁻¹ colistin (black bars). β-Galactosidase assays were performed as described in Materials and Methods. Means and standard deviation values are presented from three independent experiments. doi:10.1371/journal.pone.0021323.g004
50 μg ml⁻¹ colistin. A total of 424 genes were found to be significantly differentially expressed in the ΔgraRS mutant compared with the parental strain, with a ≥ 1.8-fold change in transcriptional levels and a P-value (Z-test) ≤ 3.5 × 10⁻⁴. Among these, 235 were positively controlled by GraSR and 189 were repressed (Table S1 and Table S2, respectively). Interestingly, among the positively controlled genes, the most highly regulated encode major virulence factors or regulators, while the remaining genes belonged to the principal categories uncovered by our in silico predictions (see Table 2; antimicrobial resistance, transport, cell envelope modification, oxidoreduction processes) as well as stress response genes, and multiple regulatory and metabolic pathways (acetate, purine and pyrimidine, pyridoxal, xanthine) (Tables S1 and S2). We chose to focus our attention on positively controlled genes involved in the classes uncovered by our in silico analysis, as well as virulence, regulation and stress response which are listed in Table 3.

The most strongly regulated genes encode haemolysins, the AgrBDCA peptide quorum-sensing signal transduction pathway, members of the Sar family of virulence regulators, several host interaction proteins and virulence factors, (fibrinogen binding protein, ClfB, CHIPS, haemolysins, Sbi, SdhH), autolysins, as well as quinol oxidases (Table 3). This is the first report linking GraSR and the AgrCA major S. aureus virulence regulatory system.

Among the regulatory genes, we note those encoding the LytSR TCS, involved in autolysis and biofilm formation [42,43]. Most of the genes involved in cell envelope modification encode autolysins, including the Ata major bifunctional autolysin, the SccD and IsaA transglycosylases, as well as seven genes encoding potential amidases with CHAP domains (Cysteine, Histidine-dependent Amidohydrolases/Peptidases), such as Slc1 or SsaA (Table 3). Interestingly, eight of the GraSR-dependent autolysin genes also belong to the WalKR regulon [44,45] (indicated by an asterisk in Table 3) suggesting a significant regulatory overlap between the two cell envelope signal transduction pathways. Indeed, thirteen other members of the GraSR regulon have also been predicted as belonging to the WalKR regulon as they are preceded by a consensus binding site for the WalR response regulator [44,46], such as the qoxABCD and SAOUHSC_00669-SAOUHSC_00670 operons (indicated by asterisks in Tables 2 & 3, S1 & S2).

In order to validate our microarray data, we chose several relevant genes (qoxA, ssaA, SAOUHSC_00669 and agrB) and compared their relative expression levels in the parental HG001 strain and the ΔgraRS mutant by qRT-PCR. As shown in Fig. 7, we confirmed by qRT-PCR that all of these genes are positively controlled by GraSR, with factors higher than those seen in the transcriptome analysis, ranging from approximately 3- to 29-fold. Results obtained using the two methods showed a linear correlation (Fig. S2; see Supplementary Material).

GraXSR are involved in S. aureus resistance to oxidative stress

Since GraSR control the expression of genes that appear to involved in oxidoreduction processes, we compared the sensitivity to oxidative stress of the parental HG001 strain with that of the ΔgraRS and ΔgraX mutants. Cells were grown in TSB in the presence or absence of 40 mM paraquat (methylviologen). No significant difference in growth between the strains was observed in the presence of paraquat (Fig. 8, open symbols). However, as shown in Fig. 8 (closed symbols), the ΔgraX
and ΔgraRS mutants were much more strongly affected by the presence of paraquat than the HG001 parental strain. Moreover, similar results were obtained in the presence of H$_2$O$_2$ for the ΔgraRS mutant (data not shown). These results reveal a novel function for the GraSR system in resistance of *S. aureus* to oxidative stress.

The GraSR system is required for growth of *S. aureus* at high temperature

The GraSR system is involved in cell envelope modifications through regulation of *mprF*, the *dlt* operon and autolysin genes, and also controls the expression of stress response genes (Tables 3, S1 & S2). We therefore tested the ability of the ΔgraRS mutant to grow

