Conformational features of the \textit{Staphylococcus aureus} AgrA-promoter interactions rationalize quorum-sensing triggered gene expression

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1. Introduction

The accessory gene regulator mechanism (Agr) coordinates the expression of cytolytic toxins with a quorum stimulus in \textit{Staphylococcus aureus}. The Agr quorum sensing system comprises of four components- the histidine kinase AgrC, a response regulator AgrA and a permease AgrB that processes AgrD to generate the auto-inducing peptides (AIP) that vary in sequence (AIP I-IV) [1]. AIP binding to the ecto-domain of AgrC triggers an intracellular signal transduction cascade that couples a quorum stimulus with a transcriptional response. The response regulator AgrA governs transcriptional re-engineering by binding cognate DNA sequences leading to the up-regulation or repression of gene expression. AgrA also modulates the expression of RNAIII, a pleiotropic effector involved in the up-regulation of exotoxins like alpha-haemolysin and thus has a direct role in the virulence and pathogenicity of \textit{S. aureus} [2]. While genes in the \textit{agr} operon are transcribed from the P2 promoter, RNAIII transcription is driven from the P3 promoter. The AgrA binding sites preceding these promoter elements are present in the intergenic region of the \textit{agr} operon and the RNAIII locus. Another AgrA interacting sequence, referred to as the P1 promoter, governs the expression of \textit{agrA}. While the P1 promoter was the first to be reported in the initial characterization of the \textit{agr} locus, very little is known about AgrA-P1 interactions [3].

The response regulator AgrA has two domains- an N-terminal CheY-like receiver domain (residues 1-130) that is connected by a flexible linker to a DNA binding domain (AgrADBD: residues 138-238). The activation of the histidine kinase AgrC upon binding AIP initiates the phosphotransfer reaction from His239 of AgrC onto Asp59 of AgrA. AgrA is predominantly a monomer in solution and dimerizes upon phosphorylation [4]. This finding differs from the more common mechanism wherein a conformational change due to the exposure of a hydrophobic pocket in the receiver domain upon phosphorylation influences DNA binding [5]. The AgrADBD domain (a representative of the LytTR domain family) adopts a ten-stranded β-scaffold with an interspaced α-helix and a short 310 helix [6]. Transcription factors with the LytTR domains have been noted to govern virulence gene expression as well as regulate house-keeping functions in bacteria [7–11]. The LytTR domain of AgrA interacts with imperfect direct repeats of DNA with a consensus sequence- [TA][AC][CA]GTTN[AG][TG] [12]. These sequence motifs, separated by 12–13 basepairs, are located upstream of the promoter element that is recognized by the RNA polymerase holoenzyme to initiate transcription. Activation by AgrA was
suggested to be crucial for transcription from the P3 promoter whereas expression from the P2 promoter can occur independent of AgrA [13]. AgrA-mediated activation of transcription from the P1 promoter remains to be characterized. The crystal structure of an AgrA-DNA complex (referred to as P2_S2 in this manuscript) revealed that AgrA binding induces substantial conformational changes in the promoter DNA [6]. While this feature could not rationalize promoter strength, the structure revealed residues in AgrA that were important for DNA binding and conformational features that can influence promoter specificity. AgrA-promoter interactions are also sensitive to reductin stimuli [14]. More recently, a post-transcriptional mechanism was suggested to regulate intracellular levels of AgrA. This mechanism, that involves the selective degradation of AgrA mRNA by CshA, is also likely to influence the temporal response to a quorum stimulus [15].

The structure of AgrA<sub>IRD</sub> in complex with promoter DNA (P2_S2) was first reported by Sidote et al. [6]. Here we describe the crystal structures of the AgrA<sub>IRD</sub> complexes with different DNA sequences. These structures revealed that the LytTR domain of AgrA makes fewer interactions with the DNA binding site located proximal to the −35 element of the promoter. We discuss the impact of these observations on the functional role of AgrA as an activator of gene expression. The structural and biochemical data presented in this manuscript suggest that phosphorylation induced dimerization of AgrA plays an important role in the selective enhancement of RNA polymerase occupancy at sub-optimal promoter elements.

