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CodY-Mediated Regulation of the *Staphylococcus aureus* Agr System Integrates Nutritional and Population Density Signals

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The *Staphylococcus aureus* Agr system regulates virulence gene expression by responding to cell population density (quorum sensing). When an extracellular peptide signal (AIP-III in strain UAMS-1, used for these experiments) reaches a concentration threshold, the AgrC-AgrA two-component regulatory system is activated through a cascade of phosphorylation events, leading to induction of the divergently transcribed *agrBDCA* operon and the RNAIII gene. RNAIII is a posttranscriptional regulator of numerous metabolic and pathogenesis genes. CodY, a global regulatory protein, is known to repress *agrBDCA* and RNAIII transcription during exponential growth in rich medium, but the mechanism of this regulation has remained elusive. Here we report that phosphorylation of AgrA by the AgrC protein kinase is required for the overexpression of the *agrBDCA* operon and the RNAIII gene in a *codY* mutant during the exponential-growth phase, suggesting that the quorum-sensing system, which normally controls AgrC activation, is active even in exponential-phase cells in the absence of CodY. In part, such premature expression of RNAIII was attributable to higher-than-normal accumulation of AIP-III in a *codY* mutant strain, as determined using ultrahigh-performance liquid chromatography coupled to mass spectrometry. Although CodY is a strong repressor of the *agr* locus, CodY bound only weakly to the *agrBDCA*-RNAIII promoter region, suggesting that direct regulation by CodY is unlikely to be the principal mechanism by which CodY regulates *agr* and RNAIII expression. Taken together, these results strongly suggest that cell population density signals inducing virulence gene expression can be overridden by nutrient availability, a condition monitored by CodY.

Bacteria can adapt to complex and rapidly changing environments. They have diverse and sophisticated systems for sensing their surroundings and regulating their gene expression accordingly. Because most pathogens can obtain nutrients from their host organisms, at least in part by producing virulence factors whose activities can also be deleterious to the host, synthesis of such factors is tightly regulated. For instance, the opportunistic pathogen *Staphylococcus aureus*, which produces a plethora of secreted and cell wall-associated pathogenicity factors, can sense and respond to environmental cues to optimize the expression of virulence genes. Indeed, the synthesis of staphylococcal pathogenicity factors changes in response to variations in growth phase (1–3), cell population density (4), pH (5, 6), glucose availability (7, 8), NaCl concentration (9), and exposure to antibiotics (10).

S. aureus virulence is centrally regulated by the accessory gene regulator (Agr) system (4). The *agr* locus consists of two divergent transcription units (Fig. 1). The P2 promoter drives the transcription of the *agrBDCA* operon, producing an mRNA called RNAII, whereas the P3 promoter drives the synthesis of RNAIII, an RNA molecule that is both an mRNA for delta-hemolysin and a regulatory factor for a large number of genes. RNAIII reduces the expression of genes encoding surface adhesins and increases the synthesis of capsule, toxins, and proteases (4). The *agrB* and *agrD* gene products are involved in the synthesis, transport, and maturation of a cyclic thiolactone that acts as an autoinducing peptide (AIP), whereas the *agrC* and *agrA* genes encode, respectively, the AIP-sensing membrane kinase and response regulator of a two-component transcriptional regulatory system. When the extracellular concentration of AIP reaches a threshold level, AIP binds to AgrC, inducing autophosphorylation and leading to the activation-by-phosphorylation of AgrA. Phosphorylated AgrA stimulates both the transcription of the operon that encodes it and the transcription of RNAIII by binding to the P2-P3 promoter region. SarA, a small DNA-binding protein, also regulates the expression of many *S. aureus* virulence genes (11) and functions in part by activating the transcription of the *agr* locus (12–14). The detailed molecular mechanism by which SarA regulates virulence gene expression is unclear (15–19).

Many staphylococcal virulence genes are regulated by nutritional signals. CodY, a global transcriptional regulator present in most low-G+C Gram-positive bacteria, senses directly the intracellular concentrations of branched-chain amino acids (BCAAs) and GTP (20). These effectors couple CodY activity to the availability of rapidly metabolized carbon and nitrogen sources and, specifically, to amino acid pools (21). CodY is most active during the exponential-growth phase in rich medium, when the intracellular concentrations of BCAAs and GTP are high (20). In *S. aureus*, as well as in several other pathogenic bacteria, CodY regulates both metabolic and virulence genes (22–37). Interestingly, the *S. aureus agr* locus is overexpressed in a *codY* mutant during exponential phase, suggesting that CodY is a repressor of transcription from the *agr* P2 and P3 promoters (25).

Although CodY represses *agr* expression, we report here that CodY regulates *agr* transcription even though it binds at best

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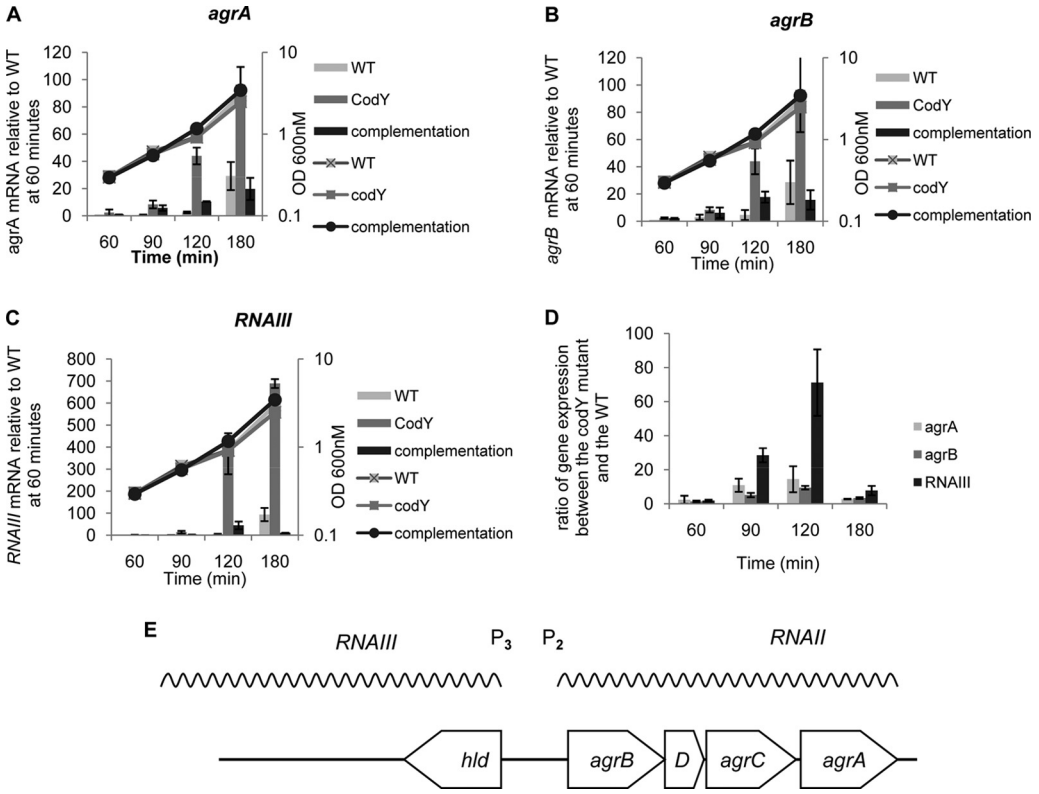


FIG 1 CodY is a negative regulator of the *agr* genes during the exponential-growth phase. (A through C) Two biological replicates of exponential-phase culture samples of UAMS-1 and MS-1 (Δ*codY*) stains were collected after 60, 90, 120, and 180 min of growth in TSB medium. RNA samples were prepared, and levels of *agrA* (A), *agrB* (B), and RNAIII (C) RNAs were determined by real-time quantitative PCR. For each target gene at each time point, the ratio of the transcript to 16S rRNA was determined and was normalized to the transcript/16S rRNA ratio for the wild-type strain at 60 min. (D) Data from panels A through C were converted to ratios of expression in the *codY* mutant relative to expression in the parent strain at each time point. Error bars correspond to the standard errors of the means.

weakly to the P2-P3 promoter region and to a putative binding site within *agrC*. Additionally, we found that the overexpression of the entire *agr* locus seen in exponential-phase cells of a *codY* mutant is correlated with increased accumulation of AIP and depends on the presence of the phosphorylated form of the response regulator AgrA as well as on the kinase AgrC. On the other hand, CodY-dependent regulation of the *agr* locus was independent of SarA and of any other regulatory protein known to affect *agr* expression. These results suggest that CodY regulates the expression of the *agr* genes indirectly. Moreover, these results indicate that the Agr quorum-sensing system is active under conditions of relatively low cell population density but that the effects of intercellular signaling are masked by CodY-mediated repression under nutrient-replete conditions. Thus, CodY-dependent regulation of the *agr* genes serves to integrate cell population density and nutritional signals that control virulence.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α (*recA*) was used for plasmid construction and was cultivated in Luria-Bertani (LB) medium. *S. aureus* strains were grown in tryptic soy broth (TSB; Becton, Dickinson Co.). Cultures were incubated with shaking (250 rpm) at 30°C, 37°C, or 42°C, with a flask-to-medium volume ratio of 10:1. Chloramphenicol (10 μg/ml) and ampicillin (50 μg/ml) were added for plasmid maintenance when needed. Chloramphenicol was used at 2.5 μg/ml when a chloramphenicol resistance gene was integrated into the chromosome. Growth was monitored as an increase in the absorbance at 600 nm in an Ultraspec II UV-visible spectrophotometer (LKB Biochrom).