### Table 2. Identification of potential new GraR regulon members by in silico genome scanning.

| NCTC B325* (SAOUHSC) | Gene or operon       | DNA strand | Sequence$^b$                          | Function$^c$                           |
|-----------------------|----------------------|------------|---------------------------------------|----------------------------------------|
|                       | dlt operon           | +          | ACAAAATTTG...\(N_{186}\)...TTG       | Antimicrobial resistance               |
|                       | mprF                 | +          | ACAAAAGTGT...\(N_{87}\)...ATG        | Lysylphosphatidylglycerol synthetase   |
|                       | prnB                 | +          | ACAAAATTTG...\(N_{76}\)...ATG        | Para-nitrobenzyl esterase              |
|                       | vraFG operon         | +          | ACAAAATTTG...\(N_{159}\)...GTG       | ABC transporter                        |
| 00167                 | oppF                 | –          | ACACATTGT...\(N_{128}\)...ATG        | Oligopeptide transporter ATP-binding protein |
| 02815                 | ycbE (B. amylophilfaciens) | –       | ACAATTGTTG...\(N_{86}\)...ATG        | Probable glucosate transporter         |
| 00669                 | +                    | ACAAAATTTG...\(N_{84}\)...ATG        | Putative Pit family transporter        |
| 00181                 | ald                  | –          | ACAAAATTTG...\(N_{53}\)...ATG        | Alanine dehydrogenase                  |
| 01002*                | qaXBACD operon       | –          | ACAAAATTTG...\(N_{158}\)...ATG       | Quinol oxidase AA3 subunit II          |
| 00035                 | yrkE (B. pumilus)    | +          | ACAAAATTTG...\(N_{73}\)...ATG        | Probable multidomain redox protein     |
| 01907                 | ytbE (B. subtilis)   | –          | ACAAAATTTG...\(N_{276}\)...ATG       | 2,5-didehydrogluconate reductase       |
| 00882                 | +                    | ACAAAATTTG...\(N_{250}\)...ATG       | NADH dehydrogenase-like                |
| 01637                 | comYC                | –          | ACAAAATTTG...\(N_{86}\)...ATG        | Probable competence protein            |
| 02500                 | rplE operon          | –          | ACAAAATTTG...\(N_{82}\)...TTG        | 50S ribosomal protein L5               |
| 02257                 | sdrH                 | –          | ACAAAATTTG...\(N_{50}\)...ATG        | Atypical serine-aspartate rich (sdr) protein |
| 00903                 | spsB                 | +          | ACAAAATTTG...\(N_{251}\)...ATG       | Type-1 signal peptidase 1B             |
| 00776                 | uvrB operon          | +          | ACAAAATTTG...\(N_{224}\)...ATG       | Excinuclease                           |
| 01819                 | +                    | ACAAAATTTG...\(N_{69}\)...ATG        | Universal stress protein UspA-like     |
| 02816                 | +                    | ACAAAATTTG...\(N_{466}\)...ATG       | Similar to alkaline phosphatase        |
| 00991                 | ykrP (L. monocytogenes) | –       | ACAAAATTTG...\(N_{58}\)...ATG        | Probable acyltransferase               |
| 00344                 | –                    | ACAAAATTTG...\(N_{43}\)...ATG        | Conserved hypothetical protein         |
| 00146                 | –                    | ACAAAATTTG...\(N_{104}\)...ATG       | Probable transmembrane protein         |
| 00971                 | –                    | ACAAAATTTG...\(N_{247}\)...TTG       | Probable transmembrane protein         |
| 01242                 | +                    | ACAAAATTTG...\(N_{233}\)...ATG       | Conserved hypothetical protein         |
| 01851                 | –                    | ACAAAATTTG...\(N_{260}\)...ATG       | Hypothetical protein                   |
| 02320                 | –                    | ACAAAATTTG...\(N_{129}\)...ATG       | Hypothetical protein                   |

$^a$Gene names correspond to the annotation of the *S. aureus* NCTC 8325 genome sequence [69]. Only the first gene is indicated for operons.

$^b$Positions of the inverted repeats of the potential GraR-binding sites are indicated for the given DNA strand with respect to the translation initiation codon.

$^c$Known and putative functions for each regulon member based on genome annotations are indicated. Based on these predictions, the potential regulon members were divided in six categories (antimicrobial resistance, transport, cell envelope modification, oxidoreduction processes, other functions and unknown function).

*Indicates genes known or predicted to be controlled by the WalKR TCS [44,46].

doi:10.1371/journal.pone.0021323.t002
at high temperatures using a plate spotting assay. Strains ST1120 (HG001 pMK4-Pprot), ST1117 (ΔgraRS pMK4-Pprot) and the complemented ΔgraRS mutant, ΔgraRS+c (Strain ST1116 ΔgraRS pMK4-Pprot-graR) were grown in TSB at 37°C and diluted to an OD₆₀₀ of 0.2. Serial dilutions were then carried out, spotted on TSA plates and incubated at 37°C or 44°C for 48 h. As shown in Fig. 9, growth of the ΔgraRS mutant was strongly impaired at 44°C as compared to the parental strain, whereas no differences were observed between the two strains at 37°C. Resistance to high temperatures was almost completely restored in the complemented ΔgraRS+c strain carrying the graR gene on a multicopy plasmid, indicating that this phenotype can be compensated by overproducing the response regulator alone. These results demonstrate an important role for the GraSR system in growth of *S. aureus* at high temperatures.

