Regulation and characterization of *rot* transcription in *Staphylococcus aureus*

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The pathogenesis of *Staphylococcus aureus* infections is dependent upon expression of various virulence factors, which are under the control of multiple regulatory systems, including two-component regulatory systems and transcriptional regulators such as the SarA family of proteins. As a part of a continuing effort to understand the regulatory mechanisms that involve the SarA protein family, the regulation and physical characterization of *rot* transcription is described here. The *rot* gene, a member of the *sarA* family of genes, was previously characterized and has been shown to regulate a large number of genes. The *rot* locus is composed of multiple overlapping transcripts as determined by primer extension and was proposed to encode an open reading frame of 133 residues. Transcription of *rot* was significantly increased in the *sarA* mutant. Gel shift and transcriptional studies revealed that SarA could bind to the *rot* promoter region, probably acting as a repressor for *rot* transcription. The data indicate that the expression of *rot* transcription is significantly repressed only by SarA among the *sarA* family of mutants tested at the post-exponential phase of growth.

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INTRODUCTION

Staphylococcus aureus is a major pathogen in both community and hospital settings, and continues to be a serious public concern due to the lack of an effective vaccine and the emergence of multiple antibiotic-resistant strains (Arvidson & Tegmark, 2001; Boyce, 1997; Lowy, 1998). The spectrum of diseases caused by this organism is extremely broad, ranging from localized skin suppuration to life-threatening septicaemia. Despite antimicrobial therapy, morbidity and mortality associated with *S. aureus* infections remains high, due, in part, to the organism's ability to develop resistance to virtually all antibiotics, including vancomycin (Arvidson & Tegmark, 2001; Lowy, 1998). Within infection sites, *S. aureus* produces several virulence factors that include surface-associated proteins, enzymes, exotoxins, capsular polysaccharides and gene products that facilitate tissue colonization, tissue destruction and immune evasion. The expression of many of these virulence genes is coordinately controlled by regulatory loci, such as two-component regulatory systems (*agr*, *saeRS*, *srrAB* and *arlSR*) and global transcriptional regulators (*sarA*, *sigB*, *sarA* paralogues, *tcaRA*, etc.) (Arvidson & Tegmark, 2001; Cheung *et al.*, 1992; Cheung & Zhang, 2002; Luong *et al.*, 2003; Manna & Cheung, 2001, 2003, 2006a, b; Manna *et al.*, 2004; McNamara *et al.*, 2000; McNamara & Bayer, 2005; Novick, 2003; Projan & Novick, 1997; Truong-Bolduc *et al.*, 2003).

The *sarA* locus, a well-studied transcriptional regulator in *S. aureus*, is important for the regulation of various genes

involved in virulence, regulatory and metabolic processes (Bayer *et al.*, 1996; Cheung *et al.*, 1992; Chien & Cheung, 1998; Chien *et al.*, 1999; Dunman *et al.*, 2001). The *sarA* locus consists of a major open reading frame, SarA (124 residues), driven by three distinct promoters, resulting in three overlapping transcripts with a common terminating end (Bayer *et al.*, 1996). The *sarA* locus regulates the expression of selected cell-wall-associated proteins and exoproteins (e.g. α - and β -haemolysins). SarA binds to several target gene promoter regions including regulatory systems such as *agr*, *sarS* and *sarV* (Cheung *et al.*, 2001; Chien & Cheung, 1998; Manna *et al.*, 2004; Rechlin *et al.*, 1999) and virulence genes such as *hla*, *spa*, *cna*, *bap*, *ica* and *fmbA* to modulate gene transcription (Blevins *et al.*, 1999; Chien *et al.*, 1999; Tormo *et al.*, 2005) by both direct and indirect pathways. Microarray analyses demonstrated that a *sarA* mutation altered the expression of over 120 genes, with 76 upregulated and 44 downregulated (Dunman *et al.*, 2001).

