**Staphylococcus aureus** Methicillin-Resistance Factor fmtA Is Regulated by the Global Regulator SarA

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**Abstract**

fmtA encodes a low-affinity penicillin binding protein in *Staphylococcus aureus*. It is part of the core cell wall stimulon and is involved in methicillin resistance in *S. aureus*. Here, we report that the transcription factor, SarA, a pleiotropic regulator of virulence genes in *S. aureus*, regulates the expression of fmtA. *In vitro* binding studies with purified SarA revealed that it binds to specific sites within the 541-bp promoter region of fmtA. Mutation of a key residue of the regulatory activity of SarA (Arg90) abolished binding of SarA to the fmtA promoter, suggesting that SarA binds specifically to the fmtA promoter region. *In vivo* analysis of the fmtA promoter using a *lux operon* reporter fusion show high level expression following oxacillin induction, which was abrogated in a sarA mutant strain. These data suggest that SarA is essential for the induction of fmtA expression by cell wall-specific antibiotics. Further, *in vitro* transcription studies show that SarA enhances fmtA transcription and suggest that regulation of fmtA could be via a SigA-dependent mechanism. Overall, our results show that SarA plays a direct role in the regulation of fmtA expression via binding to the fmtA promoter.

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**Introduction**

*Staphylococcus aureus* is a versatile gram-positive pathogen capable of causing a wide range of diseases, ranging from superficial abscesses to pneumonia, endocarditis, and sepsis [1]. In the pre-antibiotic era, serious systemic staphylococcal infection was associated with 80% mortality [2]. When penicillin was introduced in 1944, over 94% of *S. aureus* isolates were susceptible. However by 1950, 50% of *S. aureus* isolates were penicillin-resistant, further demonstrating the remarkable ability of *S. aureus* to rapidly adapt to antibiotic pressure.

In 1960, outbreaks of virulent *S. aureus* that were resistant to penicillin occurred in many hospitals. These could be treated successfully with methicillin and other newly available penicillinase-stable penicillins. However, by 1961, two years after the introduction of methicillin, methicillin-resistant *Staphylococcus aureus* (MRSA) had emerged. Since then, a number of distinct MRSA strains have emerged and spread throughout the world [3–5]. MRSA, in addition to an intrinsic resistance to virtually all β-lactams, has an ability to accumulate and develop resistance to other, unrelated antibiotics [6–9]. Some variants of MRSA have developed resistance to glycopeptide antibiotics, including vancomycin, which is the sole antibiotic that can still be used to successfully treat most *S. aureus* [9].

*S. aureus* resistance to methicillin has been attributed to the acquisition of the mecA gene, which encodes a penicillin-binding protein (PBP) that is resistance to β-lactam inactivation, namely PBP2a; the four native PBPs of *S. aureus* are sensitive to β-lactams [6,10,11]. Despite the efficiency with which expression of PBP2a confers resistance to β-lactams, the PBP2a expression level does not correlate with high levels of methicillin resistance [12–15]. Other factors are suggested to play essential roles in the phenotypic expression of methicillin resistance [12]. Screening for methicillin-resistance factors has resulted in the identification of auxiliary genes including *fem*, *fmtA*, *llm*, sigma factor, *pbpD*, and *vraSR* [16–23]. These genes reside outside the *mecA* determinants [17] and have been shown to have direct or indirect roles in the biosynthesis or autolysis of peptidoglycan. The *fem* genes are involved in the biosynthesis of the peptide core of the peptidoglycan precursor subunit [24–26] and *pbpD*, which encodes PBP4 and is involved in the synthesis of highly cross-linked peptidoglycan [20].

Genome-based studies of the *S. aureus* response to cell wall-specific antibiotics identified fmtA as being part of the core cell wall stimulon [27]. The expression level of fmtA increases in the presence of cell wall inhibitors and when genes involved in biosynthesis of peptidoglycan are inactivated [27–31]. Insertions in fmtA reduce MICs of methicillin, celoxatin and imipenem 8 to 16 fold for different MRSA strains. This effect is more pronounced in the presence of Triton X-100 [32,33]. Insertions in fmtA also impair polysaccharide intercellular adhesion production and result in significantly reduced biofilm formation [34,35]. Furthermore, the fmtA mutants exhibit reduced peptidoglycan cross-linking [32] and reduced attachment of wall teichoic acids to cell wall [34]. The fmtA gene product (FmtA) is capable of forming stable acyl-enzyme species with β-lactams, but the interaction is weak [36].
Upregulation of the fmtA expression by perturbation of peptidoglycan biosynthesis suggests presence of a regulatory mechanism capable of coordinating fmtA expression with cell wall biosynthesis. Here, we investigate the factors involved in regulation of fmtA.

In this study, we identified SarA as a transcription factor responsible for regulation of fmtA. SarA is a global regulator of S. aureus involved in the regulation of many virulence factors and was previously reported to be involved in m ethicillin resistance [37]. We report the DNA-binding sites of SarA in the fmtA promoter. The binding specificity of SarA to fmtA promoter region was probed by mutating a key functional residue of SarA (Arg90). In addition, fmtA-lux operon reporter constructs were used to investigate the regulation of fmtA expression in vivo in response to antibiotic stress. The activation of fmtA transcription by SarA was confirmed by in vitro run-off transcription assays. Together, our results show that SarA binds directly to the fmtA promoter and plays a direct role in the regulation of fmtA expression.

Materials and Methods

Growth Media and Chemicals

Chemicals were purchased from Sigma (Oakville, Canada) or Thermo-Fisher (Whitby, Canada), unless otherwise stated. Chromatography media and columns were purchased from GE Healthcare (Quebec, Canada). The [γ-32P] ATP (3000 Ci/mmol) was purchased from Perkin Elmer LAS Canada Inc. (Toronto, Canada) or GE Healthcare (Quebec, Canada). The ProteoExtract All-in-One Trypsin Digestion Kit was purchased from EMD4 Biosciences.

