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Staphylococcus aureus CcpA Affects Virulence Determinant Production and Antibiotic Resistance

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Carbon catabolite protein A (CcpA) is known to function as a major regulator of gene expression in different gram-positive organisms. Deletion of the *ccpA* homologue (*saCOL1786*) in *Staphylococcus aureus* was found to affect growth, glucose metabolization, and transcription of selected virulence determinants. In liquid culture, deletion of CcpA decreased the growth rate and yield; however, the effect was only transient during the exponential-growth phase as long as glucose was present in the medium. Depletion of glucose and production of lactate was delayed, while the level of excretion of acetate was less affected and was even higher in the mutant culture. On solid medium, in contrast, growth of the Δ*ccpA* mutant resulted in smaller colonies containing a lower number of CFU per colony. Deletion of CcpA had an effect on the expression of important virulence factors of *S. aureus* by down-regulating *RNAIII*, the effector molecule of the *agr* locus, and altering the transcription patterns of *hla*, encoding α-hemolysin, and *spa*, encoding protein A. CcpA inactivation markedly reduced the oxacillin resistance levels in the highly methicillin-resistant *S. aureus* strain COLn and the teicoplanin resistance level in a glycopeptide-intermediate-resistant *S. aureus* strain. The presence of CcpA in the capsular polysaccharide serotype 5 (CP5)-producing strain Newman abolished capsule formation and decreased *cap* operon transcription in the presence of glucose. The staphylococcal CcpA thus not only is involved in the regulation of carbon metabolism but seems to function as a modulator of virulence gene expression as well.

Carbon catabolite repression (CCR) in bacteria is a widespread, global regulatory phenomenon that allows modulation of the expression of genes and operons involved in carbon utilization and metabolization in the presence of preferred carbon source(s). In CCR, the presence of a preferred carbon source represses the expression of genes and operons whose products are involved in the metabolism of alternative, less-preferred carbon sources. In low-GC gram-positive bacteria, CCR is achieved via transcriptional control, inducer exclusion, and induction prevention (reviewed in references 55 and 60). In this group of bacteria, a common mechanism for transcriptional control has evolved that is mediated via the proteins phosphotransferase HPr, the bifunctional HPr kinase-phosphatase (HPrK/P), and the pleiotropic regulator CcpA (catabolite control protein A). CCR in *Bacillus subtilis* has been studied extensively and is thought to serve as the prototype of CCR-regulated gene expression in gram-positive organisms (reviewed in reference 52). In *B. subtilis*, regulation of transcription of catabolite-repressive genes is exerted mainly through the binding of CcpA to specific *cis*-acting DNA sequences called catabolite-responsive elements (CREs). The DNA-binding activity of CcpA itself is triggered by HPr or its regulatory paralog Crh, which, in the presence of glucose, are phosphorylated by HPrK/P on regulatory seryl residues, in which state they act as cofactors for CcpA. Depending on the localization of the CRE, CcpA may function either as an activator or as a repressor of gene expression. Whole-transcriptome analyses suggest that 10% of all genes in *B. subtilis* are affected in their regulation by glucose by a factor of more than 3, with repressed genes outnumbering activated genes by three to one (3, 39). The majority (80%) of these genes depend on CcpA for regulation, and a recent study indicated that CcpA required interaction with RNA polymerase to inhibit transcription (31).

Although CCR by the catabolite control protein CcpA has been demonstrated in *Staphylococcus xylosus* (reviewed in reference 26), only a little is known about this element in the closely related, pathogenic *Staphylococcus aureus*. However, indications that glucose affects gene expression in *S. aureus* (12, 24, 27, 44, 45, 49), the identification of a potential CRE in the promoter region of the glucose-repressible *pckA* (49) that is highly homologous to the CRE consensus of *B. subtilis* (38), and the presence of HPr (SaCOL1091), HPrK (SaCOL0825), and CcpA (SaCOL1786) homologues in *S. aureus* suggest that a similar mechanism might be present in this pathogen.

Site-directed inactivation of *ccpA* showed here that the lack of CcpA, although causing only minor effects on growth of *S. aureus*, affected oxacillin and glycopeptide resistance and had a significant impact on the ability of *S. aureus* to express virulence factors such as *RNAIII*, *hla*, and *spa* in either the presence or absence of glucose.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and relevant phenotypes are listed in Table 1. When not otherwise specified, bacteria were grown in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) buffered using 50 mM HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid [pH 7.5]) and a flask volume/culture volume ratio of 5:1 at 200 rpm and 37°C. Where