Analysis of published *S. aureus* genomes reveals that at least 10 major SarA paralogues are present (Kuroda *et al.*, 2001). SarA is the first characterized member of the SarA family; others characterized are SarR (Manna & Cheung, 2001), SarS (also called SarH1) (Cheung *et al.*, 2001; Tegmark *et al.*, 2000), SarT (Schmidt *et al.*, 2001), Rot (McNamara *et al.*, 2000), SarU (Manna & Cheung, 2003), SarV (Manna *et al.*, 2004), MgrA (Luong *et al.*, 2003; Truong-Bolduc *et al.*, 2003) and SarX (Manna & Cheung, 2006b). The SarA family

represents a group of DNA-binding proteins that share homology with each other (20–45 % identity and 45–65 % similarity to SarA protein) as well as with the MarR family of proteins involved in regulating multi-drug resistance in Gram-negative bacteria (Cheung & Zhang, 2002). Although some members of the *sarA* family of genes are only partially characterized, the known attributes of nine *sarA*-like genes suggest that most SarA paralogues function as both repressors and activators. They regulate one another and other regulatory and target genes either by binding directly to promoter regions or by acting indirectly via other regulators. Structural analysis suggests that smaller SarA proteins exist as dimers (e.g. SarA, SarR, MgrA), while the larger proteins with two homologous domains are monomers (e.g. SarS) and are also homologous to the winged-helix family of transcription factors with unique variations (Cheung & Zhang, 2002; Liu *et al.*, 2001, 2006; Manna & Cheung, 2006a). While many of these genes either activate or repress the expression of virulence and regulatory genes, the exact mode of regulation is not well defined.

The *rot* gene, a member of the *sarA* family of genes, has been characterized and is suggested to be a global regulator in *S. aureus*. Rot, a 166-residue SarA paralogue (or 153-residue as predicted in published *S. aureus* genomes) represses haemolysins (*hla* and *hld*), toxin and lipase (*geh*), and positively regulates cell-surface-associated protein synthesis [clumping factor, protein A, putative cell-surface adhesin (*sdnC*)] (McNamara *et al.*, 2000; McNamara & Bayer, 2005; Said-Salim *et al.*, 2003). Transcriptional profiling suggests that inactivation of *rot* affects the transcription of 168 genes in *S. aureus* including *sarS*, another member of the *sarA* family of genes (Said-Salim *et al.*, 2003). Although *rot* has been shown to be a global regulator for virulence gene expression, the regulation of *rot* transcription, particularly by other members of the *sarA* family of genes, is not known and physical mapping of *rot* transcript has not been performed. However, recently it has been shown that the translation of *rot* transcript is inhibited by the *agr* RNAIII molecule (Geisinger *et al.*, 2006).

In our continuing effort to understand the underlying mechanism by which the SarA protein family functions at various levels, we report here the regulation of expression of *rot* transcription by other known *sarA* family mutants and the physical mapping of the *rot* transcripts.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in the text. Routinely used standard *Escherichia coli* DH5 α and BL21(DE3) strains were employed for manipulation of DNA and overexpression of protein. Plasmid DNA manipulated in *E. coli* was transferred to the restriction-deficient *S. aureus* strain RN4220 by electroporation (Schenk & Laddaga, 1992). *S. aureus* cells were grown at 37 °C with constant aeration (220 r.p.m.) in tryptic soy broth (TSB) or on tryptic soy agar (TSA) supplemented with antibiotics when necessary. Luria-Bertani (LB) medium was used for growing *E. coli*. When required, antibiotics were added to the culture media in the following

concentrations: for *S. aureus*, 5 μ g erythromycin ml⁻¹, 3 μ g tetracycline ml⁻¹, and 10 μ g chloramphenicol ml⁻¹, or for *E. coli*, 50 μ g ampicillin ml⁻¹ and 30 μ g chloramphenicol ml⁻¹.

Genetic manipulation in *E. coli* and *S. aureus*. Construction and characterization of most of the mutants was published earlier (Manna & Cheung, 2001, 2006b), except for the *sarZ* (SA2174 of *S. aureus* N315) mutant (unpublished).

Isolation of RNA and Northern blot hybridization. Total RNA isolation from different *S. aureus* strains and Northern blot hybridization were performed as previously described (Bayer *et al.*, 1996; Manna & Cheung, 2001).

Transcriptional fusion studies of *rot* promoter linked to the *gfp_{uvr}* reporter gene. A 317 bp *rot* promoter fragment (nt 204–520; GenBank accession no. AF189239 or positioned from +1 to 317 bp of the *rot* promoter) (see Fig. 2) containing the upstream promoter region of the *rot* gene was amplified by PCR, using chromosomal DNA of *S. aureus* strain RN6390 and primers with flanking *EcoRI* or *XbaI* sites. An *EcoRI* and *XbaI* fragment containing the *rot* promoter region was cloned into shuttle plasmid pALC1484, generating a transcriptional fusion of the *rot* promoter to the *gfp_{uvr}* reporter gene (Kahl *et al.*, 2000). Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter reporter gene construct. The recombinant plasmid containing the *rot* promoter region and a promoterless plasmid as a control were first introduced into *S. aureus* strain RN4220 by electroporation. Plasmids purified from RN4220 transformants were then electroporated into RN6390, its various isogenic mutants, and single-copy complemented *sarA* (ALC812) strains as required.