Preparation of Cell Extracts

S. aureus strain RN4220 (Cedarlane, Burlington, Canada) was grown to an optical density (OD) at 600 nm of approximately 0.9

**Figure 1.** The fmtA promoter region in the S. aureus strain Mu50. (A) The open reading frames flanking fmtA operon are highlighted, and the strategy of division of the promoter region is also highlighted. The numbering of the upstream and downstream elements on the fmtA promoter is based on the putative transcription starting point denoted by an asterisk and indicated in bold in panel B. The putative −10 box is also in bold. doi:10.1371/journal.pone.0043998.g001

| Primer Name | Primer Sequence |
|-------------|----------------|
| DirPseq1 | 5’ AGGAAATTC AGAACCATGC TAGAAGGAATCCA 3’ |
| RevPseq1 | 5’ CCGATATCGA CATACATATAAACTGCGAT 3’ |
| DirPseq2 | 5’ AGGAATTCCTTA AAATTTAAAAATG CTAATAT 3’ |
| RevPseq2 | 5’ CGGATATCGA CATACATATAAACTGCGAT 3’ |
| DirPseq3 | 5’ AGGAATTCCTTA AAATTTAAAAATG CTAATAT 3’ |
| RevPseq3 | 5’ CGGATATCGA CATACATATAAACTGCGAT 3’ |
| LuxA | 5’ TACCAAAATTTCCAACATTATCAT 3’ |
| FmtAT1GDir | 5’ AAGTGAATAATTTTGGTGAATTAGATACAT 3’ |
| FmtAT1GRev | 5’ ATGTATCATACATACACAAAAATATTATACCT 3’ |
| FmtAT1GDir | 5’ TACCAAAATTTCCAACATTATCAT 3’ |
| FmtAT1GRev | 5’ CAACAAATTTATATATCCTATTACATTGTA 3’ |
| 16S-RNADir | 5’ GCTAAGTTTGATGGTGGTTC 3’ |
| 16S-RNARrev | 5’ TCCAACCTTGCCTGACT 3’ |
| SarAdv | 5’ TGTTGACATGAGATGTTTCT 3’ |
| SarARev | 5’ CCTTGTGAAACACCAACATGTGAA 3’ |
| FmtARev | 5’ ATTTATACCTATCTATGTTATAATCCTA 3’ |
| FmtARrev | 5’ CAAAAGAGAAGCCCCGTTAAATG 3’ |

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in tryptic soy broth with or without oxacillin (1.2 μg/mL) and incubated for 1.5 h at 37°C. The cells were harvested at 4°C (11,000 xg) and washed with cold TEG buffer (25 mM Tris-HCl, pH 8.0, and 5 mM EGTA) and re-suspended in TEG buffer. After two freeze-thaw cycles, cells were lysed with lysozyme (0.3 mg/mL) followed by sonication. Lysates were centrifuged at 4°C (21,000 xg) and 20% glycerol was added to the supernatants. The supernatants were dialyzed overnight in 1 L dialysis buffer (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM DTT, 50 mM NaCl, and 20% (v/v) glycerol).

Electro-mobility Shift Assay (EMSA)

The predicted promoter region of fmtA (FmtA) lying between the open reading frames (orf) denoted SAV1056 and fmtA, encompassing 540 bp, was divided into three DNA fragments of 270 bp, each designated seq1, seq2, or seq3 (Figure 1). The seq1, seq2, and seq3 fragments were amplified from the S. aureus Mu50 genome (Cedarlane) using primers as follows (Table 1): seq1 (−342 to −70); the numbering of the upstream and downstream elements in the fmtA promoter is based on a putative transcription starting
point (Figure 1B) was amplified using Dirseq1 and Revseq1, seq2 (-70 to +199) with Dirseq2 and Revseq2, and seq3 (-207 to +63) with Dirseq3 and Revseq3. The PCR amplified fragments were gel-purified, 5'-end labeled with [γ-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase, and purified by passage through ProbeQuant G-50 columns (GE Healthcare). Binding reactions were performed by mixing oxacillin-induced or uninduced cell extracts with 2 ng 5'-32P-labeled double-stranded DNA fragments in the presence of 1 μg poly (dI-dC) and 200 ng sheared herring sperm DNA for 30 min in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM diethiothreitol (DTT), 5 mM MgCl2, and 2.5% (v/v) glycerol. The reaction mixtures were loaded into 6% native polyacrylamide gels. Following electrophoresis, gels were dried and imaged using an electronic radiography instant imager (Packard). Quantitative analysis of the bands was performed using ImageJ (v 1.3, NIH). Dissociation constants were determined as described above for the EMSA as described above for the cell extracts.

**Screening Protocol for Isolation of Regulatory Proteins**

The PfnlA derived sequence seq3 (-207 to +63) was amplified by using 5' biotinylated primers and their respective reverse primers (Table 1). An aliquot of 200 μg streptavidin-coated magnetic beads (10 μg/μL Dynal® Biotech) was washed three times with 20 μL washing buffer (10 mM Tris-HCl buffer pH 7.5, supplemented with 1 mM EDTA and 2 mM NaCl) for 15 min at room temperature. A 40-μL aliquot of the seq3 target DNA at 25 ng/μL was incubated with the beads for 15 min. The supernatant was removed and the beads were washed twice with the washing buffer. The process of loading target DNA on the beads was repeated two more times, with a washing step after each loading step. This process ensured 90% loading of the beads with the target DNA. Prior to incubation with DNA-bound beads, oxacillin-induced cell extracts were incubated with 200 μg streptavidin-coated beads in binding buffer (10 mM Tris buffer, pH 7.5, supplemented with 150 mM KCl, 0.1 mM EDTA, and 0.1 mM DTT) on ice for 30 min. The pre-treated cell extracts (40 μL) were then incubated with the DNA-loaded beads for 30 min at room temperature in the presence of 100 ng/μL herring sperm DNA.

