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CodY in *Staphylococcus aureus*: a Regulatory Link between Metabolism and Virulence Gene Expression▽†

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The repressor CodY is reported to inhibit metabolic genes mainly involved in nitrogen metabolism. We analyzed *codY* mutants from three unrelated *Staphylococcus aureus* strains (Newman, UAMS-1, and RN1HG). The mutants grew more slowly than their parent strains in a chemically defined medium. However, only *codY* mutants were able to grow in medium lacking threonine. An excess of isoleucine resulted in growth inhibition in the wild type but not in the *codY* mutants, indicating that isoleucine plays a role in CodY-dependent repression. Prototypic CodY-repressed genes including the virulence regulator *agr* are repressed after up-shift with isoleucine. The CodY-dependent repression of *agr* is consistent with the concomitant influence of CodY on typical *agr*-regulated genes such as *cap*, *spa*, *fnbA*, and *coa*. However, some of these virulence genes (e.g., *cap*, *fnbA*, and *spa*) were also regulated by CodY in an *agr*-negative background. Microarray analysis revealed that the large majority of CodY-repressed genes were involved in amino acid metabolism; CodY-activated genes were mainly involved in nucleotide metabolism or virulence. In summary, CodY in *S. aureus* not only acts as a repressor for genes involved in nitrogen metabolism but also contributes to virulence gene regulation by supporting as well as substituting for *agr* function.

Staphylococcus aureus asymptomatically colonizes the nares of healthy individuals but also causes a variety of infections in humans. Regulatory loci are necessary for the adaptation of the organism to the different nutrient limitations and stress conditions encountered in vivo. This allows the pathogen to survive and/or multiply in different compartments during colonization and infection processes. However, knowledge of the environmental conditions encountered in vivo is still incomplete, and the interaction of regulatory circuits leading to metabolic adaptation and differential expression of virulence factors remains poorly understood. The in vitro expression of most virulence factors is tightly related to the growth phase. For instance protein A (encoded by *spa*), fibronectin-binding proteins (encoded by *fnbA* and *fnbB*), and coagulase (encoded by *coa*) are expressed during the exponential growth phase, whereas most secreted proteins (e.g., hemolysins, enterotoxins, and proteases) and the capsule (enzymes encoded by the *capA-capP* operon) are expressed mainly during the postexponential phase (22, 37). In many bacteria, the transition to postexponential growth is accompanied by a profound reprogramming of gene expression. Several underlying mechanisms are thought to be involved in such a transition. Quorum sensing allows the bacteria to detect their own density. Basically, bacteria secrete small diffusible molecules (autoinducers) which are also effectors of their own synthesis. Upon passing a critical concentration threshold, the autoinducers activate specific transcriptional regulators, leading to the differential expression of target genes. The *agr* locus of *S. aureus* is a prototypic quorum-sensing system mainly involved in the regulation of virulence genes (37). At high cell densities, the regulatory RNAIII is expressed, leading to the inhibition of *spa*, for instance, and to the activation of genes encoding secreted virulence factors and the capsular polysaccharide. Besides quorum sensing, additional mechanisms have to be triggered for the growth phase transition in *S. aureus* since the expression of certain virulence factors remains growth phase dependent in *agr* mutants (42, 51, 53).

In *Bacillus subtilis*, the CodY repressor has been described as a central regulator important for the transition to stationary phase and sporulation (40, 46). Homologs of *codY* could be identified in the genome of most gram-positive species, including pathogenic staphylococci and streptococci (23, 46). In *B. subtilis*, GTP reaches its highest concentration during the exponential phase, when it binds to CodY and leads to the repression of late gene expression. This mechanism is linked to the stringent response since the synthesis of (p)ppGpp by Rel leads to a lowering of the GTP pool (28). The central role of GTP in gene regulation was further proven by the use of decoyinine, an inhibitor of GMP synthetase (40). Depletion of the GTP pool by this inhibitor leads to the activation of typical late genes already in the exponential phase. CodY represses genes which are primarily involved in nitrogen metabolism (proteases, oligopeptide transporters, and genes for amino acid synthesis) and activates transcription of genes of the carbon overflow pathway (46, 47). Interestingly, in *Lactococcus lactis* CodY does not bind GTP. Here, branched-chain amino acids (BCAAs) are bound by CodY and constitute the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
TOP10	Competent *E. coli* for plasmid transformation	Invitrogen, Karlsruhe. Germany
CYL316	RN4220 harboring pYL112Δ19 and L54 *int* gene; r^-	31
RN6112	RN6390 *agr*A::Tn*551*	Richard Novick
RN4220	Restriction-deficient *S. aureus* strain	29
RN4220-21	RN4220 Δ*codY*::*tet*(M)	This work
Newman	Wild type	15
Newman-21	Newman Δ*codY*::*tet*(M)	This work
Newman-agr	Newman *agrA*::Tn*551*	This work
Newman-21/-agr	Newman *agrA*::Tn*551* Δ*codY*::*tet*(M)	This work
UAMS-1	Osteomyelitis isolate	19
UAMS-1-21	UAMS-1 Δ*codY*::*tet*(M)	This work
UAMS-1-agr	*agrA*::Tn*551*	17
UAMS-1-21/-agr	UAMS-1 *agrA*::Tn*551* Δ*codY*::*tet*(M)	This work
RN1HG	*rsbU* restored RN1	This work
RN1	8325	NARSA strain collection[a]
RN1HG-21	RN1HG Δ*codY*::*tet*(M)	This work
Plasmids		
pALC2073	*E. coli-S. aureus* shuttle vector with tetracycline-inducible promoter	3
pMAD	Shuttle vector for gene replacement mutagenesis	1
pCG29	pMAD with cloned *clpY-tet*(M)-*rpsB* fragment for *codY* mutagenesis	This work
pCG30	pALC2073 with *codY* integration via EcoRI restriction site	This work

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

primary signals for *codY* repression (23, 39). The difference between the two species is probably due to sequence variations in the proposed GTP binding domain of CodY (39). It has been shown that CodY of *B. subtilis* can also use BCAAs in addition to GTP as signaling molecules (39, 45). A conserved CodY-binding site (AATTTTCWGAAATT) was first described for *L. lactis* (12, 24) and later confirmed in *B. subtilis* (5).

The metabolic regulatory cascades for gram-positive pathogens are only partly understood, and knowledge about nitrogen metabolism and amino acid availability during infection remains limited. However, there is growing evidence that CodY is an important regulatory link between metabolism and virulence gene expression in pathogenic bacteria (6, 14, 26, 32, 35, 46). The complete set of genes necessary for amino acid synthesis has been predicted to be present in the *S. aureus* N315 genome (25). Nevertheless, *S. aureus* usually requires a complex mixture of amino acids for growth, probably because of repression of the corresponding pathways (25). It can be assumed that during infection the bacterium recruits at least some of the amino acids from the host. Interestingly, in a whole-genome screen, genes coding for oligopeptide transporters were shown to be essential for infection or to be specifically activated during infection (10, 36). *S. aureus* is also equipped with several genes coding for proteases, whose activity could provide amino acids or peptides during infection. The analysis of other gram-positive organisms together with our own preliminary data led to the hypothesis that in *S. aureus* CodY may play a central role not only in metabolic adaptation but also in virulence gene expression. Indeed, it was recently shown that a *codY* mutant shows enhanced expression of the *agr* effector molecule RNAIII (34). Additionally, *codY* influences biofilm formation, albeit with opposite effects depending on the strain analyzed (16, 34). Our results obtained at the transcriptome level clearly demonstrate that CodY is involved in the regulation of metabolic genes and also influences gene expression of virulence genes in an *agr*-dependent and -independent manner.