2. Materials and methods

2.1. Cloning, expression and purification of recombinant AgrA DNA binding domain (AgrA<sub>IRD</sub>)

AgrA<sub>IRD</sub> was amplified from Staphylococcus aureus genomic DNA using forward (5′ CCTACATATGCTCCATATGATAACCGTTGAAAGCATTGAAAT 3′) and reverse (5′ GAACCCCTGAGTTATATTTTTTTAAGATTTTTTTTAAACGTTTCACTATGACGTCATAGCAGA 3′) primers. These amplicons were ligated between the Ndel and XhoI restriction sites of the pET22b expression vector. Insertion of a stop codon at the 5′ end of the reverse primer resulted in an expression construct without the poly-histidine affinity tag. The E. coli Rosetta (DE3)pLysS cells (Novagen, Inc.) were transformed with the plasmid encoding AgrA or AgrA<sub>IRD</sub> and grown to an OD<sub>600</sub> of 0.5 at 37 °C in Luria broth with 2.5 mg/ml of kanamycin. Overexpression of the proteins was initiated by induction with 0.5 mM IPTG (Isopropyl-β-D-thiogalactopyranoside, Gold Biotechnology Inc.) and the culture was further grown for 16 h at 18 °C. All purification steps were carried out at 4 °C. The cells were lysed by sonication in a buffer containing 20 mM HEPES pH 7.4, 300 mM KCl, 10% glycerol and 1 mM PMSF. The lysate was subjected to centrifugation at 26,500 × g for 45 minutes to remove the cell debris. The supernatant was incubated with His-select nickel affinity beads (Sigma-Aldrich, Co) for one hour before loading onto a column. The protein was eluted by a gradient of imidazole (60–500 mM) in buffer containing 20 mM HEPES pH 7.4, 300 mM KCl and 10% glycerol. Subsequently, the partially purified protein was loaded onto a Sephacryl S-200 (HiPrep 16/60) column (GE Healthcare) pre-equilibrated with 20 mM HEPES pH 7.4, 300 mM KCl, 10% glycerol and 5 mM β-mercaptoethanol. The purity of the protein was analyzed on a 12% SDS-PAGE gel.

2.3. Prediction of the P1 promoter

Gene fusion analysis aided in the identification of the P1 promoter between the PvuII and RsaI restriction sites in the agr operon [3]. The region between these two restriction sites was used as an input sequence for the BPROM server, a web-based bacterial promoter prediction server, (Softberry, Inc., Mount Kisco, NY, USA; http://linux1.softberry.com) to map the −10 and −35 promoter elements. Based on the sequence characteristics of LytTR recognition motifs, the putative AgrA binding sites at the P1 promoter were identified (Fig. 1) and further characterized in this study [12].

2.4. Oligonucleotides for structural and biochemical studies

The oligonucleotides used for the crystallization, fluorescence anisotropy (Table S1) and surface plasmon resonance experiments (Table S2) were obtained from Sigma Aldrich, Co. The complementary oligonucleotides were mixed in equimolar ratios and annealed in a Bio-Rad MyCycler. In this step, the oligos were heated to 96 °C and temperature was gradually decreased to 4 °C with 1 °C/min fall in every cycle and stored at −20 °C until use.