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Reference or source
Strains		
S. aureus		
COLn	Tc^s strain from COL, homogeneous methicillin-resistant strain, Mc^r	30
Newman	Clinical isolate (ATCC 25904), CP5 producer	13
RN4220	NCTC8325-4 r^- m^+ *rsbU*	32
KS30	NM143 Δ*ccpA*::*tet*(L), Tc^r	This study
MST04	RN4220 Δ*ccpA*::*tet*(L), Tc^r	This study
MST14	Newman Δ*ccpA*::*tet*(L), Tc^r	This study
MST23	COLn Δ*ccpA*::*tet*(L), heterogeneous methicillin-resistant strain; Mc^r Tc^r	This study
MST31	COLn pAW17; Mc^r Kan^r	This study
MST35	MST23 pAW17; Mc^r Tc^r Kan^r	This study
MST36	MST23 pMST1; Mc^r Tc^r Kan^r	This study
NM143	Newman GISA derivative, in vitro step-selected mutant with a teicoplanin MIC of 24 μg ml^{-1}	N. McCallum; unpublished data
Escherichia coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tc^r)]	Stratagene
Plasmids		
pAW17	*E. coli-S. aureus* shuttle plasmid with *oris* pAMα1 and ColE1, Kan^r	46
pEC1	pUC19 derivative containing the 1.45-kb ClaI *erm*(B) fragment of Tn*551*; Ap^r Em^r	4
pMR2	pEC1 with a 2.7-kb PCR fragment covering the *ccpA* flanking regions and the *tet*(L) cassette fully replacing the *ccpA* coding region; Amp^r Em^r Tc^r	This study
pMST1	pAW17 with a 1.7-kb PCR fragment covering *ccpA* and its proposed promoter, Kan^r	This study

[a] Abbreviations: Ap^r, ampicillin resistant; CP5, capsular polysaccharide type 5; Em^r, erythromycin resistant; GISA, glycopeptide intermediate resistant *S. aureus*; Kan^r, kanamycin resistant; Mc^r, methicillin resistant, Tc^r, tetracycline resistant.

indicated, mutant strains were grown on antibiotic-supplemented media containing 100 μg of ampicillin, 10 μg of erythromycin, or 10 μg of tetracycline per ml.

DNA manipulations. DNA sequencing, PCR, and plasmid isolation were performed using standard procedures (1) or according to manufacturers' instructions.

Construction of *S. aureus* Δ*ccpA*. A 2.7-kp fragment containing the *ccpA* gene and its flanking regions was amplified by PCR from chromosomal DNA of *S. aureus* COL by use of primer pair ccpABamHI-F/ccpAEcoRI-R (Table 2), digested, and cloned into the BamHI/EcoRI-digested vector pEC1 (4) to generate plasmid pMR1 (Fig. 1). The plasmid was used in a second step to amplify a 5.8-kb

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Location (GenBank accession no. NC_002951) or reference
capA167	AGGGTGACAATCCTCAGTTTATGG	153571–153594
capA501	GACTTTAACTGCTGTACCGTCTGCT	153881–153905
capA710	TTGAACCCAATACAGGCAATCC	154035–154056
ccpA-F	CAGCATCCATTGCACTATGC	1830520–1830539
ccpA-R	TCTCTATGGCCACAGTGTCG	1831235–1831254
ccpABamHI-F	gcggatccTAGAATTGCAACAGGTGACG	1829196–1829215
ccpAEcoRI-R	gcgaattCTGTTGCACTTAGTGATGCG	1831924–1831943
ccpAko-F	AATTTCCTCCTTGTAAACG	1831295–1831313
ccpAko-R	ATGGGTGTTGGAAGAATGCC	1830246–1830265
gyr297	TTAGTGTGGGAAATTGTCGATAAT	20
gyr574	AGTCTTGTGACAATGCGTTTACA	20
gyr864	GTACGATTTAATACCGCCCTCATA	20
hla-F	AGAAAATGGCATGCACAAAAA	50
hla-R	TGTAGCGAAGTCTGGTGAAAA	50
MST12	gagtctagaACCAACTGCGAAAGCAGC	1831994–1832011
MST14	gaggatccGGCATTCTTCCAACACCCA	1830246–1830264
pckA-F	CCATCAACTTCTGGATCTGC	1893626–1893645
pckA-R	GGATGTCAGTAGACACATAC	1893129–1893148
RNAIII-F	GTGATGGAAAATAGTTGATGAG	5
RNAIII-R	GTGAATTTGTTCACTGTGTCG	5
spa-F	TgAATTCGTAAACTAGGTGTAGG	46
spa-R	cggTaCCAGGCTTGTTATTGTCTTCC	46
tetL-F	CCTGTTATAAAAAAAGGATC	17
tetL-R	CCATATTGTTGTATAAGTG	17
T7-capA59	taatacgactcactatagggagAATGGAAAGTACATTAGAA	153404–153422
T7-gyr	taatacgactcactatagggagATTATGGTGCTGGGCAAATACA	20

[a] Lowercase letters represent nucleotide additions.