After overnight incubation, *S. aureus* strains harbouring the recombinant plasmids were diluted 1:100 in TSB containing chloramphenicol and grown at 37 °C with shaking at 220 r.p.m. Aliquots (200 μ l) were transferred in triplicate hourly for 10 h to microtitre plates to assay for OD₆₅₀ and green fluorescent protein (GFP) fluorescence with an FL800TB1 fluorescence reader (BioTek Instruments). Promoter activity was plotted as mean fluorescence per OD₆₅₀ over time, using the mean values from triplicate readings calculated by subtracting the values for promoterless control.

Purification of SarA protein and gel shift analysis. The cloning and purification of His₆-SarA protein were described previously (Chien *et al.*, 1999). The purity of His fusion SarA protein was checked in a 12 % polyacrylamide-SDS gel and found to be more than 98 % pure. The concentration of the purified protein was determined by using the Bio-Rad protein estimation kit and BSA as the standard.

In order to determine if the recombinant SarA protein binds to the *rot* promoter region, a 317 bp fragment used for transcriptional fusion studies, representing the *rot* promoters region, was end-labelled with [γ -³²P]ATP by using T4 polynucleotide kinase. The labelled fragment (0.1 ng) was incubated at room temperature for 20 min with various amounts of purified SarA protein in 25 μ l binding buffer (25 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol and 5 % glycerol) containing 0.5 μ g calf thymus DNA (Invitrogen). The reaction mixtures were analysed in an 8.0 % non-denaturing polyacrylamide gel. The band shifts were detected by exposing dried gels to X-ray films.

Primer extension analysis. Mapping of the 5' end of the *rot* transcripts by primer extension was performed by using the primer 5'-ATTGCGTTTCAATCTCGCTGAAAATT-3', complementary to the *rot* coding strand and located from nucleotide positions 72 to 97 downstream from the putative proposed start codon ATG (nt

Fig. 1. Promoter analysis of the *rot* gene in *S. aureus*. Primer extension of the *rot* transcripts by using total RNA isolated from the wild-type RN6390 and the *sarA* mutant strains at the post-exponential phase of growth. P1, P2, P3 and P4 are designated according to the smallest length to the largest length of *rot* transcripts.

626–651; GenBank accession no. AF189239). Primer extension was carried out by using 30 µg total RNA isolated from wild-type RN6390 and *sarA* mutant strains in post-exponential-phase culture as described elsewhere (Ausubel *et al.*, 2000; Bayer *et al.*, 1996). The same radiolabelled primer was used for the DNA sequencing reactions.

RESULTS

Characterization of the *rot* transcripts

Although the phenotypic characterization of the *rot* gene has been performed thoroughly, the regulation and characterization of *rot* transcription are not well defined (McNamara *et al.*, 2000; McNamara & Bayer, 2005; Said-Salim *et al.*, 2003; Tseng & Stewart, 2005). Therefore, in order to determine the transcriptional start site and the promoter structure of the *rot* transcript and the probable length of the *rot* open reading frame, primer extension analysis was performed with total RNA isolated from the wild-type RN6390 and *sarA* mutant strains (Fig. 1). The primer extension results were interesting between the two isogenic strains analysed as two primer extension bands (designated P1 and P4) were detected in the case of the wild-type strain, and four bands (designated P1, P2, P3 and P4) were detected in the case of the *sarA* mutant. A significant elevation of P1 and P4 transcript signals was also observed in the case of the *sarA* mutant as compared to the wild-type strain, which indicates the probable role of *sarA* in regulating *rot* transcription. The transcriptional start sites of all four transcripts were mapped to 105, 172, 212 and 255 nt downstream from the proposed Rot (133-residue) initiation codon ATG (Fig. 2), respectively. Based upon the transcriptional start sites, the predicted putative promoter regions (–35 and –10) are shown in Fig. 2; they have close homology with the *E. coli* –35 and –10 consensus sequences (TTGACA–N_{14–21}–TATAAT) of σ^A -dependent promoters. The published translational start codon (ATG) for 166-residue Rot was located at the +7 nt position from the mapped transcriptional start site +1 of the smallest transcript P1 and the region lacked any defined ribosome-binding sequences (RBS). These results suggest that the *rot* open reading frame may encode less than a 166-residue polypeptide. Secondly, the computer-generated previously published translational start codon ATG for Rot was located