MATERIALS AND METHODS

Strains and growth conditions. Strains and plasmids are listed in Table 1. *S. aureus* strains were grown in tryptic soy broth, CYPG (10 g/liter Casamino Acids, 10g/liter yeast extract, 5 g/liter NaCl, 20% glucose, and 1.5 M phosphoglycerate), or in a chemically defined medium (CDM). For strains carrying resistance genes, antibiotics were used only in precultures at the following concentrations: kanamycin, 50 μg/ml; erythromycin, 10 μg/ml; and tetracycline, 5 μg/ml. CDM was composed as follows (final concentration in mg/liter in brackets): group 1 amino acids from a 10× stock consisting of L-tryptophan (100), L-tyrosine (100), and L-phenylalanine (100); group 2 amino acids from a 10× stock consisting of L-cysteine (50), L-histidine (100), and L-methionine (100); group 3 amino acids from a 100× stock consisting of L-glutamine (200), L-glutamic acid (100), glycine (100), and L-proline (100); group 4 amino acids from a 100× stock consisting of L-isoleucine (100), L-leucine (100), L-threonine (200), and L-valine (100); group 5 amino acids from a 100× stock consisting of DL-alanine (100), L-arginine (100), L-aspartic acid (100), L-lysine (100), hydroxy-L-proline (100), and L-serine (100); group 6 vitamins from a 50× stock consisting of *p*-aminobenzoic acid (0.2), biotin (0.2), folic acid (0.8), niacinamide (1), β-NAD (2.5), pantothenate calcium salt (2), pyridoxal (1), pyridoxamine dihydrochloride (1), riboflavin (2), thiamine hydrochloride (1), and vitamin B_{12} (0.1); group 7 nucleotides from a 100× stock (predissolved in 2 N HCl) consisting of adenine (20), guanine hydrochloride (20), and uracil (20); group 8 and 9 salts from a 50× stock consisting of K_2HPO_4 (200) and KH_2PO_4 (1,000); group 9 consisting of NaH_2PO_4 (3,195), $MgSO_4$ (700), and $CaCl_2$ (10); group 10 consisting of a 100× stock of Na_2HPO_4 (9,214); and group 11 carbohydrate from a 20× stock of glucose (10,000). Single amino acids were omitted in some experiments, as indicated in the figure legends. The pH of the medium was buffered to 7.0.

Bacteria from an overnight culture were diluted to an initial optical density at 600 nm (OD_{600}) of 0.05 in fresh medium without antibiotics and grown with shaking (222 rpm) at 37°C to the indicated OD_{600}. In the complemented strains, *codY* was induced with anhydrotetracycline (IBA GmbH, Göttingen) (0.05 μg/ml). For up-shift experiments, strains were grown in CDM without isoleucine to an OD_{600} of 0.4. The cultures were divided into aliquots and supplemented with isoleucine and grown for 1 h.

Construction of mutant strains and complementation. The *codY* locus was replaced by a tetracycline resistance cassette [*tet*(M)]. Briefly, two fragments flanking *codY* and the *tet*(M) gene were amplified and annealed by overlapping PCR using oligonucleotides (see Table S2 in the supplemental material). The

amplicon was cloned into pMAD using the BamHI/BglII restriction sites of pMAD to gain pCG29. Mutagenesis of strain RN4220 was performed as described previously (1). The obtained *codY* gene replacement mutant strain (RN4220-21) was verified by PCR (for oligonucleotides, see Table S1 in the supplemental material). *codY* and *agr* mutants of different *S. aureus* strains were obtained by transduction using ϕ11 lysates of strains RN4220-21 and RN6112, respectively. Transductants were verified by PCR and pulsed-field gel electrophoresis. All *agr* mutants were negative for δ-hemolysin and RNAIII expression. For complementation, *codY* was amplified with oligonucleotides (see Table S1 in the supplemental material) containing EcoRI sites and cloned in the EcoRI site of the tetracycline-inducible vector pALC2073, yielding plasmid pCG30. The plasmid was used to transform strain RN4220, from which it was transduced, into the *codY* mutant strains. Strain RN1HG is an *rsbU*-restored derivative of *S. aureus* strain RN1 (8325) obtained by site-directed mutagenesis using pMAD *rsbU* as described previously (50).

RNA isolation, Northern blot hybridization, and real-time reverse transcription-PCR. RNA isolation and Northern blot analysis were performed as described previously (20). Briefly, bacteria were lysed in 1 ml of Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (0.1 mm-diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of Trizol. Digoxigenin-labeled probes for the detection of specific transcripts were generated using a DIG-Labeling PCR Kit following the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). Oligonucleotides used for probe generation are as described previously (48) or are listed in Table S1 in the supplemental material.

Microarray manufacturing and microarray design. The microarray was manufactured by in situ synthesis of 60-base-long oligonucleotide probes (Agilent, Palo Alto, CA), selected as previously described (8). The array covers >98% of all open reading frames (ORFs) annotated in strains N315 and Mu50 (30), MW2 (2), COL (18), NCTC8325 and USA300 (13), and MRSA252 and MSSA476 (27), including their respective plasmids.

Preparation of labeled nucleic acids for expression microarrays. Total RNA was purified from bacteria grown in CDM to an OD_{600} of 0.5. For each strain RNA of three independently grown cultures was analyzed. After additional DNase treatment, the absence of remaining DNA traces was confirmed by quantitative PCR (SDS 7700; Applied Biosystems, Framingham, MA) with assays specific for 16S rRNA (41, 43). Batches of 5 μg of total *S. aureus* RNA were labeled by Cy3-dCTP using SuperScript II (Invitrogen, Basel, Switzerland) following the manufacturer's instructions. Labeled products were then purified onto QiaQuick columns (Qiagen).

Purified genomic DNA from the different sequenced strains used for the design of the microarray was extracted (DNeasy; Qiagen), labeled with Cy5 dCTP using the Klenow fragment of DNA polymerase I (BioPrime, Invitrogen, Carlsbad, CA) (8), and used for the normalization process (49). Cy5-labeled DNA (500 ng) and a Cy3-labeled cDNA mixture were diluted in 50 μl of Agilent hybridization buffer and hybridized at a temperature of 60°C for 17 h in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA). Slides were washed, dried under nitrogen flow, and scanned (Agilent, Palo Alto, CA) using 100% photon multiplier tube power for both wavelengths.

Microarray analysis. Fluorescence intensities were extracted using Feature Extraction software (version 8; Agilent). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of the labeled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of two independent biological experiments were expressed as log 10 ratios and analyzed using GeneSpring, version 8.0 (Silicon Genetics, Redwood City, CA). A filter was applied to select oligonucleotides mapping ORFs in the Newman genome, yielding approximately 92% coverage. Statistical significance of differentially expressed genes was calculated by analysis of variance (9, 43) using GeneSpring, including the Benjamini and Hochberg false discovery rate correction of 5% (*P* value cutoff, 0.05) and an arbitrary cutoff of twofold for expression ratios.

Microarray data accession number. The complete microarray data set has been posted on the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE12340 for the platform design and GPL7137 for the original data set.

RESULTS

Molecular organization of the *codY* operon. To characterize the CodY regulon, *S. aureus* gene replacement mutants were constructed in three clonally distinct *S. aureus* strains (Newman, RN1HG, and UAMS-1). In the mutant strains, *codY* was replaced by a *tet*(M) resistance cassette, as verified by Southern hybridization and PCR. To evaluate whether the mutation exerted a polar effect on the surrounding genes, Northern analysis was performed using probes specific for *codY* as well as for the neighboring genes (Fig. 1A). *codY* is part of a polycistronic operon encompassing *xerC*, *clpQ* (*hslV*), *clpY* (*hslU*), and *codY* (4.1 kb) (Fig. 1B). An additional 1.4-kb transcript codes for *codY* only. In the mutant, *codY* was replaced by *tet*(M), leading to termination of the transcript (*xerC* and *clpQY*) in front of *tet*(M). The expression levels of *xerC* and *clpQY* in the mutant were similar to those in the wild-type strains. However, there was a slight, but reproducible increase in the *xer* and *clpQY* transcript levels in the *codY* mutant. Microarray analysis also suggests that *xerC* is slightly affected by the mutation although the difference was not significant. *rpsB* located downstream of the *codY* operon was not affected in the mutant. Overall, the *codY* mutation showed minimal polar effects on the surrounding genes. Thus, for complementation analysis only, the *codY* ORF was cloned into an inducible vector (pCG30).

