

## *Staphylococcus aureus* CodY Negatively Regulates Virulence Gene Expression<sup>∇</sup>

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**CodY is a global regulatory protein that was first discovered in *Bacillus subtilis*, where it couples gene expression to changes in the pools of critical metabolites through its activation by GTP and branched-chain amino acids. Homologs of CodY can be found encoded in the genomes of nearly all low-G+C gram-positive bacteria, including *Staphylococcus aureus*. The introduction of a *codY*-null mutation into two *S. aureus* clinical isolates, SA564 and UAMS-1, through allelic replacement, resulted in the overexpression of several virulence genes. The mutant strains had higher levels of hemolytic activity toward rabbit erythrocytes in their culture fluid, produced more polysaccharide intercellular adhesin (PIA), and formed more robust biofilms than did their isogenic parent strains. These phenotypes were associated with derepressed levels of RNA for the hemolytic alpha-toxin (*hla*), the accessory gene regulator (*agr*) (*RNAII* and *RNAIII/hld*), and the operon responsible for the production of PIA (*icaADBC*). These data suggest that CodY represses, either directly or indirectly, the synthesis of a number of virulence factors of *S. aureus*.**

A global transcriptional regulator, CodY, controls the expression of *Bacillus subtilis* genes whose products allow the cell to adapt to nutrient limitation and metabolic stress (53). CodY represses the synthesis of catabolic enzymes (15, 59), genetic competence factors (46), biosynthetic enzymes for certain amino acids (35, 49), antibiotics (22), sporulation proteins (35), and transport systems for amino acids, peptides and sugars (35, 47, 49, 50). CodY is also a positive regulator of some carbon overflow pathways (48). *B. subtilis* CodY senses nutrient availability through interaction with two types of effector molecules, branched-chain amino acids (BCAAs; isoleucine, valine, and leucine) and GTP (44, 49). The effectors act independently and additively to increase the affinity of CodY for DNA (49). Thus, when the bacterium encounters nutrient-depleted conditions, the intracellular BCAA and GTP pools decrease, CodY loses its activity as a DNA-binding protein, and CodY-repressed genes are induced. In *Lactococcus lactis*, CodY regulates genes for amino acid biosynthesis, nitrogen uptake, peptide degradation, transporters, intermediary carbon metabolism, and some regulatory proteins (21). The *L. lactis* CodY is activated by BCAAs in vivo and in vitro but not by GTP (10, 43).

Homologs of CodY are encoded in the genomes of nearly all of the low-G+C gram-positive bacteria, including *Staphylococcus aureus* (28, 44). In several of these species, a *codY* mutation affects virulence. In *Listeria monocytogenes*, a  $\Delta relA$  mutant is highly attenuated for virulence, likely due to an inability to induce the stringent response (3). As a result, the GTP pool is thought to remain high, despite amino acid limitation (3).

Introduction of a  $\Delta codY$  mutation into a  $\Delta relA$  mutant strain restores virulence to the  $\Delta relA$  mutant, suggesting that the reduced virulence of the  $\Delta relA$  mutant is due, at least in part, to failure to control the activity of CodY (3). In *Streptococcus pyogenes*, CodY has a broad impact on virulence gene expression during growth in laboratory culture or in human blood (33, 34). Although the direct CodY targets in *S. pyogenes* are not known, analysis of 51 selected transcriptional units showed that 23 regulatory genes, virulence genes, transporters, and metabolic enzymes are differentially regulated in the *codY* mutant (33). In *Clostridium difficile*, CodY directly regulates transcription of the gene for the alternative sigma factor, TcdR, which is essential for the activation of toxin synthesis (11). A *codY* mutation in *C. difficile* leads to significant derepression of *tcdR* and toxin-encoding genes (11).

*S. aureus* causes an array of diseases, ranging from localized skin lesions and tissue damage to systemic infections, such as pneumonia, endocarditis, and exotoxin syndromes (14). A contributing factor to the diversity of *S. aureus*-associated disease is its ability to adapt efficiently to varying environmental conditions and to produce a plethora of secreted and cell wall-associated virulence factors. *S. aureus* regulates expression of virulence genes in response to signals reflecting both cell population density and nutritional availability. The well-characterized, quorum-sensing, two-component regulatory system (TCS) *agr* (for accessory gene regulator) plays a critical role in the growth-phase-dependent regulation of *S. aureus* virulence factor synthesis (5, 13, 60). The *agr* locus is transcribed from bidirectional promoters P2 and P3 (23, 42). The transcript originating from P2, known as RNAII, encodes four proteins, AgrB, AgrD, AgrC, and ArgA, which are the functional components of the quorum-sensing TCS (5, 37). High cell population density causes the activation of AgrA, the response regulator of the TCS, which induces transcription of the P3

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant genotype and/or characteristic(s)	Source or reference
Plasmids		
pTS1	Shuttle vector, pE194ori <sup>ts</sup> , ColE1 <i>bla</i> cat	20
pCD7-d	Derivative of pTS1, with $\Delta$ <i>codY::ermC</i> fragment, and deletion of plasmid-encoded 3' region of <i>ermC</i>	This study
pCL15	Expression shuttle vector, derivative of pSI-1, P <sub>SPAC</sub> - <i>cat</i>	29
pTL6936	pCL15, P <sub>SPAC</sub> - <i>codY</i>	This study
pGEM-T Easy	Cloning vector	Promega
Strains		
RN4220	<i>S. aureus</i> restriction-deficient derivative of RN450	26, 39
UAMS-1	<i>S. aureus</i> clinical isolate	18
MS1	UAMS-1 $\Delta$ <i>codY::ermC</i>	This study
SA564	<i>S. aureus</i> clinical isolate	52
CDM7	SA564 $\Delta$ <i>codY::ermC</i>	This study
MS2	UAMS-1, pTL6936	This study
MS3	MS1, pTL6936	This study
1457	<i>S. epidermidis</i> biofilm +	31
TM300	<i>S. carnosus</i> biofilm -	19

promoter (37). P3 drives transcription of RNAIII, a regulatory RNA that is both a positive and a negative regulator of virulence factor production (38). RNAIII also serves as an mRNA for the synthesis of delta-toxin, a hemolysin (23). Activation of RNAIII transcription in response to an increase in cell population density marks a transition in gene expression correlated with a need for metabolic and stress adaptations (5). Although several TCS and other regulatory proteins, such as the SarA family of proteins, have been shown to regulate virulence genes (5), there is little known about the mechanisms by which *S. aureus* senses and responds to the nutritional status of the environment.