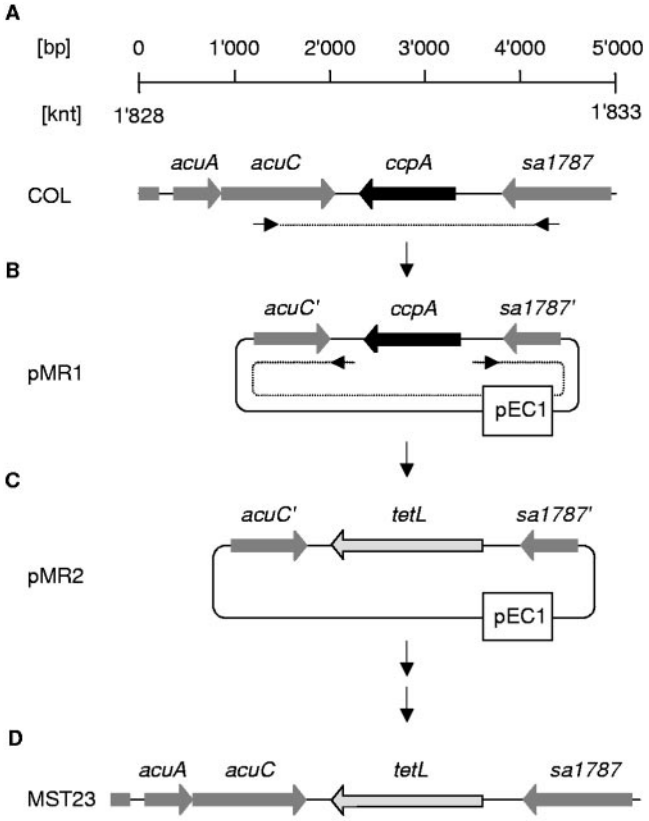


FIG. 1. Schematic representation of the *ccpA* region of *S. aureus* and of the strategy used to obtain MST23 (COLn Δ*ccpA*). The genetic organization of the *S. aureus* COL *ccpA* region (A), pMR1 (B), pMR2 (C), and MST23 (D) is shown. Open reading frame notations and nucleotide numbers correspond to those of the respective genomic regions of strain COL (GenBank accession no. NC_002951). Primers used to amplify the *ccpA* region (A), the *ccpA*-flanking regions including the backbone of pEC1 (B), and the respective PCR products (dotted lines) are indicated.

fragment containing the pEC1 backbone and the regions flanking *ccpA* by use of the primer pair ccpAko-F/ccpAko-R. The 5.8-kb fragment was ligated to a PCR-amplified and phosphorylated 1.7-kb *tet*(L) cassette obtained from plasmid pBT (17) by use of primer pair tetL-F/tetL-R to generate the suicide vector pMR2, and the plasmid was subsequently electroporated into *S. aureus* RN4220. Mutants with the allelic replacement were selected for tetracycline resistance and screened for loss of erythromycin resistance, yielding MST04 (RN4220 *ccpA*::*tet*[L]), which was subsequently used as a donor for transducing the *ccpA* deletion into other *S. aureus* strains.

Construction of plasmid pMST1. A 1.76-kp fragment, covering the *ccpA* gene and 770 bp of its upstream region, was amplified by PCR from chromosomal DNA of *S. aureus* COL by use of primer pair MST12/MST14 (Table 2), digested, and cloned into the BamHI/XbaI site of vector pAW17 (46) to generate plasmid pMST1. The plasmid was first electroporated into *S. aureus* RN4220 and then transduced into the various *ccpA* mutants.

Determination of acetate, glucose, and lactate levels. Aliquots (2 ml) of bacterial cultures were harvested at the indicated time points and centrifuged for 2 min at 16,000 × *g*. The supernatants were incubated at 80°C for 15 min and stored at −20°C until use. Acetate, glucose, and lactate levels were determined with kits from R-Biopharm (Darmstadt, Germany) according to the manufacturer's directions.

Adherence studies. *S. aureus* strains were grown in brain heart infusion (BHI) at 37°C in a shaking water bath at 250 rpm for 3 h to midexponential phase (A_{600} of 1) and used to inoculate 1 ml prewarmed BHI in 24-well plates containing presterilized polyethylene terephthalate (Thermanox) 13-mm disks (Life Technologies, Basel, Switzerland) to a starting A_{600} of 0.05 and incubated without shaking at 37°C for 15 h before fixation for scanning electron microscopy. Fixation and electron microscopy were carried out as described earlier (21).