Unbound protein was removed from the beads by three washes with 100 μL binding buffer. The beads were then resuspended in 25 μL water and subjected to trypsin digestion (EMD® Biociences). The samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) at the Advanced Protein Technology Centre of Hospital for Sick Children (Toronto, Canada). Alternatively, the beads were resuspended in 25 μL water, mixed with 0.5 μL 10% sodium dodecyl sulfate (SDS) and boiled for 5 min. The supernatant was loaded into SDS-polyacrylamide gels.

**Cloning of sarA**

The 375 bp sarA coding region was amplified from S. aureus strain Mu50 genome by PCR using primers SarADir and SarARrev (Table 1) which incorporate Nadl and HindIII restriction enzyme sites at the 5'-end and 3'-end respectively. The amplicon was digested with Nadl and HindIII and cloned into vector pET22b digested with NdeI and HindIII. The resulting plasmid was digested with Nadl (to remove the pelB leader sequence between the Nadl site of pET22b and the sarA gene), religated, and transformed into E. coli NovaBlue cells. The pET22b:sarA construct was confirmed by sequence analysis, and transformed into E. coli BL21(DE3) cells.

**Site-directed Mutagenesis of Arg90 to Ala in SarA**

The Arg90 residue of SarA was mutated to Ala using the QuickChange® Site-Directed Mutagenesis protocol (Agilent, Mississauga, Canada). The recombinant DNA construct pET22b:sarAR90A was generated using pET22b:sarA as template. PCR Thermus™ DNA polymerase (Agilent) and two mutagenic primers: SarAMDir and SarAMRev (Table 1). The PCR product was treated with restriction endonuclease DpnI and the resulting mixture was used to transform E. coli NovaBlue cells. Successful mutation of arginine to alanine was verified by DNA sequencing and the pET22b:sarAR90A plasmid was used to transform E. coli BL21(DE3) cells.

**Purification of SarA and SarAR90A Mutant Proteins**

All steps of purification were carried out at 4°C. Seed cultures in BL21 (DE3) cells were grown overnight in Luria-Bertani media (Thermo-Fisher). A 1% starter culture was used to inoculate 1 L Terrific Broth (TB), supplemented with 100 μg/mL ampicillin. The culture was grown at 37°C with shaking until the OD600 nm reached 0.7. SarA expression was initiated by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG; Rose Scientific, Edmonton, Canada). The induced cells were harvested and resuspended in 50 mM sodium phosphate buffer (pH 6.8). The protein was liberated by sonication and cellular debris removed by centrifugation at 20,000 ×g for 60 min. The clarified supernatant was loaded onto a SP-Sepharose FF column and the protein was eluted with a linear gradient of 1.0 M NaCl in 50 mM sodium phosphate buffer (pH 6.8). The fractions containing protein were concentrated and the buffer was changed to 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM DTT using a HiPrep desalting column (26/10, GE Healthcare). The protein mixture solution was then loaded to a Heparin Sepharose 6FF column (26/10, GE Healthcare). The protein was eluted with a linear gradient of 1.5 M NaCl. The fractions containing protein were concentrated and loaded onto a SuperdexTM75 (10/300 GL) column (GE Healthcare) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Following elution by the same buffer, the protein-containing fractions were analyzed with 15% SDS-polyacrylamide gel electrophoresis (PAGE). The molecular mass of the purified SarA and SarAR90A mutant was confirmed by electrospray ionization mass spectrometry in the Advanced Protein Technology Centre of Hospital for Sick Children (Toronto, Canada).

The oligomerization state of SarA was determined by size exclusion chromatography using a gel filtration LMW calibration kit from GE Healthcare and Superdex™ 75 (10/300) column (GE Healthcare). The column was equilibrated and run with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.4 mL/min.

**DNase I Footprinting Assay**

The seqA and seqB promoter sequences were amplified by PCR from the S. aureus Mu50 genome using specific primers (Table 1). The primer of interest (Dir or Rev) was 5′-end labeled with [γ-32P]ATP (3000 Ci/mmol). Binding reactions were performed as described above for the EMSA, but with 8 ng DNA. Binding reactions were incubated for 30 min at 37°C and then subjected to D Nasel (0.024 units) for 2 min at 25°C after supplementing binding buffer with 10 mM CaCl2 and 5 mM MgCl2. The
reaction was terminated by adding stop solution (200 mM NaCl, 1% [w/v] SDS, 20 mM EDTA, and 250 µg/mL tRNA). The digested DNA samples were extracted with phenol:chloroform and ethanol precipitated. They were then resuspended in formamide containing loading dye, denatured at 95°C and loaded onto 0% sequencing gel. The control A, T, G, and C were prepared by using Thermolob DNA polymerase (New England Biolabs Canada) and a set of acyclonucleotides (acyATP, acyCTP, acyGTP, and acyTTP; New England Biolabs Canada). Four separate reactions (one for each acyNTP terminator) generated DNA sequencing termination ladders. Reactions contained template DNA, 50 nM [γ-32P] ATP labeled sequencing primer, 50 nM dNTP, 20 mM Tris–HCl, pH 8.8 at 25°C, 10 mM KCl, 2 mM MgSO4, 10 mM (NH4)2SO4, 0.1% Triton X-100, and 0.1 U/µL of E. coli RNA polymerase holoenzyme (Epitome Biotechnologies, Madison, Wisconsin).