DNA manipulations and transformations. All molecular biology techniques, including *E. coli* transformations, were performed as described by Sambrook and Russell (38). Oligonucleotide primers were obtained from Integrated DNA Technologies and are listed in Table 2. All cloned fragments were verified by sequencing by the Tufts University Nucleic Acids and Protein Core Facility. A rapid boiling procedure was used to prepare template DNA from *S. aureus* as described previously (39).

Deletion mutants. Three unmarked deletion mutants were constructed: one with a deletion of P2 and P3 (ΔP2-P3), one with an in-frame deletion (Δ*agrC*), and one with an out-of-frame deletion (Δ*agrA*). PCR-based mutagenesis followed by splicing overlap extension (SOEing) (40) was carried out using UAMS-1 genomic DNA and primer sets oAR39/oAR69 and oAR70/oAR71 for Δ*agrA*, oAR95/oAR97 and oAR96/oAR98 for Δ*agrC*, and oAR88/oAR85 and oAR87/oAR86 for the ΔP2-P3 promoters. The fragments were cloned in the thermosensitive integrative plasmid pTS1 (26) by using the restriction sites SacI and XmaI. The resulting plasmids, pAR12, pAR31, and pAR23, were introduced by electroporation into the restriction-deficient *S. aureus* strain RN4220 according to methods described previously (41), but with incubation of the transformants at 30°C. The plasmids were then transferred to strain UAMS-1 using the transducing phage ϕ11, as described previously (42). Transductants were grown in TSB medium supplemented with chloramphenicol for 12 h at 30°C. A 1:100 dilution of the culture was then grown in TSB containing chloram-

TABLE 1 Plasmids and strains used in the study

Plasmid or strain	Relevant genotype and/or characteristic(s)	Source or reference
Plasmids		
pTS1	Shuttle vector; pE194*ori*(Ts); ColE1 *bla cat*	74
pNL9164	Shuttle vector; pT181 *cop-634*(Ts) *repC4 ori*	75
pKM1	*S. aureus codY* cloned into pBAD30 with 5 additional histidine codons at the C terminus	K. McCarty and A. Sonenshein, unpublished data
pCL15	Expression shuttle vector, derivative of pSI-1; P_{SPAC} *cat*	44, 46
pAR12	DNA regions surrounding *agrA* cloned in pST1	This study
pAR23	DNA regions surrounding the P2-P3 *agr* promoters cloned in pST1	This study
pAR28	Cloned intron DNA targeted to *SACOL2585* in pNL9164	This study
pAR31	DNA regions surrounding *agrC* cloned into pST1	This study
pTL6936	pCL15 P_{SPAC}-*codY*	26
P_{SPAC}-*agrA*	pCL15 P_{SPAC}-*agrA*	This study
S. aureus strains		
UAMS-1	Clinical isolate	76
MS1	UAMS-1 Δ*codY*::*ermC*	26
AR87	UAMS-1 *SA2585*::intron	This study
AR89	UAMS-1 Δ*codY*::*ermC* *SA2585*::intron	This study
CM18	UAMS-1 *agr*::*tetM*	25
CM20	UAMS-1 *sarA*::*kan*	25
CM22	UAMS-1 Δ*codY*::*ermC sarA*::*kan*	25
AR45	UAMS-1 Δ*agrA*	This study
AR48	UAMS-1 Δ*codY*::*ermC* Δ*agrA*	This study
AR70	UAMS-1 ΔP2-P3	This study
AR71	UAMS-1 Δ*codY*::*ermC* ΔP2-P3	This study
AR145	UAMS-1 Δ*agrC*	This study
AR147	UAMS-1 Δ*codY*::*ermC* Δ*agrC*	This study

TABLE 2 Primers used in the study

Primer name	Sequence (5′→3′)
oAR39	AATAATCCCGGGCGCAAGTTCCGTCATGATTA
oAR69	GTCATGCTTACGAATTTCAC
oAR70	GAAATTCGTAAGCATGACCAAAATCTCACAGACTCATTGC
oAR71	GGGGAGCTCCATTATGGGATAACGCAGAAG
oAR95	GATTGGTAGAACTATAAACACGC
oAR97	GGGCCCGGGCACCTGCAGCTACTAAGAAG
oAR96	GCGTGTTTATAGTTCTACCAATCGATGCATCAACAATCAAACAG
oAR98	GGGGAGCTCATTCACATCCTTATGGCTAG
oAR88	GGGCCCGGGGTATTAAATTAGATTTGTATAAATAAAAAGCAC
oAR85	CGACACAGTGAACAAATTCAC
oAR87	GGGGAGCTCGCACAATATAAAATGATTTGAGTAAC
oAR86	GTGAATTTGTTCACTGTGTCGGAGGAGAGTGGTGTAAAATTG
oAR134	AAAAGCTTTTGCAACCCACGTCGATCGTGAAGAAATAATAATAG TGCGCCCAGATAGGGTG
oAR135	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATAATAAGTA ACTTACCTTTCTTTGT
oAR136	CGCAAGTTTCTAATTTCGGTTATTTCTCGATAGAGGAAAGTGTCT
oAR174	CTATTTTCCATCACATCTCTGTG
oCM43	TGAATTTGTTCACTGTGTCGAT
oCM114	AGTAAGGATCCTTTGGATCGTCTTCGCAAAT
oAR154	CAATTTTACACCACTCTCCTC
p-ilvD2	CAGAAATAGGACTTAAAGCGTTTAG
oCM123	ATTGAATTCCGCAAGTTCCGTCATGATTA
oAR187	TGACCAGTTTGCCACGTATCTTCA
oAR188	GCTAAGACCTGCATCCCTAATCGT
oAR52	CCTATGGAAATTGCCCTCGC
oAR64	GCCTAATTTGATACCATTAATATCAG
oAR185	GTGAATTTGTTCACTGTGTCGATAATCC
oAR186	GGAAGGAGTGATTTCAATGGCACA
oAR16	CGTGTCTCAGTTCCAGTGTG
oAR17	CTTCTCTGATGTTAGCGGCG
oAR157	GGGGGATCCAGAGGAGAAATTAACTATGAAAATTTTCATTT GCGAAGACG
oAR158	GGGGAGCTCAATTGAATACGCCGTTAAC

phenicol for 12 h at 42°C, a nonpermissive temperature for pTS1 replication, thereby selecting for integration into the chromosome by homologous recombination. The cultures were then shifted to 30°C, and the bacteria were passaged four times in TSB without antibiotics. Bacteria were finally plated on tryptic soy agar (TSA) and were screened for chloramphenicol-sensitive clones.

The *SACOL2585* insertion mutation was constructed using the *S. aureus* TargeTron gene knockout system from Sigma-Aldrich according to the manufacturer's instructions. Briefly, primers oAR134, oAR135, and oAR136 were designed using the TargeTron design website (Sigma-Aldrich) and were used to amplify a PCR fragment encoding a mutated intron designed to specifically target the *SACOL2585* gene. The fragment was then cloned into the thermosensitive TargeTron vector pNL9164 by using the HindIII and BsrGI restriction sites. The resulting plasmid was first introduced by electroporation in RN4220 according to methods described previously (41) and then transferred to strain UAMS-1 by using the transducing phage ϕ11 (42). The transformants and transductants were selected and cultivated at 30°C. Insertion mutants were finally cultivated at 42°C, and plasmid-free, erythromycin-sensitive mutants were selected.

The chromosomal mutation Δ*codY*::*erm* was transferred into appropriate strain backgrounds by using transducing phage ϕ11, as described previously (42).

Detection of AIP-III. *S. aureus* strains UAMS-1, MS1 (Δ*codY*), and CM18 (Δ*agr*) were grown overnight in TSB medium, diluted in 10 ml fresh TSB to an initial optical density at 600 nm (OD_{600}) of 0.05, and incubated with shaking at 37°C until they reached an OD_{600} of about 0.5. The cultures were then diluted in 60 ml of TSB to give an initial OD_{600} of 0.05 and were incubated with shaking at 37°C. Ten-milliliter samples were removed at 60-min intervals and were subjected to centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatants were sterilized by passage through a 0.22-μm filter and were frozen on dry ice.

