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Global Regulation of *Staphylococcus aureus* Genes by Rot

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***Staphylococcus aureus* produces a wide array of cell surface and extracellular proteins involved in virulence. Expression of these virulence factors is tightly controlled by numerous regulatory loci, including *agr*, *sar*, *sigB*, *sae*, and *arl*, as well as by a number of proteins with homology to SarA. Rot (repressor of toxins), a SarA homologue, was previously identified in a library of transposon-induced mutants created in an *agr*-negative strain by screening for restored protease and alpha-toxin. To date, all of the SarA homologues have been shown to act as global regulators of virulence genes. Therefore, we investigated the extent of transcriptional regulation of staphylococcal genes by Rot. We compared the transcriptional profile of a *rot agr* double mutant to that of its *agr* parental strain by using custom-made Affymetrix GeneChips. Our findings indicate that Rot is not only a repressor but a global regulator with both positive and negative effects on the expression of *S. aureus* genes. Our data also indicate that Rot and *agr* have opposing effects on select target genes. These results provide further insight into the role of Rot in the regulatory cascade of *S. aureus* virulence gene expression.**

Staphylococcus aureus is an important human pathogen that causes a wide spectrum of diseases ranging from benign skin infections to life-threatening endocarditis and toxic shock syndrome (20, 32). The large array of virulence determinants produced by *S. aureus* is central to the pathogenesis of this organism. The role of these factors in pathogenesis has been demonstrated mainly by comparing the virulence of a mutant defective in one gene with that of its wild-type parent strain in specific animal models of infection. Recently, the use of signature-tagged mutagenesis (STM) identified several factors that play a role in pathogenesis, as their disruption made the mutants unable to survive within the host (10, 25). These virulence factors can be broadly subdivided into cell surface and secreted proteins. Cell surface proteins include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which enable the bacteria to colonize host tissues, and factors that facilitate the establishment of the infection by allowing the bacteria to evade the host immune system. Secreted proteins include various enzymes that degrade host components with antimicrobial activity, as well as different hemolysins that damage the host tissue (32).

Production of *S. aureus* virulence determinants is controlled by several global regulatory loci, which include *agr* (1), *sar* (5), *sigB* (3, 41), *sae* (14), *arl* (13), and six SarA homologues (2, 24). These regulators are parts of an important network modulating the expression of *S. aureus* virulence genes. One target virulence gene can be under the influence of several regulators that "cross talk" to ensure that the specific gene is expressed only when conditions are favorable. For instance, in vitro studies have demonstrated that *agr* negatively regulates the expression of *spa*, encoding protein A (32), whereas SarS binds to the *spa* promoter and activates its expression (38). Interestingly, *agr* downregulates *sarS* expression (6, 38). It has thus been proposed that *agr* downregulates *spa* expression by downregulating the expression of its activator, *sarS* (38). Therefore, virulence gene regulators could affect the expression of target genes directly, by binding to their promoters, or indirectly, via other regulators.

The specific role and/or importance of a virulence factor or a virulence gene regulator in *S. aureus* pathogenesis may vary from one infection type to another. For instance, fibronectin-binding proteins have been shown to mediate invasion of mammalian cells by *S. aureus* (36). In contrast, McElroy et al. have demonstrated that fibronectin-binding proteins decrease virulence in *S. aureus*-induced pneumonia (22). Similarly, the *agr* locus has been shown to be important in *S. aureus* pathogenesis in various animal infection models such as the mouse arthritis and abscess models (28, 29). However, a recent study by Yarwood et al. has shown that *agr* expression is not necessary for the development of toxic shock syndrome (40). Taken together, these observations illustrate the multifactorial aspect of *S. aureus* pathogenesis. In view of the fact that an increasing number of *S. aureus* strains are becoming more difficult to treat due to their resistance to multiple antibiotics (39), elucidation of *S. aureus* pathogenesis at the molecular level is crucial in the battle against this important human pathogen, as it may help in the design of new therapeutic strategies.

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Among the regulators described above, SarA and *agr* are the best characterized. SarA is a transcriptional regulator that binds to a consensus motif in the promoter of its target genes (8). Recent studies have shown that SarA is a member of a large family of transcriptional regulators that share a common amino acid motif, KXRXXXDER (2). The *agr* system is self-activating in a cell density-dependent manner (29). Expression of the *agr* effector molecule, RNAIII, correlates with down-regulation of select genes encoding cell surface proteins, such as protein A and fibronectin-binding proteins, and upregulation of genes encoding secreted proteins, such as alpha- and beta-toxins, lipases, and proteases (32). In vitro, RNAIII-regulated factors follow a temporal trend, with surface proteins being produced during the early-exponential phase of growth and secreted proteins synthesized predominantly during the post-exponential phase. The sequential expression of these determinants correlates with the production of RNAIII, whose levels peak during the post-exponential phase (20, 32). The mechanism by which RNAIII regulates the expression of these different genes is not completely understood. Several studies have suggested the existence of cofactors that interact directly or indirectly with RNAIII to control the expression of the different target genes (18, 24, 30). These factors include members of the Sar family of transcriptional regulators (2).

Recently, a novel SarA homologue, Rot, was identified as a repressor of toxins based on the following observation. An *agr*-negative strain produces low levels of alpha-toxin and protease activity. However, in a *rot agr* double mutant, expression of these proteins is increased due to the lack of repression by Rot. Understanding how Rot regulates these and other virulence factors is important in order to further discern *S. aureus* virulence gene regulation. Most *S. aureus* virulence gene modulators have both negative and positive effects on their target genes (8, 32, 38). For these reasons, we hypothesized that Rot may have both up- and downregulatory functions. To identify additional target genes regulated by Rot, we compared the transcriptional profile of a *rot agr* double mutant to that of its parental *agr* strain during the late-exponential phase of growth by use of the Affymetrix GeneChip. The GeneChip used in this study covers 86% of the *S. aureus* COL genome and contains approximately 4,600 DNA fragments, representing 2,339 individual *S. aureus* genes (12).