The regulation of the virulence factors of *S. aureus* in response to growth phase and nutrient availability is reminiscent of regulation by CodY in other gram-positive bacteria. The CodY homologue of *S. aureus* shares 64% overall amino acid similarity with the *B. subtilis* protein (16); the regions predicted to be important for dimer association, isoleucine interaction, and DNA binding share 100, 70, and 97% amino acid similarity, respectively (25, 28). Taken together, these observations suggest conservation of function between the homologues of the CodY proteins in *B. subtilis* and *S. aureus*. Although *S. aureus* CodY has not been studied extensively, a transposon mutagenesis screen in the clinical isolate S30, selecting for defects in biofilm formation, yielded an insertion in the *codY* gene (55). By constructing *codY*-null mutants of two additional clinical isolates of *S. aureus*, UAMS-1 and SA564, we show here that CodY regulates virulence gene expression. Strains UAMS-1 and SA564 were chosen because they represent two genetically and phenotypically distinct lineages (51, 61). In both clinical isolates, a *codY*-null mutation resulted in an increase in hemolytic activity of stationary-phase culture supernatants. Transcript analysis revealed that the gene encoding alpha-toxin, *hla*, was overexpressed in both the exponential and the stationary phases of growth in the *codY* mutants. In addition, the levels of the *agr* locus transcripts, RNAII and RNAIII, were derepressed during the exponential phase of growth. In contrast to what was seen for the clinical isolate S30 (55), the *codY* mutants of UAMS-1 and SA564 displayed a

TABLE 2. Primers used in this study

Primer	Nucleotide sequence (5'→3')
P-codY	GATCGACCCGGGTCTCAAAGCAATAATCC
P+codY	TCGTCACCCGGGCATCTGCCATTTTAGCA
pTS1dErm-r	AGAGTGTCTTGTGACAGACCTAAATTC
pTS1dErm-f	CGACACGGATATATAGTGGATGTG
codY22	GGATCCACAAAAGGAGAAAATTCATGAGC
codY27	CCGCGGACATCGTCACGACTAGGACAT
P-hla	GCATTTCAATTTTCGAGGG
OCD14	GAACCGCATGGTTCAAAA
OCD15	CATTTACCGCTACACATGG
OCD23	ACCCACAATATGTTGTATGATAAAAAATTGA
	ATGAGACATGCTAC
OCD24	TGTCCAGACTCATCGACAAAACCTGTTTAA
	GCCGAC
OCM43	TGAAATGTTCACTGTGTGTCGAT
OCM49	CATTGAACAAGAGCCTGACA
OCM50	GAGTGCCTGGCTTACCTC
OCM51	CAGAAAATTCCTCAGGCGTA
OCM52	AAGGGGTATGACGGTACAACA
OCM57	CAGAGATGTGATGAGAAAATAGTTGA
OCM58	TGACCAGTTTGCCACGTATC
OCM59	CCAAAAGGAAGAAGGTGCAT

greatly increased ability to form biofilms, apparently resulting from high-level expression of the *icaADBC* operon and accumulation of polysaccharide intercellular adhesion (PIA).

## MATERIALS AND METHODS

**Strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. *S. aureus* strains UAMS-1 and SA564 are clinical isolates. *S. epidermidis* 1457 is a biofilm-producing strain, and *S. carnosus* TM300 is a control strain that does not produce biofilm. The *Staphylococcus* strains were grown in tryptic soy broth (TSB; Becton-Dickinson Co.) with shaking (250 rpm) at 37°C, maintaining a flask-to-medium volume ratio of 10:1. Antibiotics needed for plasmid maintenance were used at the following concentrations: erythromycin, 10 µg/ml; and chloramphenicol, 10 µg/ml. When indicated, IPTG (isopropyl-β-D-thiogalactopyranoside; Diagnostic Chemicals, Ltd.) was added to the growth medium to a final concentration of 1 mM.

**Allelic replacement of *codY*.** A *codY* mutation was generated by using the following strategy. A 4.2-kb amplification product, consisting of a 2.2-kb sequence encoding the *ermC* gene, flanked by 1 kb of sequence normally found upstream of the *codY* gene and 1 kb of sequence normally downstream of *codY*, was generated using overlap PCR. The *ermC* gene was amplified from pCN51 (7) by using the primers OCD23 and OCD24 (Table 2), which also incorporated sequences homologous to the regions immediately flanking the *codY* gene in *S. aureus*. Genomic DNA (gDNA) from strain SA564 was used as the template for amplification of the gDNA regions flanking *codY* using the primers P-codY and P+codY and the *ermC* fragment generated as described above. The primers specific for *codY* were generated based on the sequence of *S. aureus* strain 8325 (GenBank ID ABD30331.1). The engineered fragment, which also contained XmaI sites on the 5' and 3' ends, was ligated to the XmaI site of pTS1 (20) and introduced by electroporation into *E. coli* DH5α. To eliminate intraplasmid recombination with the plasmid-encoded 3' region of the *ermC* gene, a derivative of the plasmid was made by PCR and self-ligation by using the primers pTS1dErm-f and pTS1dErm-r. The resulting plasmid, pCD7-d, was introduced by electroporation into the restriction-deficient strain RN4220 according to methods previously described (45). After isolation of pCD7-d from RN4220, the plasmid was used to transform the clinical isolates by the same method. An erythromycin resistant transformant was grown in TSB medium supplemented with erythromycin and chloramphenicol for 12 h at 30°C. A 1:100 dilution of the culture was grown at 42°C for 12 h in TSB with no added antibiotics. After six 12-h passages at 42°C, each representing a 1:100 dilution of the previous culture, the cultures were shifted to 30°C, and the bacteria were passaged six additional times without antibiotics. Bacteria were then plated on tryptic soy agar (TSA) with erythromycin, and erythromycin-resistant, chloramphenicol-sensitive clones were selected.

**Construction of the *codY* complementation strains.** The *codY* gene and its ribosome-binding site were amplified by using the primers codY22 and codY27. The PCR fragment was introduced into pGEMT-easy (Promega) and then re-cloned in pCL15 (29), using the restriction sites SstI and BamHI. In the resulting plasmid, pTL6936, the *codY* gene is under the control of the P<sub>SPAC</sub> promoter.

pTL6936 was introduced into the strain RN4220 as described above. After plasmid isolation from RN4220, pTL6936 was introduced into UAMS-1 and SA564 by electroporation, as described above. Chloramphenicol-resistant colonies were selected and characterized.