An approach used previously for the detection of AIP-I (43) was modified to facilitate measurements of the accumulation of AIP-III. AIP-III was analyzed in the filtered culture fluids using reversed-phase liquid chromatography (LC) (C_{18} column with T3 endcapping; Waters Corporation) in an Acquity ultrahigh-performance liquid chromatograph (UPLC; Waters Corporation) coupled to an Orbitrap mass spectrometer (MS) with an electrospray source (LTQ Orbitrap XL; Thermo Scientific) over a scan range of *m/z* 300 to 2,000. The mass spectrometer settings were as follows: tube lens voltage, 110 V; source voltage, 4.50 kV; source current, 100 μA; heated capillary voltage, 20.0 V; heated capillary temperature, 300.0°C; sheath gas flow rate, 20.0; auxiliary gas flow rate, 0.00. Samples (5 μl) were injected and eluted at a flow rate of 0.25 ml per min with the following binary gradient, where solvent A is 0.1% formic acid in H_2O and solvent B is 0.1% formic acid in acetonitrile: 0 to 6 min, from 80% A to 20% A; 6.0 to 6.5 min, from 20% A to 80% A; 6.5 to 7.0 min, 80% A (isocratic). For tandem MS (MS-MS) analysis, the precursor mass of 819.41 (the predicted mass of AIP-III) was subjected to collision-induced dissociation with an activation energy of 35%.

P_{SPAC}-*agrA* plasmid construction. The *agrA* gene was amplified from UAMS-1 genomic DNA using primers oAR157, containing the strong

ribosome binding site (RBS) of the pQE plasmid (Qiagen), and oAR158. The PCR fragment was introduced into pCL15 (44) using the BamHI and SstI restriction sites. In the resulting plasmid, P_{SPAC}-*agrA*, the *agrA* gene is under the control of the P_{SPAC} promoter.

Gel mobility shift assay. To generate a ^{32}P-end-labeled probe, the P2-P3, P2-P3-plus-CodY motif, *ilvD*, and *agrC* regions were PCR amplified from strain UAMS-1 genomic DNA using ^{32}P-labeled oAR174, oCM43, oAR175, and oCM114, respectively, and unlabeled oAR154 (for both P2-P3 region versions), p-ilvD2, and oCM123, respectively. The ^{32}P-labeled primer was 5′ end labeled with [γ-^{32}P]ATP (PerkinElmer) using T4 polynucleotide kinase (New England BioLabs) according to the manufacturer's recommendations and was then purified with the QIAquick nucleotide removal kit (Qiagen). Following amplification, 3,000 cpm of the end-labeled PCR product was used for each gel shift reaction. CodY with a 6-histidine tag at the C terminus was purified as described previously (25) and was mixed at various concentrations (25 to 200 nM) with the ^{32}P-end-labeled promoter DNA in binding buffer (20 mM Tris-HCl [pH 8], 50 mM KCl, 2 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 0.05% Nonidet P-40, 25 μg/ml salmon sperm DNA) supplemented with 10 mM (each) isoleucine, leucine, and valine and 2 mM GTP. After 20 min of incubation at room temperature, the reaction mixtures were loaded onto an 8% nondenaturing polyacrylamide gel prepared in a buffer (10 mM Tris-HCl [pH 8], 77 mM glycine, 0.2 mM EDTA) supplemented with 10 mM (each) isoleucine, leucine, and valine and were subjected to electrophoresis for 2 h at room temperature. The electrophoresis buffer contained 3.5 mM HEPES and 4.3 mM imidazole. Following electrophoresis, the gel was dried under a vacuum, exposed to a phosphorimager screen, and analyzed using an Applied Biosystems phosphorimager and Image Quant software (GE Healthcare).

DNase I protection experiment. To generate a ^{32}P-end-labeled probe, the P2-P3, P2-P3-plus-CodY motif, and *ilvD* regions were PCR amplified from strain UAMS-1 genomic DNA using the same primers as those used for the gel mobility shift experiment with oAR174, 0AR154, and pilvD2 as ^{32}P-labeled primers. CodY was incubated with the ^{32}P-end-labeled promoter DNA fragments in binding buffer (see above) supplemented with 10 mM (each) isoleucine, leucine and valine and 2 mM GTP. The total volume was 10 μl. After incubation for 20 min at room temperature, 1 μl of binding buffer containing 0.06 U of RQ1 DNase I (Promega), 10 mM $MgCl_2$, and 20 mM $CaCl_2$ was added to the samples. After 1 min of incubation, 4 μl of stop solution (20 mM EDTA and 95% formamide dye solution) was added, and the samples were heated at 80°C for 5 min. The samples were then loaded without further purification onto 7 M urea–5% polyacrylamide DNA sequencing gels. G+A sequence ladders were created by incubating 80,000 cpm of each radioactive fragment in 20 μl of 60% stop solution containing 1 μl of a 5% formic acid solution and boiling for 20 min. Following electrophoresis, the gel was dried under a vacuum, exposed to a phosphorimager screen, and analyzed using an Applied Biosystems phosphorimager and Image Quant software (GE Healthcare).

RNA sample collection and preparation. *S. aureus* colonies from TSA plates were used to inoculate 3-ml overnight cultures in TSB. These cultures were then used to inoculate 10 ml of fresh TSB to a starting OD_{600} of 0.05. After the bacteria reached an OD_{600} between 0.5 and 0.8, they were diluted again in fresh TSB to an OD_{600} of 0.05. During subsequent incubation, samples (5 ml) of exponentially growing cultures (OD_{600}, between 0.5 and 0.8) were rapidly withdrawn from each flask and were transferred to 15-ml conical tubes (Becton, Dickinson) containing an equal volume of an ice-cold (1:1 [vol/vol]) mixture of ethanol and acetone. The tubes were agitated vigorously and were immediately stored at −80°C until RNA extraction. Thawed samples were spun for 10 min at 4°C and 3,200 × *g*, and the pellets were washed twice with 0.5 ml TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) with centrifugation at 3,200 × *g* and 4°C for 10 min. The pellets were then resuspended in 1 ml of Qiagen RLT buffer supplemented with 1% (vol/vol) 2-mercaptoethanol, mixed with 0.25 ml of 0.1-mm-diameter silica beads (Biospec Products, Inc.), and disrupted in a Mini-Beadbeater (Biospec Products, Inc.) for two 60-s intervals at the maximum disruption frequency (setting, 48) separated by a 5-min incubation on ice. The suspensions were then centrifuged at 13,000 × *g* for 15 min at 4°C, and the supernatant fluid was used for RNA isolation by using RNeasy minicolumns (Qiagen) according to the manufacturer's recommendations, but without the on-column DNase treatment. Genomic DNA was eliminated by using the Turbo DNA-free DNase treatment and removal kit (Ambion) according to the manufacturer's instructions, using 5 μg of nucleic acid. RNA concentrations were determined by absorbance at 260 nm using a computer-controlled NanoDrop ND-1000 spectrophotometer.

cDNA synthesis and quantitative real-time reverse transcriptase PCR (RT-PCR). A 1-μg sample of total RNA was used to synthesize cDNA by using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Primers for quantitative PCR (qPCR) were designed using the online PrimerQuest tool from Integrated DNA Technologies and are listed in Table 2. We performed qPCR using the LightCycler 480 system and associated SYBR green I chemistry (Roche Applied Science) to analyze transcript abundance from prepared cDNA samples. Each 20-μl reaction mixture contained 600 nM specific primers (oAR187 and oAR188 for *agrB*, oAR64 and oAR52 for *agrA*, oAR185 and oAR186 for RNAIII, and oAR16 and oAR17 for 16S rRNA). Thermal cycling was performed according to the LightCycler 480 SYBR green I template protocol, except that we used annealing temperatures of 50°C and 55°C as the minimum temperatures for melting curve analysis. Standard curves were generated for each target by using purified DNA fragments amplified by PCR from the *S. aureus* chromosome. Serial dilutions spanning at least 4 orders of magnitude were analyzed. Standard curves as well as standard PCR controls (including no-template and no-reverse-transcriptase reactions) were run on each plate along with test reactions. Single amplification products were verified by melting curve analysis (melting temperature [T_m]-calling algorithm). The results of quantitative PCRs were calculated using the second-derivative maximum analysis algorithm. Data are presented either as numbers of copies of target transcripts per 10^6 copies of 16S rRNA or as the target/rRNA ratio of a mutant sample relative to the target/rRNA ratio of the wild-type (WT) sample cultivated under the same conditions. We used 16S rRNA for normalization because its abundance was not expected to change significantly under the conditions tested.