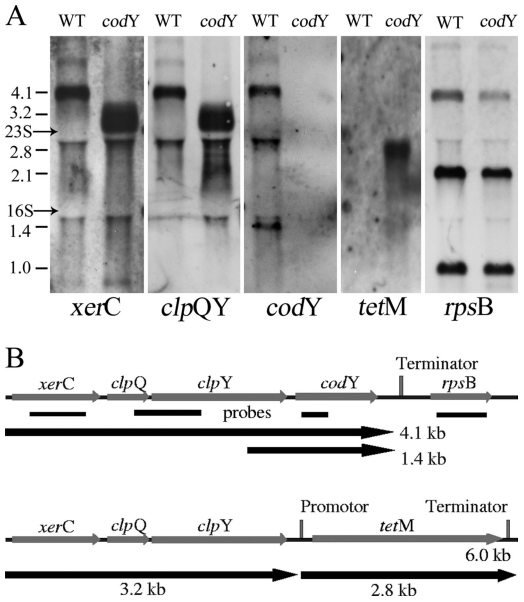

FIG. 1. Genetic organization of the *codY* locus. (A) Total RNA from strain Newman and its *codY* mutant Newman-21 was hybridized with probes specific for *xerC*, *clpQY*, *codY*, *tet*(*M*), and *rpsB*. Transcript size was estimated by comparison with an RNA marker. Note that slight hybridization bands below the ribosomal rRNA are probably due to comigration of the mRNA with rRNA and were not taken as distinct mRNA species. (B) Scheme of the genetic organization of the *codY* operon. Probes used for Northern blot analysis are indicated with black lines. Putative transcriptional units are indicated by black arrows in the wild type (upper part) and the *codY* mutant (lower part). WT, wild type.

Effect of *codY* on selected genes with putative CodY binding motifs. For other organisms, it has been shown that CodY functions as a repressor via binding to a proposed CodY-binding motif (AATTTTCWGAAAATT) (5, 12, 24). We performed a stringent search for the occurrence of the proposed motif (two mismatches accepted) within 1,000 bp upstream of

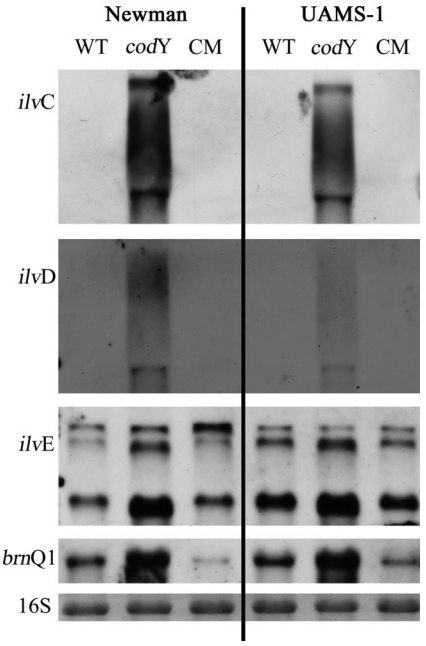

FIG. 2. Detection of *codY*-regulated operons: *ilvDBC-leuABC-ilvA* (CodY box in front of *ilvD*, detected with *ilvD* and *ilvC*), *ilvE*, and *brnQ1* (MWMN_0180, BCAA transport system II carrier protein). RNA was isolated from strains Newman and UAMS-1, their *codY* mutants (Newman-21 and UAMS-1-21), and the complemented strains ([CM] Newman-21/pCG30 and UAMS-1-21/pCG30). Bacteria were grown to an OD_{600} of 0.5 in CDM. The 16S rRNAs detected in the ethidium bromide-stained gels are indicated as loading controls in the lower lane. WT, wild type.

putative ORFs in three *S. aureus* genomes (Col, N315, and Newman) (http://xbase.bham.ac.uk/pattern.pl?id=1327). A total of 68, 76, and 71 genes preceded by a putative CodY box were identified in strains Col, N315, and Newman, respectively. The CodY boxes were mainly localized 50 to 300 bp upstream of the translational start site (see Fig. S1 in the supplemental material). Many of the identified genes code for enzymes involved in amino acid metabolism or transport. We selected four genes containing CodY boxes for Northern blot analysis: *ilvC* and *ilvD* (part of the *ilvDBC-leuABC-ilvA* operon, with the CodY box in front of *ilvD*), *ilvE*, and *brnQ1* (MWMN_0180, branched-chain amino acid transport system II carrier protein). It could be shown that all four genes were strongly upregulated in the *codY* mutants of strains Newman and UAMS-1 in comparison to the wild-type or *codY*-complemented mutant strains (Fig. 2). These results suggest that CodY in *S. aureus* binds to the same conserved motif as described for *B. subtilis* and *L. lactis*.

Effect of *codY* on growth in CDM. In a first attempt to characterize the *codY* phenotype, we performed growth analysis in CDM containing all three BCAAs (100 mg/liter each) and threonine (200 mg/liter). In this medium *S. aureus* strains did not grow without glucose, suggesting that amino acids were not being used as the primary carbon source. This is further supported by the lowering of the pH after prolonged growth, which also is indicative of glucose consumption. No growth of wild-type or mutant strains occurred if any of the five groups of amino acids was omitted from the medium. Growth analysis revealed that *codY* mutants grew more slowly in complete CDM than the wild-type strains (Fig. 3A and B). The *codY* mutants were also delayed in growth in complex medium such as CYPG (data not shown). Thus, one may assume that gene repression by CodY might be favorable for the organism under conditions of amino acid surplus.

Next, growth in CDM that had been depleted either of one of the BCAAs (valine, leucine, or isoleucine) or of threonine was analyzed. Wild-type strains and *codY* mutants failed to grow in medium lacking either valine or leucine (data not shown). In medium lacking isoleucine, there was no growth difference between the wild type and *codY* mutants in contrast to growth in CDM containing 100 mg/liter of isoleucine. We hypothesized that isoleucine may be a major signal for CodY regulation. If isoleucine is indeed a natural ligand of CodY, then maximal repression of CodY-regulated genes should occur under isoleucine-rich conditions. Thus, an excess of isoleucine may lead to growth inhibition, e.g., if some amino acids become limiting but the biosynthetic pathways remain repressed. In fact, a high concentration of isoleucine (500 mg/liter) in the medium resulted in diminished growth in the wild type in comparison to growth in medium with moderate isoleucine (100 mg/liter) (Fig. 3C). This growth inhibition is CodY dependent since in the *codY* mutant no effect of isoleucine excess on growth was observed. Similar results were obtained with strain UAMS-1 and strain RN1HG (data not shown). A predominant role of isoleucine in comparison to other BCAAs for signaling is emphasized by the observation that an excess of valine or leucine did not result in growth inhibition of the wild-type strains (data not shown).

Interestingly, in medium without threonine the *codY* mutants were able to grow but not the wild type (Fig. 3D). This indicates that, under these growth conditions, CodY represses genes necessary for threonine synthesis. The growth of the mutant in medium lacking threonine was characterized by a typical lag phase. Thus, the mutant is obviously able to adapt to the CDM conditions. When the *codY* mutant was subcultured from the exponential growth phase (OD_{600} of 0.5) into fresh medium, growth continued without lag (Fig. 3D), indicating adaptation to the medium. However, when the mutants were subcultured in complete CDM and again inoculated into medium without threonine, the typical lag phase was again apparent. Thus, the adaptation of the *codY* mutants to threonine-depleted medium seems to be due a regulatory adaptation in metabolism and is not mediated by the generation of suppressor mutations in genes of the biochemical pathways.