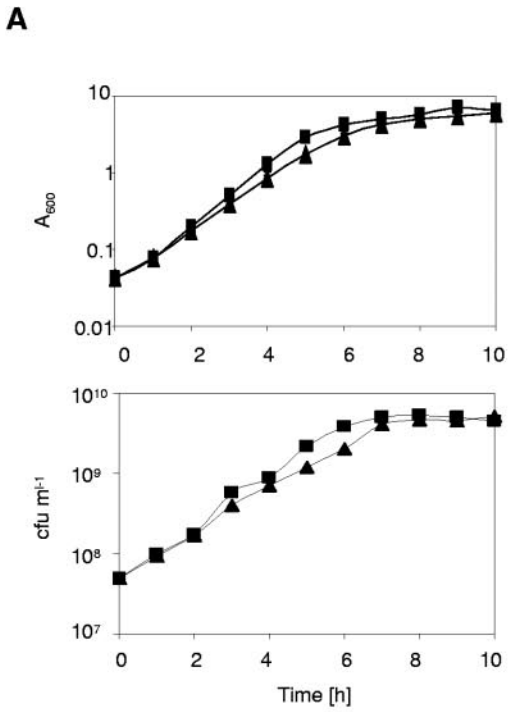
Susceptibility testing. For testing antibiotic resistance on gradient plates, plates were prepared by pouring 35 ml of LB agar containing 1,000 μg ml^{-1} oxacillin into a rectangular inoculum dish (Dynatech, Dübendorf, Switzerland) that was reposed on one side to allow the solidifying LB agar to form a wedge. In a second step, the solidified LB agar plate was placed horizontally and 35 ml of LB agar lacking the antibiotic was poured onto the first layer and allowed to solidify for 3 h, thereby allowing the antibiotic of the lower layer to diffuse into the upper layer to form a gradient. Cells of the strains to be tested were resuspended in physiological NaCl solution to a density of 0.5 McFarland (McF 0.5) and swabbed onto the plate along the gradient. Growth was read after 24 h and 48 h of incubation at 35°C. For E-tests, bacterial suspensions (McF 0.5 for oxacillin and McF 2 for teicoplanin) were swabbed onto the surface of either Mueller-Hinton agar plates supplemented with 2% NaCl (oxacillin) or BHI agar plates (teicoplanin) according to the manufacturer's instructions (AB-Biodisk, Solna, Sweden). Determinations of MICs by broth microdilution were performed as recommended by the CLSI (formerly NCCLS) (9). For population analysis profiles, appropriate dilutions of an overnight culture were plated on LB agar plates containing increasing concentrations of oxacillin (0 to 2,048 μg ml^{-1}) and the numbers of CFU were determined after 48 h of incubation at 35°C.

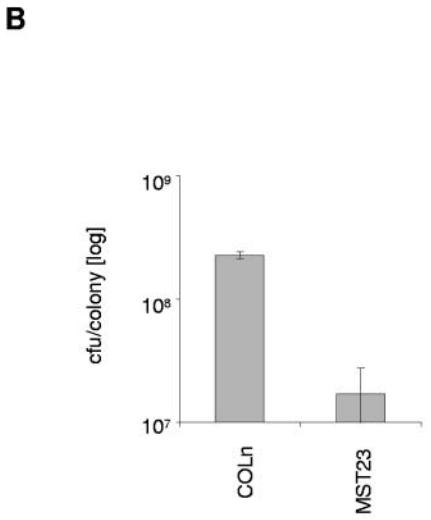
Northern blot analyses. For in vitro growth studies, overnight cultures of *S. aureus* were diluted 1:100 into fresh prewarmed, HEPES-buffered LB medium (pH 7.5). Cells were grown either with or without 10 mM glucose, samples were removed from the cultures after 1, 3, 5, and 8 h of growth and centrifuged at 13,000 × *g* and 4°C for 5 min, and the cell sediments were snap-frozen in liquid nitrogen. In a second approach, cells were grown in HEPES-buffered LB to an A_{600} of 1, the cultures were split in two, and 10 mM glucose added to one half. Aliquots were sampled at 0, 10, 20, and 30 min and harvested at 16,000 × *g* at room temperature for 1 min, and the cell sediments were snap-frozen in liquid nitrogen. Total RNAs were isolated according to the method of Cheung et al. (6). Blotting, hybridization and labeling were performed as previously described (17). The intensities of the 23S and 16S rRNA bands stained with ethidium bromide were verified to be equivalent in all the samples before transfer. Primer pairs ccpA-F/ccpA-R, hla-F/hla-R, pckA-F/pckA-R, RNAIII-F/RNAIII-R, and spa-F/spa-R (Table 2) were used, respectively, to generate digoxigenin-labeled *ccpA*-, *hla*-, *pckA*-, *RNAIII*-, and *spa*-specific probes by PCR labeling. Data shown were confirmed in at least two independent experiments.