Fig. 2. Nucleotide sequence of the 354 bp promoter region of the *rot* gene; putative promoter recognition sites (–35 and –10) are bold and underlined. The transcriptional start sites (+1) were identified based on a primer extension and are in bold and underlined. The ribosome-binding sequences (RBS) and the putative predicted translational start codon (ATG) in various *S. aureus* genomes are marked in bold. The putative consensus SarA-binding regions (B1 to B4) on the promoter are marked by bold and arrows.

at the +46 nt position from the mapped transcriptional start site of the P1 transcript and its adjacent region displays a relatively weak putative RBS (TTGGGATT). However, a strong RBS, GGGAGA, is located at the +92 nt position from the transcriptional start site of the P1 transcript. Based on the mapped transcriptional start sites and the presence of a strong putative RBS, we suggest that the ATG located at the +92 nt position (position 352; Fig. 2) is probably the Rot translational start codon and that Rot is a 133-residue polypeptide. Indeed, recent transcriptional fusion analysis of the upstream promoter region of the *rot* gene suggested that translation of *rot* initiated from the ATG codon located at the +92 nt position (Geisinger *et al.*, 2006). Analysis of the downstream sequence of the *rot* gene disclosed the presence of a potential hairpin structure or *rho*-independent transcriptional terminator sequence from a position 13–48 bp immediately after the stop codon (TAA).

To further confirm, we affinity-purified approximately 16 kDa Rot protein from a *sarA* mutant harbouring pSK236 plasmid carrying a 780 bp DNA fragment, which contains a 350 bp upstream sequence of the *rot* gene plus *rot* coding region with six histidine codons inserted before the stop codon. MALDI-TOF-MS and N-terminal amino acid sequencing verified the authentication of the purified Rot-His₆ protein band and the starting amino acid methionine (M) at the 34-residue position (data not shown). This result was supported by the observation that the 15.6 kDa Rot protein (corresponding to 133 residues) co-eluted with SarA, MgrA and SarR in an affinity pull-down assay from whole-cell lysates with a *spa* promoter fragment (Oscarsson *et al.*, 2005). In addition, studies by Geisinger *et al.* (2006) with the C-terminal histidine fusion of the *rot* gene in a multicopy plasmid and immunoblot analysis with anti-histidine antibody indicated the production of a functional Rot of 17 kDa in the various isogenic *S. aureus* strains. The published *S. aureus* N315 genome reveals at least ten proteins with significant homology to SarA (Kuroda *et al.*, 2001), of which nine have been characterized. Recently, we determined the length of SarZ (SA2174 of *S. aureus* N315) by primer extension and found it to be 148 residues (unpublished data) as predicted in the published *S. aureus* genomes. Therefore, the lengths of all ten SarA paralogues, including Rot as determined in this report, are known. An alignment of all ten SarA paralogues, including the 133-residue Rot, with other SarA paralogues is much more ordered and of higher degree than the alignment of the 166- or 153-residue counterparts.

Analysis of *rot* transcription in wild-type RN6390 and in an assortment of isogenic mutants

Northern blot assays were performed with a *rot* probe (425 bp within the coding region) with the various regulatory mutants in our collection to determine if the transcription of the *rot* gene is affected by inactivation of other regulatory systems, including those of the *sarA* family of genes. Results demonstrated that the level of *rot* transcript

was increased only in the *sarA* and *sarS* mutants, whereas no significant changes were observed in other mutants at the post-exponential phase of growth (Fig. 3a). A diffused pattern of transcripts appeared particularly in the late-exponential and post-exponential phases, probably due to the presence of the multiple transcripts as evidenced in the primer extension experiment. Alternatively, it may have been due to cleavage of the *rot* transcripts by the base pairing of the *agr* RNAIII molecule as suggested by Geisinger *et al.* (2006) or the degradation of the *rot* transcripts at the post-exponential phase of growth. In addition, it is consistent with the primer extension results that the *sarA* gene product acts as a repressor for *rot* transcription.

Similarly, we performed Northern blot analysis to determine the variation of *rot* transcription within the various growth phases by isolating total RNA from different time points in various isogenic strains, as shown in Fig. 3(b) and (c). Maximum expression of *rot* transcription was observed during the exponential phase of growth (between 3 and 4 h) in the wild-type strain, whereas the transcription decreased during the early and post-exponential phase of growth. The inactivation of *sarA* yielded a significantly higher level of *rot* transcription in all phases of growth, whereas the introduction of a single copy of the *sarA* gene into the lipase locus (*geh*) of the respective *sarA* mutant yielded expression of *rot* transcripts to near-parental levels, suggesting that the expression of *rot* is truly repressed by the *sarA* gene product. Nevertheless, the cause of or reason for the apparent discrepancy in the expression of *rot* transcripts (less for *sarA* mutant at 3 h, higher for *sarA*-complemented *sarA* strain at 4 h, or less for *agr* mutant at 5 h) is unknown. This observation is consistent with the repeated experiments.