Influence of isoleucine on CodY target genes. To gain further insight into putative CodY ligands in *S. aureus*, we analyzed whether isoleucine in the medium affects the transcription of *codY* target genes (Fig. 4). Strains were precultured in CDM without isoleucine and then supplemented with isoleucine for 1 h. Isoleucine resulted in a dose-dependent repression of the CodY target gene *ilv*C (Fig. 4B). The addition of 5 μg/ml isoleucine was already sufficient to cause a severe downregulation of *ilvC*. The repression is mediated by CodY since the *codY* mutants were not responsive to isoleucine under the tested conditions (Fig. 4A). However, the *ilvC* level observed in the wild type grown without isoleucine was lower than that in the *codY* mutant. This may be due to a baseline level of isoleucine produced by the bacteria or by other putative signaling

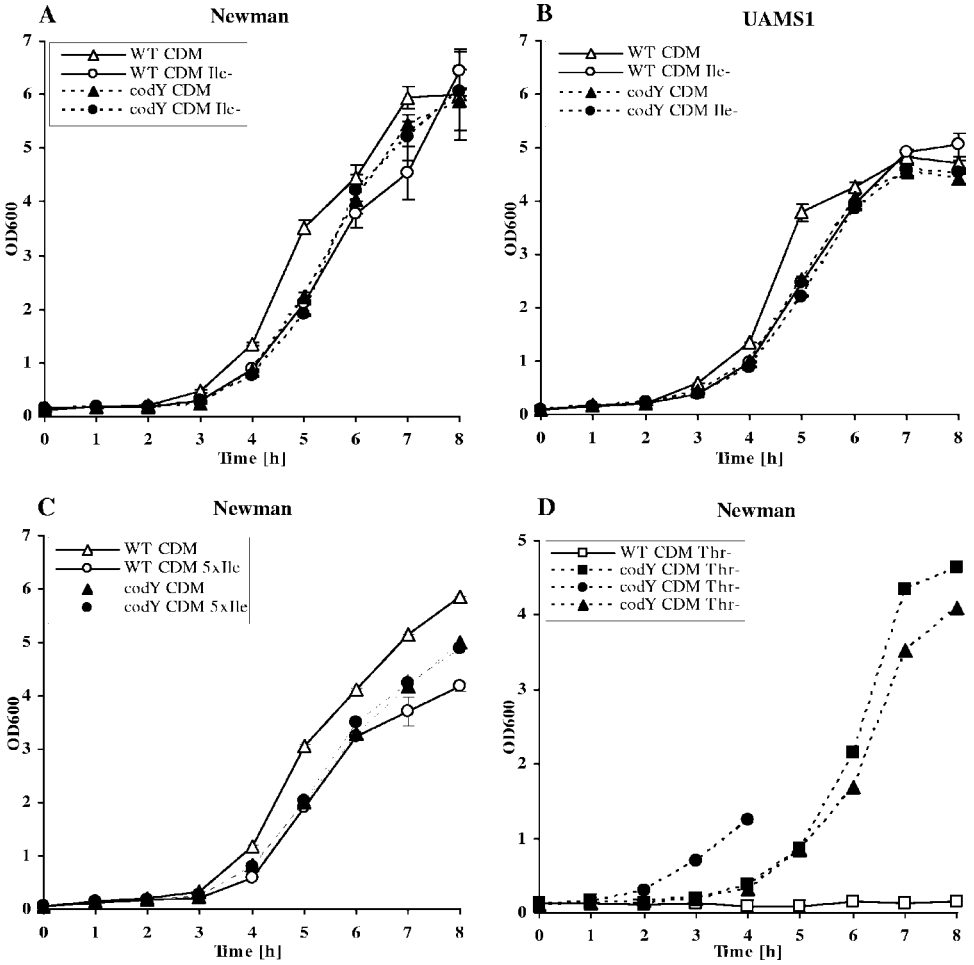

FIG. 3. Growth analysis of wild-type and *codY* mutants in CDM. (A and B) Growth of *S. aureus* strain Newman and the *codY* mutant Newman-21 (A) and of UAMS-1 and the *codY* mutant UAMS-1-21 (B) in CDM and in CDM lacking isoleucine (Ile^-). (C) Growth of *S. aureus* strain Newman and the *codY* mutant Newman-21 in CDM and CDM with an excess of 500 μg/ml isoleucine (5× Ile). (D) Newman-21 was grown in medium lacking threonine (Thr^-; triangle). For analysis of the observed lag phase, the *codY* mutant was subcultured from threonine-depleted CDM. Bacteria from CDM without threonine grown to the exponential phase (OD_{600} of 1) were then subcultured in medium lacking threonine (square). In addition, the bacteria were recultured in complete CDM overnight and again inoculated in CDM without threonine (circle). The lag phase was omitted after preadaptation of the bacteria to threonine depletion but was restored after intermittent growth in medium with threonine. WT, wild type.

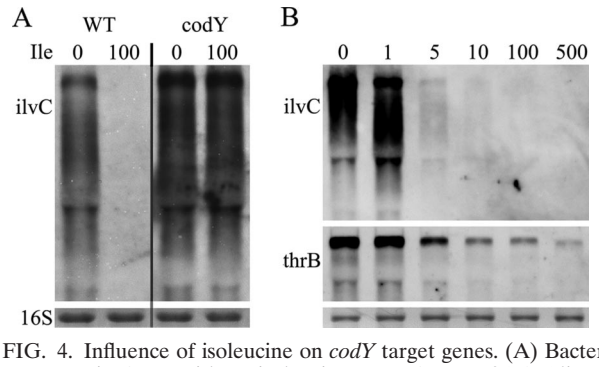

FIG. 4. Influence of isoleucine on *codY* target genes. (A) Bacteria were grown in CDM without isoleucine to an OD_{600} of 0.4. Aliquots were then supplemented with isoleucine (200 mg/liter) and grown for another 1 h. (B) Strain Newman was grown without isoleucine to an OD_{600} of 0.4. Aliquots were then supplemented with increasing concentrations of isoleucine and further grown for 1 h. RNA were hybridized with a probe specific for *ilvC* or *thrB*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as a loading control in the lowest panel. WT, wild type.

molecules such as GTP. Since only the *codY* mutant was able to grow in medium lacking threonine, we also tested whether genes involved in threonine biosynthesis are similarly repressed via CodY. We could show that *thrB* transcription is also dependent on the isoleucine concentration. However, repression of *thrB* by isoleucine was less pronounced than that of *ilvC*.

CodY as a virulence regulator in *S. aureus*. The repressor CodY may be essential not only for the regulation of metabolic genes but also for the fine-tuning of virulence-associated genes, as recently proposed by Majerczyk et al. (34). Indeed, the transcriptional pattern of RNAIII of the virulence regulator *agr* was identical to that of the prototypic CodY-repressed *ilvC* in strain Newman: repression after growth was observed with isoleucine in the wild-type and complemented strains but not in the *codY* mutant (Fig. 5). In contrast, genes coding for the cell surface proteins *fnbA* and *spa* were upregulated by isoleucine in a *codY*-dependent manner. No significant effect

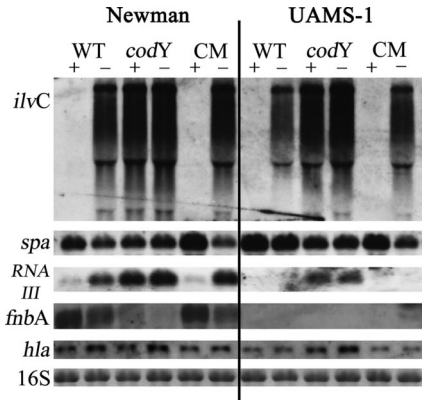

FIG. 5. Influence of isoleucine on virulence gene expression. RNA from strain Newman and UAMS-1, their *codY* mutants (Newman-21 and UAMS-1-21, respectively) and the complemented strains ([CM] Newman-21/pCG30 and UAMS-1-21/pCG30, respectively) isolated from bacteria grown to an OD_{600} of 0.5 in CDM with (+) or without (−) isoleucine. They were then hybridized with probes specific for *ilvC*, *spa*, *agr* (RNAIII), *fnbA*, and *hla*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as a loading control in the lowest panel. WT, wild type.