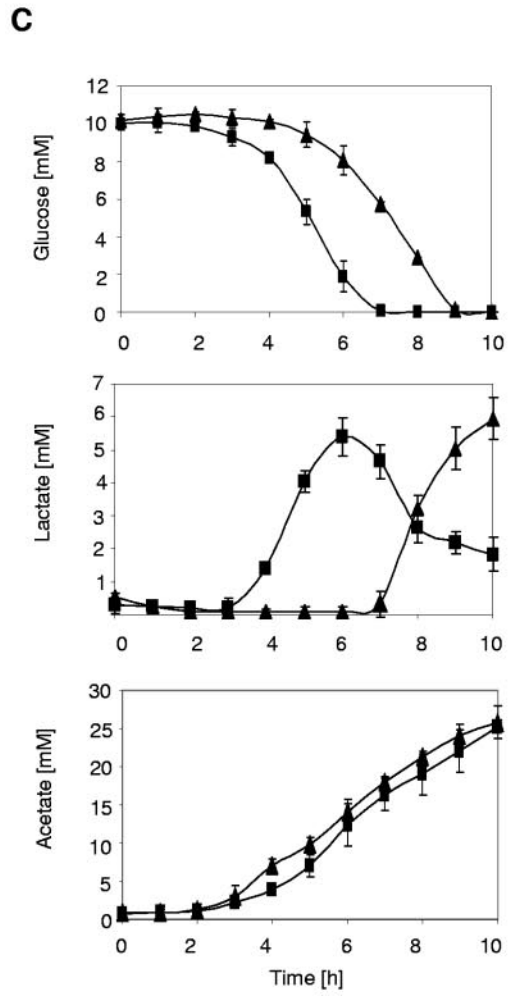
RNA quantification by LightCycler RT-PCR. For quantification of transcripts by LightCycler reverse transcription-PCR (RT-PCR), RNA preparations were performed as described earlier (19). Briefly, approximately 10^9 *S. aureus* cells were lysed in 1 ml TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (BioSpec Products, Bartlesville, OK) (0.1 mm diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, N.Y.). RNA was isolated as described in the instructions provided by the manufacturer of TRIzol. Contaminating DNA was degraded by digesting RNA samples with DNase as described before (19). Sequence-specific RNA standards for quantitative RT-PCR were engineered as described previously (20) using primer pair T7-gyr/gyr864 for *gyrB* and primer pair T7-capA59/capA710 for *capA* (Table 2).

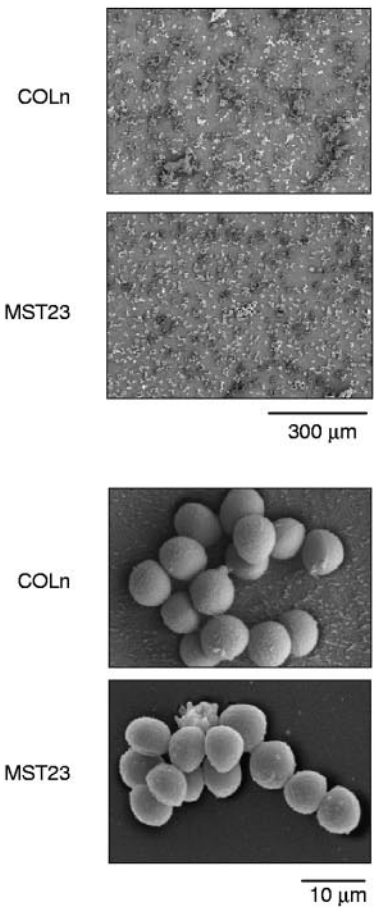
LightCycler RT-PCR was carried out using a LightCycler RNA amplification kit for hybridization probes or with a LightCycler RNA amplification kit and SYBR green I (Roche Biochemicals). Master mixes were prepared following the manufacturer's instructions using the oligonucleotides gyr297/gyr574 for *gyrB* (20) and primer pair capA167/capA501 for *capA* (Table 2). Specific primers were selected in such a way that they bound to an internal part of the respective RNA standard. Standard curves were generated using 10-fold serial dilutions (10^4 to 10^8 copies/μl) of the specific RNA standards. The number of copies of each sample transcript was then determined with the aid of the LightCycler software. At least two independent RT-PCR runs were performed for each sample. The specificity of the PCR was verified by ethidium bromide staining on 3% agarose gels. To check for DNA contamination each sample and RNA standard was subjected to PCR using a LightCycler DNA amplification kit and SYBR green I (Roche Biochemicals). No amplification product was detectable in any of the cases.