**Discussion**

Cationic antimicrobial peptides (CAMPs) are a major component of host innate immune defense systems, produced by all living organisms, and have emerged as promising therapeutic antimicrobial agents [47,48]. Part of the success of some major human pathogens such as *Staphylococcus aureus* can be attributed to efficient CAMP resistance. One such mechanism involves incorporation of positively charged residues into the envelope, effectively increasing electrostatic repulsion of CAMPs from the cell surface. In *S. aureus*, this is accomplished through D-alanylation of teichoic acids, mediated by the DltABC enzymes, as well as by MpfF-dependent lysylation of phosphatidylglycerol [10]. Expression of mpfF and the dlt operon is induced by the presence of CAMPs and specifically controlled by the GraSR TCS, which has attracted growing interest in recent years [16,17,18,19]. GraSR also control expression of the vraFG operon, located directly downstream of the graXRS genes and encoding an ABC transporter also playing a role in CAMP resistance [16,17,18,19].

In this study we wished to further define the GraSR regulon and its function. We determined that unlike other TCSs such as VraSR or AgrCA [13,30], GraSR do not autoregulate their own synthesis. We demonstrated that graX, cotranscribed with graRS, specific to *S. aureus* and also involved in CAMP resistance, encodes a essential regulatory cofactor of the GraSR signaling pathway, effectively constituting a three-component system.

Noting that an imperfect palindromic sequence upstream from the vraFG operon had been suggested as a potential regulatory target in a multi-genome analysis of low G+C% Gram-positive bacteria [37], we found this inverted repeat as being highly conserved upstream from two other well-studied GraSR target genes/operons, mprF and dltXABCD. Extending our analysis through detailed genome scanning of the *S. aureus* NCTC 8325 genome, we were able to derive a highly conserved ten base pair perfect palindromic sequence (5' ACATA TGTG 3') upstream from 29 potential GraSR regulon members (Table 2). By a genetic approach combining deletions and point mutations, we were able to conclusively demonstrate that this sequence is essential for transcriptional regulation by GraR and induction in response to CAMPs, indicating it is the likely GraR operator binding site. Despite multiple attempts, we were unable to purify the GraR response regulator in an active form in order to show DNA-binding *in vitro*. However, our proposed GraR operator binding site is similar to that suggested for the closely related VirR response regulator of *Listeria monocytogenes* which was shown to bind to DNA [35]. The two proteins share 46% overall amino acid sequence identity, rising to 75% for the winged helix-turn-helix domain, with 9 out of 11 identical residues in the DNA recognition helix, indicating that they must bind to similar DNA sequences.

Of the 29 potential GraSR regulon members we identified with this binding site present in their upstream regions, we showed that 13 of these are indeed controlled by the GraSR system *in vivo* under our conditions. Of these, nine are positively regulated (dltXABCD, mprF, vraFG, tsaM, sdrH, ssaA, SAOUHSC_00669, qoxABCD, spaE) and four were found to be repressed (SAOUHSC_00146, SAOUHSC_00991, SAOUHSC_00882, SAOUHSC_02816). This suggests that for the remaining 16 predicted target genes, either the potential GraR binding site is not appropriately located with respect to the promoter in order to allow transcriptional activation/repression, or that additional genetic control mechanisms exist for these genes, preventing their expression under our specific experimental conditions.

During a phenotypic analysis, we observed that the ΔgraRS mutant displayed increased sensitivity to oxidative stress. This may in part be due to positive control by GraSR of the mtaABC manganese transporter genes (Table 3), which have been shown to play a role in *S. aureus* resistance to superoxide radicals [49]. Furthermore, these data suggest that the GraSR system may also respond to other signals in *S. aureus*, and not only to the presence of CAMPs. The combined sensitivity of the ΔgraRS mutant to oxidative stress and antimicrobial peptides could explain the important role of this system in staphylococcal survival in human neutrophils [23,24].

Virulence gene expression in *S. aureus* involves a complex regulatory network, with at least four two-component systems (AgrCA, ArtSR, SaeSR and SrrAB) and several accessory transcription factors (SarA, SarS, SarT, SarR, and Rot) [13]. In this study, our transcriptome analysis allowed us to unveil previously unsuspected connections between the GraSR TCS and the AgrCA signal...
Table 3. Expression profiling of the ΔgraRS mutant.