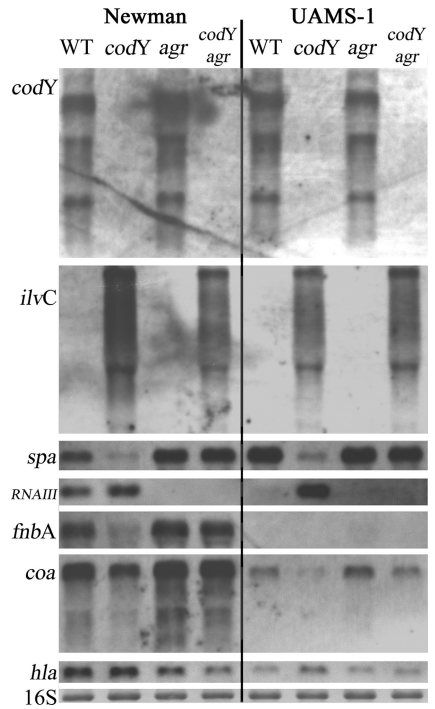

FIG. 6. Influence of CodY and *agr* on virulence gene expression in bacteria from exponential growth phase. RNA from the wild type (WT), *codY*, *agr*, and *codY agr* mutants was isolated from bacteria grown in CDM to the exponential (OD_{600} of 0.5) growth phase. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the lowest panel.

on *hla* expression was observed in strain Newman under these growth conditions. The diminished expression of *spa* and enhanced expression of RNAIII in the *codY* mutant compared to the wild type were also evident in strain UAMS-1. However, there were some differences between the results obtained from strain UAMS-1 and those from strain Newman. First, *fnbA* transcription was not detectable, which agrees with the results from genome sequencing showing that *fnbA* is not present in strain UAMS-1 (unpublished observation). Second, the effects of isoleucine in the wild-type and complemented strains were less pronounced. In addition, in this genetic background, the expression of the *agr*-regulated gene *hla* was enhanced in the *codY* mutant compared to the wild-type and complemented strains.

Effect of *codY* on virulence gene expression in *agr*-negative background. The virulence gene *hla* is known to be activated by RNAIII, whereas *spa*, *coa*, and *fnbA* are inhibited by RNAIII (37, 52, 53). To clarify whether the effect of CodY on virulence genes is solely due to RNAIII upregulation in the *codY* mutant, we analyzed virulence gene expression in *agr* and *codY agr* double mutants (Fig. 6). Interestingly, *codY* expression was not affected in the *agr* mutants of *S. aureus*. This is in contrast to *Staphylococcus epidermidis*, in which *agr* leads to elevated *codY* transcription (4). As expected in the *agr*-negative background, increased levels of *spa*, *fnbA*, and *coa* transcripts were detectable. In the *agr*-negative background, no significant effect of CodY was observed on these genes in bacteria during the exponential growth phase. However, when we analyzed bacteria from the postexponential phase, it could be shown that *fnbA* and *spa* were activated by CodY, independently of *agr* (Fig. 7). The lack of *spa* transcription in the *agr*-positive background is due to the strong repressive effect of RNAIII on *spa* transcription. Surprisingly, growth in CDM also allowed transcription of *fnbA* in the late growth phase. This is usually not seen using complex medium, in which *fnbA*, like *coa*, is repressed during postexponential growth independently of *agr* (42, 53).

Global effect of *codY* on gene expression in *agr*-positive and *agr*-negative backgrounds. In order to acquire a more comprehensive understanding of the *codY* regulon, we performed microarray analysis. On the basis of the results obtained up to that point, we expected to detect the most pronounced effects of CodY in bacteria grown with isoleucine during the expo-

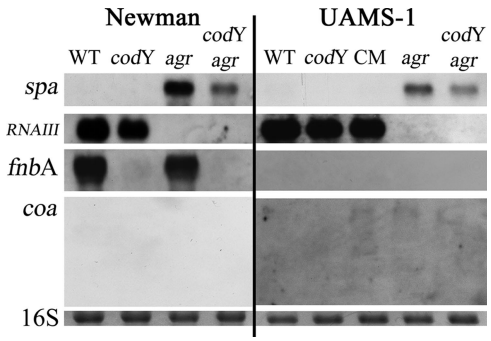

FIG. 7. Influence of CodY and *agr* on virulence gene expression in bacteria from postexponential growth phase. RNA from wild-type (WT) strains (Newman or UAMS-1) and their *codY*, *agr*, and *codY agr* mutants was isolated from bacteria grown in CDM with an excess of isoleucine (500 mg/liter) to the postexponential phase. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the lowest panel.

nential phase. Under these conditions, CodY should be saturated with its ligand(s) and thus display full repression.

First, we determined the *codY* regulon by comparing gene expression in the parental strain with that in the *codY* mutant. A total of 124 genes (5% of the genome) showed differential expression in the *codY* mutant compared to its parent (Table 2). These genes were predicted to be contained in 71 operons (operon structure was predicted by the public database http://www.microbesonline.org). For each gene with significant difference in gene expression, the neighboring genes within the operons were also analyzed (see Table S3 in the supplemental material). In most cases genes that were predicted to be located in an operon were coregulated although this correspondence did not always reach the level of significance.

The vast majority of targets (106/124) appeared to be repressed by CodY (upregulated in the mutant). Sixteen of these genes were preceded by a CodY box as predicted above (see Table S2 in the supplemental material). Fifty-eight of the *codY*-repressed genes encompassed genes involved in amino acid transport and metabolism, e.g., the *ilv* operon and the peptide transporters *opp* and *brnQ1* encoding proteins involved in the metabolism or the transport of BCAAs. Besides *agr* only one other regulatory gene was significantly affected by CodY. The transcription activator of the glutamate synthase operon *gltC* is repressed by CodY, suggesting that the CodY-repressed genes *gltB* and *gltD* are indirectly influenced via *gltC*.

Only 18 genes seemed to be activated by CodY (downregulated in the mutant). None of these genes contained a putative CodY binding motif, supporting the hypothesis that upregulation of these genes is probably not related to a direct interaction with CodY. Interestingly, six CodY-activated genes were predicted to be involved in nucleotide transport and metabolism.

Genes encoding virulence and defense factors were either up- or downregulated by CodY. For instance, MWMN_1084, which codes for phenole-soluble moduline, appeared to be strongly downregulated. The *cap* operon coding for enzymes in capsular biosynthesis and genes for katalase (*katA*) and superoxide dismutase (*sodM*) were also downregulated. In contrast, expression of genes coding for the cell surface proteins *fnbA*, *sasG*, and *coa* were positively influenced by CodY.

We next asked which of the 124 *codY*-dependent genes determined in the *agr*-positive background are still *codY* regulated in an *agr*-negative background. To determine this, we compared gene expression of the *agr* mutant versus the *agr codY* double mutant. Ninety-six of the 124 genes appeared to be regulated by *codY* independent of the *agr* background, and most of these were involved in amino acid metabolism and transport. In contrast, many of the genes categorized as virulence and defense factors were not significantly influenced by CodY in the *agr*-negative background. However genes within the *cap* operon, *katA* and *sodM*, were still *codY* repressed independent of *agr*. These results confirm the results of Northern blot analysis indicating that CodY acts in an *agr*-independent and *agr*-dependent manner on virulence genes, whereas the influence on metabolic genes is mostly *agr*-independent.