Capsular polysaccharide serotype 5 (CP5) detection by indirect immunofluorescence. CP5 production was determined from cultures grown for 24 h in LB medium. Slides with heat-fixed bacteria were washed three times with phosphate-buffered saline (PBS)–0.05% Tween 20 and incubated with 0.2 mg ml^{-1} human immunoglobulin (IgG) (Sigma, Deisenhofen, Germany) diluted in PBS–0.05% Tween 20 for 30 min to prevent unspecific binding of IgG by cell-wall-associated protein A. The slides were incubated with mouse IgM monoclonal antibodies to CP5 (22) diluted 1:50 in PBS–0.05% Tween 20 for 1 h followed by incubation with CY3-conjugated anti-mouse F(ab)$_2$ fragment (Dianova, Hamburg, Ger-









many) diluted 1:500 in PBS–0.05% Tween 20 for 1 h. Bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (2 μg ml^{-1}) for 5 min, washed three times with water, and air dried. The slides were then mounted with fluorescent mounting medium (DakoCytomation, Hamburg, Germany), and positively stained bacteria were detected using fluorescence microscopy.

RESULTS AND DISCUSSION

Growth and carbohydrate utilization of *S. aureus* Δ*ccpA* mutants. The *ccpA* gene was deleted in strain RN4220 by allelic replacement (Fig. 1) and transduced therefrom into the capsular polysaccharide serotype 5 (CP5)-producing strain Newman and the methicillin-resistant strain COLn, yielding strains MST14 and MST23, respectively. Analysis of the transcript sizes of the genes surrounding *ccpA*, i.e., *acuAC*, encoding acetoin utilization protein A and C, and *saCOL1787*, thought to encode a chorismate mutase–phospho-2-dehydro-3-deoxyheptonate aldolase, yielded identical patterns for Newman and its Δ*ccpA* mutant MST23, indicating that the genetic manipulations leading to the deletion of *ccpA* did not affect the integrity of the adjacent genes (data not shown).

The growth characteristics and metabolite production of strain COLn and its Δ*ccpA* mutant MST23 were monitored in HEPES-buffered LB supplemented with 10 mM glucose. Comparison of the wild-type and mutant cultures revealed a clear difference only for the mid- to late-exponential-growth phases (i.e., hours 3 to 6). During these growth periods, the mutant displayed slower growth, yielding cell densities lagging approximately 1 h behind the wild-type culture densities. However, the mutant culture reached almost the same A_{600} values after 10 h of growth (Fig. 2A), and no differences in A_{600} values were observable between the wild type and the mutant after 16 and 24 h of growth (data not shown). CFU determinations of the growing cultures showed the same tendency, being different only during the mid- to late-exponential-growth phase, and again, no differences in growth yield were observed after an incubation period of 24 h (data not shown). During the exponential-growth phase, differences in growth rate were also visible with respect to doubling times; the wild type yielded a significantly lower doubling time (44.34 ± 0.65 min) than MST23 (51.67 ± 0.75 min; $P < 0.01$). A clearer difference was observed between the wild type and the mutant on solid media. MST23 produced significantly smaller colonies on sheep blood agar or on Muller-Hinton plates, with lower CFU numbers per colony than COLn after 48 h of incubation (Fig. 2B). No differences were observed when COLn and MST23 were checked for cell size and adherence properties (Fig. 2D), signaling that the differences in growth on solid media were likely to be due to a reduced growth rate of the mutant.

In addition to their growth kinetics, growing COLn and MST23 cultures were further analyzed for glucose metabolism and breakdown. The glucose level of the wild-type culture visibly decreased from hour 3 on and glucose was depleted after hour 7. The glucose level of the MST23 culture started to decrease from hour 5 and was depleted only after 9 h of growth (Fig. 2C). Simultaneously with glucose degradation, the lactate level of the wild-type culture started to increase from hour 3 on, reached its maximum around hour 6, and significantly decreased thereafter. In contrast, the lactate level of the mutant culture started to increase only after 7 h of growth, representing a delay of more than 2 h compared with glucose consumption, and reached its maximum after 10 h. Interestingly, only slight differences were observable between the wild type and the mutant with respect to acetate accumulation; the acetate level seen with the mutant culture preceded that of the wild-type culture by approximately 0.5 h. After 24 h of growth, no lactate was present in either wild-type or mutant media anymore, while acetate levels were reduced to similar amounts, namely, 3.8 ± 0.86 mM for COLn and 3.75 ± 0.76 mM for MST23, signaling that the *ccpA* mutation did not affect the ability of the mutant to assimilate acetate and lactate that was excreted into the media during earlier growth stages.