These results were also confirmed by promoter fusion studies using a 317 bp DNA fragment (positioned from 1 to 317; Fig. 2) containing all four promoters of the *rot* gene with a promoterless *gfp_{uvr}* reporter gene in the various isogenic strains of RN6390. A substantial increase in mean GFP fluorescence for the *sarA* mutant as compared to that for the wild-type RN6390 and the single-copy complemented *sarA* mutant was found as shown in Fig. 4. However, the twofold increment of mean GFP activity in the *sarS* mutant is probably due to the indirect effect, which is yet to be verified by performing more confirmative experiments. We consistently observed a partial decrease or increase in *rot* promoter fusion activity in some of the mutants, which we speculate might be due to a direct or an indirect effect, which is yet to be verified.

Binding of SarA protein to the *rot* promoter region

The data presented in earlier sections clearly suggest that expression of *rot* transcription was affected only by *sarA* mutation among the mutants tested; we therefore speculated that SarA might bind to the *rot* promoter region to repress *rot* transcription. To test this, we employed the 317 bp *rot* promoter fragment used in transcriptional fusion

Fig. 3. Northern analysis of the *rot* transcript in the wild-type, various isogenic mutants, and single-copy-complemented strain (cp) at post-exponential phase (OD₆₀₀ ~ 1.7) of growth in (a), various time points of growth in (b) and growth curves of the wild-type RN6390, RN6390 *sarA* mutant, complemented *sarA* mutant and RN6911 *agr* mutant strains in (c). A 425 bp DNA fragment containing the *rot* gene was used for hybridization. The 723 bp internal fragment of 16S rDNA was used for hybridization as a loading control in (a) and (b). The OD₆₀₀ of various cultures was measured in a Spectronic 20 spectrophotometer. Note that the time scale in (c) is non-linear.

studies for DNA-binding assay. The γ -³²P-radiolabelled DNA fragment was used in gel shift assays with varying amounts of purified SarA protein (Fig. 5). A retarded

DNA–protein complex was detected with as little as 0.2 µg SarA. The retarded protein–DNA complex band became predominant with an increasing concentration of SarA protein, with complete conversion occurring at ~0.5 µg. The unlabelled promoter fragment competed effectively with labelled fragment, whereas an unlabelled non-specific DNA fragment (185 bp *P**sarX* fragment; Manna & Cheung, 2006b) did not compete significantly with the radiolabelled fragment, suggesting that the DNA–protein interaction was specific.

Previously, we mapped the consensus DNA-binding sequences of the SarA protein to upstream of various target promoter regions based on DNase I footprinting, and we found SarA consensus binding sequences to be 5'-ATTTgTATtTAATATTTataTAATT-3' (Chien & Cheung, 1998; Chien *et al.*, 1999; Manna *et al.*, 2004; Manna & Cheung, 2006a). In aligning the 26 bp SarA consensus binding sequences with the upstream *rot* promoter region, we found four matching regions. Of these four matching regions, three were in top-strand positions 60–84 (B1), 86–110 (B2) and 174–198 (B3), and one was in the bottom-strand position 113–88 (B4) (Fig. 2). Of the 26 nucleotides, 18, 17, 19 and 16 matched, respectively. The location of SarA-binding sites within the P2, P3 and P4 promoter regions is consistent with the results of the primer extension

Fig. 4. Promoter expression of the combined *rot* promoter region fused to a *gfp_{uvr}* reporter gene in the wild-type and various isogenic mutants at the post-exponential (7 h) phase of growth. To minimize variations in fluorescence attributable to cell density, the data are presented as the mean of triplicate samples in fluorescence units per OD₆₅₀.

Fig. 5. Autoradiogram of an 8.0% polyacrylamide gel showing gel shifts for purified SarA protein with a γ - ^{32}P -labelled, 317 bp *rot* promoter fragment. Mobility of the DNA band in the presence of increasing amounts of SarA protein, as indicated on the top. Cold competition lanes with 50-fold excess (molar ratio) of unlabelled 317 bp *rot* fragment (specific) and 185 bp *sarX* promoter fragment (non-specific; Manna & Cheung, 2006b) are indicated and contained 1.0 μg of the purified SarA protein.

and Northern blot analyses of the *sarA* mutant as compared to the wild-type strain.