RESULTS

CodY-dependent regulation of the *agr* locus. The Agr quorum-sensing system directly or indirectly regulates a large number of virulence genes (4). Interestingly, microarray analysis demonstrated that the expression of the *agr* genes is derepressed in a *codY* mutant, compared to that in its parent strain, during the exponential-growth phase of strains UAMS-1 and Newman (25, 27), suggesting that CodY is a repressor of *agr* gene expression. To confirm and quantify this regulatory effect for strain UAMS-1, we performed quantitative, real-time RT-PCR experiments at several time points during the exponential-growth phase to compare the levels of *agrA* and *agrB* mRNAs (reflecting P2 activity) and RNAIII (reflecting P3 activity) in the parental strain and the *codY* mutant strain (MS-1). As expected, expression of the *agr* locus in the wild-type strain increased significantly only at 180 min, i.e., near the end of exponential phase, but the *codY*-null mutation led to levels of all three RNAs significantly higher than those in the parent strain starting at 90 min (Fig. 1). Additionally, introduction into the *codY* mutant strain of the wild-type *codY* gene cloned in a low-copy-number plasmid (44) suppressed the overexpression phenotype (Fig. 1A). As reported by others (45), we found that the level of RNAIII was higher than the levels of *agrA* and *agrB* mRNAs. Interestingly, we found that CodY regulates P3 promoter expression to a greater extent than P2 promoter expression, sug-

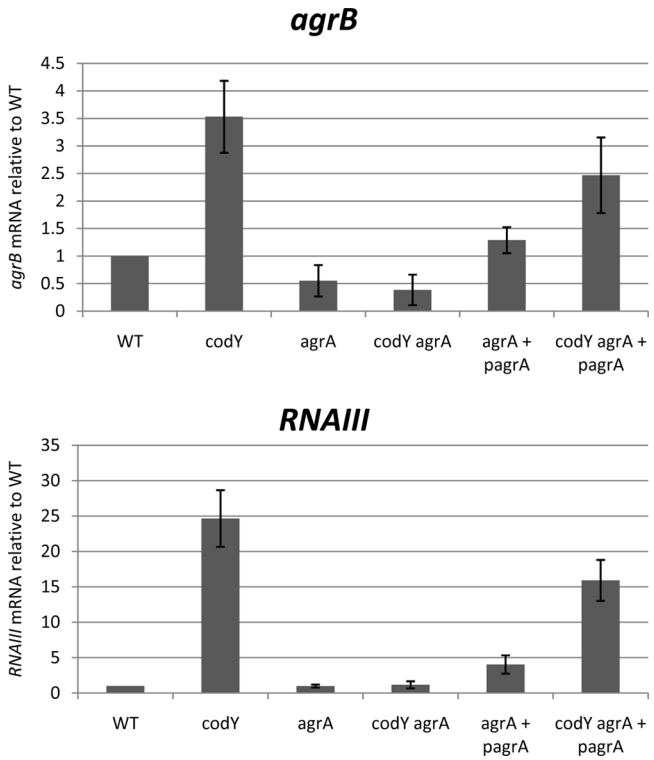


FIG 2 AgrA is required for overexpression of the *agr* locus in a *codY* mutant. Three biological replicates of exponential-phase culture samples (OD_{600}, 0.5 to 0.8) of strains UAMS-1, UAMS-1 Δ*agrA*, UAMS-1 Δ*agrA* (P_{SPAC}-*agrA*), and their respective *codY* mutants were collected, and RNAs were prepared. The relative levels of *agrB* and RNAIII RNAs were determined by real-time quantitative PCR. For each target gene, the ratio of the transcript to 16S rRNA was normalized to the corresponding ratio obtained for wild-type cells. Error bars correspond to the standard errors of the means.

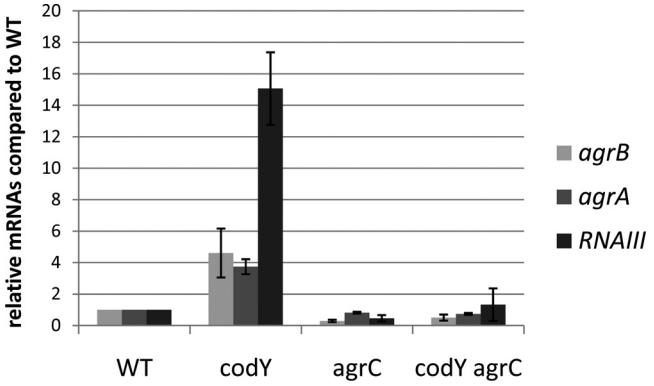


FIG 3 AgrC is required for overexpression of the *agr* locus in a *codY* mutant. Three biological replicates of exponential-phase culture samples (OD_{600}, 0.5 to 0.8) of strains UAMS-1, MS-1 (Δ*codY*), UAMS-1 Δ*agrC*, and UAMS-1 Δ*codY* Δ*agrC* were collected, and RNAs were prepared. The relative levels of *agrA*, *agrB*, and RNAIII RNAs were determined by real-time quantitative PCR. For each target gene, the ratio of the transcript to 16S rRNA was normalized to the corresponding ratio obtained for wild-type cells. Error bars correspond to the standard errors of the means.

gesting that CodY regulates the two promoters differentially (Fig. 1D). These results confirm that CodY is a negative regulator of the *agr* genes and also show that *agr* transcription is not strictly dependent on high cell population density; when CodY is inactive, the *agr* locus is expressed even early in the exponential-growth phase.

AgrA is required for hyperexpression of the *agr* locus in a *codY* mutant. To determine whether the overexpression of the *agr* locus in a *codY* mutant depends on the normal Agr autoregulatory system, we constructed an *agrA* deletion mutant strain and measured the effect of a *codY* mutation on *agrB* and RNAIII expression in this strain. As expected, deletion of *agrA* prevented the activation of *agr* gene expression in both the exponential (Fig. 2) and the stationary (data not shown) phase. Interestingly, the introduction of a *codY* mutation did not cause any derepression of the *agr* locus in the *agrA* mutant background (Fig. 2), showing that the overexpression of the *agr* locus in the *codY* mutant during the exponential-growth phase requires the presence of AgrA. To confirm this result, we cloned *agrA* under the control of the P_{SPAC} promoter in a low-copy-number plasmid (44, 46). To limit the expression of *agrA* in *trans* and to prevent the induction of the P2 and P3 promoters in the $codY^+$ strain, we took advantage of the leakiness of the P_{SPAC} promoter and cultivated the strains in the absence of an inducer. The expression of *agrA* from the plasmid induced greater increases in *agrB* and RNAIII expression in the *agrA codY* double mutant than in the *agrA* single mutant (Fig. 2). (In fact, we may have underestimated the effect of the *codY* mutation in this case, because the transcription of *agrA* from the plasmid was unexpectedly lower in the *codY* mutant strain than in the wild-type strain [data not shown].) These results suggest that the effect of the CodY protein on *agr* locus expression depends on the presence of the AgrA response regulator.

Phosphorylation of AgrA by AgrC is required for *agr* overexpression in the *codY* mutant. Phosphorylation of AgrA is required for the activation of the P2 and P3 promoters in stationary-phase cells (45, 47). Both the phosphorylated and unphosphorylated forms of AgrA can bind to the *agr* promoter region with high affinity *in vitro*, but the affinity and stability of the binding are higher for the phosphorylated form (45). Because CodY is active mostly during the exponential phase of growth in rich media, we investigated whether AgrA phosphorylation is required for *agr* overexpression in a *codY* mutant during exponential phase. To do so, we constructed an in-frame deletion of the histidine kinase-encoding gene *agrC*. Such a strain should synthesize AgrA but should not be able to mediate its phosphorylation. To check that the deletion of *agrC* was not affecting the expression of the downstream *agrA* gene, we compared, by RT-PCR, the expression of *agrA* in the constructed strain UAMS-1 Δ*agrC* and in the P2-P3 promoter deletion mutant strain, where *agrA* expression is not driven by the P2 promoter. We found that whereas the deletion of the P2-P3 promoter region leads to very low expression of *agrA*, the deletion of *agrC* allows a basal level of *agrA* expression identical to the level seen in the wild-type strain (data not shown). This result confirms that *agrA* is expressed at a wild-type level in the UAMS-1 Δ*agrC* strain. We then measured the effect of *codY* deletion on the levels of *agrB*, *agrA*, and RNAIII transcripts using RT-PCR analysis in exponential-phase cells of strains UAMS-1, MS-1 (Δ*codY*), UAMS-1 Δ*agrC*, and UAMS-1 Δ*agrC* Δ*codY*. We found that the deletion of *codY* led to the overexpression of RNAII (*agrB* and *agrA*) and RNAIII in $agrC^+$ cells, as expected, but not in *agrC* mutant strains (Fig. 3). Therefore, we infer that phosphorylation of AgrA by AgrC is required for the overexpression of *agr* in a *codY* mutant. These results also imply that no kinase other than