Although the growth rate and yield of the *ccpA* mutant MST23 were only slightly affected by the deletion, the slower glucose consumption and delayed lactate secretion of MST23, paired with the slightly increased acetate production, suggested that CpA seemed to exert a positive effect on lactate production and secretion whereas acetate production and secretion seemed to be negatively affected by this protein, taking into account that MST23 possessed lower cell densities and consumed glucose slower than the wild type at almost all time points analyzed. Preliminary Northern analyses supported the hypothesis that CcpA might stimulate lactate and suppress acetate formation in the presence of glucose. Expression of *ldh1* (*saCOL0222*), thought to encode L-lactate dehydrogenase 1 (EC 1.1.1.27) that is believed to catalyze the conversion from pyruvate to lactate when *S. aureus* is grown in the presence of a rapidly catabolizable carbon source under anaerobic conditions, was highly induced in wild-type cells grown in the presence of glucose but was not detectable in the absence of glucose or in the Δ*ccpA* mutant under either growth condition. Expression of genes encoding enzymes involved in acetate formation, such as *pdhABCD*, encoding components of the pyruvate dehydrogenase multienzyme complex thought to catalyze the conversion of pyruvate to acetyl coenzyme A, and the *adhE* homologue (*saCOL0135*) thought to encode alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10) that catalyzes the formation of acetyl-coenzyme A to acetaldehyde, on the other hand, was found to be repressed in the wild type in the presence of glucose, while no differences in expression were observed in the Δ*ccpA* mutant in either the presence or absence of glucose (K. Seidl, unpublished data).

Effect of *ccpA* on antibiotic resistance. CcpA was among a series of auxiliary factors reported to reduce methicillin resistance in strain COL upon Tn*551* inactivation (11). We

FIG. 2. Growth characteristics of COLn (squares) and its Δ*ccpA* mutant MST23 (triangles) in LB supplemented with 10 mM glucose. (A) Absorbances at 600 nm (A_{600}) and CFU over the growth cycle. (B) Cfu per colony of COLn and MST23 grown on sheep blood agar for 48 h at 35°C. (C) Glucose, acetate, and lactate concentrations in the culture supernatants corresponding to panel A. The data presented are mean values of three independent experiments. (D) Scanning electron microscopy images of COLn and MST23 adhering to polyethylene terephthalate (Thermanox) disks.