**Hemolytic assays.** *S. aureus* colonies from TSA plates were used to inoculate a 3-ml culture in TSB. In addition, four successive 100-fold dilutions of the initial inoculum were incubated overnight. The next day, flasks were selected that contained cultures with an optical density at 600 nm ( $OD_{600}$ ) of 3 to 7 and used to inoculate fresh TSB to a starting  $OD_{600}$  of 0.05. After the bacteria reached an  $OD_{600}$  between 0.5 and 2, they were diluted again in fresh TSB to an  $OD_{600}$  of 0.05 to be sure that the experimental cultures were well adapted to steady-state exponential growth phase. During subsequent growth, culture supernatant fluids were harvested from bacterial cells by centrifugation at 6,000 rpm for 10 min at 4°C. Supernatant samples were frozen immediately and stored at -20°C. The hemolytic activity of culture supernatants was determined by using a 2% suspension of rabbit red blood cells (rRBCs) prepared from rabbit defibrinated blood (HemoStat, Dixon, CA) in phosphate-buffered saline (PBS). The rRBCs were washed three times prior to resuspension in PBS. Serial dilutions (1.5-fold) of bacterial supernatant fluids were made in PBS plus 0.5% bovine serum albumin (MP Biomedicals, Inc.) in 96-well V-bottom plates (Corning, Inc.). PBS plus 0.5% BSA was used as a negative control, and 1% saponin (Sigma-Aldrich) was used as a positive control. An equal volume of 2% rRBCs was added to each sample and allowed to incubate for 10 min at 37°C. rRBCs were pelleted at 1,000 rpm for 5 min at 4°C, and the  $OD_{540}$  of the supernatant fluid was determined. For each sample, the dilution values were plotted ( $\mu$ l of culture fluid versus  $OD_{540}$ ), and the slope was calculated for the linear range of the lysis reaction. Using the linear equation, the hemoglobin release ( $OD_{540}$ ) for 50  $\mu$ l of the lysis reaction fluid was determined for each sample. This value was then used to determine the hemolytic units (HU) per sample by using the equation  $HU = \text{test } OD_{540}/50\% \text{ total lysis } OD_{540}$ . This equation defines one HU as 50% of the total RBC lysis.

**Western blot analysis.** *S. aureus* strains were grown, and supernatant samples were prepared in the same manner as for the hemolytic assay. Samples (4  $\mu$ l) of supernatant fluid were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel after boiling in Laemmli sample buffer (27). The Western blot protocol was performed as described by Ausubel et al. (1) and the product guide for Amersham ECL Western blotting detection reagents (GE Healthcare). Alpha-toxin was detected with rabbit anti-staphylococcal alpha-toxin (Sigma-Aldrich) according to the manufacturer's recommendations. Human immunoglobulin G (Sigma-Aldrich) at 25  $\mu$ g per 10 ml total volume was added during all antibody incubations and washes to block nonspecific binding by protein A (4).

**Primer extension and reverse transcription-PCR (RT-PCR) analysis.** *S. aureus* strains were grown in the same way as for the hemolytic assay. For RNA isolation,  $10^9$  bacteria from exponential ( $OD_{600} = 0.6$  to  $0.8$ , 100 min) or stationary ( $OD_{600} = 8$  to  $11$ , 330 min)-phase cultures were mixed with an equal volume of a 1:1 mixture of ice-cold acetone and ethanol and frozen at -80°C for 24 h. The suspensions were thawed on ice and centrifuged at 6,000 rpm for 10 min. The samples were washed twice with TE (10 mM Tris [pH 8.0], 1 mM EDTA) and resuspended in 1 ml of RLT (plus 1%  $\beta$ -mercaptoethanol) from the RNeasy kit (Qiagen). Cells were transferred to assembled 2-ml screw-cap tubes (Sarstedt) filled to 1/4 volume with 0.1-mm silica beads (BioSpec Products, Inc.) and broken by agitation in a Mini-Beadbeater (BioSpec Products, Inc.) for two 60-s intervals at the maximum disruption frequency (setting of 48) separated by a 5-min incubation on wet ice. The suspensions were then centrifuged at  $13,000 \times g$  for 15 min at 4°C, and the supernatant fluid was used for RNA isolation with an RNeasy mini-column (Qiagen), according to the manufacturer's recommendations, without the on-column DNase treatment. RNA concentrations were determined from the  $OD_{260}$ , and the RNA was run on an RNase-free 2% agarose gel to test for generalized degradation.

For primer extension analysis, 10 to 20  $\mu$ g of total RNA was used per reaction. Radioactive [ $\gamma$ - $^{32}$ P]ATP (Perkin-Elmer) was used to label the primers P-hla for the *hla* gene and OCM43 for the RNIII/*hld* gene with T4 polynucleotide kinase (New England Biolabs, Inc.) according to the manufacturer's recommendations for primer extension analysis. cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations and then collected by precipitation with ethanol. Samples were run on a denaturing 6% urea-polyacrylamide gel according to the procedure suggested by the Sequa-Gel sequencing system kit (National Diagnostics). The dried gel was exposed to a Phosphor Screen (Kodak) and analyzed with a Storm 820 Scanner and software (Amersham Biosciences). Using ImageQuant TL (Amersham Biosciences), the appropriate bands were quantified by using the rolling-ball method and normalized to the total amount of RNA in the sample.

For RT-PCR analysis, gDNA contamination was removed from the RNA

samples by treatment with Turbo DNase (Ambion) according to the manufacturer's recommendations. Removal of DNA was confirmed by the absence of a product in a PCR using the treated RNA as a template. A 0.5- $\mu$ g portion of gDNA-free RNA was then subjected to cDNA synthesis using Superscript II reverse transcriptase (see above). Subsequent PCRs used *Taq* DNA polymerase (Invitrogen) and incubation in the PTC-100 programmable thermal controller (MJ Research, Inc.). A total of 1  $\mu$ l of the cDNA reaction was used for each sample when testing for *icaA* and *icaR* transcripts; 1  $\mu$ l of a 1:10 dilution of the cDNA reaction was used for each sample when testing for 16S rRNA, RNIII, and RNIII. To determine the linear range of the PCR for each cDNA sample, samples were removed after cycles 15, 20, 25, 30, and 35. Then, 10  $\mu$ l of the PCR product was run on a 0.8% agarose gel containing ethidium bromide for detection. After determination of the linear range of the PCR for each transcript, samples from each cDNA synthesis reaction were chosen for comparison within the same cycle number. The following primers were used for cDNA synthesis: OCD15 (16S rRNA), OCM50 (*icaA*), OCM51 (*icaR*), OCM43 (RNIII), and OCM59 (RNIII). The following additional primers were used for PCR amplification: OCD14 (16S rRNA), OCM49 (*icaA*), OCM52 (*icaR*), OCM57 (RNIII), and OCM58 (RNIII).