In this study, we demonstrate that Rot is a global regulator that negatively regulates the transcription of several known virulence genes. Some of these genes encode secreted proteins such as lipase, hemolysins, and proteases, all of which are postulated to play a role in tissue invasion. Rot also positively regulates the expression of a number of genes including those encoding cell surface adhesins. In addition, Rot modulates the expression of genes not previously implicated in pathogenesis. A relationship between Rot and *agr* in the regulation of *S. aureus* virulence genes is proposed.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All *S. aureus* strains used in this study are listed in Table 1. Bacteria were grown in 0.3 GL agar and CY broth, supplemented with β-glycerophosphate (GP), at 37°C (27). Bacterial cell cultures were grown with rotary agitation at 200 rpm. All mutants were maintained on erythromycin or tetracycline at a concentration of 5 μg ml^{-1}.

TABLE 1. Bacterial strains used in this study

Strain	Description	Reference or source
RN6390	*agr*$^+$ laboratory strain	29
RN6911	*agr*Δ::*tetM* (Tc^r)	29
KT201	*sarH1*::pKT200 (Em^r)	38
PM614	*agr*-null *chr*::Tn*917*::*rot* (Tc^r Em^r)	24
BK9986	*tetM*::*agr* Tn*917*::*rot* (Tc^r Em^r)	This study
BK9987	*tetM*::*agr sarH1*::pKT200 (Tc^r Em^r)	This study

Construction of *rot agr* and *sarH1* (*sarS*) *agr* mutants. The *rot agr* double mutant (BK9986) was constructed by bacteriophage ϕ11-mediated transduction of the *rot*::Tn*917* insertion from strain PM614 into the *agr* mutant strain RN6911. The *sarH1 agr* double mutant (BK9987) was made by transduction of the *agr*::*tmn* mutation from RN6911 into KT201 (*sarH1* mutant). Transfer of the *rot* and *agr* mutations into strains BK9986 and B9987 was verified by Southern blot analysis (data not shown).

RNA isolation and Northern blot analysis. Overnight cultures of *S. aureus* were diluted 1:50 in CY-GP medium and grown to the late-exponential (5 h) phase of growth. Since our aim was to determine genes whose expression is affected by Rot at any time point, we chose the late-exponential-growth phase because at this time point, we can still observe transcripts of genes expressed early, such as cell surface protein genes, as well as those expressed later, such as genes encoding secreted enzymes and toxins. Cells were harvested by centrifugation, and RNA was isolated by the FastPrep system (developed by Savant [Farmingdale, N.Y.] and Bio 101 [Vista, Calif.]) (4). RNA samples (10 μg) were electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer. RNA was blotted onto a Hybond N^+ membrane (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) by using the VacuGene XL blotting system (Amersham Pharmacia). The transfer was performed with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate),, pH 7.0, for 2 h. After the RNA transfer, the RNA ladder (Invitrogen, Carlsbad, Calif.) was manually marked before the hybridization step. Membranes were hybridized overnight with a PCR-amplified probe derived from the target gene (Table 2). Specific transcripts were detected by using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia). In all the Northern blot experiments, the same RNA samples were hybridized with 16S rRNA as an internal control (38). New membranes were used for each Northern blot experiment.

RNA labeling. The integrity of RNA preparations was analyzed by electrophoresis in 1.2% agarose–0.66 M formaldehyde gels. Samples with defined 16S and 23S rRNA bands were subjected to a series of reactions that resulted in 5′ biotin-labeled fragmented RNA for GeneChip analysis, as previously described (12). Briefly, total bacterial RNA was first enriched for mRNA. This was accomplished by reverse transcription with rRNA-specific primers, followed by RNase H digestion to remove the rRNA of RNA-cDNA hybrid molecules and DNase I treatment. Enriched mRNA was then chemically fragmented and 5′-thiolated by T4 polynucleotide kinase treatment in the presence of γ-S-ATP. Samples were then biotinylated by addition of polyethylene oxide (PEO)-iodoacetyl-biotin.

GeneChip analysis. The custom-made *S. aureus* GeneChips used in this study have been described previously (12). Each chip contains 4,528 open reading frames (ORFs), 12 tRNAs, and 3 rRNAs, with an average of 25 probe sets per ORF. Due to the preliminary state of the genome sequence at the time of design, many genes are represented on the chip as partial, duplicate, or overlapping fragments. Subsequent analyses based on the updated *S. aureus* genomic sequences suggest that these 4,528 tilings represent approximately 2,700 to 2,900 individual genes and that >86% of the *S. aureus* COL genome is represented on the chip. The missing genes (14%) include those encoding staphylococcal accessory regulator A (*sarA*), the sensor histidine kinase SaeS (*saeS*), D-alanine-D-alanyl carrier protein ligase (*dltA*), serine protease (*sspA*), leukotoxin D (*lukD*), sigma factor B regulators (*rsbU*, *rsbV*, and *rsbW*), and staphylococcal accessory regulator R (*sarR*).

GeneChip analysis was performed by the method described by Dunman and colleagues (12). Accordingly, to address the issue of reproducibility, two separate RNA samples from each strain were prepared from two different experiments, and for each sample, 1.5 μg of biotinylated RNA was hybridized to at least two separate GeneChips for 14 h at 45°C. GeneChips were then washed and subjected to a series of staining procedures that included the successive binding of streptavidin, biotinylated anti-streptavidin, and phycoerythrin-conjugated streptavidin. Each GeneChip was washed and scanned at a 570-nm wavelength