In vitro Run-off Transcription Assay

Single round transcription with E. coli RNA polymerase holoenzyme (RNAP; Epitome Biotechnologies, Madison, Wisconsin) was carried out using a 500-bp fragment of the fmtA promoter region (Pnad, nucleotide position −342 to +239 of fmtA promoter region) as template, in the absence or presence of SarA. The Pnad fragment was amplified using pfu Turbo DNA polymerase and primers Dir-Pnad and Rev-Pnad. All four DNA fragments, Pnad, Pseq1, Pseq2, and Pseq3, were PCR amplified and digested using restriction enzymes EcoRI and BamHI. The DNA fragments were ligated to similarly digested vector pXEN1. pXEN1 harbors the luxABCDE operon from Photobacterium luminescens that has been modified for lux expression in Gram positive bacteria [7]. The ligation mix was transformed into E. coli DH5α and transformants were selected on medium containing ampicillin (100 µg/mL). Putative clones obtained were confirmed to harbor the inserts by sequence analysis using a primer derived from fmtA and the forward primer used to amplify the insert fragment. The pXEN1 plasmids carrying different inserts are referred to as lux fusion plasmids.

The pXEN1 plasmid and lux fusion plasmids were introduced into the restriction-deficient S. aureus strain RN4220 by electroporation (26). Transformants were plated on tryptic soy broth (TSB)-agar plates supplemented with 5 µg/mL chloramphenicol (selectable marker in pXEN1). Positive colonies were confirmed by sequencing of the plasmids using the primers described above. The S. aureus strain RN4220 carrying pXEN1 is referred to as RN::lux, and RN4220 strains carrying the pXEN1 fusion plasmids, constructed herein, are referred to as lux fusion strains RN(Pseq1::lux), RN(Pseq2::lux), RN(Pseq3::lux), and RN(Pseq4::lux).

In S. aureus PC1839 (parent strain S. aureus RN4220), the sarA gene has been knocked out and replaced with a kanamycin resistance cassette [43]. PC1839 was transformed with the lux promoter constructs Pseq1, Pseq2, Pseq3, and Pseq4, and pXEN1 isolated from strain RN4220. The resultant colonies were resistant to kanamycin and chloramphenicol. The S. aureus strain PC1839 carrying the empty vector pXEN1 is referred to as PC::lux, and PC1839 strains carrying the pXEN1 fusion plasmids are referred to as lux fusion strains PC(Pseq1::lux), PC(Pseq2::lux), PC(Pseq3::lux), and PC(Pseq4::lux). The effect of pXEN1 and lux fusion plasmids on the growth of S. aureus RN4220 and PC1839 fusion strains was investigated by monitoring the growth profiles. Briefly, lux fusion strains RN::lux, PC::lux, RN4220, and PC1839 were inoculated into TSB and grown overnight at 37°C with 5 µg/mL chloramphenicol, except for RN4220 and PC1839. Next, 1% culture was inoculated in fresh TSB supplemented with 5 µg/mL chloramphenicol for the lux fusion strains except for RN4220 and PC1839. The OD(600 nm) was measured at 1 h intervals for the next 7 h.

Measuring Bioluminescence from S. aureus Strains

After overnight growth, RN4220, PC1839, and PC::lux, were diluted into fresh TSB and grown at 37°C with shaking at 200 rpm. The strains were grown to an OD(600 nm) of approximately 0.3 and induced with oxacillin at 10 µg/mL or 100 µg/mL for 1 h. The optical densities were measured at 600 nm for all the samples. The cultures with higher OD(600 nm) were diluted such that the cell density in each culture was the same. A 300-µL aliquot from each sample (in triplicates) was transferred to opaque 96-well optiplates and analyzed in a HT-Analyst (Molecular Devices, California). Bioluminescence was measured immediately after dispensing the samples into the plates over a period of 10 min. The data points collected over 10 min were averaged for each strain at each oxacillin concentration, and

Construction of luxABCDE Fusion Strains with Derivatives of fmtA Promoter Region

The three fmtA-derived sequences (seq1, seq2 and seq3) were amplified by PCR from fmtA operon using specific primer pairs designed to introduce EcoRI and BamHI restriction sites at the 5’ and 3’ ends, respectively (Table 1). The predicted full length fmtA control region encompassing nucleotides −342 to +199 (referred to as seq1–2) was also amplified using primers DirPseq1 and RevPseq2. All four DNA fragments, Pseq1, Pseq2, Pseq3, and Pseq4, were PCR amplified and digested using restriction enzymes EcoRI and BamHI. The DNA fragments were ligated to similarly digested vector pXEN1. pXEN1 harbors the luxABCDE operon from Photobacterium luminescens that has been modified for lux expression in Gram positive bacteria [7]. The ligation mix was transformed into E. coli DH5α and transformants were selected on medium containing ampicillin (100 µg/mL). Putative clones obtained were confirmed to harbor the inserts by sequence analysis using a primer derived from fmtA and the forward primer used to amplify the insert fragment. The pXEN1 plasmids carrying different inserts are referred to as lux fusion plasmids.

The pXEN1 plasmid and lux fusion plasmids were introduced into the restriction-deficient S. aureus strain RN4220 by electroporation (26). Transformants were plated on tryptic soy broth (TSB)-agar plates supplemented with 5 µg/mL chloramphenicol (selectable marker in pXEN1). Positive colonies were confirmed by sequencing of the plasmids using the primers described above. The S. aureus strain RN4220 carrying pXEN1 is referred to as RN::lux, and RN4220 strains carrying the pXEN1 fusion plasmids, constructed herein, are referred to as lux fusion strains RN(Pseq1::lux), RN(Pseq2::lux), RN(Pseq3::lux), and RN(Pseq4::lux).

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the standard deviations were determined from three independent measurements. The promoter activity was plotted as luminescence over time using the mean values from the readings taken over a period of 10 min calculated by subtracting values from blank medium controls. These experiments were repeated three times.