**PIA immunoblot assay.** PIA accumulation was determined as previously described (58). Briefly, TSB medium supplemented with 0.25% dextrose was inoculated with equal numbers of *S. aureus* bacteria from a 13-h preculture. The starting inoculum was determined by using OD (i.e.,  $OD_{600}$ ) to measure the amount of wild-type bacteria in a 1:200 dilution of the culture. The starting inoculum for non-wild-type strains was then normalized to the wild-type inoculum. Every 2 h, equal numbers of cells (equivalent to an absorbance of 0.5  $OD_{600}$  units) were harvested by centrifugation, and PIA was extracted in 0.5 M EDTA (pH 8.0) by boiling for 5 min. Samples of PIA-containing extracts were applied to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) and blocked with 5% skim milk overnight. The polyvinylidene difluoride membrane was incubated for 2 h with PIA-specific antiserum (kindly provided by Michael Otto) and then for 2 h with an anti-rabbit immunoglobulin G-peroxidase conjugate. The presence of PIA-antibody complexes was detected by using SuperSignal West Pico chemiluminescent substrate (Pierce). The integrated density values of bands on autoradiograms were determined with TotalLab software (Nonlinear Dynamics, Ltd.). Error bars reflect replicates from two separate experiments. The amount of PIA produced by a wild-type sample at 2 h in one experiment was used as a standard (100%) for relative PIA accumulation.

**Static in vitro biofilm assay.** The static biofilm assay was performed as previously described (2). Briefly, overnight cultures of *Staphylococcus* strains were grown in TSB. Samples (100  $\mu$ l) of 1:100 dilutions of the overnight cultures in fresh TSB supplemented with 0.5% glucose and 3% sodium chloride were used to inoculate wells of a sterile, polystyrene 96-well, flat-bottom microtiter plate (Corning, Inc.) and then incubated at 37°C for 24 h. Microtiter plates were not coated with human plasma. In each experiment, each strain was represented at least four times in the microtiter plate. *S. epidermidis* strain 1457 was included as a biofilm-producing positive control, and *S. carnosus* strain TM300 was included as a non-biofilm-producing control. After incubation, the medium and nonadherent bacteria were decanted, and the dishes were washed two times with PBS. Adherent bacteria were fixed to the microtiter plate with 100% ethanol for 20 min. The fluid was then removed by aspiration, and the plate was exposed to 100% crystal violet (CV) for 8 min. After the CV was decanted, the plate was washed two times with PBS and then incubated with 95% ethanol for 10 min. The  $OD_{600}$  of the CV released from adherent bacteria was determined. All samples were normalized to the negative control, *S. carnosus* TM300, and 100% biofilm formation was determined based on the values determined for *S. epidermidis* 1457.

## RESULTS

**Construction of the *codY* deletion mutants.** *codY*-null mutations were created in two clinical isolates, UAMS-1 and SA564, by allelic replacement of the *codY* gene with the *ermC* gene. The *ermC* gene was cloned in a temperature-sensitive plasmid and was flanked by 1-kb regions upstream and downstream of the *codY* gene. The replacement of *codY* with *ermC* and the loss of the plasmid backbone were verified by PCR and Southern blot analysis (data not shown). Western blot analysis showed that in the parental strains, UAMS-1 and SA564, *CodY* is produced at similar levels during exponential and

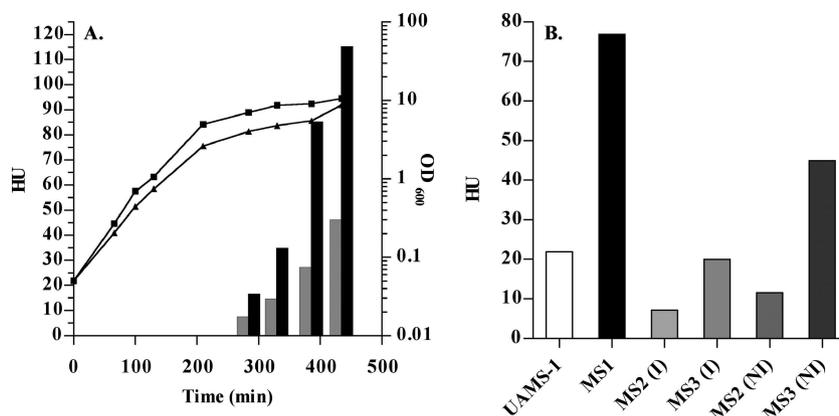


FIG. 1. Effect of a  $\Delta codY$  mutation on the hemolytic activity of the culture fluid of *S. aureus*. The hemolytic activity of the culture fluid of each strain was tested on rabbit erythrocytes as described in Materials and Methods. One HU is defined as 50% total erythrocyte lysis per 50  $\mu$ l of culture fluid. (A) The culture fluids of CDM7 (SA564  $\Delta codY::ermC$ ) (■) and SA564 (wild type) (□) were tested throughout growth for hemolytic activity. The growth of strains SA564 (■) and CDM7 (▲) were measured as the OD<sub>600</sub>. (B) The culture fluids of MS1 (UAMS-1  $\Delta codY::ermC$ ), UAMS-1 (wild type), MS2 (UAMS-1 containing pTL6936), and MS3 (MS1 containing pTL6936) were tested in the late stationary phase for hemolytic activity. Strains carrying the *codY*<sup>+</sup> complementation plasmid, pTL6936, were grown with (I) or without (NI) IPTG to induce *codY* expression.

stationary phases and confirmed the absence of CodY in the mutant strains (data not shown). In TSB medium, strains MS1 ( $\Delta codY::ermC$  derivative of UAMS-1) and CDM7 ( $\Delta codY::ermC$  derivative of SA564) showed a slight growth defect compared to UAMS-1 and SA564, respectively (Fig. 1A and data not shown). The *codY* mutants also had similar, yet smaller, colony morphology compared to the wild-type isolates (data not shown).