DISCUSSION

The fact that *S. aureus* exhibits complex patterns of protein expression in response to various environmental conditions (Arvidson & Tegmark, 2001; Cheung & Zhang, 2002; Lowy, 1998; Novick, 2003; Projan & Novick, 1997) is consistent with the notion that large numbers of complex regulatory systems coordinate expression of most virulence factors that are involved in the infectious processes. The transcriptional regulators, particularly the SarA protein family, are emerging as an important regulatory system for potential interference and therefore control of *S. aureus* infections. In prior studies, we and others have characterized nine SarA paralogues, including SarA, SarR, SarS, Rot, SarT, SarU, MgrA, SarV and SarX, all of which are involved in regulation of both regulatory genes including the *sarA* family of genes, and virulence genes in *S. aureus*.

The predicted lengths of the 10 major SarA paralogues in *S. aureus* genomes are consistent with the lengths determined by primer extension, with the exception of the SarX and Rot proteins. The predicted length of Rot was reported in 11 published *S. aureus* genomes to be 166 residues (*S. aureus* strains RF122, COL, 252, 476, USA300), 153 residues (*S. aureus* strains JH1, JH9, MW2, Mu50, N315), or 133 residues (*S. aureus* strain NCTC 8325), but our various experimental analyses, along with data of Oscarsson *et al.* (2005) and Geisinger *et al.* (2006), indicate that the length of Rot is likely to be 133 residues. The Rot protein is the tenth

member of the SarA protein family found to have low sequence homology with SarA protein (*e*-value of 0.003 with 25% identity and 50% similarity), but the degree of conserved residues throughout the entire length of all paralogues is significant. Interestingly, many basic residues are conserved within members of the SarA protein family, consistent with the notion that the DNA-binding domains are highly conserved, and detailed mutational analysis of SarR and SarA indicated that many of the conserved basic residues (e.g. K52, K80, R82, R88, etc., of SarR) are indeed involved in DNA binding (Liu *et al.*, 2006; Manna & Cheung, 2006a).

The transcription of *sarA*-like genes is highly variable in various *S. aureus* strains including strain RN6390. For instance, *sarA*, *sarR*, *mgrA* and *sarX* transcripts are highly expressed, whereas the transcripts of *sarU*, *sarT*, *sarV* and *sarS* are not readily detectable under normal laboratory growth conditions, probably due to the repression of other regulatory gene products. We found the *rot* gene to be poorly transcribed in the early (lag) and post-exponential phases of growth, whereas transcription was significantly higher in the exponential phase in the wild-type strain. This finding is consistent with the earlier observation that the *rot* gene is transcribed maximally in the exponential phase of growth (McNamara *et al.*, 2000). Northern analysis of *rot* transcription in mutants of *agr*, *sae*, *sigB* and nine *sarA* family genes revealed that transcription of *rot* remains unaltered in these mutants at the post-exponential phase of growth, with the exception of *sarA* and *sarS*. Rot transcription is increased in the *sarA* mutant and is nearly restored upon single-copy complementation of the *sarA* gene. Similar results were observed with a combined *rot* promoter *gfp_{uvr}* fusion. However, *rot* transcription was increased nearly twofold in the *sarS* mutant as determined from Northern and transcriptional fusion assays, probably due to an indirect effect of *sarS*. Alternatively, we are not ruling out the direct involvement of *sarS*, which has yet to be established. Regulation of the *sarA* family of genes is intertwined in nature (Manna & Cheung, 2006a, b). It should be stressed that *sarS* is transcribed in the early growth phase and tapers off in transcription at the post-exponential and stationary phases of growth in RN6390 strain (Cheung *et al.*, 2001; Tegmark *et al.*, 2000). In the future, we will be analysing various *sarA* family mutant strains of *S. aureus* from different genetic backgrounds to determine if there is any variability of the data presented in this report.

In the primer extension experiment, we found that four transcripts encoding the major Rot polypeptide originated from different locations. The abolished transcription of the P2 and P3 transcripts and reduced levels of the P1 and P4 transcripts in the wild-type strain, as compared to the *sarA* mutant, clearly indicated that SarA is involved in negative regulation of *rot* transcription. The pattern of *rot* transcription in various growth phases does not correlate with the expression of the SarA protein in *S. aureus* as

SarA is expressed more in the exponential than in the post-exponential phase of growth (Manna & Cheung, 2001). This means that SarA may not be solely involved in repression of *rot* transcription, which may interact with one or more other intermediary transcription factors, probably with Rot, as it has been suggested that Rot may act as an autorepressor (Geisinger *et al.*, 2006). We believe the regulation of the target genes by the SarA paralogues is complex, and may involve more than one protein. The details of the SarA-mediated *rot* transcription remain to be determined. It should be noted that the involvement of the *agr* RNAIII molecule in the regulation of *rot* expression at the translational level (Geisinger *et al.*, 2006) has been reported.