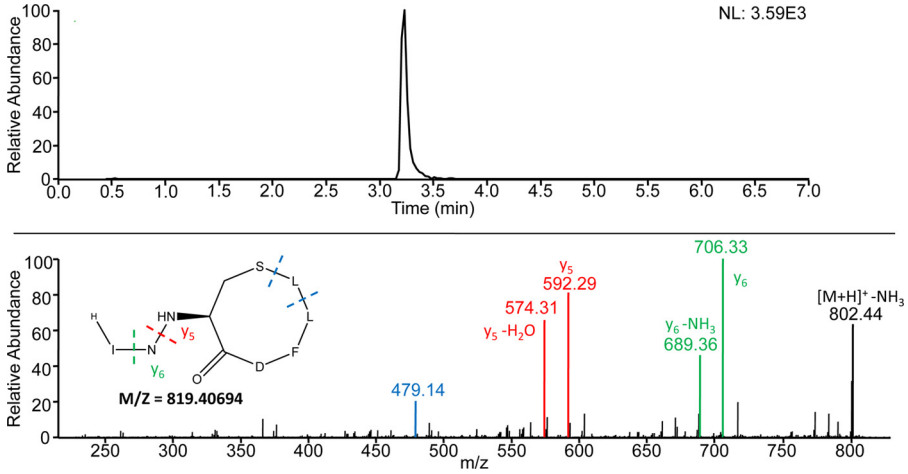


FIG 4 Detection of AIP-III. The filter-sterilized culture fluid of strain MS1 (Δ*codY*) was subjected to reversed-phase liquid chromatography coupled to mass spectrometry. (Top) Extracted ion chromatogram for the transition from *m/z* 819.4 to 592.2, the y5 fragment of AIP-III. Filtering the data in this way enabled the peak corresponding to AIP-III to be clearly visualized at a retention time of 3.23 min. (Bottom) The $[M+H]^+$ ion for AIP-III (*m/z* 819.4) was subjected to collision-induced dissociation to generate the MS-MS spectrum shown. The predicted structure and calculated mass of AIP-III are shown on the left. Product ion y6 corresponds to loss of the N-terminal isoleucine residue and y5 to loss of the N-terminal isoleucine-asparagine dipeptide. The product ion with a mass of 479.14 corresponds to cleavage on both sides of the leucine residue at position 7.

AgrC and no metabolite found in exponential-phase cells is able to phosphorylate AgrA sufficiently to stimulate *agr* gene expression in exponential-phase cells.

AIP-III accumulation in a *codY* mutant strain. The dependence on AgrA and AgrC of *agr* locus hyperexpression in a *codY* mutant strain (Fig. 2 and 3) implies that an autoinducing peptide is involved in the derepression. Moreover, this result suggests that, even in exponential phase, when the cell density is relatively low, there is a sufficient level of autoinducing peptide to promote *agr* expression. To compare the levels of autoinducing peptide in the parental strain and the *codY* mutant, we prepared culture fluid samples from exponential-phase and early-stationary-phase cells grown in TSB. The samples were subjected to coupled liquid chromatography-mass spectrometry. There are at least four different types of AIPs produced by different *S. aureus* strains (48). All are short peptides (7 to 9 amino acids) that assume a thiolactone configuration (48). Strain UAMS-1 has been shown to encode AIP-III (49). To verify that the isolate of UAMS-1 used for our experiments did indeed code for AIP-III, the *agrD* gene was sequenced and found to encode within it the heptapeptide INCDFLL, as predicted (S. R. Brinsmade, personal communication). Figure 4 shows that we detected by mass spectrometry a heptapeptide thiolactone with the anticipated sequence and structure. As far as we can determine, this represents the first physicochemical assay for AIP-III in culture fluid. The amount of AIP-III in the culture fluid increased during growth for both the parental strain and the *codY* mutant, but at early times, the amount was considerably higher in the *codY* mutant (Table 3). At 180 min of growth, the *codY* mutant had accumulated about 15 times more AIP-III in the culture fluid than the parental strain (Table 3 and Fig. 5). The control strain, CM18 (Δ*agr*), did not accumulate any detectable AIP-III at any time tested.

CodY integrates cell density and nutritional signals. To assess the impact of overaccumulation of AIP-III, we measured RNAIII levels at several points during growth in the parental strain and the *codY* mutant (harvested at an OD_{600} of 0.05), which were resuspended in filtered culture fluids of later-exponential-phase cultures (180 min) of strain UAMS-1, CM18 (Δ*agr*), or MS-1 (Δ*codY*). (The culture fluids were harvested at 180 min so as to allow the resuspended cells to continue growing for several generations.) We found that growth in the culture fluid from the parental strain led to an increase in *agr* expression even in the parental strain (compare Fig. 6A and C), presumably due to the presence of some AIP-III in the 180-min culture fluid. In parental culture fluid, a *codY*-null mutant showed a statistically significant higher level of expression of RNAIII than the parental strain (Fig. 6A). As expected, resuspension of parental strain or *codY* mutant cells in the culture fluid of a *codY* mutant caused even higher levels of RNAIII expression (Fig. 6B). These results suggest that overproduction of AIP-III in a *codY* mutant is responsible, at least in part, for the hyperexpression of the *agr* operon and the RNAIII gene. Moreover, CodY normally prevents the expression of RNAIII even after the population density threshold has been reached. Interestingly, we could not detect this effect for the P2 promoter (data not shown), confirming that the P2 and P3 promoters are differentially regulated by CodY.

TABLE 3 Accumulation of AIP-III in culture fluid of parental and mutant strains[a]

Time of sampling (min)	Amt of AIP-III (arbitrary units)[b] in culture fluid of strain:		
	UAMS-1	MS1 (Δ*codY*)	CM18 (Δ*agr*)
60	ND	ND	ND
120	ND	ND	ND
180	30 ± 30	503 ± 186	ND
240	1,728 ± 788	4,365 ± 1,241	ND
360	7,449 ± 322	11,007 ± 1,321	ND

[a] All strains were grown in TSB medium in three biological replicates, and samples were removed for centrifugation at the times indicated. The amount of accumulated AIP-III in the culture fluid was determined by LC-MS, as described in Materials and Methods.
[b] ND, no peak detected for the ion corresponding to AIP-III (with the threshold for detection set at a signal/noise ratio of 3:1).

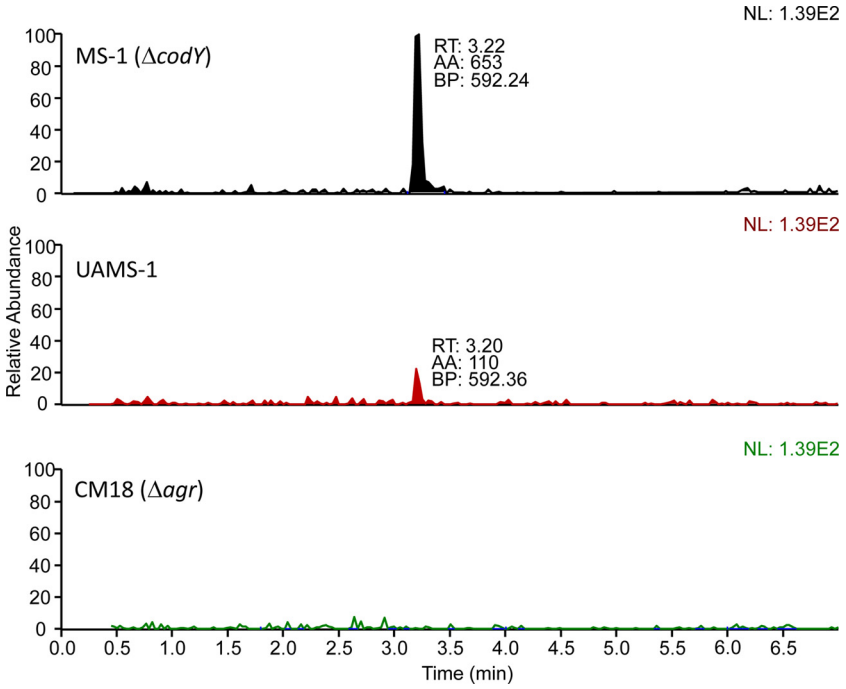


FIG 5 Comparison of AIP-III accumulation in the parental and *codY* mutant strains. Filter-sterilized culture fluid from 180-min cultures of the parent strain UAMS-1, the *codY*-null mutant MS1, and the *agr* deletion mutant CM18 were subjected to coupled LC-MS, as described in Materials and Methods. Extracted ion chromatograms for the 819.4-to-592.2 transition of the $[M+H]^+$ ion of AIP-III are shown for the three different cultures. All chromatograms were normalized to the same value (1.39×10^2) to facilitate comparison of the relative abundances of AIP-III. A peak representing AIP-III was detected at a retention time (RT) of 3.20 min, with base peak (BP) at *m/z* 592.36 and area (AA) of 110 (arbitrary units).