| Category/Gene name* | Function/similarityb | HG001/ΔgraRS transcription ratioc | P-value |
|---------------------|----------------------|---------------------------------|---------|
| **Antimicrobial resistance** | | | |
| SAOUHSC_02611 | HP similar to lysostaphin resistance protein A | 3.7 | <1.0E-16 |
| SAOUHSC_01359 | mprF Phosphatidylglycerol lysyltransferase | 2.8 | <1.0E-16 |
| SAOUHSC_00867 | HP | 2.7 | <1.0E-16 |
| SAOUHSC_00868 | dtx D-alanine-D-alanyl carrier protein ligase | 2.0 | 1.04E-11 |
| SAOUHSC_00869 | dta D-alanine carrier protein | 2.0 | 1.78E-11 |
| SAOUHSC_00872 | dtd D-alanine-activating enzyme/transfer protein | 2.1 | 1.38E-14 |
| SAOUHSC_02866 | HP similar to drug exporter of the RND superfamily | 2.1 | 8.93E-14 |
| SAOUHSC_02629 | Putative EmrB/QacA family drug resistance transporter | 1.8 | 1.16E-09 |
| SAOUHSC_02630 | HP similar to multidrug resistance protein A | 1.9 | 3.42E-10 |
| SAOUHSC_01866 | HP similar to aminoglycoside resistance associated protein | 1.8 | 5.96E-10 |
| **Transport** | | | |
| SAOUHSC_02516 | HP similar to major facilitator transporter permease | 3.6 | <1.0E-16 |
| SAOUHSC_00060* | HP similar to Na-Pi cotransporter family protein | 2.5 | <1.0E-16 |
| SAOUHSC_00136 | HP similar to ABC transporter ATP-binding protein | 2.5 | <1.0E-16 |
| SAOUHSC_00137 | HP similar to sulfonate/nitrate/taurine transport system substrate-binding protein | 2.0 | 1.41E-12 |
| SAOUHSC_00138 | HP similar to sulfonate/nitrate/taurine transport system permease | 2.2 | 7.11E-15 |
| SAOUHSC_00367 | HP similar to proton/sodium-glutamate symporter | 2.2 | 1.55E-15 |
| SAOUHSC_02576* | HP CHAP domain-containing protein | 18.2 | <1.0E-16 |
| SAOUHSC_02855 | HP CHAP domain-containing protein | 15.6 | <1.0E-16 |
| SAOUHSC_00427* | sle1 N-acetylmuramoyl-L-alanine amidase | 14.9 | <1.0E-16 |
| SAOUHSC_02571* | ssaA Secretory antigen SsaA precursor | 8.0 | <1.0E-16 |
| SAOUHSC_00671* | HP CHAP domain-containing protein | 5.8 | <1.0E-16 |
| SAOUHSC_02333* | sseD Transglycosylase | 4.9 | <1.0E-16 |
| SAOUHSC_00994* | atlA Bifunctional autolysin | 4.0 | <1.0E-16 |
| SAOUHSC_02887* | isaA Immunodominant antigen A, transglycosylase | 5.5 | <1.0E-16 |
| SAOUHSC_00256 | HP CHAP domain-containing protein | 2.3 | <1.0E-16 |
| SAOUHSC_02883* | HP CHAP domain-containing protein | 2.2 | 3.11E-15 |
| SAOUHSC_00974 | tarM wall teichoic acid glycosylation | 1.8 | 1.17E-08 |
| **Cell envelope modification** | | | |
| SAOUHSC_02576* | HP CHAP domain-containing protein | 18.2 | <1.0E-16 |
| SAOUHSC_02855 | HP CHAP domain-containing protein | 15.6 | <1.0E-16 |
| SAOUHSC_00427* | sle1 N-acetylmuramoyl-L-alanine amidase | 14.9 | <1.0E-16 |
| SAOUHSC_02571* | ssaA Secretory antigen SsaA precursor | 8.0 | <1.0E-16 |
| SAOUHSC_00671* | HP CHAP domain-containing protein | 5.8 | <1.0E-16 |
| SAOUHSC_02333* | sseD Transglycosylase | 4.9 | <1.0E-16 |
| SAOUHSC_00994* | atlA Bifunctional autolysin | 4.0 | <1.0E-16 |
| SAOUHSC_02887* | isaA Immunodominant antigen A, transglycosylase | 5.5 | <1.0E-16 |
| SAOUHSC_00256 | HP CHAP domain-containing protein | 2.3 | <1.0E-16 |
| SAOUHSC_02883* | HP CHAP domain-containing protein | 2.2 | 3.11E-15 |
| SAOUHSC_00974 | tarM wall teichoic acid glycosylation | 1.8 | 1.17E-08 |
| **Oxidoreduction processes** | | | |
| SAOUHSC_00999* | qoxD Quinol oxidase, subunit IV | 4.0 | <1.0E-16 |
| SAOUHSC_01000* | qoxC Cytochrome c oxidase subunit III | 3.5 | <1.0E-16 |
| SAOUHSC_01001* | qoxB Quinol oxidase subunit I | 4.3 | <1.0E-16 |
| SAOUHSC_01002* | qoxA Quinol oxidase AA3 subunit II | 6.9 | <1.0E-16 |
| SAOUHSC_01103 | sdhC Succinate dehydrogenase cytochrome b-558 subunit | 1.8 | 2.66E-15 |
| SAOUHSC_01104 | sdhA Succinate dehydrogenase flavoprotein subunit | 1.9 | 3.73E-11 |
| SAOUHSC_01105 | sdhB Succinate dehydrogenase iron-sulfur subunit | 1.8 | 1.99E-09 |
| **Stress** | | | |
| SAOUHSC_00634 | mntC Putative ABC transporter substrate-binding protein | 3.9 | <1.0E-16 |
| SAOUHSC_00636 | mntB Putative iron (chelated) ABC transporter permease | 4.3 | <1.0E-16 |
transduction network. Indeed, expression of the sarR regulon is itself repressed by GraSR, increasing approximately 2.6-fold in the graRS mutant (Table S2). This is the first report linking the GraSR and AgrCA TCSs, which could in part explain the numerous results implicating the GraSR system in S. aureus virulence using several experimental models [18,23–26]. It is likely this connection could not be detected in a previous GraSR transcriptome analysis as it was carried out using strain SA113, an agr mutant, in the absence of CAMPs as an inducer [16,27]. However, comparing the two sets of transcriptome data obtained during exponential growth revealed similar numbers of GraSR-regulated genes using the same cutoff values, although significant differences in the genes controlled were observed. Indeed, only 63 genes were common to the two experiments, and 19 differentially regulated in each condition, which may be attributed to differences in the genetic backgrounds of the two strains (HG001 and SA113) or to a different behaviour of the GraSR system under basal growth conditions or upon induction in the presence of CAMPs.