DISCUSSION

CodY has been described as a conserved repressor of genes involved in the biosynthesis and transport of amino acids in several gram-positive species. Here, we analyze the role of CodY in the human pathogen *S. aureus*. In *S. aureus codY* is cotranscribed with three genes located upstream of *codY* (*xerC*, *clpQ*, and *clpY*). *xerC* codes for tyrosine recombinase, and *clpQY* codes for the ATP-dependent heat shock protease HslVU. The same gene order is present in the genome of *B. subtilis*, *Listeria monocytogenes*, and *Enterococcus faecalis*, indicating a physiological link between CodY and the heat shock protease Hs1VU in these bacteria. This must remain speculative, however, since little is known about the function of Hs1VU in stress response or pathogenesis (7). In streptococcal species, *L. lactis* or *Clostridium difficile*, *codY* is located in a different genetic context. Alignments of the CodY sequences (http://www.ebi.ac.uk/Tools/clustalw/) from a selected set of gram-positive bacteria revealed two clusters: cluster one containing CodY from *S. aureus*, *B. subtilis*, *L. monocytogenes*, and *C. difficile* and cluster two with CodY from *E. faecalis*, streptococci, and lactococci. Thus, the evolution of the genetic context is not congruent with sequence differences within CodY. For instance, *E. faecalis* resembles *S. aureus* with respect to the gene context, whereas from the CodY amino acid sequence, *E. faecalis* is very similar to the streptococcal species.

From studies of other bacteria, it was postulated that BCAAs are major ligands of CodY, resulting in severe repression of target genes. We could show that in *S. aureus* isoleucine, although not required for growth, resulted in strong repression of CodY target genes. Furthermore, an excess of isoleucine resulted in growth inhibition in wild-type bacteria. This suggests that certain target genes that are needed for optimal growth are repressed under these conditions. Since an excess of leucine or valine had no effect on *codY* target gene repression or growth, we conclude that isoleucine is the major ligand in *S. aureus* as proposed for CodY from *L. lactis* (11).

Interestingly, only *codY* mutants but not wild-type *S. aureus* strains were able to grow without threonine, indicating that threonine synthesis requires enzymes whose transcription is efficiently repressed by CodY. Indeed, *thrC* (coding for threonine synthase) was shown to be repressed by CodY (shown by microarray analysis and Northern analysis). Since threonine auxotrophy was also seen under conditions without isoleucine, it can be assumed that besides isoleucine, other ligands (or CodY without ligand) are sufficient to mediate the CodY-dependent repression of the threonine biosynthetic genes.

Functionally, there are also clear differences between different species in the GTP-binding capacity of CodY (39). CodY from *S. aureus* is similar to its homolog in *B. subtilis* with respect to a proposed GTP-binding motif derived from structure and sequence analyses (33). For an in-depth analysis of the role of GTP as a signaling molecule, mutants with defects in internal GTP synthesis and/or the stringent control are currently being examined.

Overall, it can be assumed that CodY of *S. aureus* functions in a manner similar to that which has been shown for other gram-positive organisms. This is probably also true for the proposed CodY binding motif since we were able to detect CodY target genes based on a stringent motif search allowing