**CodY represses the production of hemolytic activity in culture supernatants.** To characterize the *codY* mutants, the culture fluids of UAMS-1 (*codY*<sup>+</sup>), MS1 ( $\Delta codY::ermC$ ), SA564 (*codY*<sup>+</sup>), and CDM7 ( $\Delta codY::ermC$ ) were assayed for hemolytic activity toward rabbit erythrocytes. Hemolytic activity in the culture fluid of SA564 was undetectable during exponential phase but increased to 46 HU in stationary-phase cultures (Fig. 1A). CDM7 showed increased levels of hemolytic activity compared to the isogenic wild-type strain, SA564. No hemolytic activity was detected in exponential-phase cultures of CDM7 but reached 115 HU in stationary phase (Fig. 1A). The culture fluids of UAMS-1 and its *codY* mutant, MS1, showed no hemolytic activity in exponential phase but increased to 22 and 77 HU in the stationary phase, respectively (Fig. 1B and data not shown). In summary, postexponential-phase and stationary-phase culture supernatants of CDM7 and MS1 had two-fold-higher hemolytic activities than did their parent strains.

The *codY* mutation was complemented in the UAMS-1 background with pTL6936, a plasmid containing the *codY* gene under the control of P<sub>SPAC</sub>, an IPTG-inducible promoter. Upon induction, strain MS3 (MS1 carrying pTL6936) produced CodY protein as determined by Western blot analysis (data not shown). The hemolytic activities of MS2 (UAMS-1 carrying pTL6936) and MS3 were tested under inducing (I) and noninducing (NI) conditions (Fig. 1B). The hemolytic activity of the stationary-phase culture fluid of MS3 (complemented *codY* mutant treated with IPTG) was fourfold less than that of the culture fluid of MS1 (uncomplemented *codY* mutant). Even without IPTG, the presence of the wild-type *codY*

gene on a plasmid caused a reduction in the hemolytic activity of the *codY* mutant (compare MS1 and MS3 [NI]). Thus, when CodY is supplied in *trans*, the phenotype of increased hemolytic activity in strain MS1 is suppressed. Similarly, when MS2 (*codY*<sup>+</sup> plus pTL6936) was treated with IPTG, the hemolytic activity of this strain decreased compared to UAMS-1 and MS2 (NI) (Fig. 1B), suggesting that increased levels of CodY protein result in repressed hemolytic activity even in a wild-type background.

**CodY represses alpha-toxin levels in the culture fluid.** To determine whether the increased hemolytic activity of the culture supernatant of the *codY* mutants was due to increased production of alpha-toxin, the culture fluid samples were subjected to Western blot analysis (Fig. 2). It has previously been reported that UAMS-1 has a nonsense mutation in the *hla* gene (6). Anti-alpha-toxin Western blot analysis of culture fluid from strains UAMS-1 and its *codY* mutant derivative MS1 in stationary phase confirmed that no alpha-toxin was made in these strains (Fig. 2, lanes 7 and 8), suggesting that the hemolytic activity of these strains is due to a different hemolysin. In contrast, Western blot analysis showed that SA564 and CDM7 produced alpha-toxin. This result correlates well with the observation that SA564 and CDM7 had higher HU values than did UAMS-1 and MS1. During exponential phase, there was no detectable alpha-toxin antigen in the culture fluids of SA564 or CDM7. In postexponential phase, CDM7 had much more alpha-toxin in the culture fluid than did SA564. In stationary-phase culture fluids, alpha-toxin was present at similarly high levels in SA564 and CDM7.

***codY* represses *hla* transcription.** To determine whether the increased hemolytic activity of the *codY* mutants was due to an increase in *hla* transcription, primer extension analysis was used to quantitate *hla* mRNA in the various strains (Fig. 3). In exponential-phase cells, CDM7 had 4.2-fold more *hla* transcript than did SA564. Similarly, MS1 was derepressed for *hla* transcription in the exponential phase, having 5.2-fold more transcript than did UAMS-1. In stationary-phase cells, the

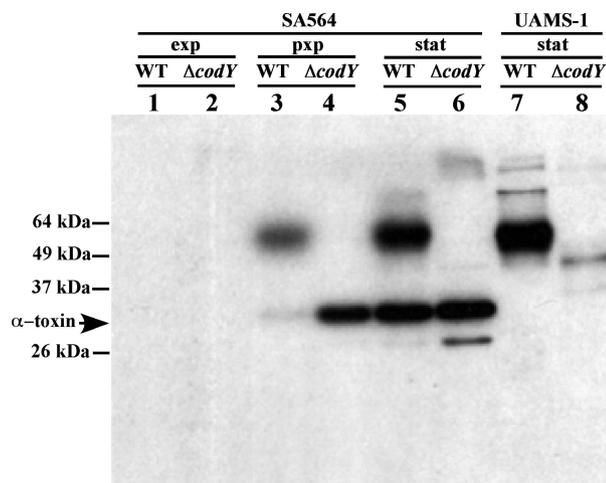


FIG. 2. Effect of growth phase on alpha-toxin production in the culture supernatants of SA564, CDM7, UAMS-1, and MS1. Proteins in equal volumes of culture fluids of wild-type and *codY* mutant strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, and probed with rabbit antibody to alpha-toxin. Lanes 1 to 6 contained culture fluids from SA564 (wild type [WT]) and CDM7 (SA564  $\Delta codY::ermC$ ); lanes 7 to 8 contained culture fluids from UAMS-1 (wild type) and MS1 (UAMS-1  $\Delta codY::ermC$ ). Culture fluids in lanes 1 and 2 were from exponentially growing cells (exp), culture fluids in lanes 3 and 4 were from postexponentially growing cells (pxp), and culture fluids in lanes 5 through 8 were from stationary-phase cells (stat). The bands appearing above 50 kDa are of unknown origin but appear to be unrelated to alpha-toxin.

*codY* mutants continued to have more *hla* transcript than did the WT samples. CDM7 had 2.5-fold more *hla* transcript than did SA564, and MS1 had 1.8-fold more *hla* transcript than did UAMS-1. We note that strains UAMS-1 and MS1 expressed an *hla* transcript even though the *hla* gene contains a nonsense mutation (6) and produces no alpha-toxin protein.