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)	GenBank accession no.	Nucleotide positions
hlbF	GGTGCACTTACTGAC	X13404	824–838
hlbR	CGCATATACATCCCATGGC		1678–1660
16S rRNAF	GCGTGGGGATCAAACAGG	X68417	777–794
16S rRNAR	CCCCAATCATTTGTCCCACC		1500–1481
splAF	CGAAGGAGGTAAAATATGAATAAAAATG	AF271715	987–1013
splAR	GTGGTGTGAAATAAACACCGAAATTC		1670–1645
sspAF	CAACGAATGGTCATTATGCACCC	AF309515	598–620
sspAR	GGTACACCGCCCCAATGAATTCC		1123–1101
gehF	GCACAAGCCTCGG	M12715	808–820
gehR	GACGGGGGTGTAG		1280–1268
ureCF	CTCGTGTAACACGTGATGACGTGAACG	AP003136	251461–251487
ureCR	GGCGCATGACCGCCACCAGCACCTTC		252139–252114
spaF	AGACGATCCTTCGGTGAGCAAAGA	U54636	829–852
spaR	GCTTTTGCAATGTCATTTACTGTAT		1203–1179
sarSF	GTCAAGCCTGAAGTCGATATG	AB035454	911–931
sarSR	GCATGGTCTTGCTGCGCGTC		1518–1499
rotF	GTTTTGGGATTGTTGGGATG	AF189239	478–497
rotR	GCATTGCTGTTGCTCTACTTGC		924–903

and a 3-μm resolution in an Affymetrix GeneChip scanner. Affymetrix Microarray Suite 4.0 algorithms calculated signal intensities (average difference) and present or absent determinations for each ORF (19). GeneChips were then normalized, and background was defined by using GeneSpring 4.0 (Silicon Genetics) as previously described (12). GeneSpring software was used to further analyze the transcription patterns of genes. Genes negatively regulated by Rot were identified as ORFs with transcript titers at least twofold higher in BK9986 (*rot* negative) than in RN6911 (*rot* positive). Genes whose transcript levels were at least twofold higher in RN6911 (*rot* positive) than in BK9986 (*rot* negative) were categorized as being positively regulated by Rot. These lists were further filtered to include only genes with signal intensities above background level in the *rot agr* (*rot*-negative) strain for Rot-downregulated genes and in the *agr* (*rot*-positive) strain for Rot-upregulated genes. Genes represented more than once on the chip are represented only once on the list.

Urease activity. Urease production was assayed on urea agar slants (Remel, Lenexa, Kans.) as described by the manufacturer. In this test, urease-positive strains show a color change from orange to pink while urease-negative strains have an intact orange color.

RESULTS

Rot is a transcriptional modulator. GeneChip results indicate that Rot behaves as both a positive and a negative modulator of numerous virulence genes. We have identified 60 genes whose expression is negatively regulated by Rot (Table 3) and 86 genes whose expression is positively regulated by Rot (Table 4). From both categories of regulated genes, we chose seven genes with a well-known or postulated virulence function for further validation (Table 5).

Virulence genes negatively regulated by Rot. Initial characterization of Rot has indicated that it is a repressor of *hla*, encoding alpha-toxin (24). Our GeneChip data showed that Rot negatively regulates another toxin gene, *hlb*, which encodes beta-toxin (Tables 3 and 5). The GeneChip result for *hlb* was confirmed by Northern blot analysis using the *rot agr* double mutant (*rot* negative) and its parental *agr* strain (*rot* positive). As shown in Fig. 1A, the *hlb* transcript is present at higher levels in the *rot agr* strain than in the *agr* mutant. The detection of two bands that hybridize with *hlb* is consistent with previous findings (7, 41). The 16S rRNA transcript (internal control) is present at similar levels in the two strains (Fig. 1B).

Rot was previously shown to have a phenotypic effect on protease production. (24). Unlike alpha-toxin and beta-toxin, which are solely encoded by *hla* and *hlb*, respectively, staphylococcal protease activity is associated with the gene products of at least two operons, *splABCDEF* (33) and *sspABC* (34). Our GeneChip results demonstrated that Rot negatively regulates both operons (Tables 3 and 5). The *sspA* gene, encoding V8 protease (34), is not listed in Table 3 because it is not represented on the GeneChip used in this study.

The GeneChip data for *spl* were confirmed by Northern blot analysis, using *splA* as a probe. The transcript of the *spl* operon (5.5 kb) is detectable only in the *rot agr* strain, BK9986, not in the *agr* strain, RN6911 (Fig. 1C). We tested *ssp* operon expression in the two strains by Northern blot analysis, using *sspA* as a probe, and found that the transcript levels of the *ssp* operon (2.8 kb) were higher in the *rot agr* double mutant than in the *agr* strain (Fig. 1D).

Rot also negatively regulates the expression of *geh*, encoding lipase (Tables 3 and 5). The negative regulation of *geh* expression by Rot was confirmed by Northern blot analysis in which RNA samples from the *rot agr* double mutant and the *agr* strain taken at late-exponential growth were hybridized with a *geh*-specific probe. The results demonstrated that *geh* mRNA is present at higher levels in the *rot agr* strain than in the *agr* strain (Fig. 2A).

As shown in Table 3, GeneChip data indicated that Rot negatively regulates various urease genes such as *ureA*, *ureB*, and *ureC*, encoding different subunits of the urease enzyme, as well as accessory genes such as *ureD* and *ureE*, required for the enzyme activity (17) (GenBank accession number AP003136). We performed Northern blot analysis using the *ureC* gene as a probe and found that the *rot agr* strain had higher levels of *ureC* transcripts than the *agr* strain (Fig. 2B). One of the urease genes downregulated by Rot, *ureD*, was previously identified as a potential virulence gene, because a *ureD* transposon mutant was strongly attenuated in an animal model (10). We also tested for urease activity on urea agar slants for the two strains. As shown in Fig. 2C, the *rot agr* strain was urease positive but the *agr* strain was urease negative.

