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Conformational features of the *Staphylococcus aureus* AgrA-promoter interactions rationalize quorum-sensing triggered gene expression

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ABSTRACT

The intracellular trigger for the quorum sensing response mechanism in *Staphylococcus aureus* involves the phosphorylation of the response regulator AgrA by the membrane anchored histidine kinase AgrC. AgrA activates transcription from three promoter sequences (P1–P3). The promoter strength, conditional association of AgrA with these promoter elements and temporal delay in AgrA-mediated changes in gene expression contribute to the diversity of the quorum sensing response in different *S. aureus* strains. AgrA promoters comprise of imperfect direct repeats of DNA with a consensus sequence- [TA][AC][CA]GTTN [AG][TG]. Here we describe crystal structures of the DNA-binding (LytTR) domain of AgrA with different cognate DNA sequences that reveal a hitherto unanticipated feature of AgrA-DNA interactions. AgrA promoter interactions are asymmetric with fewer interactions at the binding site proximal to the −35 promoter element. Biochemical assays to evaluate AgrA-promoter interactions suggests that phosphorylation induced dimerization of AgrA can compensate for the asymmetry in AgrA-DNA interactions. The structures also provide a basis to rationalize mutations that were noted to alter AgrA activity without affecting protein-DNA interactions. Put together, the structural data, gene expression and mutational analysis reveal that promoter strength and AgrA phosphorylation enable quorum-sensing triggered transcriptional changes leading to a transition from the persistent to virulent phenotype.

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1. Introduction

The accessory gene regulator mechanism (Agr) coordinates the expression of cytolytic toxins with a quorum stimulus in *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 autoinducing 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 *S. aureus* [2]. While genes in the *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 *agr* operon and the RNAIII locus. Another AgrA interacting sequence, referred to as the P1 promoter, governs the expression of *agrA*. While the P1 promoter was the first to be reported in the initial characterization of the *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 ($AgrA_{DBD}$: 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 $AgrA_{DBD}$ domain (a representative of the LytTR domain family) adopts a ten-stranded β-scaffold with an interspaced α-helix and a short 3_{10} 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 −35 promoter element that is recognized by the RNA polymerase holoenzyme to initiate transcription. Activation by AgrA was

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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 redox 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 $AgrA_{DBD}$ in complex with promoter DNA (P2_S2) was first reported by Sidote et al. [6]. Here we describe the crystal structures of the $AgrA_{DBD}$ 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 ($AgrA_{DBD}$)

$AgrA_{DBD}$ was amplified from *Staphylococcus aureus* genomic DNA using forward (5′ CCTAACATATGATCCATATGGATAATAGCGTTGAAACGATTGAATT 3′) and reverse (5′ GAACCCTCGAGTTATATTTTTTTAACGTTTCTCACCGATGCATAGCA 3′) primers. These amplicons were ligated between the *NdeI* and *XhoI* restriction enzyme 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 containing $AgrA_{DBD}$ and grown at 37 °C in Luria broth with 100 μg/ml of ampicillin till the OD reached 0.5 at 600 nm. 0.5 mM IPTG (Isopropyl-β-D-thiogalactopyranoside, Gold Biotechnology Inc.) was used to induce over-expression of the protein and the culture was grown further for 16 h at 16 °C. All the purification steps were carried out at 4 °C. The cells were lysed by sonication in a buffer containing 20 mM sodium potassium phosphate pH 6.0, 100 mM NaCl and 2 mM PMSF. The lysate was subjected to centrifugation at 26,500 g for 45 min to remove the cell debris. The supernatant was added to a 80% saturated solution of ammonium sulphate with constant stirring for 1 h followed by centrifugation at 4850 g for 15 min. The pellet was re-suspended in a buffer containing 20 mM sodium potassium phosphate pH 6.0 and 100 mM NaCl and dialysed against the same buffer to remove excess ammonium sulphate. Ion exchange chromatography was performed on the dialysed lysate using a 5 ml in-house packed SP sepharose (GE healthcare) column pre-equilibrated with 20 mM sodium potassium phosphate pH 6.0 and 100 mM NaCl. The protein was eluted in a gradient of 0.7–1.5 M NaCl and analyzed on a 12% SDS-PAGE gel. Elution fractions containing the protein were pooled, concentrated and applied onto a Sephacryl S200 (HiPrep 16/60) size exclusion chromatography (GE healthcare) column pre-equilibrated with either buffer A (20 mM Bis-tris pH 6.0 and 100 mM NaCl) or buffer B (20 mM MES monohydrate pH 6.0 and 100 mM NaCl). The protein eluted as a monomer and the elution fractions were analyzed on a 12% SDS-PAGE gel and concentrated to 16 mg/ml using a 3 kDa MWCO amicon ultra centricon (Millipore, Inc.). The mass of the protein was confirmed by MALDI-TOF mass spectrometry.

2.2. Cloning, expression and purification of recombinant AgrA and $AgrA_{D59A}$

AgrA was amplified from *Staphylococcus aureus* spp COL genomic DNA using forward (5′ GAATCATATGATGAAAATTTTCATTTGCGAAGACGATCC 3′) and reverse (5′ GAACCCTCGAGTTATATTTTTTTAACGTTTCTCACCGATGCATAGCA 3′) primers. These amplicons were ligated between the *NdeI* and *XhoI* restriction enzyme sites of the pET28b expression vector. This clone was subsequently used as a template to generate the $AgrA_{D59A}$ mutant by site directed mutagenesis (double primer method- partially overlapping primers). *E. coli* Rosetta(DE3)pLysS cells (Novagen, Inc.) were transformed with the plasmid encoding AgrA or $AgrA_{D59A}$ and grown to an OD_{600} 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

$AgrA_{DBD}$ (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 $AgrA_{DBD}$ with different

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