Although the *codY* mutants showed significant derepression of *hla* transcription in the exponential phase, *hla* transcription in the *codY* mutants still showed growth-phase-dependent regulation. The parental strains UAMS-1 and SA564 showed 18-

and 55-fold stationary-phase inductions of *hla* transcription, respectively, whereas MS1 and CDM7 showed 6.2- and 32-fold stationary-phase inductions of *hla* transcription, respectively. These data suggest that, even in the *codY* mutants, the *hla* promoter is subject to CodY-independent growth-phase-dependent regulation.

**CodY represses synthesis of RNAII and RNAIII.** RNAIII positively regulates *hla* transcription (13) and translation (36, 38) and also encodes a hemolysin, delta-toxin (5, 23). RNAIII transcription peaks in postexponential and early stationary phase (57). Because RNAIII is expressed in a growth-phase-dependent manner, contributes to alpha-toxin activity, and also encodes delta-toxin, we speculated that synthesis of RNAIII might be CodY-regulated. Transcript analysis using primer extension revealed that the level of RNAIII in exponential-phase cells was 40-fold higher in MS1 than in its parent UAMS-1 (Fig. 4A). In stationary-phase cells, RNAIII transcript was greatly increased in both MS1 and UAMS-1, showing a <2-fold difference in expression (Fig. 4A).

RT-PCR analysis was performed on the wild-type and *codY* mutant strains of SA564 and UAMS-1 to determine RNAII transcript levels and to further characterize RNAIII transcription. RT-PCR analysis showed that RNAIII transcript levels were derepressed in exponential-phase cells of CDM7 compared to its parent SA564 (Fig. 4B). Similarly, RT-PCR confirmed the primer extension analysis results, which showed that RNAIII was derepressed in exponential-phase cells of MS1 compared to UAMS-1 (Fig. 4B). In addition, in exponential-phase cells, RNAII transcript levels were derepressed in the *codY* mutants, MS1 and CDM7 compared to the parental strains, UAMS-1 and SA564, respectively (Fig. 4B). In summary, transcript analysis of the *agr* locus showed that in exponential phase, P2 (RNAII) and P3 (RNAIII) were both derepressed in the *codY* mutants of the clinical isolates UAMS-1 and SA564.

**CodY represses biofilm formation in a static biofilm assay.** Biofilm formation on implanted medical devices and mammalian tissues is a significant contributor to the pathogenicity of *S. aureus* (12). Although there are many environmental and bacterial factors that influence biofilm attachment and accumula-

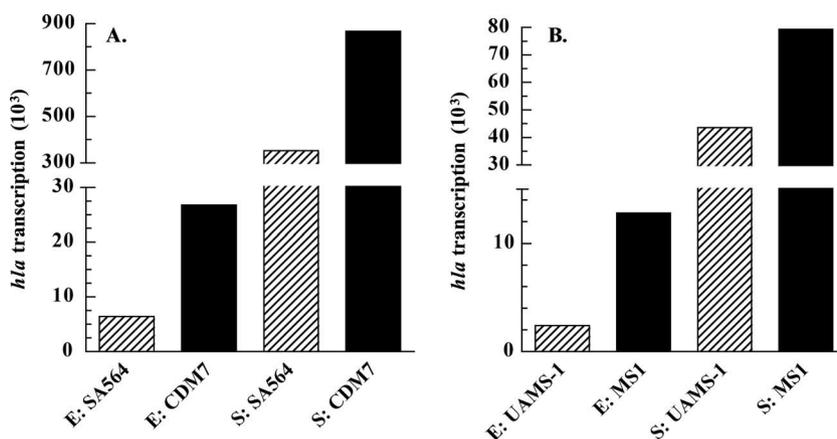


FIG. 3. Effect of a  $\Delta codY$  mutation on *hla* transcription. *hla* transcription values represent quantified reverse transcripts normalized to total 16S rRNA from primer extension analysis of the *hla* promoter. RNA was extracted from cells harvested in the exponential (E) or stationary (S) phases of growth. (A) SA564 (wild type) and CDM7 (SA564  $\Delta codY::ermC$ ); (B) UAMS-1 (wild type) and MS1 (UAMS-1  $\Delta codY::ermC$ ).

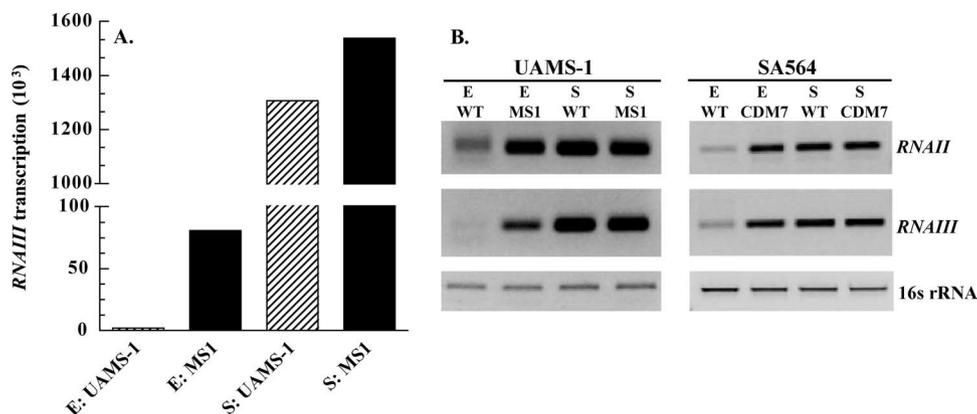


FIG. 4. Effect of a  $\Delta codY$  mutation on transcription of the *agr* locus. Transcript analysis was done on RNA extracted from exponential-phase (E) or stationary-phase (S) cells. (A) RNAPIII transcription values represent quantified reverse transcripts normalized to total 16S rRNA from primer extension analysis of the RNAPIII promoter of the strains UAMS-1 (wild type [WT]) and MS1 (UAMS-1  $\Delta codY::ermC$ ). (B) RT-PCR analysis of RNAII, RNAIII, and 16S rRNA from strains UAMS-1, MS1, SA564 (wild type), and CDM7 (SA564  $\Delta codY::ermC$ ) harvested during the exponential phase (E) or the stationary phase (S).

tion, the PIA, also known as polymeric *N*-acetyl-glucosamine, is the best understood contributor to *S. aureus* biofilm formation (30, 32, 40). The enzymes responsible for PIA production are encoded by the genes of the *ica* operon; an *ica* mutant of *S. aureus* is deficient in PIA production and biofilm formation (9). Divergent from the promoter of the *icaADBC* operon is a promoter driving transcription of the *icaR* gene. IcaR is a potent repressor of *icaADBC* transcription, limiting the production of PIA (24). Other factors, such as SarA (2), TcaR (24), Spx (41), and general stress responses (40) also regulate biofilm formation.