Virulence genes upregulated by Rot. Although Rot was originally identified as being a repressor of certain virulence fac-

TABLE 3. Genes downregulated by Rot

Chip ORF no.[a]	N315 ORF no.[b]	N315 gene[b]	N315 description[b]	Fold change[c]	*agr* or *sar* effect[d]	Role category[d]
4877	SA1811	*hlb*	Truncated beta-hemolysin	6.9		
4218	SA0143	*adhE*	Alcohol-acetaldehyde dehydrogenase	3.8		
1726	SA0471	*cysK*	Cysteine synthase (*o*-acetylserine sulfhydrylase) homologue	4.6		
1664	SA2312	*ddh*	*D*-specific D-2-hydroxyacid dehydrogenase	11.5	*agr*, down	Carbohydrate metabolism
904	SA1267	*ebhA*	HP, similar to streptococcal adhesin Emb	567.4		
1885	SA1268	*ebhB*	HP, similar to streptococcal adhesin Emb	Upregulated	*agr*, up	Virulence factors
1434	SA1091	*fmhC* (*eprh*)	FmhC protein	Upregulated		
1527	SA0309	*geh*	Glycerol ester hydrolase	8.4	*agr*, up	Virulence factors
4046	SA2294	*gntK*	Gluconokinase	3.1		
5281	SA2293	*gntP*	Gluconate permease	4.6	*sar*, down	Transport
1928	SA2209	*hlgB*	Gamma-hemolysin component B	31.9	*agr*, up	Virulence factors
1927	SA2208	*hlgC*	Gamma-hemolysin component C	19.6	*agr*, up	Virulence factors
1700	SA1881	*kdpA*	Probable potassium-transporting ATPase A chain	2.8		
4715	SA1879	*kdpC*	Probable potassium-transporting ATPase C chain	3.7		
4583	SA1090	*lytN*	LytN protein	Upregulated		
5046	SA0549	*mvaK2*	Phosphomevalonate kinase	Upregulated		
2268	SA2334	*mvaS*	3-Hydroxy-3-methylglutaryl CoA synthase	8.1		
3524	SA2185	*narG*	Respiratory nitrate reductase alpha chain	6.2	*agr*, down	Electron transport
431	SA2435	*pmi*	Mannose-6-phosphate isomerase	3.0		
4537	SA1659	*prsA*	Peptidyl-prolyl *cis/trans* isomerase homolog	6.5		
3551	SA2326	*ptsG*	PTS system, glucose-specific IIABC component	2.7		
1071	SA1589	*ribD*	Riboflavin-specific deaminase	Upregulated		
5329	SA1631	*splA*	Serine protease SplA (pathogenicity island SaPIn3)	Upregulated	*agr*, up	Virulence factors
2929	SA1630	*splB*	Serine protease SplB (pathogenicity island SaPIn3)	Upregulated	*agr*, up	Virulence factors
3742	SA1629	*splC*	Serine protease SplC (pathogenicity island SaPIn3)	62.5		
324	SA1628	*splD*	Serine protease SplD (pathogenicity island SaPIn3)	Upregulated	*agr*, up	Virulence factors
325		*splE*	Serine protease SplE	133.4		
2377	SA1627	*splF*	Serine protease SplF (pathogenicity island SaPIn3)	Upregulated	*agr*, up	Virulence factors
2174	SA0900	*sspB*	Cysteine protease precursor	10.4	*agr*, up	Virulence factors
2175	SA0899	*sspC*	Cysteine protease	5.2	*agr*, up	Virulence factors
4823	SA2082	*ureA*	Urease gamma subunit	Upregulated		
4822	SA2083	*ureB*	Urease beta subunit	Upregulated		
1900	SA2084	*ureC*	Urease alpha subunit	Upregulated		
4817	SA2088	*ureD*	Urease accessory protein UreD	2.9		
1899	SA2085	*ureE*	Urease accessory protein UreE	6.7		
1898	SA2086	*ureF*	Urease accessory protein UreF	6.0		
1897	SA2087	*ureG*	Urease accessory protein UreG	23.7		
401	SA0124		HP, similar to glycosyltransferase TuaA	5.6		
657	SA2455		Capsular polysaccharide biosynthesis, CapC	11.1		
849	SA2414		HP, similar to glutathione peroxidase	10.8		
1386	SA0368		HP, similar to proton/sodium-glutamate symport protein	6.4	*agr*, up	Transport
1574	SA2062		HP	3.5		
1815	SA2200		HP, similar to ABC transporter, ATP binding subunit	3.0		
1816	SA2201		HP, similar to ABC transporter, permease protein	3.2		
1994	SA1812		HP, similar to synergohymenotropic toxin precursor of *Staphylococcus intermedius*	3.1		
2064	SA2218		Reverse complement of HP	4.4		
2067	SA1437		Conserved HP	3.6		
2238	SA2434		Fructose phosphotransferase system enzyme FruA homolog	5.4		
2543	SA1725		Staphopain, cysteine proteinase	11.9		
2612	SA1093		DNA topoisomerase I TopA homolog	Upregulated		
2860	SA0989		Conserved HP	7.2		
2926	SA0663		HP	3.0		
3017	SA0127		HP, similar to capsular polysaccharide synthesis protein 14L	50.7		
3078	SA0165		HP, similar to alpha-helical coiled-coil protein SrpF	2.7		
3373	SA0904		HP, probable autolysin transcription regulator	Upregulated		
3768	SA0850		HP, similar to oligopeptide ABC transporter oligopeptide-binding protein	Upregulated		
4061	SA1007		Alpha-hemolysin precursor	24.5	*agr*, up	Virulence factors
4069	SA0123		HP, similar to UDP-glucose 4-epimerase (Gale-1)	Upregulated		
4530	SA0710		Conserved HP	Upregulated		
5131	SA2297		HP, similar to GTP-pyrophosphokinase	2.8		

[a] *S. aureus* GeneChip ORF number.

[b] Based on the published sequence of strain N315 (accession no. NC_002745). For genes not present in N315, the gene name and description are from the COL genome, available from The Institute for Genomic Research Comprehensive Microbial Resource website (http://www.tigr.org). HP, hypothetical protein; CoA, coenzyme A; PTS, phosphotransferase system.

[c] Normalized values in the *agr rot* mutant over values in the *agr* strain. "Upregulated" denotes genes highly downregulated in the *agr* strain, such that the transcripts were below detectable levels and the fold change could not be accurately calculated.

[d] As described by Dunman et al. (12). up, upregulated; down, downregulated.