In addition to AgrCA, GraSR also appear to interact with the WalKR TCS [44,46], providing increasing evidence for TCS signal transduction network-gene regulatory modules. Regulatory overlap with the WalKR regulon is particularly extensive, with at least 21 WalR regulon genes also known to be controlled by GraSR in S. aureus [50]. Indeed, expression of sarR, agr and hla [50].

Table 3. Cont.

| Category/Gene name | Function/similarity | HG001/graRS transcription ratio | P-value |
|--------------------|---------------------|-----------------------------|---------|
| SAOUHSC_00637      | mntA                | Putative manganese/iron ABC transporter ATP-binding protein | 2.5 | <1.0E–16 |
| SAOUHSC_03045      | cspB                | Cold shock protein CspB | 2.3 | <1.0E–16 |
| SAOUHSC_01730      | csbD                | Sigma-B mediated bacterial general stress response protein | 2.1 | <1.0E–16 |
| SAOUHSC_00819      | cspC                | Cold shock protein C | 1.9 | 1.18E–13 |
| SAOUHSC_01403      | cspA                | Cold shock protein | 1.9 | 1.13E–10 |

Legend:
- SG: hypothetical protein.
- a: Gene names correspond to the annotation of the S. aureus NCTC 8325 genome sequence [69].
- b: Fold-change of selected positively regulated genes determined as the ratio of the signal values between strain HG001 and the graRS mutant.
- *: Indicates genes known or predicted to be controlled by the WalKR TCS [44,46].

doi:10.1371/journal.pone.0021323.t003
controlled by GraSR, suggesting a significant level of interaction between the two cell envelope signal transduction pathways. These include eight autolysin genes (atlA, sceD, isaA, ssaA, sle1, SAOUHSC_00671, SAOUHSC_02576, SAOUHSC_02883) that have all been shown to be transcriptionally controlled by WalKR [45] (Delaune et al., in preparation). Thirteen other members of the GraSR regulon have also been predicted as being controlled by WalKR as they are preceded by a consensus binding site for the WalR response regulator, including the qoxABCD quinol oxidase biosynthesis operon, the SAOUHSC_00669-SAOUHSC_00670 operon, prs, encoding a putative ribose-phosphate pyrophosphokinase, the manA mannose-6 phosphate isomerase gene, the SAOUHSC_00738 and vraFG ABC transporter genes as well as a gene of unknown function, SAOUHSC_00060 (Tables 3, S1 & S2) [44,46]. This is reminiscent of genes under multiple regulatory controls, such as the B. subtilis degQ gene, which is preceded by tandemly arranged binding sites for both the DegU and ComA response regulators [53], and it will be interesting to determine the respective contributions of WalKR and GraSR to expression of their co-regulated genes.

During this investigation, we also showed that GraSR are required for growth of S. aureus at high temperatures. This may be linked to their role in modification of wall teichoic acids, which are known to be required for growth under these conditions [54]. This is the first report revealing a function for GraX as a regulatory cofactor of the GraSR TCS, and showing a role for this system in staphylococcal high temperature and oxidative stress survival. We have shown that the GraSR system controls genes involved in stress response, cell wall metabolism and pathogenesis control pathways in addition to its primary role in CAMP resistance, significantly enhancing its importance as a major signal transduction pathway in S. aureus.

Materials and Methods

Bacterial strains and growth procedures

Bacterial strains and plasmids are listed in Table 4. Escherichia coli K12 strain DH5α™ (Invitrogen Life Technologies) was used for cloning experiments. Plasmid constructs were first passaged through the restriction modification deficient S. aureus strain RN4220 [55].
before introduction into S. aureus strain HG001, a rbsU+ variant of strain NCTC 8325 [27] and its derivatives. HG001 is a genetically tractable, clinically relevant non mutagenized strain and was used for all genetic studies. E. coli strains were grown in LB medium with ampicillin (100 μg ml⁻¹) when required. S. aureus was grown in Trypticase Soy Broth (TSB; Difco) with shaking (180 rpm) at 37 °C; for plasmid selection, chloramphenicol (10 μg ml⁻¹) or erythromycin (2 μg ml⁻¹) were added as required. E. coli and S. aureus strains were transformed by electroporation using standard protocols [56] and transformants were selected on LB or Trypticase Soy Agar (TSA; Difco) plates, respectively, with the appropriate antibiotics.

Colistin sulfate, nisin and indolicidin (Sigma-Aldrich) were used as MCS; Difco) plates, respectively, with the appropriate antibiotics.