Having observed that the *codY* mutants were much more adherent than their parents when cultured in glass tubes and flasks, we tested the *codY* mutants and wild-type strains in a static assay that is used as a surrogate for tests of biofilm formation (2). Cultures in TSB supplemented with 0.5% glucose and 3% NaCl were used to inoculate wells of polystyrene plates, which were then incubated for 24 h at 37°C. After several washing steps, adherent bacteria were detected by using CV. By this assay, the *codY* mutant MS1 produced 7.5-fold more biofilm than did its parent UAMS-1 (Fig. 5). SA564 produced no detectable biofilm, but the *codY* mutant of this isolate, CDM7, showed high biofilm formation (Fig. 5). The data in Fig. 5 represent four independent experiments for UAMS-1 and MS1 and three independent experiments for SA564 and CDM7.

#### CodY represses PIA production during exponential growth.

PIA is a major component of *S. aureus* biofilm formation. Cultures of UAMS-1 and MS1 were tested for PIA production by using a PIA immunoblot assay. MS1 showed derepressed PIA synthesis that peaked at 560% relative PIA accumulation in late exponential phase (time [*t*] = 4 h) (Fig. 6). At this time, the level of PIA accumulation for MS1 was eightfold higher than for UAMS-1. In postexponential phase (*t* = 6 h), PIA accumulation for MS1 was ~20-fold higher than for UAMS-1. After *t* = 6 h, the extent of derepression in MS1 was reduced; at *t* = 12 h, PIA production in both the MS1 and the UAMS-1 strains had decreased to ca. 100% relative PIA accumulation (Fig. 6). In summary, PIA production was derepressed in late

exponential to early stationary phase in the *codY* mutant compared to the isogenic wild-type strain.

**codY represses *icaA* transcription.** To know whether CodY regulates PIA production at the transcriptional level, we analyzed the transcript levels of *icaA*, the first gene in the *icaADBC* operon. RT-PCR analysis was performed on RNA isolated from samples grown to exponential or stationary phases. In both strain backgrounds, a *codY* mutation resulted in strong derepression of *icaA* transcription in exponential-phase cells (Fig. 7). In stationary-phase cells, little to no *icaA* transcript could be detected in the wild-type or *codY* mutant strains. The *codY* mutant strains showed no difference in *icaR* transcript levels compared to the wild-type isolates in either the exponential or the stationary phases (Fig. 7). These data indicate that the exponential-phase derepression of *icaA* in the *codY* mutants is independent of the levels of *icaR* transcript

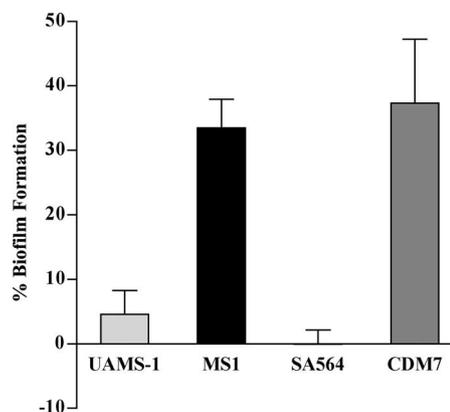


FIG. 5. In vitro static biofilm formation. All strains were grown in TSB medium supplemented with glucose and sodium chloride. The percent biofilm formation was determined as described in Materials and Methods after 24 h of growth of the strains UAMS-1 (wild type), MS1 (UAMS-1  $\Delta codY::ermC$ ), SA564 (wild type), and CDM7 (SA564  $\Delta codY::ermC$ ). The non-biofilm-producing strain *S. carnosus* TM300 was used as a negative control. One-hundred percent biofilm production was defined as that produced by *S. epidermidis* strain 1457.

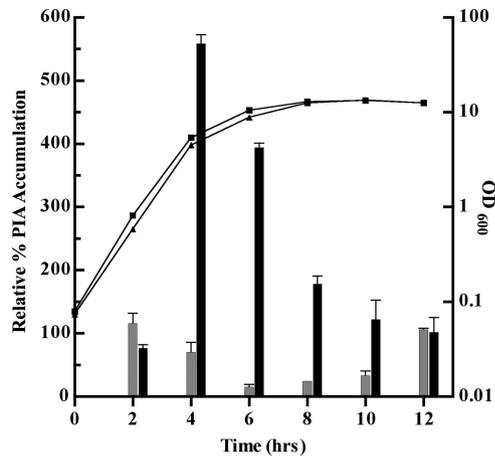


FIG. 6. Relative PIA accumulation. Samples were taken throughout growth and prepared for PIA release. A PIA immunoblot assay was performed on samples prepared from equal cell numbers, and the results were quantified. PIA accumulation of a wild-type sample at  $t = 2$  h was set as a 100% standard for relative PIA accumulation. UAMS-1 (wild type) (□) and MS1 (UAMS-1  $\Delta codY::ermC$ ) (●).

and also that a CodY-independent regulator is responsible for the stationary-phase repression of *icaA* transcription and PIA production.

## DISCUSSION

The present study shows that introduction of a *codY* mutation in two clinical isolates of *S. aureus*, UAMS-1 and SA564, resulted in derepressed synthesis of *hla* mRNA and its product, alpha-toxin, derepressed transcription of the accessory gene regulator (*agr*) locus, and derepressed PIA-dependent biofilm formation. Although transcript analysis of the genes encoding alpha-toxin (*hla*) and delta-toxin (RNAIII/*hld*) showed that their transcription was most significantly derepressed during exponential-phase growth, increased hemolytic activity and alpha-toxin production could only be detected in the culture fluids of postexponential-phase cells. Because both RNAIII and *hla* transcripts are derepressed in the *codY* mutant strains and RNAIII is an activator of *hla* translation (36, 38), it is unclear why the appearance of hemolytic activity and alpha-toxin production is delayed. It is possible that the hemolytic assay and Western blot analysis are not sensitive enough to detect differences in low levels of hemolysin(s) present in culture fluids from low numbers of bacteria. Since UAMS-1 does not produce alpha-toxin, in keeping with a nonsense mutation in the UAMS-1 *hla* gene (6), the hemolytic activity of the culture fluid of MS1 is likely due to the derepression of another hemolysin, such as delta-toxin. The culture fluids of strains SA564 and CDM7 did contain alpha-toxin. Thus, the high HU values seen in the culture fluid of CDM7 are likely due to derepression of both alpha-toxin and delta-toxin. In addition, although the *codY* mutants showed significant exponential-phase derepression of *hla* and RNAIII/*hld* transcription, both genes were still subject to growth-phase-dependent regulation. This is not surprising since these loci are known to be regulated by a number of other factors, such as numerous