2.5. Crystallization and structure determination

AgrA<sub>IRD</sub> (0.67 mM) was incubated with 0.8 mM of DNA for one hour at 4 °C prior to setting up crystallization experiments using the vapor diffusion method. Crystals for AgrA<sub>IRD</sub> with different
Fig. 1. The *S. aureus* agr operon. A. Schematic of agr operon. AgrA mediated change in gene expression occurs from three characterized promoters segments P1–P3. While P1 governs the expression of AgrA, P2 controls the expression of the entire *agr* operon. P3 dependent expression leads to up-regulation of the effector RNAIII thus providing an additional indirect mode of transcriptional re-engineering upon a quorum stimulus. B. Sequence features of P1, P2 and P3 promoter segments. The AgrA binding sites (imperfect sequence repeats) proximal and distal to transcriptional initiation site are referred to as Px_S1 and Px_S2 in the text. C. The structure of AgrADBD domain in complex with the P3 promoter sites. AgrADBD interacts with promoter DNA by inserting loops into two successive major grooves and an intervening minor groove. Extensive mutational analysis revealed residues which confer structural stability to the protein (green spheres) and residues which are important for protein-DNA interactions (blue spheres) [28]. The red spheres represent residues which form base specific interactions with the promoters. D. AgrADBD-P3_S1 promoter interactions. In this representation, the AgrADBD is shown to be involved in three base specific interactions with P3_S2 alongside several hydrogen and non-hydrogen bonded interactions with the phosphate backbone.
promoters were obtained in various conditions (Table S3). All the crystals were grown at 18 °C. The crystals were soaked in cryo-protectant (25% Ethylene glycol) for 30 s before flash freezing in liquid nitrogen. The data were collected at the BM-14 beamline (ESRF, Grenoble) at 100 K. The diffraction images were processed using iMosfet and scaled with SCALA [16,17]. The AgrA LyrTR domain promoter complex (PDB code 3BS1) was used as a search model for molecular replacement [6]. The program phaser from CCP4 suite or phenix-MR from the Phenix interface were used for the molecular replacement calculations [18,19]. The structures were refined in Refmac5 and the fit of the model to electron density was examined using COOT [20,21]. All the models were validated using MOLPROBITY and illustrative figures were prepared in PyMOL (Schrödinger, LLC) [22].

2.6. Surface Plasmon Resonance spectroscopy

The interaction kinetics of P1, P2 and P3 promoters with AgrA was analyzed by Surface Plasmon Resonance (SPR: BIACORE 2000, GE Healthcare). 5′-biotinylated promoters (Table S2) (Sigma-Aldrich, Co.) were immobilized on a Streptavidin (SA) chip (GE Healthcare). The first flow cell in the SA chip was used as a reference channel. These experiments were performed in a buffer containing 20 mM HEPES pH 7.4, 200 mM KCl, 2% glycerol and 5 mM β-mercaptoethanol. Varying concentrations of AgrA and AgrAΔN389 were used as analytes. Both AgrA and AgrAΔN389 were also incubated with 300 μM of acetyl phosphate for 30 min prior to passing on the SA chip. The binding kinetics for AgrA and AgrAΔN389 with different promoters (P1, P2 and P3) was analyzed using the BIA evaluation software (BIACORE, GE Healthcare). The rates of association and dissociation were obtained by fitting the data to a 1:1 Langmuir interaction model.

The relative specificity of AgrA for the P1, P2 and P3 promoters was evaluated using a SPR-based competition assay [23]. In this assay, biotinylated P1, P2 and P3 promoters were immobilized on a streptavidin chip (SA chip, GE Healthcare). 1 μM of AgrA was pre-incubated with 5 μM of P1, P2 and P3 promoters (non-biotinylated) at room temperature for 20 min. 1 mM acetyl phosphate was added to AgrA (1 μM) to obtain the phosphorylated protein. Both analyte samples (AgrA and AgrA pre-incubated with promoters) were evaluated for their interaction with the biotinylated promoters immobilized on the SA chip. The change in the response units due to binding of AgrA and AgrA pre-incubated with promoters were recorded. In these experiments, a competition between the immobilized promoter and a non-biotinylated promoter for AgrA results in a change in the response units. A decrease in the response units can thus be correlated with stronger interactions between AgrA and the pre-incubated non-biotinylated DNA than the immobilized DNA on the chip.