CodY binds weakly to the *agr* P2-P3 promoter region *in vitro*. A consensus motif, AATTTTCWGAAAATT ("CodY box"), has been shown to play an important, but not necessarily sufficient, role in the interaction of CodY with DNA in *Lactococcus lactis* and *Bacillus subtilis* (50, 51). In *S. aureus*, a conserved motif highly reminiscent of the canonical CodY box was strongly associated with sites of CodY binding (25). Sequence analysis showed the presence of one CodY box with 3 mismatches near the beginning of the RNAIII gene, whereas no CodY box with fewer than 5 mismatches was identified in the intergenic region that includes the P2 and P3 promoters of the *agr* locus (Fig. 7A). Moreover, unlike hundreds of bona fide CodY binding sites, neither P2 nor P3 was copurified with CodY in a genomewide screen for CodY targets (25). To analyze further the ability of CodY to bind within the *agr* locus *in vitro*, we performed an electrophoretic mobility shift experiment using DNA fragments containing either the P2-P3 promoter intergenic region alone (P2-P3 *agr*) or both the P2-P3 promoter region and the putative CodY motif present within RNAIII (P2-P3 *agr* plus CodY motif) (Fig. 7A). As shown in Fig. 7C, *S. aureus* CodY was able to bind with high affinity to the *ilvD* promoter region (K_D [equilibrium dissociation constant], $\leq$25 nM; K_D is estimated as the concentration of CodY that causes 50% of the DNA fragments to be shifted), used here as a positive control, but bound with low affinity to the *agr* P2-P3 promoter region (K_D, 100 to 200 nM). Interestingly, we found that CodY was able to bind with higher affinity to the DNA fragment containing the putative CodY box present within RNAIII in addition to the P2-P3 promoter region (K_D, 50 to 100 nM) (Fig. 7C). This result raised the possibility that the putative binding motif present within RNAIII is a physiologically relevant binding site for CodY. To try to verify the apparent binding, we performed DNase I footprinting analyses of the various DNA fragments. As expected, we found that CodY protected two regions in the *ilvD* promoter fragment, corresponding to the regions identified in a genome-wide pulldown assay (25) (Fig. 7D). However, we were unable to detect protection with the P2-P3-containing fragments, even the fragment with the apparent CodY box, at CodY concentrations below 400 nM (Fig. 7D). The relatively weak interaction of CodY with the P2-P3 promoter region and the absence of a footprint for this region strongly suggest that the relatively weak binding observed *in vitro* does not reflect meaningful interaction *in vivo* and that CodY is likely to regulate the transcription of the *agr* genes indirectly.

CodY does not regulate the expression of *agrA* through binding within *agrC*. Given that CodY binds weakly to the P2 promoter region, which normally drives *agrA* transcription, a possible explanation for the AgrA dependence of *agr* overexpression in *codY* mutant cells would be that, in such cells, CodY controls the expression of *agrA* by binding to a DNA region located inside the *agrBDCA* operon. Such a mechanism would also explain the derepression of P3-dependent transcription in *codY* mutant cells. Interestingly, *in vitro* binding experiments showed previously that CodY binds to a site located within *agrC* (25). To confirm this result, we performed a gel shift experiment using a fragment of the *agrC* region containing the putative CodY binding site (Fig. 7B) in the presence of GTP and BCAAs. As shown in Fig. 7C, CodY bound with intermediate affinity to the *agrC* region (K_D, 50 to 100 nM). Because *agrC* is located upstream of *agrA*, CodY bound at that site might function as a transcription roadblock, preventing the transcription of *agrA*

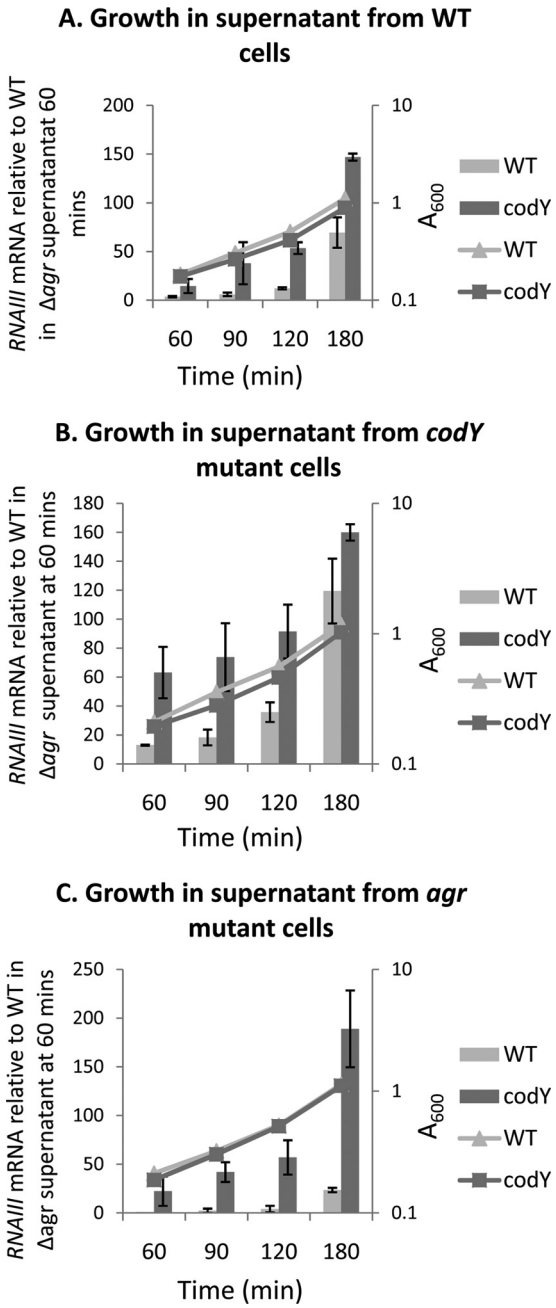


FIG 6 CodY integrates cell density and nutritional signals. Cultures of strains UAMS-1 (A), MS-1 (Δ*codY*) (B), and CM18 (Δ*agr*) (C) were harvested at 180 min of growth in TSB medium (late-exponential phase), filtered, and used to resuspend exponential-phase cells of strain UAMS-1 or MS-1 (Δ*codY*) at an initial A_{600} of 0.05. Samples of the latter cultures were harvested at 60, 90, 120, and 180 min of growth in the culture fluids, RNA samples were prepared, and relative levels of RNAIII were determined by real-time quantitative PCR. For each target gene, the ratio of the transcript to 16S rRNA was normalized to the corresponding ratio obtained for wild-type cell RNA harvested at 60 min of growth in culture fluid from Δ*agr* cells. The data represent the results of two biological replicates. Error bars correspond to the standard errors of the means.

from P2. To test that hypothesis, we compared the absolute levels of *agrB* and *agrA* mRNAs in the wild-type strain during exponential growth. We found that *agrB* and *agrA* were expressed at the same basal level (Table 4). Thus, we could find no evidence that any transcriptional roadblock exists downstream of *agrB*.

Another possible explanation for the CodY-dependent regulation of *agr* expression would be that an additional promoter, controlled by CodY, was driving *agrA* expression. In fact, the initial characterization of the *agr* locus reported the presence of a promoter, P1, that lies within the *agrC* coding sequence and could, in principle, contribute to the expression of *agrA* (52). The role, if any, played by this promoter in the regulation of the *agr* locus has never been determined. Interestingly, the *agrC* region bound by CodY *in vitro* overlaps with the putative P1 promoter (25). To examine the potential regulatory effect of CodY on transcription from P1, we measured the effect of a *codY* deletion on *agrA* gene expression in a strain that lacks the P2-P3 promoter region and therefore can express *agrA* only from P1. To that end, we constructed a mutant strain carrying a deletion of the P2-P3 promoter region and monitored the expression levels of *agrA* in the ΔP2-P3 and Δ*codY* ΔP2-P3 mutant strains by quantitative RT-PCR. Very low expression of *agrA* was detected in the *codY*$^+$ strain, and *agrA* expression did not increase in the *codY* mutant strain lacking the P2-P3 promoter region (Table 4). Thus, P1 is not likely to contribute significantly to *agrA* expression under the conditions tested and therefore is not likely to be the target of regulation by CodY.

Neither SarA nor SACOL2585 is the mediator of the CodY effect on *agr* gene expression. CodY might control *agr* expression indirectly by controlling the expression of another regulatory protein that acts directly on the *agr* operon. In addition to CodY, at least 15 other proteins have been reported to regulate the *agr* locus, including AgrA, SarA (11), SarT (53), SarU (54), SarX (55), SarZ (56), MgrA (55–57), CcpA (58), ArlRS (59), ClpP (60, 61), CvfA (62, 63), Msa (64), Rsr (65), SrrAB (66), and RpiR homologs (67). Other than AgrA, however, none of the known transcriptional regulators of the *agr* operon was found to be differentially expressed more than 2-fold in a *codY* mutant by microarray analysis (25, 27) or by transcriptome sequencing (RNA-seq) analysis (A. Roux, unpublished data). Since SarA is a particularly well-studied regulator of *agr* and binds directly to the P2 and P3 promoter regions (12, 13, 68), we pursued further the possibility that the activity of SarA, rather than its synthesis, might mediate the CodY effect. Previous results, obtained using semiquantitative RT-PCR analysis, showed that RNAII and RNAIII were overexpressed in both the *codY* single mutant and the *codY sarA* double mutant (25). To address this question more rigorously, we used quantitative RT-PCR to compare the accumulation of *agrA* and *agrB* mRNAs (from P2) and RNAIII (from P3) during the exponential-growth phase in strains UAMS-1, MS-1 (Δ*codY*), UAMS-1 Δ*sarA*, and UAMS-1 Δ*codY* Δ*sarA*. As expected, the levels of all three RNAs were significantly higher in the *codY* mutant and significantly lower in the *sarA* mutant than in the parental strain. In a *sarA* mutant, however, there was still significant derepression when a *codY* mutation was introduced (Fig. 8). These results demonstrate that while SarA is required for maximal *agr* expression, the derepressing effect of a *codY* mutation does not depend on SarA. Thus, SarA and CodY appear to be independent regulators of the *agr* promoters.