Plasmid pMK4Pprot, a derivative of vector pMK4 carrying a constitutively expressed Gram-positive promoter sequence [58] was used for gene complementation experiments. Complementation of the ST1096 (AgrRS) strain was carried out using a DNA fragment corresponding to the graR gene coding sequence, amplified with oligonucleotides MF118-MF119, generating BamHI/PstI restriction sites at the extremities, and cloned in the replicative plasmid pMK4-Pprot.

Plasmid pSA14 was used to measure the expression of S. aureus genes by constructing transcriptional fusions between gene promoter regions and the E. coli lacZ reporter gene. The pSA14 plasmid was constructed by cloning a 3.2 kb EcoRI-PstI DNA fragment from plasmid pHT304-18Z [59] between the corresponding restriction sites of the pMK4 shuttle vector [60]. The insert carried the promoterless E. coli lacZ gene fused to the B. subtilis vraFG ribosome binding site [61,62]. In the absence of an upstream promoter, the pSA14 vector displays no detectable β-galactosidase activity, making it a highly useful transcriptional lacZ fusion reporter tool for in vivo expression analysis. For constructing transcriptional lacZ fusions, promoter regions of the mprF gene and the graRS and vraFG operons, and truncated promoter regions of mprF gene and the vraFG operon were amplified by PCR using oligonucleotides introducing BamHI/PstI restriction sites, except for the vraFG-lacZ fusion, constructed using a forward oligonucleotide containing seven mismatches generating point mutations (see Table 5). The corresponding DNA fragments were then cloned between the corresponding restriction sites of the pSA14 vector, yielding plasmids listed in Table 4.

The strong constitutive promoter of the tufA gene was used as a control.

**MIC determinations**

MIC determinations were performed in a 96-well microtiter plate with a 100 μl culture volume. Bacterial cultures were grown for eight hours in TSB at 37 °C, diluted to an OD₆₀₀ of 0.05 and used to inoculate wells containing TSB with standard two-fold

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**Figure 9. GraSR are required for growth of Staphylococcus aureus at high temperature.** The effect of high temperature was tested on growth of S. aureus strains ST1120 (HG001 pMK4-Pprot), ST1117 (ΔgraRS pMK4-Pprot) and the complemented ΔgraRS mutant, ΔgraRS-c (strain ST1116; ΔgraRS pMK4-Pprot-protGraR). Strains were grown at 37 °C in TSB and diluted to an OD₆₀₀ of 0.2. Serial dilutions were then carried out and 10 μl of each dilution was spotted on TSA plates, and incubated at 37 °C or 44 °C for 48 h.

doi:10.1371/journal.pone.0021323.g009
were performed in triplicate. Software (BioTek Instruments Inc., Winooski, VT). All experiments
increments of colistin concentration (v/v). Plates were incubated for
12 h with vigorous shaking at 37 °C in a Synergy 2 thermoregulated
spectrophotometer plate reader using the Gen5TM Microplate
Software (BioTek Instruments Inc., Winooski, VT). All experiments
were performed in triplicate.

| Table 4. Bacterial strains and plasmids used in this study. |
|-------------------------------------------------------------|
| **Strain or plasmid** | **Relevant genotype or description** | **Source or construction** |
|-------------------------------------------------------------|
| **Strains** |
| Escherichia coli |
| DH5αTM | F [Φ80 (d LacZΔM15) Δ (lacZYA-argF) U169 recA1 endA1 Δ (iucD)] supE44 thi-1 gyrA96 relA1 | Invitrogen Life Technologies |
| Staphylococcus aureus |
| RN4220 | Restriction deficient transformation recipient | [55] |
| HG001 | NCTC 8325 rsbU’ | [27] |
| ST1036 | ΔgraRS | pMADgraRS → HG001 |
| ST1039 | vraF-lacZ, Cm’ | pSA14vraF → HG001 |
| ST1040 | ΔAvraF-lacZ, Cm’ | pSA14AvraF → HG001 |
| ST1041 | ΔgraRS vraF-lacZ, Cm’ | pSA14vraF → ST1036 |
| ST1052 | ΔgraX vraF-lacZ, Cm’ | pSA14vraF → ST1070 |
| ST1070 | ΔgraX | pMADgraX → HG001 |
| ST1074 | mprF-lacZ, Cm’ | pSA14mprF → HG001 |
| ST1075 | ΔgraRS mprF-lacZ, Cm’ | pSA14mprF → ST1036 |
| ST1082 | graX-lacZ, Cm’ | pSA14graX → HG001 |
| ST1105 | ΔgraX mprF-lacZ, Cm’ | pSA14mprF → ST1070 |
| ST1111 | ΔAmpF-lacZ, Cm’ | pSA14AmpF → HG001 |
| ST1112 | ΔgraRS graX-lacZ, Cm’ | pSA14graX → ST1036 |
| ST1116 | ΔgraRS pMK4-PprotgraR, Cm’ | pM4K-PprotgraR → ST1036 |
| ST1117 | ΔgraRS pMK4-Pprot, Cm’ | pM4K-PprotST1036 |
| ST1120 | pMK4-Pprot, Cm’ | pM4K-Pprot → HG001 |
| ST1168 | vraF2-lacZ, Cm’ | pSA14vraF2 → HG001 |
| ST1169 | vraF2-lacZ, Cm’ | pSA14vraF2 → HG001 |
| ST1176 | ΔgraX graX-lacZ, Cm’ | pSA14graX → ST1070 |
| ST1199 | PtuA-lacZ, Cm’ | pSA14tuA → HG001 |
| **Plasmids** |
| pMAD | pE194 derivative with a thermosensitive origin of replication for deletion/replacement of genes in Gram-positive bacteria | [57] |
| pMADgraRS | pMAD derivative allowing deletion of the graRS genes | This study |
| pMADgraX | pMAD derivative allowing deletion of the graX gene | This study |
| pSA14 | pMK4 derivative carrying the promoterless E. coli lacZ gene for constructing transcriptional fusions | This study |
| pSA14vraF1 | pSA14 derivative carrying the intergenic region between graXRS and vraFG | This study |
| pSA14AvraF1 | pSA14 derivative carrying the truncated intergenic region between graXRS and vraFG | This study |
| pSA14mprF | pSA14 derivative carrying the mprF promoter region | This study |
| pSA14AmpF | pSA14 derivative carrying the mprF truncated promoter | This study |
| pSA14graX | pSA14 derivative carrying the graX promoter region | This study |
| pSA14vraF2 | pSA14 derivative carrying the vraFG promoter region | This study |
| pSA14vraF2* | pSA14 derivative carrying the truncated vraFG promoter region | This study |
| pSA14tuA | pSA14 derivative carrying the tuaA promoter region | [63] |
| pM4K-Pprot | pM4K derivative carrying a constitutive Gram-positive promoter for gene complementation | [58] |
| pM4K-PprotgraR | pM4K-Pprot derivative carrying graR | This study |