2.7. Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 2. Cloning and DNA manipulations were performed in E. coli DH5α whereas E. coli DC10B (a DNA cytosine methyltransferase deficient strain) was used as a primary recipient for plasmids to be electroporated in to S. epidermidis TU3298 using a Biorad MicroPulser following the protocol reported by Augustin et al. earlier [24,25]. While E. coli strains were grown on LB (Luria Bertani) agar or broth at 37 °C with shaking 100 μg/ml ampicillin, S. epidermidis cells with plasmids were grown on TSb (tryptic soy) agar or broth at 37 °C with shaking containing 10 μg/ml chloramphenicol and induced with 1 μg/ml of anhydrotetracycline (ATC).

2.8. GFP reporter plasmid construction

pRMC2 (an E. coli - Staphylococci shuttle vector) has one multiple cloning site between KpnI and EcoRI restriction sites. For GFP reporter plasmid assays, an additional cloning site was introduced between PstI and HindIII by the addition of a Nhel restriction site. This modified pRMC2 vector is referred to as pRMC2M in this study. Agr P1, P2 and P3 promoters were amplified from S. aureus COL genome using the primers tabulated in Table S4. GFP was amplified from the pKEN vector using primers (GFP_P1_Fw, GFP_P2_Fw, GFP_P3_Fw and GFP_Nhe_Rv). Overlap extension PCR was used to fuse the promoters with the GFP and the resulting PCR product was digested with PstI and Nhel restriction enzymes and ligated into the cloning site-2 of pRMC2M (P1GFP, P2GFP and P3GFP clones).

2.9. Cloning and site directed mutagenesis of agrA

S. aureus agrA was amplified using primers AgrA_Fw and AgrA_Rv. The amplified product was digested with KpnI, PstI and EcoRI restriction enzymes (cloning site 1) and ligated into the pRMC2M vector with P1GFP, P2GFP or P3GFP cloned at the cloning site 2. Clones were confirmed by sequencing. A single primer was used for site directed mutagenesis in which P1GFP-AgrA, P2GFP-AgrA and P3GFP-AgrA clones were used as templates (Table S4). The PCR product was treated with DpnI for 2 h at 37 °C before transforming into E. coli DH5α. Three other plasmid clones were used as controls. These had only P1GFP, P2GFP and P3GFP at cloning site 2 and a vacant cloning site 1 (no agrA). All the reporter plasmid clones were confirmed by sequencing.

2.10. RNA isolation and qRT-PCR reaction

0.5 ml of S. epidermidis cells, uninduced and induced (with ATC) were harvested after 3 hours of growth. Two volumes of RNA protect reagent (Qiagen) was added to S. epidermidis cells and incubated for 10 minutes at room temperature before centrifugation at 7000 rpm for 10 min at 4 °C. These cells were stored at ~ −80 °C till further use. RNA was isolated from S. epidermidis using previously described protocols [26]. Cells were thawed on ice and resuspended in 500 μl of acidified phenol chloroform (5:1) pH 4.5 (Ambion) and mixed with 500 μl of NAES buffer (50 mM sodium acetate pH 5.1, 10 mM EDTA, 1% SDS) at room temperature. The sample was transferred to tubes containing 0.1 ml zirconia-silica beads and vortexed (4800 rpm × 30 s × 2times) with intermittent incubation on ice using a mini bead beater (Biospec, Inc.). After this step, the tubes were centrifuged at 12,000 × g for 5 minutes at 4 °C. Around 450 μl supernatant was precipitated with 520 μl of isopropanol and 35 μl of 3 M sodium acetate. The pellet was washed with 70% ethanol and the tubes were completely dried before resuspending in 25 μl of RNase free water. RNA samples were treated with RNase-free DNase (Qiagen) for 1 hour at 37 °C and RNA was further purified using RNAeasy mini kit (Qiagen) before cDNA synthesis. 1 μg of RNA was converted to cDNA using iscript reverse transcription supermix (Biorad, Inc.). 20 ng of cDNA per reaction was used for quantitative real time PCR (qRT-PCR) with the Biorad iQ5 thermo cycler. The primers used to amplify target genes are listed in Table S5. The reaction mixtures were incubated for 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C and a final extension at 72 °C for 5 min. The chloramphenicol acetyl transferase (cat) gene which is an antibiotic marker of pRMC2 plasmid was used both as an endogenous control as well as to monitor the copy number of the plasmid per cell. The relative transcription levels of target genes were determined by the 2−ΔΔCT method by using the values from uninduced samples as control [27]. The values represent the mean standard deviation from three independent experiments.
3. Results and discussion