Two other transcription factor-encoding genes, *saeR* and *SACOL2585*, were significantly overexpressed in a *codY* mutant strain (25). The *saeR* gene encodes the response regulator protein of the SaeRS two-component system and is known to act on vir-

A

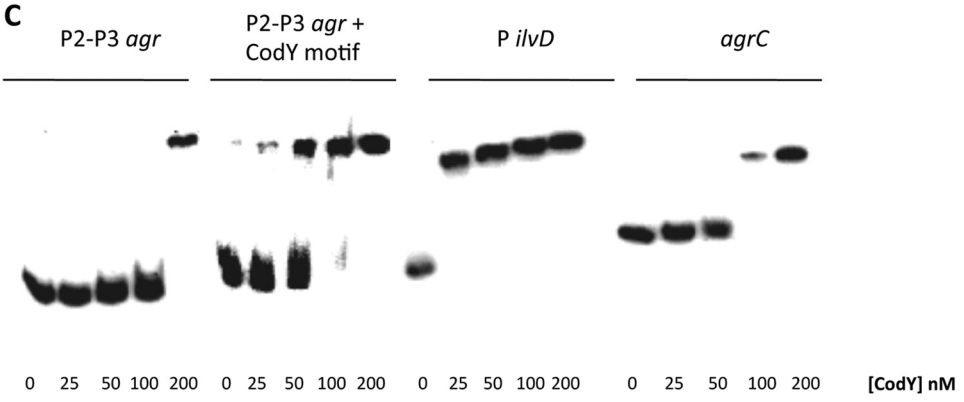
TGAATTTGTTCACTGTGTCGATAATCCATTTTACTAAGTCACCGATTGTTGAAATGATATCTTGT
GCCATTGAAATCACTCCTTCCTTAATTAAGATAaaaattcttaaaattAAACAACT***CAT***CAA**CTATTTTCCA**
TCACATCTCTGTGATCTAGTTATATTAAAACATGCTATAAGCATTTATTTTCCATTTTTTCTTAACT
AGTCGTTTTTTATTCTTAACTGTAAATTTTTTTATGTTAAAATATTAAATACAAATTACATTTAACAG
TTAAGTATTTATTTCCTACAGTTAGGCAATATAATGATAAAAGATTGTACTAAATCGTATAATAACA
GT**GAGGAGAGTGGTGTAAAA*TTG***

B

703 1051

CGCAAGTTCCGTCATGATTA....ATCATTCGCGTTGCATTTATTGAAAGTG*AAAATTCAGTAACGT*T
TATTGTTATGAATAAATGCGCTGATGATATACCACGCATTCATGAATTGTTCCAAGAAAGTTTTTCT
ACTAAAGGTGAAGGTCGTGGTTTAGGTCTATCAACTTTAAAAGaaattgctgataatgCAGACAATGTCTT
ATTAGATACAATTATCGAAAATGGTTTCTTTATTCAAAAAGTTGAAATTATTAACAAC***TAG***CCATAA
GGATGTGA***ATG***TATGAAAATTTTC**ATTTGCGAAGACGATCCAAA**

C



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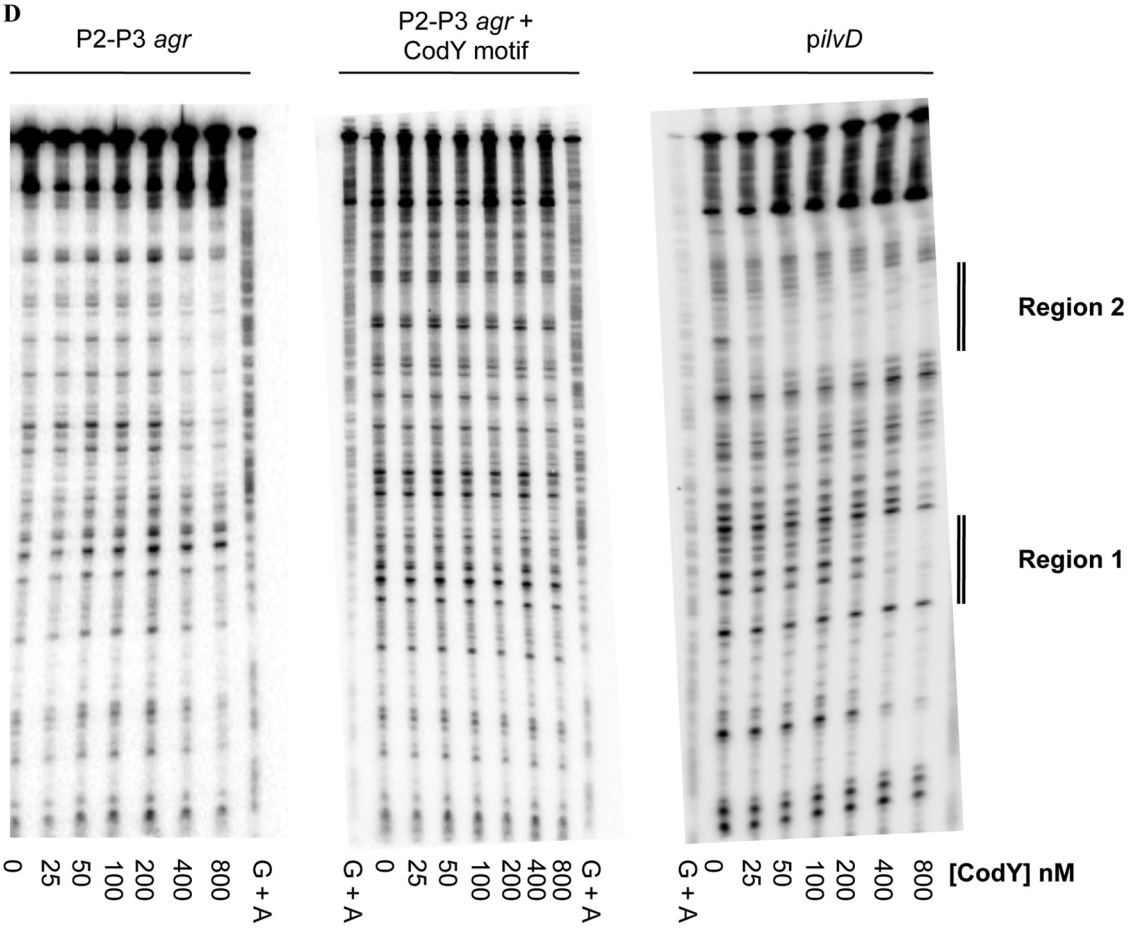

TABLE 4 CodY does not regulate the expression of *agrA* through binding within *agrC*[a]

Strain	Level of mRNA[b]	
	agrB	*agrA*
WT (UAMS-1)	17.17 ± 0.49	18.15 ± 2.19
UAMS-1 ΔP2-P3	ND	0.52 ± 0.02
UAMS-1 Δ*codY* ΔP2-P3	ND	0.51 ± 0.01

[a] Exponential-phase culture samples (OD_{600}, between 0.5 and 0.8) of UAMS-1, UAMS-1 ΔP2-P3, and UAMS-1 Δ*codY* ΔP2-P3 were collected, and RNAs were prepared. The absolute levels of *agrB* and *agrA* mRNAs were determined by real-time quantitative PCR.

[b] Expressed as the number of copies of *agr* mRNA per 10^6 copies of 16S rRNA. ND, not detectable.