TABLE 4. Genes upregulated by Rot

Chip ORF no.[a]	N315 ORF no.[b]	N315 gene[b]	N315 description[b]	Fold change[c]	*agr* or *sar* effect[d]	Role category[d]
3994	SA1736	*aldH*	Aldehyde dehydrogenase	2.1		
5372		*clfB*	Clumping factor B	2.9		
2462	SA2336	*clpL*	ATP-dependent Clp proteinase chain ClpL	2.7	*sar*, down	Adaptation
3833		*coa*	Coagulase	4.3		
2163	SA1253	*ctpA*	Probable carboxy-terminal processing proteinase CtpA	2.9		
5110	SA1164	*dhoM*	Homoserine dehydrogenase	2.3		
4873	SA0796	*dltD*	Poly(glycerophosphate chain) D-alanine transfer protein	2.0	*agr*, down	Transport
3383		*epiA*	Lantibiotic gallidermin precursor EpiA	2.6		
84	SA0602	*fhuA*	Ferrichrome transport ATP-binding protein	2.1		
4551	SA2172	*gltT*	Proton/sodium-glutamate symport protein	2.2		
4042	SA2288	*gtaB*	UTP-glucose-1-phosphate uridyltransferase	2.4		
3984	SA0189	*hsdR*	Probable type I restriction enzyme restriction chain	2.4		
2883	SA1505	*lysP*	Lysine-specific permease	2.4	*agr*, up	Transport
4486	SA0250	*lytS*	Two-component sensor histidine kinase	2.3		
1047	SA1194	*msrA*	Peptide methionine sulfoxide reductase homolog	2.4		
854	SA2410	*nrdD*	Anaerobic ribonucleoside-triphosphate reductase	2.1		
4205	SA0685	*nrdI*	NrdI protein involved in ribonucleotide reductase function	2.9		
4390	SA0374	*pbuX*	Xanthine permease	2.3		
4905	SA0923	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase PurM	2.2	*sar*, down	Nucleotide and nucleic acid metabolism
2800	SA0676	*recQ*	Probable DNA helicase	2.8	*agr*, up	DNA replication
1079	SA1583	*rot*	Repressor of toxins Rot	12.0		
2343		*sdrC*	SdrC protein	2.7	*sar*, up	Virulence factors
2119	SA0107	*spa*	Immunoglobulin G binding protein A precursor	15.6	*agr*, down	Virulence factors
5112	SA1166	*thrB*	Homoserine kinase homolog	3.9		
3239	SA1165	*thrC*	Threonine synthase	3.8		
5075	SA0373	*xprT*	Xanthine phosphoribosyltransferase	3.0		
17			HP	2.0		
18	SA0220		HP, similar to glycerophosphodiester phosphodiesterase	2.9		
237	SAS013		Reverse complement of HP (pathogenicity island SaPIn2)	2.3	*agr*, up	Unknown
392	SA0739		Conserved HP	2.4		
442	SA0651		HP	2.2		
457	SA1613		Conserved HP	2.5		
477	SA0428		Conserved HP	2.5		
493	SA2378		Conserved HP	3.2	*agr*, down	Unknown
534	SA1717		Glutamyl-tRNA^{Gln} amidotransferase subunit C	2.6		
600	SA0523		HP, similar to poly (GP) alpha-glucosyltransferase (TA biosynthesis)	2.2	*sar*, up	Cell wall
616	SA2133		Conserved HP	2.8		
678	SA0682		HP, similar to di-tripeptide ABC transporter	2.1		
748	SA2261		HP, similar to efflux pump	3.0		
873	SAS088		HP	2.2		
1027	SA2007		HP, similar to alpha-acetolactate decarboxylase	2.1		
1082	SA0620		Secretory antigen SsaA homologue	2.0		
1175	SA2284		HP, similar to accumulation-associated protein	2.5	*agr*, up	Virulence factors
1353			HP	2.7		
1356	SA2170		HP, similar to general stress protein 26	2.1		
1357	SA2171		HP	2.0		
1398	SA0914		Reverse complement of HP, similar to chitinase B	3.0	*agr*, up	Miscellaneous
1563	SA0407		Conserved HP	2.0		
1564	SA0408		HP	2.3		
1592			HP	3.0		
1597			HP	2.0		
1666	SA2303		HP, similar to membrane-spanning protein	3.2		
1765			Epidermin immunity protein F	4.7	*agr*, up	Antibiotic production
1991	SA0439		HP, similar to lysine decarboxylase	2.5		
2032	SA1059		Methionyl-tRNA formyltransferase	2.4	*agr*, up	Aminoacyl tRNA synthetases
2076				2.4		
2077	SA0291		HP	3.1		
2078	SA0292		HP	2.5		
2103	SA0271		Conserved HP	6.3	*agr*, up	Unknown
2132	SA2131		Conserved HP	4.9		
2133	SA2132		HP, similar to ABC transporter (ATP-binding protein)	2.4		
2232	SA2440		HP	2.0		
2255			HP	2.1		
2337	SA2001		HP, similar to oxidoreductase, aldo/ketoreductase family	2.0		
2454	SA1320		HP	4.4		
2507	SA0380		Conserved HP (pathogenicity island SaPIn2)	3.5		
2697	SA2442		Preprotein translocase SecA homolog	2.2		
3259	SA0675		HP, similar to ABC transporter ATP-binding protein	3.0		
3313	SA2436		HP, similar to phage infection protein	2.1	*sar*, up	Bacteriophage related

Continued on following page

TABLE 4—*Continued*

Chip ORF no.[a]	N315 ORF no.[b]	N315 gene[b]	N315 description[b]	Fold change[c]	*agr* or *sar* effect[d]	Role category[d]
3391			Conserved HP	2.0		
3426	SA1924		HP, similar to aldehyde dehydrogenase	2.5		
3599			ABC transporter, ATP-binding protein	2.6		
3698	SA0173		HP, similar to surfactin synthetase	2.9	*agr*, up	Antibiotic production
3888	SA2339		HP, similar to antibiotic transport-associated protein	2.1		
4153	SA0310		HP	2.1		
4253	SA0003		Conserved HP	2.2		
4282	SA1942		Conserved HP	2.5		
4525	SA1016		Reverse complement of conserved HP	2.4		
4550			HP	2.3		
4667	SA0973		Phosphopantetheine adenyltransferase homolog	3.2		
4752	SA1056		HP	3.5		
5065	SA2256		Conserved HP	15.8		
5245	SA0827		HP, similar to ATP-dependent nuclease subunit B	2.3		
5437	SA0246		Hypotheticl protein, similar to D-xylulose reductase	2.1		
5471	SA1131		HP, similar to 2-oxoacid ferredoxin oxidoreductase, alpha subunit	2.3		
5554	SA1679		HP, similar to D-3-phosphoglycerate dehydrogenase	2.0		