3.1. Comparison of the AgrA LytTR domain promoter DNA complexes

AgrA influences transcription from three promoter elements (P1–P3) (Fig. 1A). Each promoter comprises of imperfect direct repeats referred to as binding site 1 (proximal to –35 promoter element) and binding site 2 (distal to –35 promoter element) in this manuscript (Fig. 1B). The distal site (Site 2) is located 12 bp upstream of site 1. Crystal structures of the AgrADBD in complex with these different promoter DNA sequences were determined to evaluate conformational features that enable transcriptional activation. While all three pairs of AgrADBD complexes (corresponding to the DNA sequences at the P1, P2 and P3 elements) could be crystallized, crystals of the AgrADBD-P1 complexes diffracted poorly in comparison to the P2 and P3 complexes. The structural comparisons were thus limited to four structures—corresponding to the P2 (P2_S1 and P2_S2) and the P3 promoter sequences (P3_S1 and P3_S2). The diffraction data, refinement and model statistics for these structures are compiled in Table 1.

AgrADBD belongs to the LytTR family of response regulator proteins with ten β-strands assuming a β-β-β fold. Unlike other DNA binding proteins, AgrADBD interactions with DNA are governed by residues located in the loop segments of the protein (Fig. 1C). These interactions comprise of both direct and indirect readouts involving base specific interactions as well as interactions with the phosphodiester backbone. The structures of both apo and DNA bound AgrADBD have been described earlier [6]. However, a comparison between the structures of AgrADBD bound to different DNA complexes revealed a pattern to AgrADBD-DNA interactions that was hitherto unanticipated. A common element in all four AgrADBD-DNA complexes is a direct readout between the Guanine 13 on Strand A in the major groove with His169 and nine indirect readouts formed through hydrogen bonding and van der Waals interaction with the phosphodiester backbone. Apart from these interactions, the site 2 of both P2 and P3 promoters formed two more base specific interactions with the DNA. These include Asn201 interacting with Thymine 10 (Strand A) in P3_S2 and Agr233 which makes electrostatic interactions with Guanine 12 (Strand B) in both P3_S2 and P2_S2 (Fig. S1). The residues present in the loops form about 12–16 interactions with the phosphodiester backbone at the site 2 of P2 and P3 promoters. A comparison between the pairs of site 1 and site 2 complexes however, revealed an interesting pattern in the protein-DNA interactions (Fig. 2). The AgrA binding site proximal to the –35 element (AgrADBD:P2_S2; AgrADBD:P3_S1) has fewer protein-DNA interactions than the distal interaction segment (AgrADBD:P2_S2; AgrADBD:P3_S2) (Figs. 1D, S2 and S3). These enhanced interactions in case of AgrA with the binding site distal to the transcription start site suggest a role for this site in anchoring the AgrA dimer.

The crystal structures also rationalize mutations that were noted to substantially influence AgrA activity. These residues were classified as those that abrogate DNA binding, residues that influence protein stability and point mutants that influence transcription activation (Figs. 1C and S4) [28]. For example, His200 which was shown to affect protein DNA interactions (apart from His169, Arg233 and Asn201 which were shown to distort AgrADBD-P2_S2 promoter interactions by Sidote et al.), forms a hydrogen bond with the phosphate backbone of the P3_S1 promoter DNA. In P2_S1, His200 forms interactions with deoxyribose sugar while in P2_S2, a water molecule coordinates this interaction with the phosphate backbone. The AgrA LytTR domain-promoter complex (referred to as P2_S2 in this study) introduces a 35° bend in the conformation of the bound DNA [6]. In the four complexes that were examined, a distortion of 30–36° was observed except in the case of the P2_S1 promoter where the conformational change is