Viability Testing of the Induced Versus Uninduced Cultures

Cultures of RN4220, PC1839, RN::lux, and PC::lux were inoculated at 1:100 with an overnight culture into TSB and chloramphenicol (no antibiotic was added to RN4220 and PC1839). When the OD₅₀₀ nm of the cultures reached approximately 0.3, oxacillin was added to a final concentration of 10 or 100 μg/mL, and the cultures were grown for 1 more hour. The OD of each culture was normalized to the sample having the lowest OD. A 20-μL aliquot from each culture was diluted 5000-fold with TSB to dilute the antibiotic, and a 50-μL aliquot of the dilution was plated on TSB-agar plates without antibiotic and incubated at 37°C. These experiments were repeated three times.

RNA Extraction and c-DNA Synthesis

Overnight-grown wild-type S. aureus RN4220 and PC1839 were diluted (200-fold) separately in 30 mL tryptic soy broth (TSB) in 125-mL Erlenmeyer flask. Cultures were incubated at 37°C with shaking at 200 rpm and growth was measured at regular intervals until the optical density (OD₅₀₀ nm) reached to early-exponential phase (~0.4). Cultures were then distributed in 2 flasks (10 mL each), where one culture flask was used as control, and other was treated with 10 μg/mL of oxacillin for 15- and 60-min. Control cultures were incubated along with the treated culture for 15-min and 60-min. Aliquots of 2 mL cultures from each flask were taken, and mixed with 4 mL of bacterial RNA Protect solution (Qiagen, Valencia, CA). The mixtures were further vortex briefly for 5 s and incubated for 5 min at room temperature, followed by centrifugation at 5000×g for 20 min in a swinging-bucket rotor centrifuge to collect the cells. Cells were washed with fresh TSB and used for isolation of RNA.

Cells were further suspended in 200 μL of TE (30 mM Tris-HCl; 1 mM EDTA, pH 8.0) buffer containing lysozyme (50 μg/mL), mixed by vortexing for 10 s, and incubated at room temperature for 10 min on a shaker–incubator. Subsequently, 10 μL Proteinase K (100 μg/mL solution) was added in the solution and total RNA was extracted and purified using an RNeasy minikit (Qiagen) following the manufacturer recommendation for bacterial RNA isolation. For RNA isolation of both antibiotic treated and untreated cultures, two independent bacterial cultures were prepared. cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). RNA concentrations were determined by absorbance readings at 260 and 280 nm, using a Nanodrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was used to characterize the transcript levels in the cells in response to oxacillin treatment. qRT-PCR was performed with Rotor-Gene Real-Time PCR Cyclers (Qiagen) using SYBR green technology. RT-PCRs were performed in a 20 μL reaction volume containing 1 μL of template DNA (25 ng), 1 μL of gene specific primers (10 μM, Table 1), 10.0 μL of Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and 8.0 μL of H₂O. The following PCR conditions were used: 50°C for 2 min, 95°C for 10 min, 40 cycle of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s and one final extension step of 72°C for 10 min. The transcript levels of the fmtA and sarA genes were normalized using the 16S rRNA transcript level of S. aureus RN4220 as an internal control. Each gene assay was performed in triplicate. The data analysis was carried out with Rotor-Gene Q software (Qiagen).

Determination of MIC

Minimum inhibitory concentration (MIC) assays were performed as follows. Oxacillin dilutions were made using sterilized water and then aliquotted (1.5 μL) into 96-well plates. An overnight culture of RN4220 and sarA mutant were diluted 1/1000 into fresh TSB medium to give ca. 5×10⁵ CFU mL⁻¹, and 150 μL aliquots were added to each well of the 96-well plate. Plates were incubated at 37°C for ~18 h. MIC determinations were carried out on two different times in duplicate.

Results

Screening for the Transcription Factor(s) of fmtA

We divided the predicted 540 bp fmtA promoter region into three DNA fragments of approximately 270 bp each, referred to as seq1, seq2, and seq3, with seq3 overlapping seq1 and seq2 at the 5’ and 3’ ends respectively (Figure 1). When radioactively labeled seq1, seq2, and seq3 were incubated with oxacillin-induced and uninduced cell extracts from S. aureus RN4220, electrophoretic mobility shifts were observed (Figure 2A). Presence of oxacillin has a clear effect on the electrophoretic mobility profile of DNA considering the multitude of non-specific interactions that these DNA fragments could be subject of. Further, introduction of unlabeled DNA targets eliminated the mobility shifts of the labeled target DNA fragments (Figure 2B). These observations suggest that the promoter region of fmtA is the target of regulation by transcription factors.

We developed a screening protocol to isolate the transcription factor(s) of fmtA, using biotinylated seq3 (~207 to +63) immobilized on streptavidin-coated magnetic beads. These beads when incubated with oxacillin-induced cell extracts pulled down several proteins that were identified by analyzing LC-MS-MS data using the Mascot program and a probability based Mowse score of >45. The analyses showed the presence of staphylococcal accessory regulator A (SarA), pyruvate carboxylase, biotin carboxyl carrier protein of acetyl-CoA carboxylase, DNA-directed RNA polymerase alpha chain, DNA polymerase I, translation elongation factor Tu, translation elongation factor G, elongation factor Ts, chaperone protein dnaK, chaperonin GroEL, cell division proteins FisZ, DNA binding protein II, putative DNA-binding protein, and 30S ribosomal protein S5.

To confirm that SarA is pulled down by this protocol we incubated the oxacillin-induced cell extracts pulled down several proteins that were identified by analyzing LC-MS-MS data using the Mascot program and a probability based Mowse score of >45. The analyses showed the presence of staphylococcal accessory regulator A (SarA), pyruvate carboxylase, biotin carboxyl carrier protein of acetyl-CoA carboxylase, DNA-directed RNA polymerase alpha chain, DNA polymerase I, translation elongation factor Tu, translation elongation factor G, elongation factor Ts, chaperone protein dnaK, chaperonin GroEL, cell division proteins FisZ, DNA binding protein II, putative DNA-binding protein, and 30S ribosomal protein S5.