[a] *S. aureus* GeneChip ORF number.
[b] Based on the published sequence of strain N315 (accession no. NC_002745). For genes not present in N315, the gene name and description are from the COL genome, available from The Institute for Genomic Research Comprehensive Microbial Resource website (http://www.tigr.org). HP, hypothetical protein; TA, teichoic acid.
[c] Normalized values in the *agr* strain over values in the *agr rot* double mutant.
[d] As described by Dunman et al. (12). up, upregulated; down, downregulated.

tors, our results indicate that Rot also acts as a positive modulator of staphylococcal virulence genes. Among the genes upregulated in the presence of Rot are those encoding cell surface proteins. One of these genes is the well-studied *spa* gene, which encodes protein A (Tables 3 and 5). To validate this result, Northern blot analysis was performed on *rot agr* and *agr* strains, and the results demonstrated that *spa* mRNA is detected only in the *agr* (*rot*-positive) strain, not in the *rot agr* double mutant (*rot* negative) (Fig. 3A).

Previous studies have demonstrated that SarH1, also called SarS, positively regulates the expression of the protein A gene, *spa* (6, 38). Since our results indicate that *spa* is also positively regulated by Rot, we sought to determine if Rot positively regulates *spa* expression by positively regulating the expression of the *spa* activator SarS. Using Genespring software analysis, we found that the *sarS* transcript was considered present in the *rot*-positive strain (RN6911) but absent in the *rot* mutant (BK9986), suggesting that Rot may positively regulate *sarS* expression.

To validate the *sarS* result, and to analyze the effects of Rot and SarS on each other, we created a *sarH1 agr* double mutant

TABLE 5. Classification of Rot-regulated genes

Gene/gene product[a]	Function	Effect[b] of: Rot	*agr*
Secreted enzymes			
geh/lipase[c]	Degrades lipids	−	+
splA through *splF*/serine protease operon[c]	Degrades proteins	−	+
sspB, sspC/within serine protease operon[c]	Degrades proteins	−	+
Secreted toxins			
hla/alpha-toxin	Disrupts red blood cells	−	+
hlb/β-hemolysin[c]	Sphingomyelinase activity	−	+
Cell surface proteins			
clfB/clumping factor	Binds fibrinogen	+	NA
sdrC	Putative cell surface adhesin	+	ND
spa/protein A	Binds Fc portion of immunoglobulin	+	−
Potential virulence factors			
thrB	Threonine synthase	+	ND
ureA through *ureG*[c,d]	Urease complex	−	+
Regulator, *sarS*	Virulence gene regulator	+	−

[a] Genes are classified based on their known or postulated virulence function.
[b] +, activation; −, repression; NA, not affected; ND, not determined.
[c] Validated by Northern blot analysis.
[d] Validated by protein activity.

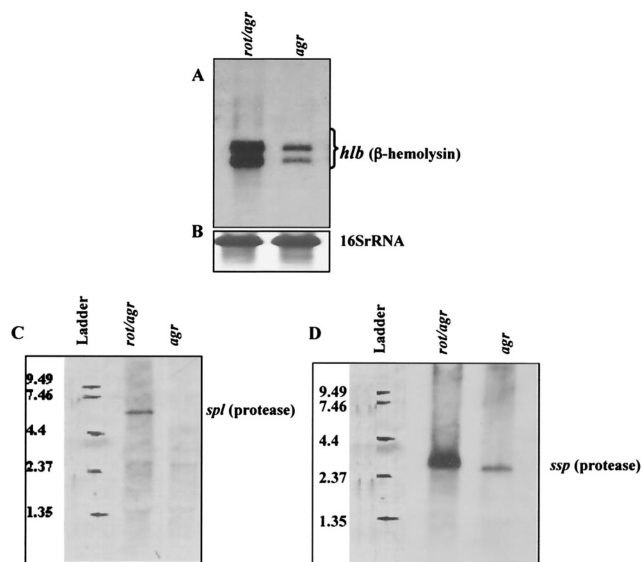

FIG. 1. Northern blot analysis of *hlb*, *spl*, and *ssp*. RNA was isolated from BK9986 (*rot agr*) and RN6911 (*agr*) at the late-exponential-growth phase and hybridized with *hlb* (A), *splA* (C), or *sspA* (D). Transcription of 16S rRNA was used as an internal control (B).

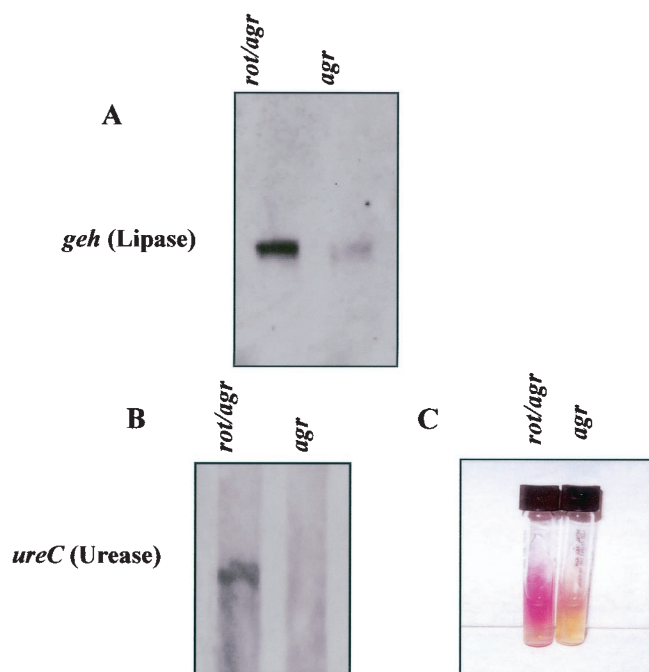

FIG. 2. Expression analysis of *geh* (A) and *ureC* (B and C). (A) RNA samples from BK9986 (*rot agr*) and RN6911 (*agr*) were taken during the late-exponential phase of growth and hybridized with a *geh*-specific probe. (B) *ureC* transcripts in BK9986 (*rot agr*) and its parental *agr* strain (RN6911) were analyzed. (C) Urease activity in both strains was tested on urea agar slants.