Utilizing gel shift studies, we found that purified SarA binds to the *rot* promoter region with high affinity. Four SarA consensus binding sequences were found within the 350 bp *rot* promoter region, three (B1–3) on the top strand and one (B4) on the complementary strand of the B2 region. B1, B2 and B4 overlapped within the P4 and P3 promoter regions, whereas the B3 site was located near the –10 region of the P2 promoter. We speculate that on the B3 site SarA probably interacts with the other SarA dimeric molecules at the B1, B2, or B4 locations to loop out the DNA, thus interfering with binding of RNA polymerase to the P2 and partially the P1 promoters. Although the mechanisms by which SarA regulates its target genes are not well understood, we now know that more than two consensus-binding sites are present in the promoter regions of most of the SarA-regulated target genes analysed, including the *agr* locus (Manna & Cheung, 2006a). These results are consistent with our earlier finding that most of the SarA paralogues regulate each other. Furthermore, the SarA paralogues involved in virulence gene regulation are intertwined: for example, *sarR* represses SarA expression (Manna & Cheung, 2001), *sarA* represses and *rot* activates *sarS* expression (Cheung *et al.*, 2001; Said-Salim *et al.*, 2003; Tegmark *et al.*, 2000), *sarA* negatively regulates *sarT* and *sarT* activates *sarS* (Schmidt *et al.*, 2001), both SarA and MgrA repress *sarV* (Manna *et al.*, 2004), while MgrA activates *sarX* transcription (Manna & Cheung, 2006b).

To further define the precise function of each of the SarA paralogues, we are in the process of analysing the effects of various combined knockouts of different *sarA* family genes on other regulatory genes, and investigating the molecular interaction of SarA paralogues with the upstream region of the target genes.

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REFERENCES

- Arvidson, S. & Tegmark, K. (2001). Regulation of virulence determinants in *Staphylococcus aureus*. *Int J Med Microbiol* **291**, 159–170.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (2000). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bayer, M. G., Heinrichs, J. H. & Cheung, A. L. (1996). The molecular architecture of the *sar* locus in *Staphylococcus aureus*. *J Bacteriol* **178**, 4563–4570.
- Blevins, J. S., Gillaspay, A. F., Rechten, T. M., Hurburt, B. K. & Smeltzer, M. S. (1999). The staphylococcal accessory regulator (*sar*) represses transcription of the *S. aureus* collagen adhesin gene (*cna*) in an *agr*-independent manner. *Mol Microbiol* **33**, 317–326.
- Boyce, J. M. (1997). Epidemiology and prevention of nosocomial infections. In *The Staphylococci in Human Diseases*, vol. 12, pp. 309–329. Edited by K. B. Crossley & G. L. Archer. New York: Churchill Livingstone.
- Cheung, A. L. & Zhang, G. (2002). Regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Front Biosci* **7**, D1825–D1842.
- Cheung, A. L., Koomey, J. M., Butler, C. A., Projan, S. J. & Fischetti, V. A. (1992). Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proc Natl Acad Sci U S A* **89**, 6462–6466.
- Cheung, A. L., Schmidt, K. A., Bateman, B. & Manna, A. C. (2001). *SarS*, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. *Infect Immun* **69**, 2448–2455.
- Chien, Y.-t. & Cheung, A. L. (1998). Molecular interactions between two global regulators, *sar* and *agr*, in *Staphylococcus aureus*. *J Biol Chem* **273**, 2645–2652.
- Chien, Y.-t., Manna, A. C., Projan, S. J. & Cheung, A. L. (1999). *SarA*, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. *J Biol Chem* **274**, 37169–37176.
- Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. & Projan, S. J. (2001). Transcriptional profiling-based identification of *Staphylococcus aureus* genes regulated by *agr* and/or *sarA* loci. *J Bacteriol* **183**, 7341–7353.
- Geisinger, E., Adhikari, R. P., Jin, R., Ross, H. F. & Novick, R. P. (2006). Inhibition of *rot* translation by RNAIII, a key feature of *agr* function. *Mol Microbiol* **61**, 1038–1048.
- Kahl, B., Goulian, M., Van Wamel, W., Herrmann, M., Simon, S., Kaplan, G., Peters, G. & Cheung, A. L. (2000). *Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. *Infect Immun* **68**, 5385–5392.
- Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K. I. & other authors (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240.
- Liu, Y., Manna, A., Li, R., Martin, W. E., Murphy, R. C., Cheung, A. L. & Zhang, G. (2001). Crystal structure of the *SarR* protein from *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **98**, 6877–6882.
- Liu, Y., Manna, A. C., Pan, C.-H., Kriksunov, I. A., Thiel, D. J., Cheung, A. L. & Zhang, G. (2006). Structural and functional analyses of the global regulatory protein *SarA* from *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **103**, 2392–2397.
- Lowy, F. (1998). *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520–532.
- Luong, T. T., Newell, S. W. & Lee, C. Y. (2003). *mgr*, a novel global regulator in *Staphylococcus aureus*. *J Bacteriol* **185**, 3703–3710.