Purification of SarA and Characterization of its Oligomerization State

SarA belongs to the family of transcription factors called the “wing-helix” proteins [45]. Full-length SarA gene was cloned,
expressed, and purified to homogeneity (Figure 3). The identity of the protein was confirmed by mass spectrometry. The observed molecular mass of SarA was 14,635 D (theoretical mass, 14,586 D).

The oligomeric state of SarA was investigated using gel filtration chromatography. Based on the elution times of four standards, the molecular mass of SarA was estimated to be 30 kD (Figure 3B), which shows that SarA is dimer in our solution as shown previously [45, 46], hence we are working with a functional protein.

**SarA Binding to PfmtA**

To elucidate whether purified SarA binds directly to the PfmtA promoter, we performed EMSA experiments with seq1, seq2, seq3, seqA and seqB (Figure 1). The dissociation constant of each DNA sequence was determined as the concentration of SarA that resulted in 50% DNA bound. We estimated the dissociation constants of SarA with respect to seq1, seq2, seq3, seqA, and seqB to be 63±9 nM, 54±12 nM, 66±7 nM, 147±18 nM, and 139±17 nM, respectively (Figure 4). As a control, we used a 45-bp sequence derived from the promoter region of agr that harbors the SarA binding consensus sequence (underlined), 

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P 5'-GTAAAATTTTTATGTTAAAATATTAACAAAATTAC-GTTTTAC-3' (47). The SarA binding affinity for the Pagr sequence was about 400 nM (Figure 4).
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Supershifts were observed in the EMSA experiments with seq1, seq2, seq3, seqA, seqB, and Pagr at SarA concentrations higher than...
Interestingly, in the case of seq3, which covers the 3’-end of seq1 and 5’-end of seq2, the supershift starts at 150 nM SarA. This observation indicates that either there is more than one SarA binding site in these sequences, which are saturated at higher protein concentrations, or there are different oligomers of SarA binding to the fragments; SarA is known to form dimers and dimers of dimers in target promoters and is proposed to oligomerize at its binding sites (45).

Identification of SarA Binding Sites on PfmtA by DNase I Footprinting

Two fragments of PfmtA spanning the regions −207 to −70 (seqA) and −70 to +63 (seqB) were used to investigate SarA binding to PfmtA (Figure 1). The SarA protection sites on the bottom strand of each sequence are shown in Figure 5. SarA provided a protection of the DNA at concentrations higher than 200 nM. A similar DNase I protection profile was observed on the top strand of each DNA sequence (data not shown). Alignment of these sequences against the proposed 26 bp SarA consensus binding site, 5’-ATTTGTATTTAATATTTATATAATTG-3’ (47), revealed that the SarA-protected sites in PfmtA encompass the SarA consensus binding site (Figure 6). Interestingly, the SarA-protected sites in PfmtA also included the 7-bp DNA sequence 5’-ATTTTAT-3’, recognized by the winged-helix proteins, and also suggested to be recognized by SarA (45, 48) (Figure 6A). The lower binding affinity for seqA1 in comparison to Pagr could be due to the removal of the four

Figure 4. EMSA-based analysis of the DNA binding activities of SarA. 5’-[32P]-end labeled seq1 (A), seq2 (B), seq3 (C) fragments of PfmtA were incubated with increasing amounts of SarA (15, 23, 30, 45, 60, 75, 90, 105, 120, 150, 225, 300, 450, 600, 900, 1200, 1500, and 1800 nM). In the bottom panels, 5’-[32P]-end labeled seqA (D) and seqB (E) fragments of PfmtA were incubated with increasing amounts of SarA (0, 15, 30, 60, 120, 240, 480, 960, and 1500 nM). The 5’-[32P]-end labeled agr promoter region (F) was incubated with 30, 60, 90, 120, 180, 240, 480, 960, and 1800 nM SarA. doi:10.1371/journal.pone.0043998.g004

Figure 5. DNase I footprint analysis of SarA binding to seqA and seqB fragments of PfmtA. A. Each DNA sequence was labeled with [γ-32P]ATP on the 5’-end of the bottom strand. doi:10.1371/journal.pone.0043998.g005
nucleotides however binding of SarA to seqA suggests that the 26 bp SarA consensus binding site identified in seqA recruits SarA and these nucleotides may be involved in the SarA binding.

Analysis of the DNA-binding Properties of SarAR90A Mutant
Arginine at position 90 is essential for the SarA regulatory activity in vivo. It is located within the winged region of SarA and is part of the conserved basic region of SarA, the motif DER (45). We mutated this residue to alanine, purified the mutant protein, and confirmed the substitution by mass spectrometry. We then analyzed the binding affinity of the SarAR90A mutant protein to seq, seq2 and seq3 by EMSA. The mutant protein failed to bind to these three DNA sequences (Figure 7).

SarA Promotes in vitro Transcription of fmtA
We used E. coli RNA polymerase holoenzyme (RNAP) in these experiments. Several lines of evidence lead us to conclude that E. coli RNAP is a good substitute for S. aureus RNAP in the case of fmtA. The E. coli RNAP is a complex of the RNAP core enzyme and the β70 factor protein. The DNA sequence of the putative −10 box in the fmtA promoter is identical to the consensus −10 box recognized by E. coli β70 (Figure 1), suggesting that the fmtA promoter may recruit a β70-like factor for transcription. The β70 homolog in S. aureus is SigA factor [49,50], in turn suggesting that regulation of fmtA could be SigA-dependent. Incidentally, SarA regulates its own promoter in a SigA-dependent mechanism [51]. Our hypothesis that E. coli RNAP is a good substitute for that of S. aureus was confirmed by in vitro run-off transcription experiments.
with PfmtA and PfmtAT14G mutated at the putative +1 and −14 sites. We observed that while E. coli RNAP could poorly initiate transcription from PfmtA, there was a complete lack of transcription for the T(+)1 to G and T(−)14 to G PfmtA variants (Figure 8). Furthermore, the presence of SarA increased the production of the transcript from the PfmtA fragment in a concentration-dependent manner (data not shown).