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Table 1: Diffraction data and refinement statistics.

| Model | Ramachandran Statistics | R.m.s. Deviations |
|-------|-------------------------|------------------|
| # | Native (PDB: 4XYO) | P2_S1 promoter (PDB: 4XXE) | P2_S2 promoter (PDB: 4XYQ) | P3_S1 promoter (PDB: 4XQJ) | P3_S2 promoter (PDB: 4XQI) | P3_S2 promoter (PDB: 4XQN) |
| Preferred (%) | 96.9 | 96 | 97 | 97 | 96.4 | 99 |
| Allowed (%) | 3.1 | 3.0 | 3.0 | 3.0 | 3.6 | 1.0 |
| Outliers (%) | 0 | 1 | 0 | 0 | 0 | 0 |
| Length (Å) | 0.009 | 0.011 | 0.012 | 0.009 | 0.011 | 0.012 |
| Angle (°) | 1.34 | 1.49 | 1.61 | 1.33 | 1.54 | 1.64 |

# Values in the parenthesis represent the outer resolution shell statistics

- $R_{	ext{free}} = \frac{\sum_i |F_{o,i} - F_{c,i}|}{\sum_i F_{o,i}}$ is the average intensity.
- $R_{	ext{free}} = \frac{\sum_i S_i |F_{o,i}| - F_{c,i}}{\sum_i S_i |F_{o,i}|}$ is the average intensity.
much lower (25.9° global bend over a 13 bp stretch calculated using Curves++ [29]). Indeed, the buried surface area for P2_S1 is only 1293 Å² when compared to other AgrA LytTR-promoter complexes that have a larger interacting interface 1400–1500 Å² [30]. The variations noted in the crystal structures of AgrADBD with different promoter complexes suggest a mechanism whereby the

Table 2

| Bacterial Strains/Plasmids | Description                                                                 | Reference |
|----------------------------|------------------------------------------------------------------------------|-----------|
| **Bacterial Strains**      |                                                                              |           |
| E. coli DH5α               | F’ (w80d Δlac2 M15) D(lacZYA-argF) U169 hsdR17(rK2M) recA1 endA1 relA1 deoR12 phoA supE44 thi-1, gyrA96 | [31]      |
| E. coli DC10B              | Δdcm in the DH10B background, Dam methylation only                           | [24]      |
| S. aureus COL              | Clinical isolate, genomic DNA                                                | [32]      |
| S. epidermidis TU3298      | Capable of being transformed with and stably maintaining recombinant plasmids. | [25]      |
| **Plasmids**               |                                                                              |           |
| pRMC2                      | Derivative of the tetracycline-inducible expression vector pALC2073; anhydrotetracycline inducible expression vector, Amp’ in E. coli, Chl’ in S. aureus. | [33]      |
| pRMC2M                     | pRMC2 vector with cloning site 2 after TetR gene having (PstI, SphI, MfeI, NheI and HindIII) restriction site. | This Study|
| P1GFP-AgrA                 | AgrA in cloning site 1 with agrP1 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P2GFP-AgrA                 | AgrA in cloning site 1 with agrP2 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P3GFP-AgrA                 | AgrA in cloning site 1 with agrP3 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P1GFP-AgrAD59A             | AgrA59A in cloning site 1 with agrP1 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P2GFP-AgrAD59A             | AgrA59A in cloning site 1 with agrP2 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P3GFP-AgrAD59A             | AgrA59A in cloning site 1 with agrP3 promoter driven GFP, cloned in cloning site 2 of pRMC2M. | This Study|
| P1GFP-ΔAgrA                | agrP1 promoter driven GFP, cloned in cloning site 2 of pRMC2M.               | This Study|
| P2GFP-ΔAgrA                | agrP2 promoter driven GFP, cloned in cloning site 2 of pRMC2M.               | This Study|
| P3GFP-ΔAgrA                | agrP3 promoter driven GFP, cloned in cloning site 2 of pRMC2M.               | This Study|

Fig. 2. Structural features of AgrADBD-promoter interactions. (A) Stereo representation of the AgrADBD-promoter interactions. (B) The residues which are involved in base specific interactions are superimposed. The structures of the AgrADBD complex with the P3_S1 and P3_S2 promoters reveal that P3_S1 interactions involve fewer protein DNA contacts that P3_S2.
imperfect direct repeats in the promoter sequences could influence AgrA function (Table 2).