(BK9987) and compared the transcription of both *rot* and *sarS* in the two strains, i.e., BK9986 (*rot agr)* and BK9987 (*sarS agr*), as well as in RN6911 (*agr*) and RN6390 (wild-type strain), in a Northern blot assay. We found that *rot* is expressed in all the strains tested except the *rot* mutant, as expected (Fig. 3B). On the other hand, *sarS* is expressed only in the presence of Rot and in the absence of RNAIII (Fig. 3C). The fact that the *sarS* transcript is detectable in RN6911 (*agr*) but not in BK9986 (*rot agr*) indicates that Rot is indeed required for *sarS* expression. The higher levels of *sarS* transcript in RN6911 compared to RN6390 are in good agreement with previous data that showed that *agr* represses *sarS* expression (6, 38).

Rot and *agr* have opposing effects on the expression of target genes. Our results indicate that Rot-regulated factors follow a general expression pattern opposite that of *agr*, that is, secreted proteins generally upregulated by *agr* are negatively regulated by Rot (Table 5). For example, Rot negatively regulates *hla* and *hlb* while *agr* positively regulates them (32). Since Rot negatively regulates the expression and activity of urease, we tested to see if *agr* will upregulate them. For this, we tested the urease activity of an *agr* mutant (RN6911) and its parental wild-type strain (RN6390) on urea agar slants and found that indeed the *agr*-positive strain was urease positive whereas the *agr*-negative strain was urease negative (data not shown). Conversely, cell surface proteins generally downregulated by *agr* appear to be positively modulated by Rot (Table 5). For instance, Rot positively regulates *spa*, encoding protein A, whereas *agr* negatively regulates it. In addition to structural genes, Rot and *agr* also have opposing effects on the expression of the virulence gene regulator *sarS*. Our results indicate that Rot positively regulated the expression of *sarS*, which has been shown to be repressed by *agr* (6, 38).

Additional Rot-regulated genes. Table 3 lists additional virulence genes negatively regulated by Rot as determined by GeneChip analysis, and Table 4 lists all genes shown to be positively modulated by Rot. These tables include genes which encode factors previously shown to promote the growth and survival of *S. aureus* in an infection model. For instance, Rot downregulates *hlgB* and *hlgC*, which encode gamma-hemolysins B and C, respectively. Gamma-hemolysin is an *S. aureus* virulence factor that has been shown to play a role in *S. aureus* endophthalmitis and corneal pathogenesis (11, 37). Rot positively regulates *thrB*, a gene involved in threonine biosynthesis. Disruption of this gene reduces virulence in animal infection models (25). From both lists, it is clear that in addition to virulence genes, Rot also regulates the expression of genes that play different biological roles, including amino acid and carbohydrate metabolism, cell wall biosynthesis, and transport.

DISCUSSION

S. aureus is a versatile human pathogen capable of causing diverse infections in different sites of the body. This versatility can be attributed to its impressive arsenal of virulence factors (20). It is now apparent that *S. aureus* has numerous regulators that, alone and in combination, control the expression of these factors. Although the list of these virulence factors is quite substantial, it is believed to be incomplete and limited to determinants that are easily detectable by available assays. The recent advent of genomewide expression profiling techniques such as DNA arrays and proteomics provides an unprece-

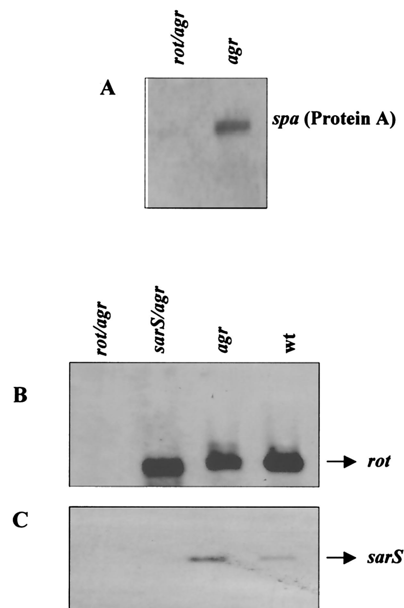

FIG. 3. Northern blot analysis of *spa* (A), *rot* (B), and *sarS* (C). (A) Analysis for the *spa* transcript in BK9986 (*rot agr*) and RN6911 (*agr*) at the late-exponential phase of growth. (B and C) RNA samples from BK9986 (*rot agr)*, BK9987 (*sarS agr)*, RN6911 (*agr*), and RN6390 (wild-type [wt] strain) at the late-exponential phase of growth were hybridized with *rot*- and *sarS*-specific probes, respectively.

dented ability to identify genes regulated in a coordinated manner, thus increasing our understanding of biological processes. In a recent study, Dunman and colleagues used DNA GeneChip technology to demonstrate that the two well-studied virulence gene regulators *agr* and SarA regulate the expression of known virulence genes as well as novel genes not previously known to play a role in *S. aureus* pathogenesis (12). Likewise, using a proteomic approach, Ziebandt et al. identified new members of the SarA regulon (41).

Virulence factors whose expression is controlled by the different regulators generally fall into two categories: cell surface proteins and secreted proteins. The *agr* system generally downregulates cell surface proteins such as protein A and upregulates secreted proteins such as alpha-toxin. By use of DNA array technology, we have clearly demonstrated in this report that Rot does not act exclusively as a repressor. The work presented here indicates that Rot also positively regulates the expression of genes, many of which have previously been determined to be virulence factors. We also demonstrated that Rot and *agr* have opposing effects on the expression of certain virulence determinants. Collectively, these findings suggest that Rot is a more global virulence regulator than previously recognized. Whether the effects of Rot on its target genes are direct or indirect is currently unknown.