SarA Regulates Expression of fmtA–lux Fusion in vivo

To investigate the role of the fmtA promoter in recruiting SarA in vivo, we fused PfmtA-derived sequences (Pseq1, Pseq2, Pseq3, and Pseq1−2) upstream of the lux operon in the reporter vector pXEN1 [52]. The lux fusion plasmids and the control vector pXEN1 were introduced into S. aureus RN4220. We hypothesized that SarA exerts direct regulation of fmtA via the SarA binding sites identified in our in vitro experiments. If this hypothesis were correct, we would expect that the disruption of SarA would reduce lux operon expression.

To test this hypothesis, we introduced the lux fusion plasmids into a SarA-deficient strain, PC1839 [53], and compared the luminescence of the RN4220 (sara+) strains and PC1839 (sara−) strains.

Figure 7. DNA binding activity of SarAR90A mutant. Binding reactions between [γ-32P]-end labeled seq1, seq2 and seq3 fragments of PfmtA and increasing amounts of SarAR90A variant.
doi:10.1371/journal.pone.0043998.g007

Figure 8. SarA activates fmtA transcription in vitro. (A) In vitro run-off transcription from the fmtA promoter region using E. coli RNAP holoenzyme and SarA. PfmtA region −342 to +199 was used as a template. The size of the transcript is compared against the qX174DNA/Hinf I molecular weight marker (M). (B) The intensities of the corresponding transcripts were measured by ImageJ software. The substitution of T1G in the proposed transcription start point of the fmtA promoter led to a significant decrease in the transcript (P = 0.0242) whereas the mutation of the T14 to G in PfmtAT14G has the most pronounced effect on the transcription (P = 0.001). The statistical analysis was carried out by student t test and GraphPad Prism software (La Jolla, California).
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strains under oxacillin-induced and uninduced growth conditions. Strains harboring different fusion constructs showed comparable growth rates (data not shown). The luminescence data are shown in Figure 9. In the absence of oxacillin, a low level of luminescence signal was observed, but the RN4220::lux strain, treated with oxacillin, showed increased luminescence signal. Presence of 100 μg/ml oxacillin increased the lux operon expression especially in the case of the Pseq3::lux fusions, along with the detection of a 5.8-fold increase in the presence of 100 μg/ml oxacillin. The newborns represent the standard deviations determined from these trials. doi:10.1371/journal.pone.0043998.g009

Deletion of sarA Enhances S. aureus Sensitivity to Oxacillin

To understand whether the lack of functional SarA protein alters the sensitivity towards oxacillin, we tested the oxacillin MICs of wild type S. aureus RN4220 and sarA mutant. The sarA mutant showed modest, but significant increase (2-fold) in MIC. The measured MICs of oxacillin for wild-type RN4220 and sarA mutant were 0.3 μg/mL and 0.15 μg/mL, respectively.

Transcript Levels of sarA and fmtA in S. aureus RN4220 and PC1839

Our data revealed that sarA transcript level in S. aureus RN4220 did not change upon oxacillin treatment compared with corresponding untreated control culture. However, fmtA transcript level was increased 3-fold upon oxacillin treatment (3.5±0.7). This finding is in good agreement with the previously published data where no change in sarA transcript level, but increased transcript level of fmtA was observed [28]. Increased expression of fmtA in response to several cell wall active antimicrobials was observed in several studies [32,54]. On the other hand, in the sarA mutant strain, PC1839, the transcript level of fmtA did not change upon oxacillin treatment compared with corresponding untreated control culture.

Discussion

Genome-based studies have shown that fmtA levels in S. aureus are upregulated in the presence of cell wall inhibitors and by the knock-out of the genes involved in peptidoglycan biosynthesis [27,28,30,55]. A study by Kuroda et al. suggested that fmtA is under the regulation of the two component signal transduction system VraSR [55]. However, the mechanism by which fmtA expression is regulated remains unknown. In our study DNA fragments derived from fmtA region showed retardation in their electrophoretic mobilities when incubated with cell extracts isolated from S. aureus RN4220 with clear differences from the instance when bacteria were subjected to oxacillin (Figure 2A). The electrophoretic mobility shifts were eliminated in the presence of a competing DNA fragment (Figure 2B).

Herein, we report a screening protocol designed to identify the transcription factor(s) involved in regulation of fmtA expression. A 269-bp fragment of the fmtA promoter region (seq3) was immobilized on streptavidin-coated beads and used to capture proteins from extracts of S. aureus cells treated with oxacillin to induce fmtA expression. Protein binding and washing steps were performed at physiological ionic strength and in the presence of non-specific DNA to reduce non-specific interactions. A relatively small number of non-DNA-binding proteins were identified in the screen, including pyruvate carboxylase and biotin carboxyl carrier protein of acetyl-CoA carboxylase which both have biotin as a cofactor and could be binding to the beads through streptavidin. The presence of RNA polymerase alpha chain, DNA polymerase I, translation elongation factor Tu, translation elongation factor G, elongation factor TS, and 30S ribosomal protein S3 could be due to the presence of the fmtA ribosomal binding site in seq3. The presence of DNA binding protein II, a protein that recognizes polyA DNA regions, could bind nonspecifically to the seq3 fragment which contains several polyA sites, and proteins such as chaperone protein DnaK, GroEL and FtsZ could bind nonspecifically to DNA or streptavidin.