Arrows indicate plasmid introduction by electroporation. doi:10.1371/journal.pone.0021323.t004

Extraction of total RNA

Cells were grown until OD₆₀₀nm = 1, and colistin was added to the medium at 50 μg ml⁻¹ or 200 μg ml⁻¹ when required. Growth was pursued during 30 min and cells were harvested by centrifuging 30 ml culture samples (4 min; 5,400 x g) and
immediately frozen at −80°C. RNA extractions were then performed as previously described [64], followed by a DNase I treatment with the TURBO DNA-free reagent (Ambion, Austin, TX) in order to eliminate residual contaminating genomic DNA.

### Primer extensions

Primer extensions were performed as previously described [65] using 30 µg of RNA, 2 pmol of oligonucleotide (previously radiolabeled with [γ-32P] ATP using T4 polynucleotide kinase, New England Biolabs), and 200 U of Superscript II reverse transcriptase (Invitrogen). Oligonucleotides were chosen so as to hybridize downstream from the translation initiation codon (see Table 3). The corresponding DNA sequencing reactions were carried out with the same oligonucleotides and PCR-amplified DNA fragments carrying the respective promoter regions, using the Sequenase PCR product sequencing kit (USB, Cleveland, OH).

### β-Galactosidase assays

Cells were grown until OD600 nm = 1, colistin was added to the medium at 50 µg ml⁻¹ or 200 µg ml⁻¹ when required and growth was pursued for 30 min. *S. aureus* strains carrying the different lacZ fusions were then harvested by centrifugation 2 ml culture samples (2 min; 20,800 x g). Cells were resuspended in 500 µl of Z buffer [66] with 0.5 mg ml⁻¹ DNase, 5 mM DTT and 0.1 mg ml⁻¹ lysostaphin added extemporaneously, and lysed by incubation at 37°C for 30 min. Cell debris were eliminated by centrifugation (2 min; 20,800 x g) and the supernatant was either used directly for assays or stored at −20°C. Assays were performed as previously described and β-galactosidase specific activity was expressed as Miller units mg⁻¹ protein [66]. Protein concentrations were determined using the Bio-Rad protein assay (BioRad, Hercules, CA) [67]. All experiments were carried out in triplicate.
Microarray experiments

RNA samples for tiling arrays were prepared as described above using cultures of *S. aureus* strains HG001 and ST1036 (*graRS*) grown in TSB with 50 μg ml⁻¹ colistin for GraSR induction, with an additional 2-fold dilution step in killing buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 20 mM Na₂SO₄) before centrifugation. RNA samples were then treated using the RNA Clean-Up kit (Norgen Biotech, Canada) according to the manufacturer’s recommendations and eluted in 40 μl of RNase-free water. RNA preparations were quantified using a spectrophotometer at 260 nm and quality was checked by electrophoregram analysis on a BioAnalyzer (Agilent).