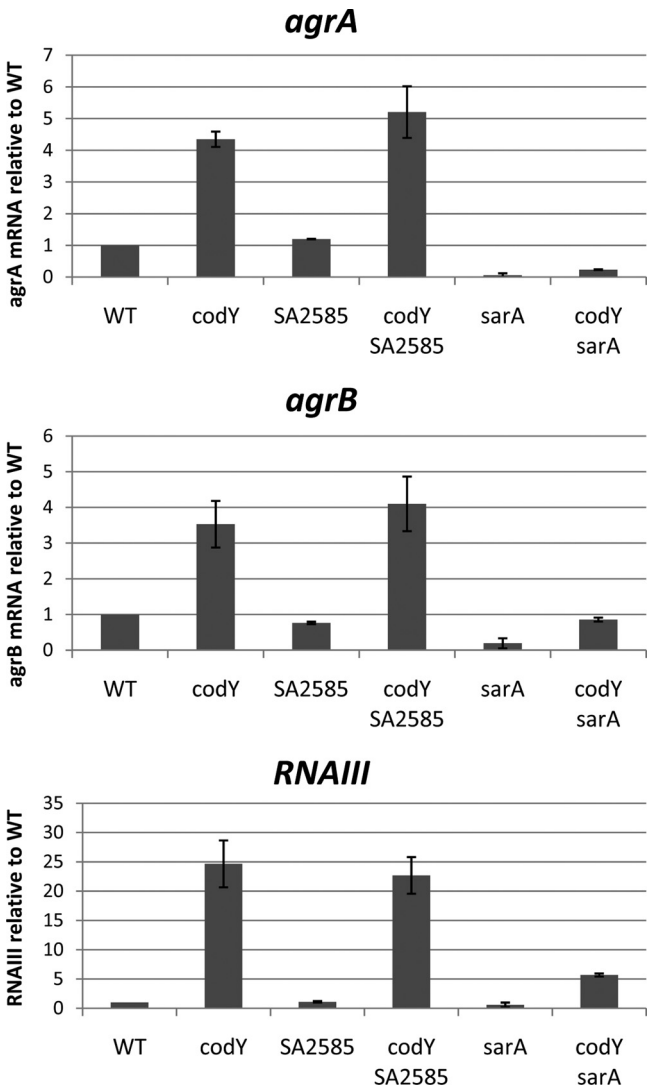

FIG 8 Neither SarA nor SACOL2585 is a mediator of CodY-dependent *agr* regulation. Three biological replicates of exponential-phase culture samples (OD_{600}, 0.5 to 0.8) of strains UAMS-1, UAMS-1 *SACOL2585*::intron, UAMS-1 Δ*sarA*, and their respective *codY* mutants were collected, and RNAs were prepared. The relative levels of *agrA*, *agrB*, and RNAIII RNAs were determined by real-time quantitative PCR. For each target gene, the ratio of the transcript to 16S rRNA was normalized to the corresponding ratio obtained for wild-type cells. Error bars correspond to the standard errors of the means.

ulence gene expression in *S. aureus*, but several studies have shown that SaeR is not an activator of *agr* expression, seeming to rule out SaeR as the regulator that links CodY to the regulation of the *agr* locus (69–71). *SACOL2585* encodes a potential transcriptional regulator of the PfoR family. To assess the role of this gene in the regulation of *agr* in a *codY* mutant, we constructed a *SACOL2585* insertion mutation and measured its effect on *agr* expression. Interruption of *SACOL2585* did not affect the expression of *agr* in the parental strain or in a *codY* mutant (Fig. 8), making it unlikely that this protein mediates CodY-dependent regulation of *agr*.

To test the possibility that a previously unidentified regulatory protein mediates the effect of CodY on the *agr* locus, we purified proteins that bind to the P2-P3 promoter region by streptavidin-affinity chromatography using biotinylated P2-P3 promoter region DNA as the bait. The same four polypeptides in the same relative amounts were found to bind to the P2-P3 region in extracts of parental and *codY* mutant cells (data not shown). Mass spectrometry of the bands excised from SDS-polyacrylamide gels identified the polypeptides as MgrA, SarZ, SarA, and enolase (note that CodY was not among the proteins in wild-type extracts that copurified with the regulatory region). Except for enolase, a glycolytic enzyme that binds nonspecifically to DNA, all of these proteins are known regulators of the *agr* locus (12, 13, 57, 68, 72). The fact that expression of the genes that encode these proteins is not affected by a *codY* mutation and that they were present in the same amounts in wild-type and *codY* mutant extracts argues against (but does not rule out) their serving as mediators of the CodY effect.

DISCUSSION

In the present study, we have analyzed the regulation of the *agr* genes by CodY and found that this mechanism is likely to be multifaceted. CodY binds to the P2 and P3 promoter regions *in vitro* but does so with low affinity. Therefore, CodY binding to the P2-P3 promoter region, if physiologically relevant, is unlikely to be the primary mode by which CodY controls *agr* expression. It is still possible that the binding of CodY to the P2-P3 promoter region is enhanced *in vivo* through simultaneous binding of an unidentified cofactor, but there is no evidence for such a cofactor in any of the dozens of other cases where CodY binding to a reg-

FIG 7 CodY binds with low affinity to the *agr* locus *in vitro*. (A and B) Sequences of the RNAIII-*agrB* intergenic region probes (A) and the *agrC* gene internal probe (B). The *agrC* probe is a 640-bp fragment that starts at position 703 of the *agrC* coding sequence, extends through the *agrC* stop codon, and ends at the 36th base of the *agrA* coding sequence. The oligonucleotides used to amplify each region for use as a probe are in boldface. The 3′ oligonucleotides at the downstream ends of each sequence are the complements of the actual oligonucleotides used. Putative CodY binding motifs are in lowercase. (A) The start codons for *agrB* and the transcription start site for RNAIII are in bold italics; the P2 and P3 promoter regions are indicated by gray letters; and the regions protected by AgrA in DNase footprinting experiments (45) are underlined. (B) The stop codon of *agrC* and the start codon of *agrA* are in bold italics. (C) Electrophoretic mobility shift experiment for *S. aureus* CodY binding to the P2-P3, *agrC*, and *ilvD* promoter regions. (D) DNase I protection assays of *S. aureus* CodY binding to the P2-P3 and *ilvD* promoter regions. Regions 1 and 2 correspond to CodY-protected sites in the *ilvD* promoter region, as described in reference 25. The lanes marked "G + A" provide sequence markers based on cleavages specific for dGMP and dAMP residues.

ulatory site has been studied. Also, in accord with a previous result (25), we showed that CodY binds with moderate affinity *in vitro* within the *agrC* gene. We found that this binding has no effect on *agrA* transcription *in vivo*, however. In fact, we could find no evidence that CodY acts as a roadblock to transcription from P2 and no evidence that CodY represses *agrA* transcription from the previously described, poorly active P1 promoter, which also resides within *agrC* (52). Taken together, these results strongly suggest that CodY does not control *agr* gene expression through direct binding within the *agr* locus.

The most surprising aspect of our results is that AgrA can be activated by AgrC-mediated phosphorylation even when the cells are at relatively low population density. Thus, our results imply that the autoinducing peptide accumulates to a sufficient level to signal to AgrC at an earlier stage of growth than previously assumed. In wild-type cells, this signaling has a minimal effect, because the *agr* locus is independently repressed by CodY. Only when CodY loses activity (that is, when the intracellular pools of the BCAAs or GTP, or both, drop sufficiently) does the release of CodY-mediated repression allow the activated AgrA to stimulate *agr* gene expression. Therefore, the apparent role of CodY is to prevent inappropriate autoinduction of *agr* until the nutrient limitation and the population density thresholds have both been reached. Thus, expression of the quorum-sensing system is itself dependent on nutritional signals that indicate amino acid availability. The fact many other proteins also contribute both positively and negatively to regulation of *agr* locus expression emphasizes the extraordinary capacity of *S. aureus* to integrate multiple signals simultaneously and to regulate Agr-dependent gene expression accordingly.

Although our conclusion is that CodY regulates the *agr* system indirectly, we have not been able to determine the mechanism of such regulation. Several hypotheses can be offered. First, the synthesis of a direct regulator of *agr* could be under CodY control, but we could find no evidence that the expression of any known or previously unknown transcriptional regulator of *agr*, other than AgrA, is controlled by CodY. In fact, overexpression of the *agr* locus in a *codY* mutant depends on the same kinase (AgrC) and the same response regulator (AgrA) as does the induction that normally occurs when cells reach high population density. Alternatively, CodY could regulate the activity of AgrA, AgrC, or another regulator through a posttranscriptional mechanism. Such a regulatory mechanism has been inferred for *Bacillus anthracis*, in which CodY has been shown to control the stability of AtxA, the master regulator of virulence (30). Thus, *S. aureus* CodY might positively regulate the synthesis of a protease that degrades AgrA or AgrC during the exponential-growth phase. In a *codY*-null mutant, the protease would not be made, and active AgrA or AgrC would accumulate to higher-than-normal levels. Alternatively, since the activity of AgrA is modulated by disulfide bond formation under oxidizing conditions (73), lack of CodY might affect the cell's redox balance. Given that the activity of AgrC is thought to depend on sensing a peptide signal outside the cell, we cannot rule out a posttranscriptional effect of CodY on AgrD or AgrB as well. In summary, the direct target of CodY that mediates its effect on the expression of the Agr system remains elusive; identifying this target will undoubtedly reveal a novel pathway of regulation that affects most virulence genes of *S. aureus*.

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