SarA was the only transcription factor identified by our screening protocol. DNase I footprinting demonstrates that SarA binds to specific sites in the fmtA promoter region. Specificity of SarA binding to fmtA was probed by mutation of one of the

Figure 9. Probing the role of SarA on fmtA expression using a lux reporter vector. Luminescence signal recorded in the absence and presence of 10 or 100 μg/mL oxacillin. Each strain is represented by the name of the promoter fused upstream of the lux operon in the pXEN1 plasmid. A) Experiments carried out with S. aureus RN4220 strain and B) experiments carried out with S. aureus ΔsarA mutant strain, PC1839. The luminescence signals were recorded in triplicates and normalized based on the viability data. The error bars represent the standard deviations determined from these trials.
conserved residues located in the winged region of SarA, Arg-90. The mutant SarAR90A protein, which is unable to regulate the SarA target promoters in vivo [45], failed to bind to the fmtA promoter region (Figure 7).

The role of SarA in regulation of fmtA was corroborated by in vivo studies. Fusion of the lux operon to the various regions of the fmtA promoter indicated that the fmtA promoter region harbors transcriptional regulatory elements and that SarA is involved in the regulation of fmtA expression. These data are corroborated by the qRT-PCR data, whereby the fmtA expression level increases by 3.5-fold in the presence of oxacillin, while sacA level remains unchanged. In the sacA mutant, fmtA level are not affected by the presence of oxacillin. Interestingly, the in vivo data (Figure 9) suggest that SarA is involved in basal expression of fmtA as well as upregulation of fmtA in the presence of cell wall stress. Further, in vitro run-off studies suggest that transcription of fmtA may require the S. aureus primary sigma factor, SigA. This is in agreement with a previous report that shows that SarA regulates promoters that are SigA-dependent [51]. Additional evidence of the involvement of SigA in the transcriptional regulation of fmtA is provided by mutagenesis studies in which mutation of the −14 position and the transcription starting site of PfmtA resulted in drastic reduction of fmtA transcription.

Sequence alignment of PfmtA-seq3 against the SarA consensus binding site [56] revealed two regions that contain this sequence, centered around −168 and −53 nucleotides. In addition, we identified three sites that harbor the 7-bp consensus binding sequence for the winged-helix-turn-helix DNA-binding proteins, which SarA is a member (Figure 6) [57]. The 7-bp consensus binding sequence appears in pairs in PfmtA; two pairs are within the 26-bp SarA binding consensus sequences and the third pair is centered on the −112 nucleotide (Figure 6). The EMSA studies with DNA fragments derived from PfmtA, harboring the 26-bp (segA1) or the 7-bp SarA binding sequences (segA2), showed that SarA is recruited to these sites. The DNA fragment that did not contain any of these sequences (segB1) failed to recruit SarA, suggesting that SarA recognize specific sequences in PfmtA, known to interact with SarA, and that it may utilize both these sites to bind to the fmtA promoter.

SarA is a global transcriptional regulatory protein linked to the regulation of numerous virulence factors [58–60]. It is a 124-residue protein that forms dimers in solution and binds as a dimer to the target promoters. SarA also belongs to the winged-helix-turn-helix family of proteins. Several S. aureus proteins share high sequence similarities with SarA and are grouped into a SarA family of proteins [44].

Based on the structural information on the SarA family of proteins, four regulatory mechanisms are proposed for SarA [44]: 1) bending of the target DNA to facilitate contact with the regulatory proteins; 2) the formation of three SarA dimers which hold the DNA in a closed configuration not amenable to transcription; 3) the formation of a heterodimer between compatible family members that may interfere with the function of the homodimer and 4) competitive displacement of one homolog by another. In the case of the fmtA promoter, it is possible that SarA may follow the first regulatory mechanism whereby SarA could bind as a dimer at the three identified sites.

The SarA family of proteins has been recently linked to S. aureus autolysis, biofilm formation, and resistance to cell wall inhibitors [61–65]. A study by McAleese et al. showed that a clinical isolate of S. aureus with intermediate resistance levels to vancomycin (a VISA strain) exhibited higher sacA expression levels than the vancomycin susceptible parent strain when grown in an antibiotic-free medium [27]. The expression levels of fmtA were also increased in the VISA strain. Interestingly, the sacA levels in susceptible or methicillin-resistant strains are not reported to increase in the presence of cell wall inhibitors; our data show the same phenomenon. By contrast, fmtA expression increases in the presence of cell wall inhibitors despite the strain background [27,28,30,52]. The question that arises is, how does SarA regulate the expression of fmtA when its protein levels are not altered in response to cell wall stress? It has been hypothesized that the pleiotropic regulatory capabilities of SarA could be due to posttranslational modifications [66]. Recently, it was reported that SarA is a phosphorylation target by Stk1 Ser/Thr kinase [67]. Perhaps regulation of fmtA in response to stress is mediated by phosphorylated SarA or other posttranslational modifications. However, we cannot exclude that another transcription factor may be involved in the regulation of fmtA during antibiotic-induced cell wall stress.

The fmtA gene is reported to be part of the VraSR regulon [55]. However, VraR was not isolated from our protocol. There could be two reasons for this: i) VraR is not involved in regulation of fmtA or ii) our screening protocol failed to identify VraR due to low binding affinity of VraR for the fmtA promoter. We have previously shown that VraR binding affinity to its own promoter is 1 μM, which is one order of magnitude higher than that of SarA for PfmtA [68]. It is possible that under our screening conditions the occupancy of PfmtA by VraR is low. A recent study by Sengupta et al. used chromatin immunoprecipitation techniques to investigate the promoters under the direct control of VraR [69]. Interestingly, VraR was not identified as the transcription factor for fmtA, but the study showed that other members of the VraSR regulon, such as phb2, marZ, and sgrB, are under the direct regulation by VraR.

In conclusion, this study links the global regulator SarA with a penicillin-binding protein that is also involved in autolysis and biofilm formation. Our findings further extend the multiple functions of SarA and establish a link between processes involved with S. aureus pathogenicity, i.e., virulence factor expression and the cell wall stress response.

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Author Contributions

Analyzed the data: YZ VV AS. Designed the in vitro run-off experiments: AB. Performed the qRT-PCR, MIC experiments: AS. Performed the EMSA experiments: MF.

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