- Manna, A. & Cheung, A. L. (2001). Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. *Infect Immun* **69**, 885–896.
- Manna, A. C. & Cheung, A. L. (2003). *sarU*, a *sarA* homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus*. *Infect Immun* **71**, 343–353.
- Manna, A. C. & Cheung, A. L. (2006a). Transcriptional regulation of the *agr* locus and the identification of DNA-binding residues of the global regulatory protein SarR in *Staphylococcus aureus*. *Mol Microbiol* **60**, 1289–1301.
- Manna, A. C. & Cheung, A. L. (2006b). Expression of SarX, a negative regulator of *agr* and exoprotein synthesis, is activated by MgrA in *Staphylococcus aureus*. *J Bacteriol* **188**, 4288–4299.
- Manna, A. C., Ingavale, S. S., Maloney, M., van Wamel, W. & Cheung, A. L. (2004). Identification of *sarV* (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*. *J Bacteriol* **186**, 5267–5280.
- McNamara, P. J. & Bayer, A. S. (2005). A *rot* mutation restores parental virulence to an *agr*-null *Staphylococcus aureus* strain in a rabbit model of endocarditis. *Infect Immun* **73**, 3806–3809.
- McNamara, P. J., Milligan-Monroe, K. C., Khalili, S. & Proctor, R. A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. *J Bacteriol* **182**, 3197–3203.
- Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **48**, 1429–1449.
- Oscarsson, J., Harlos, C. & Arvidson, S. (2005). Regulatory role of proteins binding to the *spa* (protein A) and *sarS* (staphylococcal accessory regulator) promoter regions in *Staphylococcus aureus* NTCC 8325-4. *Int J Med Microbiol* **295**, 253–266.
- Projan, S. J. & Novick, R. P. (1997). The molecular basis of pathogenicity. In *The Staphylococci in Human Diseases*, pp. 55–81. Edited by K. B. Crossley & G. L. Archer. New York: Churchill Livingstone.
- Rechtin, T. M., Gillasp, A. F., Schumacher, M. A., Brennan, R. G., Smeltzer, M. S. & Hurlburt, B. K. (1999). Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*. *Mol Microbiol* **33**, 307–316.
- Said-Salim, B., Dunman, P. M., McAleese, F. M., Macapagal, D., Murphy, E., McNamara, P. J., Arvidson, S., Foster, T. J., Projan, S. J. & Kreiswirth, B. N. (2003). Global regulation of *Staphylococcus aureus* genes by Rot. *J Bacteriol* **185**, 610–619.
- Schenk, S. & Laddaga, R. A. (1992). Improved methods for electroporation of *Staphylococcus aureus*. *FEMS Microbiol Lett* **94**, 133–138.
- Schmidt, K. A., Manna, A. C., Gill, S. & Cheung, A. L. (2001). SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. *Infect Immun* **69**, 4749–4758.
- Tegmark, K., Karlsson, A. & Arvidson, S. (2000). Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **37**, 398–409.
- Tormo, M. A., Marti, M., Valle, J., Manna, A. C., Cheung, A. L., Lasa, I. & Penades, J. R. (2005). SarA is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. *J Bacteriol* **187**, 2348–2356.
- Truong-Bolduc, Q. C., Zhang, X. & Hooper, D. C. (2003). Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. *J Bacteriol* **185**, 3127–3138.
- Tseng, C. W. & Stewart, G. C. (2005). Rot repression of enterotoxins B expression in *Staphylococcus aureus*. *J Bacteriol* **187**, 5301–5309.

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