In the light of earlier reports wherein AgrA was also demonstrated to be redox-sensitive, several attempts were made to obtain the oxidized form of this protein. However, in all the structures that were determined in this study, we could not

### Table 3

Interaction between AgrA and cognate DNA sequences monitored by Surface Plasmon Resonance spectroscopy.

|          | P1 promoter | P2 promoter | P3 promoter |
|----------|-------------|-------------|-------------|
|          | $k_a$ (X $10^4$ M$^{-1}$ s$^{-1}$) | $k_d$ (X $10^3$ s$^{-1}$) | $K_D$ (X $10^8$ M) |
| AgrA     | 1.24        | 2.38        | 19.2        |
| AgrA$\sim$P | 1.54        | 1.53        | 9.92        |
| AgrAD59A | 1.16        | 1.93        | 16.6        |
| AgrAD59A$\sim$P | 1.07        | 1.33        | 12.4        |

![Figure 3](image-url)

**Fig. 3.** Relative affinity between AgrA and the P1, P2 and P3 promoter binding sites. Protein DNA interactions monitored by SPR. Varying concentrations of AgrA were passed onto an Streptavidin chip with (A) P1 (C) P2 and (E) P3 promoters to ascertain the relative affinity. The role of phosphorylation of AgrA on (B) P1 (D) P2 and (F) P3 were also analyzed. In these experiments, phosphorylation was noted to alter AgrA P3 interactions more substantially than the others (Table 2).
observe electron density corresponding to the disulfide in the LytTR domain. Based on the structure of apo-AgrA and mutational analysis, it appears that the disulfide could substantially distort the domain conformation, thus limiting protein-DNA interactions under oxidising conditions (Fig. S5).

3.2. Promoter strength determines the stringency of AgrA-induced expression changes

The affinity of AgrA for different promoter sequences was examined using Surface Plasmon Resonance (SPR) experiments. In the SPR experiments, the promoter DNA was immobilized using a streptavidin tag. Both phosphorylated and non-phosphorylated forms of AgrA served as an analyte in these studies. The sensorgrams of phosphorylated AgrA interaction with promoters were fit to Langmuir 1:1 model. The fits were validated by both residual values which range from −10 to +10 with chi square values within 10. The association ($k_a$) and dissociation ($k_d$) constants along with the equilibrium dissociation constant ($K_D$) values for the three promoter sequences are compiled in Table 3. The strength of interaction of AgrA with P2 promoter is more than P1 and P3 promoters. The effect of phosphorylation of Asp59 (upon addition of acetyl phosphate) is more prominent in case of the P3 promoter (≈7 times) compared to the P1 and P2 promoters where the affinity increased less than two fold (Fig. 3 and Table 3). The affinity for AgrA is twice in the case of P3 when compared to P2 upon phosphorylation. The active site mutant AgrA D59A does not show considerable difference in affinity between the phosphorylated and non-phosphorylated protein (Fig. S6 and Table 3). The specific nature of these interactions was evaluated using a DNA segment corresponding to the intergenic region of 31 base pairs between the P2 and P3 binding sites. This DNA sequence did not show any interaction with AgrA or the dimeric phosphorylated protein.