TABLE 2. The *codY* regulon of *S. aureus*

Newman ORF[a]	Description	Gene	Differential expression in the indicated strains (*n*-fold)[b]		Category
			Newman vs *codY* mutant	*agr* mutant vs *agr codY* mutant	
NWMN_0128	*N*-Acetylglutamate gamma-semialdehyde dehydrogenase	*argC*	0.39	NS	Amino acid transport/metabolism
NWMN_0130	Branched-chain amino acid transport system II carrier protein	*brnQ1*	0.33	0.44	Amino acid transport/metabolism
NWMN_0144	Oligopeptide ABC transport. permease		0.03	0.15	Amino acid transport/metabolism
NWMN_0145	Peptide ABC transporter. permease		0.15	0.33	Amino acid transport/metabolism
NWMN_0146	RGD-containing lipoprotein	*rlp*	0.31	0.4*	Amino acid transport/metabolism
NWMN_0147	Gamma glutamyltranspeptidase	*ggt*	0.12	0.21	Amino acid transport/metabolism
NWMN_0348	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*metE*	0.06	0.06	Amino acid transport/metabolism
NWMN_0349	Methylenetetrahydrofolate reductase protein	*metH*	0.03	0.04	Amino acid transport/metabolism
NWMN_0350	*trans*-Sulfuration enzyme family protein		0.36	0.06	Amino acid transport/metabolism
NWMN_0351	Cys/Met metabolism PLP-dependent enzyme		0.02	0.06	Amino acid transport/metabolism
NWMN_0425	Cystathionine gamma-synthase	*metB*	0.36	0.48	Amino acid transport/metabolism
NWMN_0436	Glutamate synthase large subunit	*gltB*	0.08	0.14	Amino acid transport/metabolism
NWMN_0437	NADH-glutamate synthase small subunit	*gltD*	0.09	0.07	Amino acid transport/metabolism
NWMN_0516	Branched-chain amino acid aminotransferase	*ilvE*	0.47	0.52*	Amino acid transport/metabolism
NWMN_0831	Argininosuccinate lyase	*argH*	0.24	0.46	Amino acid transport/metabolism
NWMN_0855	Oligopeptide transport system permease protein	*oppB*	0.15	0.15	Amino acid transport/metabolism
NWMN_0858	Oligopeptide transport ATP-binding protein	*oppD*	0.16	0.19	Amino acid transport/metabolism
NWMN_0860	Hypothetical protein		0.12	0.17	Amino acid transport/metabolism
NWMN_0883	Na^+/alanine symporter family protein		0.03	0.04	Amino acid transport/metabolism
NWMN_1239	Aspartate kinase		0.06	0.06	Amino acid transport/metabolism
NWMN_1240	Homoserine dehydrogenase	*metL*	0.05	0.06	Amino acid transport/metabolism
NWMN_1241	Threonine synthase	*thrC*	0.06	0.07	Amino acid transport/metabolism
NWMN_1245	Amino acid permease		0.5*	0.5*	Amino acid transport/metabolism
NWMN_1277	Prephenate dehydrogenase	*tyrA*	0.50	0.44	Amino acid transport/metabolism
NWMN_1279	Anthranilate synthase component I		0.11	0.23	Amino acid transport/metabolism
NWMN_1280	Anthranilate synthase component II	*trpG*	0.15	0.21	Amino acid transport/metabolism
NWMN_1281	Anthranilate phosphoribosyltransferase	*trpD*	0.16	0.16	Amino acid transport/metabolism
NWMN_1282	Indole-3-glycerol phosphate synthase	*trpC*	0.10	0.11	Amino acid transport/metabolism
NWMN_1283	Phosphoriborylanthranilate isomerase	*trpF*	0.08	0.16	Amino acid transport/metabolism
NWMN_1284	Tryptophan synthase subunit beta	*trpB*	0.10	0.09	Amino acid transport/metabolism
NWMN_1304	Aspartate kinase	*lysC*	0.12	0.19	Amino acid transport/metabolism
NWMN_1305	Aspartate semialdehyde dehydrogenase	*asd*	0.12	0.09	Amino acid transport/metabolism
NWMN_1306	Dihydrodipicolinate	*dapA*	0.09	0.09	Amino acid transport/metabolism
NWMN_1307	Dihydrodipicolinate reductase	*dapB*	0.15	0.07	Amino acid transport/metabolism
NWMN_1308	Tetrahydrodipicolinate acetyltransferase	*dapD*	0.10	0.11	Amino acid transport/metabolism
NWMN_1311	Diaminopimelate decarboxylase	*lysA*	0.29	0.23	Amino acid transport/metabolism
NWMN_1348	Threonine dehydratase	*ilvA*	0.12	0.13	Amino acid transport/metabolism
NWMN_1616	Aminotransferase, class V		0.08	0.10	Amino acid transport/metabolism
NWMN_1617	D-3-Phosphoglycerate dehydrogenase	*serA*	0.07	0.10	Amino acid transport/metabolism
NWMN_1749	Glutamine transport ATP-binding protein		0.30	0.55*	Amino acid transport/metabolism
NWMN_1750	Extracellular glutamine-binding protein		0.5*	NS	Amino acid transport/metabolism
NWMN_1960	Dihydroxy acid dehydratase	*ilvD*	0.03	0.09	Amino acid transport/metabolism
NWMN_1961	Acetolactate synthase large subunit	*ilvB*	0.02	0.03	Amino acid transport/metabolism
NWMN_1962	Ketol acid reductoisomerase	*ilvC*	0.01	0.02	Amino acid transport/metabolism
NWMN_1963	2-Isopropylmalate synthase	*leuA*	0.01	0.02	Amino acid transport/metabolism
NWMN_1964	3-Isopropylmalate dehydrogenase	*leuB*	0.02	0.04	Amino acid transport/metabolism
NWMN_1965	Isopropylmalate isomerase large subunit	*leuC*	0.02	0.04	Amino acid transport/metabolism
NWMN_1966	3-Isopropylmalate dehydratase small subunit	*leuD*	0.03	0.02	Amino acid transport/metabolism
NWMN_2347	Glycine betaine/L-proline transport	*opuCA*	0.47	0.27	Amino acid transport/metabolism
NWMN_2500	Amino acid permease family protein		0.10	0.11	Amino acid transport/metabolism
NWMN_2501	4-Aminobutyrate aminotransferase		0.09	0.13	Amino acid transport/metabolism
NWMN_2571	Imidazole glycerol phosphate synthase subunit	*hisF*	0.20	0.21	Amino acid transport/metabolism
NWMN_2572	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	*hisA*	0.18	0.14	Amino acid transport/metabolism
NWMN_2573	Imidazole glycerol phosphate synthase	*hisH*	0.20	0.31	Amino acid transport/metabolism
NWMN_2574	Imidazoleglycerol phosphate dehydratase	*hisB*	0.11	0.03	Amino acid transport/metabolism
NWMN_2577	ATP phosphoribosyltransferase	*hisG*	0.18	0.31	Amino acid transport/metabolism
NWMN_2370	Putative transport protein		0.19	0.24	Amino acid transport/metabolism
NWMN_0859	Oligopeptide transport ATP-binding protein	*oppF*	0.33	0.30	Amino acid transport/metabolism
NWMN_0428	ABC transporter		0.10	0.18	Inorganic ion transport/metabolism
NWMN_1950	Ammonium transporter	*nrgA*	0.23	NS	Inorganic ion transport/metabolism
NWMN_0423	**Sodium-dependent symporter protein**		**3.03**	**3.92**	**Inorganic ion transport/metabolism**
NWMN_2288	**Nitrite transport protein**	*narK*	**2.28**	**NS**	**Inorganic ion transport/metabolism**
NWMN_0016	**Adenylosuccinate synthase**	*purA*	**2.65**	**NS**	**Nucleotide transport/metabolism**
NWMN_0379	**Xanthine permease**	*pbuX*	**2.51**	**NS**	**Nucleotide transport/metabolism**
NWMN_1110	**Uracil permease**	*pyrP*	**2.23**	**2.1***	**Nucleotide transport/metabolism**
NWMN_1111	**Aspartate carbamoyltransferase catalytic subunit**	*pyrB*	**2.35**	**2.2***	**Nucleotide transport/metabolism**
NWMN_1112	**Dihydroorotase**	*pyrC*	**2.09**	**2.1***	**Nucleotide transport/metabolism**
NWMN_1249	**GMP oxidoreductase**	*guaC*	**2.33**	**2.1***	**Nucleotide transport/metabolism**
NWMN_0163	Formate acetyltransferase activating enzyme	*pflA*	0.40	0.12	Energy production/conversion
NWMN_0162	Formate acetyltransferase	*pflB*	0.27	0.16	Energy production/conversion
NWMN_0979	Pyruvate carboxylase	*pycA*	0.37	0.31	Energy production/conversion
NWMN_1325	Dihydrolipoamide acetyltransferase	*sucB*	0.45	0.55*	Energy production/conversion
NWMN_2294	**Putative nitrate reductase gamma chain**	*narI*	**2.44**	**NS**	**Energy production/conversion**
NWMN_0167	**Acetyl-coenzyme A acetyltransferase**	*fadA*	**3.04**	**NS**	**Energy production/conversion**

Continued on following page

TABLE 2—*Continued*

Newman ORF[a]	Description	Gene	Differential expression in the indicated strains (*n*-fold)[b]		Category
			Newman vs *codY* mutant	*agr* mutant vs *agr codY* mutant	
NWMN_0435	Transcription activator of glutamate synthase operon	*gltC*	0.32	NS	Regulation
NWMN_1943	Accessory gene regulator B	*agrB*	0.42	NS	Regulation
NWMN_1946	Accessory gene regulator A	*agrA*	0.44	NS	Regulation
NWMN_0077	Superoxide dismutase	*sodM*	0.26	0.49*	Defense/Virulence factor
NWMN_0095	Capsular polysaccharide synthesis enzyme CapA	*capA*	0.32	0.33	Defense/Virulence factor
NWMN_0096	Capsular polysaccharide synthesis enzyme CapB	*capB*	0.29	0.19	Defense/Virulence factor
NWMN_0097	Capsular polysaccharide synthesis enzyme CapC	*capC*	0.35	0.16	Defense/Virulence factor
NWMN_0098	Capsular polysaccharide synthesis enzyme CapD	*capD*	0.30	0.36	Defense/Virulence factor
NWMN_0099	Capsular polysaccharide synthesis enzyme CapE	*capE*	0.32	0.31	Defense/Virulence factor
NWMN_0100	Capsular polysaccharide synthesis enzyme CapF	*capF*	0.33	NS	Defense/Virulence factor
NWMN_0101	Capsular polysaccharide synthesis enzyme CapG	*capG*	0.30	NS	Defense/Virulence factor
NWMN_0102	Capsular polysaccharide synthesis enzyme CapH	*capH*	0.34	0.45	Defense/Virulence factor
NWMN_0103	Capsular polysaccharide synthesis enzyme CapI	*capI*	0.41	0.50	Defense/Virulence factor
NWMN_0104	Capsular polysaccharide synthesis enzyme CapJ	*capJ*	0.32	NS	Defense/Virulence factor
NWMN_0105	Capsular polysaccharide synthesis enzyme CapK	*capK*	0.31	0.64	Defense/Virulence factor
NWMN_0107	Capsular polysaccharide synthesis enzyme CapM	*capM*	0.45	NS	Defense/Virulence factor
NWMN_0108	Capsular polysaccharide synthesis enzyme CapN	*capN*	0.43	NS	Defense/Virulence factor
NWMN_0262	Truncated glycerol ester hydrolase	*geh*	0.35	NS	Defense/Virulence factor
NWMN_0525	Bone sialoprotein-binding protein	*sdrE*	0.50	1.88*	Defense/Virulence factor
NWMN_1084	Antibacterial protein (phenole-soluble moduline)		0.06	NS	Defense/Virulence factor
NWMN_1246	Catalase	*katA*	0.36	0.36	Defense/Virulence factor
NWMN_1872	MHC class II analog protein	*eap*	0.48	NS	Defense/Virulence factor
NWMN_nd	Delta-hemolysin	*hld*	0.30	NS	Defense/Virulence factor
NWMN_0166	**Staphylocoagulase precursor**	*coa*	**2.49**	**NS**	**Defense/Virulence factor**
NWMN_0394	**Auperantigen-like protein 7**	*set7nm*	**3.34**	**NS**	**Defense/Virulence factor**
NWMN_2392	**Cell wall-anchored protein**	*sasG*	**2.85**	**0.5***	**Defense/Virulence factor**
NWMN_2399	**Fibronectin binding protein**	**FnBPA**	**2.01***	**NS**	**Defense/Virulence factor**
NWMN_0071	Acetoin reductase	*butA*	0.19	0.17	Miscellaneous
NWMN_0429	*N*-Acetylmuramoyl-L-alanine amidase		0.50	NS	Miscellaneous
NWMN_0721	Sigma 54 modulation protein		0.45	0.47	Miscellaneous
NWMN_1309	Hippurate hydrolase		0.14	0.19	Miscellaneous
NWMN_1618	Haloacid dehalogenase-like hydrolase		0.43	0.31	Miscellaneous
NWMN_2097	Tagatose-6-phosphate kinase	*lacC*	0.20	NS	Miscellaneous
NWMN_2448	ATP-dependent Clp protease	*clpC*	0.38	0.37	Miscellaneous
NWMN_0028	**Metallo-beta-lactamase superfamily protein**		**2.91**	**NS**	**Miscellaneous**
NWMN_0322	**Ascorbate-specific phosphotransferase system enzyme IIC**	*ulaA*	**2.35**	**NS**	**Miscellaneous**
NWMN_0220	Hypothetical protein		0.20	0.47	Function unknown
NWMN_0404	Hypothetical protein	*lpl2nm*	0.5*	0.25	Function unknown
NWMN_0667	Hypothetical protein		0.5*	0.47	Function unknown
NWMN_0896	Hypothetical protein		0.21	0.20	Function unknown
NWMN_0901	Hypothetical protein		0.07	0.24	Function unknown
NWMN_0902	Hypothetical protein		0.11	0.07	Function unknown
NWMN_1243	Hypothetical protein		0.27	0.27	Function unknown
NWMN_2221	Hypothetical protein		0.30	0.42	Function unknown
NWMN_2222	Hypothetical protein		0.10	0.16	Function unknown
NWMN_2230	Hypothetical protein		0.21	0.14	Function unknown
NWMN_2470	Hypothetical protein		0.11	0.09	Function unknown
NWMN_2578	Hypothetical protein		0.06	0.25	Function unknown
NWMN_0027	**Hypothetical protein**		**3.66**	**4.07**	**Function unknown**
NWMN_0401	**Hypothetical protein**		**2.38**	**NS**	**Function unknown**