Rot positively regulates the expression of *sarS*, a transcriptional modulator of virulence gene expression (6, 38). Arvidson and colleagues have shown that SarS binds to the promoter of *spa* (38), a gene activated by both Rot and SarS. Thus, Rot may affect *spa* transcription via SarS. Other SarA homologues have also been shown to regulate the expression of virulence gene regulators. For instance, SarR represses *sarA* expression (21) and SarA represses *sarT* expression (35). We could postulate that these modulators affect certain target genes by influencing the expression of regulators of these genes.

Besides genes with well-characterized or postulated virulence functions, Rot also regulates genes not previously linked to virulence. These genes could either be potential virulence genes or additional accessory genes with other biological functions. In the latter case, as Dunman and colleagues point out, the regulators involved in the *agr*-SarA regulatory pathway may be more global than previously recognized and may affect processes that have yet to be established in *S. aureus* pathogenesis (12). Given the ability of *S. aureus* to cause a broad range of clinical manifestations, it is likely that many of these gene products are involved in various, as yet unrecognized stages of infection.

Although this study identified numerous members of the Rot regulon, it is very likely that the list is not complete due to the fact that 14% of the staphylococcal genes are missing from the chip. For example, the *sspA* gene from the serine protease operon is missing from the GeneChip and hence from Table 3. However, Northern blot analysis showed that the whole operon is downregulated by Rot. With the complete genome available, new GeneChips are being designed to cover 100% of the genome, and these will be used in future studies.

Based on the virulence function of Rot-regulated genes, we hypothesize that Rot plays an important role in the early steps of infection. Factors positively modulated by Rot include cell surface adhesins such as ClfB (clumping factor B), which binds fibrinogen (26), and SdrC, a cell surface adhesin (15). Rot also positively regulates the expression of a gene represented by ORF 1175 on the chip, which corresponds to N315 ORF 2284 (Table 4). This ORF contains a cell wall sorting signal motif, LPXTG, which is characteristic of most adhesins (17). Rot positively regulates the production of protein A, a factor involved in evasion of the host immune system (32). Another gene upregulated by Rot is *dltD* (Table 4). This gene is a member of the *dlt* operon, which consists of *dltA*, *dltB*, *dltC*, and *dltD* (31). This operon encodes proteins involved in D-alanine incorporation into teichoic acid in the *S. aureus* cell wall. Interestingly, *dltA* is not present on the list because it is among the genes missing from the GeneChip used in this study (see Materials and Methods). In addition, *dltB* and *dltC* are not listed because their fold difference (1.3 for *dltB* and 1.4 for *dltC*) did not make the cutoff. The *dlt* operon has been shown to confer resistance to human antimicrobial agents such as defensins (31). A recent study by Collins et al. has demonstrated that *dlt* mutants are more efficiently killed by human neutrophils than wild-type strains (9). The same study has also shown that mortality and arthritis frequencies were significantly lower in mice infected with *dlt* mutants than in mice infected with the wild type strains, indicating that the strains lacking the *dlt* operon were less virulent than *dlt*-positive strains. All these factors positively modulated by Rot are hypothesized to be required at an early stage of infection. Some factors, such as the adhesins, allow the bacteria to attach to host cells, while others, such as protein A and DltA, -B, -C, and -D facilitate establishment of the infection through inhibition of the host immune response.

Simultaneously, Rot negatively regulates factors that may interfere with the establishment of the infection. For instance, Rot negatively regulates the production of proteases. One role for proteases is to degrade surface proteins such as protein A, fibronectin-binding proteins, and clumping factor B, which facilitate adherence of the bacteria to host tissues. Degradation of these factors will allow the bacteria to detach from the initial site of infection and start the infection at another location (16, 23). Rot also represses the synthesis of *hla* and *hlb*, which encode alpha- and beta-hemolysin, respectively. These hemolysins are known to disrupt a variety of mammalian cells, and because of their function, they are classified as virulence factors involved in invasion and tissue penetration (32). Thus, Rot promotes early stages of infection by stimulating factors needed at this stage and by inhibiting those that interfere with the early processes of infection or that are needed for later processes.

During late stages of infection, which correspond to the post-exponential growth phase, there is a peak of the levels of RNAIII, the *agr* effector molecule (32). Since our results indicate that Rot and *agr* have opposing effects on the expression of *S. aureus* virulence genes, it is likely that RNAIII either directly or indirectly exerts an inhibitory effect on Rot. Since *rot* is transcribed throughout the growth curve (data not shown) and the *rot* transcript is present in both RNAIII-positive and RNAIII-negative strains (Fig. 3B), Rot is likely to be inhibited by RNAIII posttranscriptionally. The inhibitory effects of RNAIII on Rot result in the repression of the cell surface proteins and the activation of the secreted proteins that promote the spread of the bacteria and allow them to initiate infection at other sites. Given that Rot was identified in an

agr-negative strain, we proceeded to determine the extent of *S. aureus* gene regulation by Rot in the same background. However, preliminary results indicate that Rot effects on select target genes such as *ureC*, *geh*, *spl*, and *ssp* operons could be validated in an *agr*-positive background as well.

In conclusion, the present body of work has employed transcription profiling technology to investigate the spectrum of genes that are regulated by the *S. aureus* repressor of toxin, Rot. The results demonstrate that Rot is a global transcriptional modulator of virulence genes and that its influence on the expression of certain target genes is opposite that of *agr*. Genes whose expression is influenced by Rot can be grouped in three major categories: (i) genes that have been extensively studied and have well-defined virulence functions, (ii) genes postulated to play a role in virulence, as their disruption results in attenuation of the mutant in a specific infection model, and (iii) genes not previously known to play a role in *S. aureus* pathogenesis. By unveiling additional regulatory levels, this study gives us better insight into the *agr*-SarA regulatory network of *S. aureus* virulence genes. Additional studies are needed to investigate the molecular interaction of Rot with its target genes and with other regulators, as well as the role of the novel genes regulated by Rot in *S. aureus* virulence.

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