To further examine the relative affinity for different promoter elements, AgrA-promoter interactions were also examined by a competitive binding assay using SPR (Figs. 4 and S7). In these experiments, the biotinylated promoter DNA was immobilized on the SPR chip. The relative change in the response units upon the passage of AgrA pre-incubated with the unlabelled promoter DNA was monitored. This competition assay suggests that AgrA binds more efficiently to the P2 than the P1 and P3 promoter sequences with the order $P_2 > P_3 > P_1$ (Fig. 4A and C). In another experiment AgrA was pre-incubated with acetyl phosphate and unlabelled promoter 30 min prior to the SPR experiment. When AgrA was incubated with unlabelled promoter in presence of acetyl phosphate and passed onto the SPR chip, the change in response units was lower suggesting that AgrA (phosphorylated) bound the unlabelled P3 promoter more tightly when compared to the other immobilized promoters. The order of affinity is changed to $P_3 > P_2 > P_1$ upon phosphorylation (Fig. 4B and D).
3.3. Role of AgrA in RNA polymerase recruitment

The finding that the binding affinity of AgrADBD for the P1, P2 and P3 promoter sequences are similar suggested that other parameters are likely to account for the diversity in AgrA induced changes in the expression profile. The influence of phosphorylation was evaluated using a cellular assay based on green fluorescent protein (GFP) markers (Fig. 5A). The premise for this experiment is that an increase in the intracellular concentration of AgrA would change the expression of genes that are located downstream of the P1, P2 or P3 promoter element. As this assay relies on the change in intracellular concentration of AgrA upon the addition of the inducer anhydrotetracycline (ATC), this aspect was verified by qRT-PCR (Fig. 5B). The AgrAD59A mutant that lacked the aspartate residue involved in phosphorylation served as a ‘constitutively inactive’ AgrA in these experiments. Changes in the expression levels of GFP under the control of P1, P2 and P3 promoter segments were examined at a similar AgrA concentration (fixed ATC concentration). The consequent change in GFP expression under the control of the P2 promoter was substantially different between that induced by AgrA and the non-phosphorylated AgrAD59A mutant (Fig. 5C). Similar results were obtained when the GFP fluorescence was measured by flow cytometry (Fig. S8). Put together, this finding suggests that non-phosphorylated AgrA can retain constitutive expression of AgrA and the agr operon. The release of secreted toxins as well as changes in expression by the RNAIII effector (that rely on the P3 promoter), however, are selectively enhanced upon AgrA phosphorylation.

Previous studies on the transcription of the agr operon revealed that the transcription competent open promoter complex (RPO) occurs more readily at P2 than at P3 [13]. This suggested a potential role for AgrA in RNA polymerase recruitment with the caveat that AgrA selectively increases the occupancy of the RNA polymerase at a promoter site depending on the cellular context. The mechanism that would enable this mode of AgrA mediated transcription initiation remained unclear. Here we propose a
mechanistic model based on biochemical data as well as the asymmetry in AgrA-promoter interactions noted from the crystal structures of the AgrA-DNA complexes (Fig. 6). In vitro biochemical assays as well as a competition assay to evaluate promoter selection reveal that AgrA binds more efficiently to the P2 than the P1 or P3 promoters. Phosphorylation affects this complex in distinct ways. Previous studies revealed that AgrA dimerization is stronger upon phosphorylation. Another relevant observation is that the P3 promoter is sub-optimal (20 bp spacing between the phosphorylation. Put together, these observations support the hypothesis that the phosphorylated AgrA dimer improves RNA polymerase recruitment at the sub-optimal P3 promoter thus enhancing the expression of toxin genes. The model thus provides a link between AgrA phosphorylation and the virulent phenotype of S. aureus that is characterized by increased levels of intracellular RNAIII as well as secreted toxins.

4. Conclusion

A comparison between the crystal structures of AgrADBD with different promoters and biochemical data on AgrA-promoter interactions suggest a mechanism that can rationalize the role of AgrA as an activator of gene expression. These studies suggest that the level of exotoxins in S. aureus is regulated by locating these genes under a sub-optimal promoter that is selectively enhanced upon AgrA activation by a quorum stimulus.

Author contributions

KR, AF and BG designed the studies, KR and AF performed the experiments, KR, AF and BG analyzed the experiments. KR and BG wrote the manuscript.

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The PDB accession codes for the deposited structures are 4XQJ, 4XQN, 4XQQ, 4XXE, 4XYO and 4XYQ.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.012.

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