The BaSysBio Sau T1 NimbleGen 385K array was designed with a total of 383,452 features using OligoWiz 2.0 [68], with long iso-thermal probes (45–65 nt) covering the entire genome of *Staphylococcus aureus* NGCT 8325 (CP000253.1; [69]) in 18 nt intervals on each strand (Hanne Jarmer, Technical University of Denmark, Lyngby, Denmark, personal communication). Tiling array experiments were performed in triplicate using RNA isolated from cultures grown in TSB with 50 μg ml⁻¹ colistin for GraSR induction, with an additional 2-fold dilution step in killing buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 20 mM Na₂SO₄) before centrifugation. RNA samples were then treated using the RNA Clean-Up kit (Norgen Biotech Corp., Canada) according to the manufacturer’s recommendations and eluted in 40 μl of RNase-free water. RNA preparations were quantified using a spectrophotometer at 260 nm and quality was checked by electrophoregram analysis on a BioAnalyzer (Agilent).

For data analysis, an aggregated expression value was computed for each Genbank annotated CDS as the median from independent cultures.

For analysis, an aggregated expression value was computed for each Genbank annotated CDS as the median log₂ intensity of probes lying entirely within the corresponding region (Pierre Nicolas, MIG INRA Jouy-en-Josas, personal communication). To control for possible cross-hybridization artefacts the sequence of each probe was BLAST-aligned against the whole chromosome sequence and probes with a SeqS value above the 1.5 cut-off were discarded (SeqS is 2 for a probe with two exact matches) [71].

Aggregated intensity values of the individual samples were normalized by median scaling using the Rosetta Resolver software (version 7.2.1, Rosetta BioSoft, CA) according to the manufacturer’s recommendations, in a 20 μl final reaction volume containing 1 μg total RNA. For qRT-PCR experiments, amplicon primers were designed using the BEACON Designer 4.02 software (Premier Biosoft International, Palo Alto, CA) (see Table 3). Quantitative real-time PCRs (qRT-PCRs), critical threshold cycles (CT) and n-fold changes in transcript levels were performed and determined as previously described and normalized with respect to 16S rRNA whose levels did not vary under our experimental conditions [45]. All experiments were performed in triplicate.

Oxidative stress

*S. aureus* strains were treated with paraquat (methylviologendichloride hydrate) or H₂O₂ (Sigma Aldrich) and growth was followed in a 96-well microtiter plate (100 μl culture volume).

Bacterial cultures were grown in TSB, diluted to an OD₆₀₀ nm = 0.05 and used to inoculate wells containing TSB with or without 40 mM paraquat, or 0.004% H₂O₂. Cultures were incubated for 14 h with vigorous shaking at 37 °C in a Synergy 2 thermoregulated spectrophotometer plate reader using the Gen5™Microplate Software (BioTek Instruments Inc., Winooski, VT). All experiments were performed at least in duplicate.

High temperature growth

The effect of high temperatures was observed on *S. aureus* strains ST1120 (pMK4-Pprot), ST1116 (*graRS*-c; *graRS* pMK4-Pprot-*graRS*), and ST1117 (*graRS* pMK4-Pprot), on cells grown in TSB at 37 °C and diluted to an OD₆₀₀ nm = 0.2. Cultures were serially diluted (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ fold) and 10 μl of each dilution was spotted onto TSA plates, which were dried for 10 min at room temperature and incubated at 37 °C or 44 °C for 48 h.

Supporting Information

**Figure S1** lacZ fusion control experiment expression analysis (A) Expression of tufA-lacZ is not induced by colistin. Expression of the tufA-lacZ fusion was measured in strain ST1189 (HG001 tufA-lacZ) during mid-exponential growth at 37 °C in TSB (grey bars) or after treatment with 200 μg ml⁻¹ colistin (black bars). β-Galactosidase assays were performed as described in Materials and Methods. (B) Indolicidin induces expression of the vraFG operon and mprF. Expression of vraFG-lacZ and mprF-lacZ fusions in *S. aureus* strain HG001 was measured during mid-exponential growth at 37 °C in TSB (grey bars) or after treatment with 5 μg ml⁻¹ indolicidin (black bars). β-Galactosidase assays were performed as described in Experimental Procedures.

**Figure S2** Linear correlation between microarray and qRT-PCR experiments for expression of GraSR-dependent genes. Fold changes in expression as measured by qRT-PCR and transcriptome analysis measured for 4 representative genes in the *S. aureus* HG001 strain relative to the ST1036 (*graRS*) strain grown in the same conditions were plotted against each other to evaluate their correlation. Data points were analyzed in triplicate by both methods.

**Table S1** Genes positively controlled by GraSR.

**Table S2** Genes negatively controlled by GraSR.

**Acknowledgments**

We are grateful to Cécile Wandesman for critical reading of the manuscript. We thank Olivier Pouget for assistance with qRT-PCR experiments, as well as Hanne Jarmer for array design, Pierre Nicolas and Aurélie LeDuc for kind assistance with generating microarray aggregated expression values, and Charlene Blanchet for numerous virulence assays.

**Author Contributions**

Conceived and designed the experiments: TM MF AH. Performed the experiments: MF AH MD. Analyzed the data: TM MF UM. Contributed reagents/materials/analysis tools: MF AH MD UM. Wrote the paper: TM MF.
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