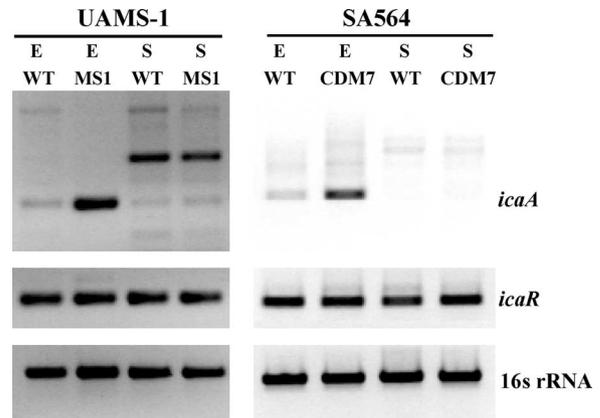


FIG. 7. Effect of a  $\Delta codY$  mutation on transcription of the *ica* locus. Transcripts corresponding to *icaA*, *icaR*, and 16S rRNA were analyzed by RT-PCR in samples extracted from exponential-phase (E) or stationary-phase (S) cells of strains UAMS-1 (wild type), MS1 (UAMS-1  $\Delta codY::ermC$ ), SA564 (wild type), and CDM7 (SA564  $\Delta codY::ermC$ ). The upper band that occurs in the RT-PCR analysis of *icaA* is likely to reflect nonspecific RT products. WT, wild type.

TCS (5, 17), SarA (8, 13), SarS (54), and other members of the SarA family (5).

Since our studies show that RNAII transcription is also altered in the *codY* mutants, the CodY-mediated regulation of RNAIII/*hld* transcription may be an indirect effect of regulation of RNAII transcription. In addition, our present results are consistent with either direct or indirect repression by CodY of *hla* transcription. Ongoing studies aim to understand how CodY participates in the regulatory network of *hla* and RNAIII/*hld* transcription, specifically by identifying the direct CodY targets. Since CodY has been shown to control many aspects of metabolism important for adaptation to nutritional and metabolic changes in other bacteria, such as *B. subtilis* (35) and *L. lactis* (21), we assume that CodY will prove to play a central role in these processes in *S. aureus* as well. Thus, the effects of *codY* mutations on virulence gene expression may reflect direct interaction of CodY with virulence gene promoters or indirect effects of alterations of metabolic activities.

CodY acts as a repressor of PIA-dependent biofilm formation. In looking at quantitative measures of biofilm formation, such as PIA production and *icaA* transcription, it is clear that the CodY-mediated control of biofilm formation in UAMS-1 and SA564 is specific to the growth phase and is likely to occur through transcriptional regulation of the *icaADBC* operon. In the postexponential phase of in vitro growth, PIA production is derepressed in the *codY* mutant of UAMS-1. Similarly, in both clinical isolates, a *codY* mutation results in derepression of *icaA* transcription during exponential phase. Interestingly, in stationary phase, the CodY-mediated derepression is abolished, as *icaA* transcript levels and PIA production in the *codY* mutant drop to the very low levels seen for wild-type cells. In the *codY* mutants, this transient, yet potent, derepression of the *icaADBC* operon during exponential phase likely leads to a substantial increase in biofilm formation after 24 h of growth. Thus, CodY is certainly not the sole regulator of this locus. IcaR, TcaR (24), SarA (2, 56), and likely numerous other

factors (40) contribute to the complex regulation of the *icaADBC* operon.

IcaR is a transcriptional repressor that binds to the promoter region of *icaADBC* (24) and represses this operon in both the exponential phase (41) and the stationary phase (24). Transcriptional analysis of *icaR* in the *codY* mutants of SA564 and UAMS-1 showed that even though *icaA* was highly derepressed in the exponential phase, there was no difference in *icaR* transcript levels between the *codY* mutants and the wild-type strains. This suggests that CodY-mediated regulation of the *icaADBC* locus is independent of IcaR and that CodY-mediated exponential-phase repression of *icaA* transcription supersedes IcaR-mediated repression.

Tu Quoc et al. recently reported that a *codY* mutation in the clinical isolate S30 results in reduced biofilm formation and PIA production (55). These data are in contrast to the effect of a *codY* mutation in UAMS-1 and SA564. Other than citing strain-to-strain variation among *S. aureus* isolates, it is difficult to explain the different results obtained by the two groups. One possibility is that CodY's regulatory role in biofilm formation is multifactorial. CodY may act, directly or indirectly, to repress *icaADBC* transcription in isolates of *S. aureus* such as SA564 and UAMS-1. However, in high-biofilm-producing isolates, such as S30 (55), the regulatory network of PIA-dependent biofilm formation may be different than in SA564 and UAMS-1. If this is the case, the effect of CodY on biofilm formation in a high-biofilm-producing strain may be an indirect reflection of its role in regulating various aspects of intermediary metabolism. As we learn more about the mechanism of CodY-mediated regulation of the *icaADBC* locus, we hope to be able to explain the different phenotypes resulting from *codY* mutations in S30, UAMS-1, and SA564.

In other gram-positive bacteria, CodY is a regulatory protein that responds to the nutrient status of the cell by sensing the intracellular pools of GTP and/or the BCAAs. Interaction with these effector molecules activates CodY as a DNA-binding protein. In this manner, CodY is an important regulator of stationary phase adaptation that is activated and inactivated by environmental cues. Amino acid sequence similarity with the *B. subtilis* protein and in vitro DNA-binding studies indicate that the affinity of *S. aureus* CodY for its binding sites is enhanced by the BCAAs and GTP (C. Majerczyk and A. L. Sonenshein, unpublished results), raising the possibility that CodY serves in *S. aureus* to link environmental nutrient availability with regulation of pathogenesis. Moreover, since transcription of RNAIII is derepressed in the *codY* mutants, *S. aureus* appears to regulate this characterized virulence gene regulator through both quorum-sensing and nutrient-sensing mechanisms.

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