[a] Based on the publically available Newman genome sequence. Boldface, genes that are repressed in the *codY* mutant.
[b] Fold changes are indicated for each comparison and displayed for genes showing statistically significant differential expression. Values correspond to expression ratios, i.e., averaged expression levels from two independent replicate experiments ($P < 0.05$). NS, not significantly different; *, limit of significance.

only two mismatches within the consensus sequence. Lowering the stringency to four or five mismatches resulted in >1,000 putative sites within the genome, which appears of little informative value. However, the high stringency may have caused us to miss several real CodY boxes as a previous study in *B. subtilis* showed that even up to five mismatches within the CodY box consensus could result in a functional element (5). In fact, a CodY box was predicted for only a subset of the *codY*-dependent genes identified by our microarray analysis. When we allowed three mismatches within 200 bp upstream of ORFs, a reasonable number (222) of additional genes with a putative *codY* box were found. For instance, in the upstream sequence of the *cap* operon and the *fnbA* gene, a *codY* motif could now be identified. Interestingly, the CodY box preceding the *cap* operon overlapped with the mapped −10 region (38). The putative CodY box in front of *fnbA* was located between a sigma B binding motif and the transcriptional start site. Thus, these two virulence-associated genes may be direct targets of CodY regulation. Direct binding assays such as chromatin immunoprecipitation with microarray analysis or gel retardation assays using purified CodY are needed to clarify this topic in the future.

From microarray analysis it became clear that, overall, the *codY* regulon is conserved between gram-positive species with regard to profound and mostly direct repression of genes involved in amino acid biosynthesis and transport. Genes that are activated via CodY are much less conserved, and a direct interaction of CodY with this activated target gene could be

shown only for *ackA* of *B. subtilis* (44). In our screen, several genes involved in nucleotide synthesis and transport were activated by *codY*. Similarly, in *L. monocytogenes*, *guaA* and *guaB*, which encode the enzymes involved in biosynthesis of GMP, which encode, were also found to be activated by *codY* (6). Thus, there may be at least an indirect link between CodY and the intracellular nucleotide pool.

The most comprehensive data on the CodY regulon and function are based on the analysis of the nonpathogenic bacteria *B. subtilis* and *L. lactis*. There is emerging evidence that CodY also has an impact on virulence gene expression in gram-positive pathogens (6, 14, 26, 32, 35, 46). Interestingly, in *S. aureus* and *L. monocytogenes*, the quorum-sensing *agr* system is affected by CodY in opposite directions: in *L. monocytogenes* CodY leads to *agr* activation, while in *S. aureus* it leads to *agr* repression (6, 34). Although a putative CodY box with three mismatches is located upstream of *agrB* in *S. aureus*, this box overlaps with *hld* and is not in proximity to the transcriptional start site or the binding site of the sensor histidine kinase ArgA. Thus, the mechanism leading to *agr* repression remains unclear. Nevertheless, repression of the *agr* system by CodY presumably enhances the tight growth phase-dependent activation of this quorum-sensing system which by definition is activated at higher cell densities. Thus, under conditions of isoleucine limitation (or in *codY* mutants), the *agr* system is prematurely activated. Consequently, *agr*-activated genes like toxins and the *cap* operon also become activated, whereas genes known to be downregulated by the *agr* system are switched off. This can be seen as an escape mechanism for the bacteria under limiting conditions. A CodY-dependent repression of *hla* and the *ica* operon (coding for the enzyme generating the polysaccharide intercellular adhesin) was shown recently by Majerczyk et al. (34). In our microarray analysis, *hla* and the *ica* operon were not significantly affected in the *codY* mutants. Both genes are known to be poorly expressed in strain Newman. However, increased expression of *hla* in the *codY* mutant could be confirmed for strain UAMS-1. Genes of the *ica* operon were below the threshold in our analysis but also showed a clear tendency toward higher expression in the *codY* mutant, as further confirmed by reverse transcription-PCR in both genetic backgrounds (strain Newman and UAMS-1) (data not shown).

Genes for cell-associated proteins such as *fnbA*, *spa*, and *coa* are activated by CodY. The role of CodY in the activation of these virulence genes might be primarily via *agr*. However, direct activation through CodY binding can be presumed for *fnbA* activation because of the presence of a putative CodY box and the observation that *fnbA* is still CodY dependent in an *agr*-negative background. Additional regulatory mechanism(s) may act primarily at the posttranscriptional level on some of the genes since, besides *agr*, no other virulence regulatory gene was differentially expressed between wild-type and mutant strains.

A link to the situation during infection or colonization is hard to draw since several studies have shown that *agr* is not activated during chronic infections and/or colonization (20, 21), and little is known about the growth conditions in vivo. However, our analysis of the *codY* regulon in an *agr*-negative background clearly shows that CodY also regulates some of the virulence factors independently of *agr*. We and others have shown elsewhere that *hla* can be transcribed independently of *agr* in vivo (21, 54). Thus, CodY may contribute to virulence gene regulation in vivo not only by supporting but also by replacing *agr* function: under limited conditions (low isoleucine) the capsule and several secreted enzymes and toxins are derepressed, whereas some of the cell surface molecules are downregulated. Overall, the impact of *codY* mutation on *S. aureus* needs to be assessed in vivo as some *codY* targets may contribute to cell adhesion and survival in hostile environments.

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