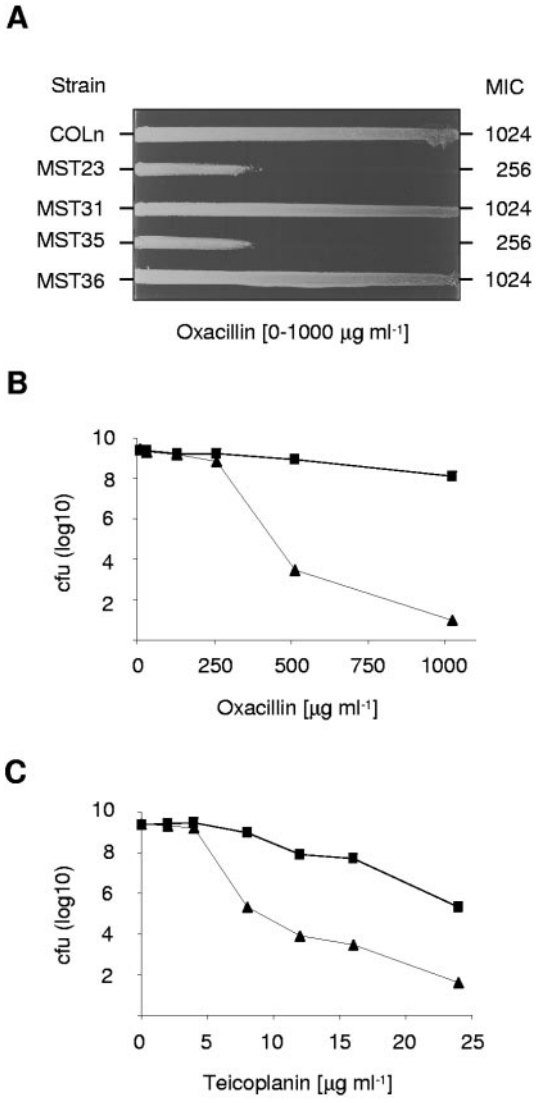


FIG. 3. Susceptibility of COLn and NM143 and their isogenic Δ*ccpA* mutants. (A) Effects of *ccpA* inactivation and complementation on oxacillin resistance. The methicillin-resistant strain COLn, its Δ*ccpA* mutant MST23, control strains MST31 (COLn pAW17) and MST35 [COLn Δ*ccpA*::*tet*(L) complemented with control plasmid pAW17], and strain MST36 [COLn Δ*ccpA*::*tet*(L) complemented with plasmid pMST1] were swabbed along a plate containing an oxacillin gradient. The corresponding MICs of oxacillin (in micrograms per milliliter as determined by E-Test and broth microdilution) for the strains are indicated. (B) Population analysis profiles of COLn (squares) and MST23 [COLn Δ*ccpA*::*tet*(L); triangles] on oxacillin. (C) Population analysis profiles of the step-selected glycopeptide-intermediate-resistant *S. aureus* derivative NM143 (squares) and its Δ*ccpA* mutant KS30 (triangles) on teicoplanin.

demonstrated here a fourfold reduction of the oxacillin MIC for strain COLn upon *ccpA* inactivation and full restoration to its original level by *trans*-complementation with the wild-type *ccpA* allele under the control of its native promoter (Fig. 3A), thus confirming experimentally that the effect was indeed solely CcpA dependent. Moreover, the population analysis profile (Fig. 3B) showed that the homogenous oxacillin resistance of strain COLn was reduced to heterogeneous resistance by *ccpA* inactivation.

Transduction of *ccpA*::*tet*(L) into NM143, the step-selected teicoplanin-resistant derivative of strain Newman, yielding strain KS30, had a similar negative effect on teicoplanin resistance. The *ccpA* inactivation reduced the MIC of teicoplanin from 24 to 12 μg ml^{-1}, and the population analysis profile teicoplanin showed that the number of more highly resistant variants was reduced by a magnitude of over 10^3 (Fig. 3C).

Effect of glucose and *ccpA* on virulence determinant production. Previous studies showed that fermentation of glucose and/or the accompanying decrease in pH affected expression of the global regulator *agr* and of virulence factors such as α-hemolysin (*hla*) and the staphylococcal enterotoxins A, B, and C (*sea*, *seb*, and *sec*) (12, 24, 27, 44, 45). More recently, Weinrick and coworkers (62) showed that mildly acidic conditions (pH 5.5) influenced the expression of a variety of genes, including *agr*, *hla*, and *spa*, encoding protein A, and concluded that changes in staphylococcal gene expression formerly thought to represent a glucose effect might be largely the result of declining pH of the growth medium due to the fermentation of the supplemented carbon source. The effect of glucose on *hla* and *spa* expression is further complicated by the fact that both genes are affected by a complex regulatory network including *agr* and further regulatory elements such as ArlRS (15), MgrA (25), MsrR (46), Rot (37, 47), SaeRS (18, 20), SarA (reference 8 and references therein), SarS (7, 36, 53), SarT (48), SvrA (16), TcaR (36), and the alternative transcription factor σ^B (2, 20, 23), with *rot*, *sae* and *tcaR* expression being pH dependent as well (62).

To elucidate whether *agr*, *hla*, and *spa* transcription was affected by glucose independently from the fermentation-dependent pH and to find out whether CcpA may be involved in mediating such a glucose effect, we monitored the expression levels of these genes in COLn and its *ccpA* mutant MST23, grown in buffered LB in the presence or absence of glucose (Fig. 4). *pckA*, encoding a phosphoenolpyruvate carboxykinase, shown to be affected by glucose, and predicted to be regulated by CcpA in *S. aureus* (49), was included in this study as well. The 50 mM HEPES concentration used here to buffer the medium to pH 7.5 had no inhibiting effect on the growth kinetics and kept the pH fairly constant (Fig. 4A), while concentrations higher than 50 mM were growth inhibitory (data not shown). No changes in pH were observed when COLn and MST23 were grown in LB in the absence of glucose. In the glucose-supplemented wild-type culture, the pH started to drop slightly after 5 h to a final pH of 7, while the pH of the glucose-supplemented MST23 culture dropped from hour 7 on to pH 7.25. Growth of COLn in unbuffered glucose-supplemented LB medium would have caused a drop in pH to 5.5 already after 3 h of growth (data not shown). Addition of glucose produced a higher growth rate and growth yield in the wild type from hour 5 on (Fig. 4A). Surprisingly, glucose seemed to have a slightly inhibiting effect on the growth rate of the mutant MST23 during exponential-growth phase, as seen in the lower A_{600} values for MST23 grown in LB supplemented with glucose compared to those in LB alone; this effect might be due to a slightly increased lag phase.

Expression of *ccpA*, *pckA*, *RNAIII*, *spa*, and *hla* was monitored after 1, 3, 5, and 8 h of growth. In COLn grown in LB,

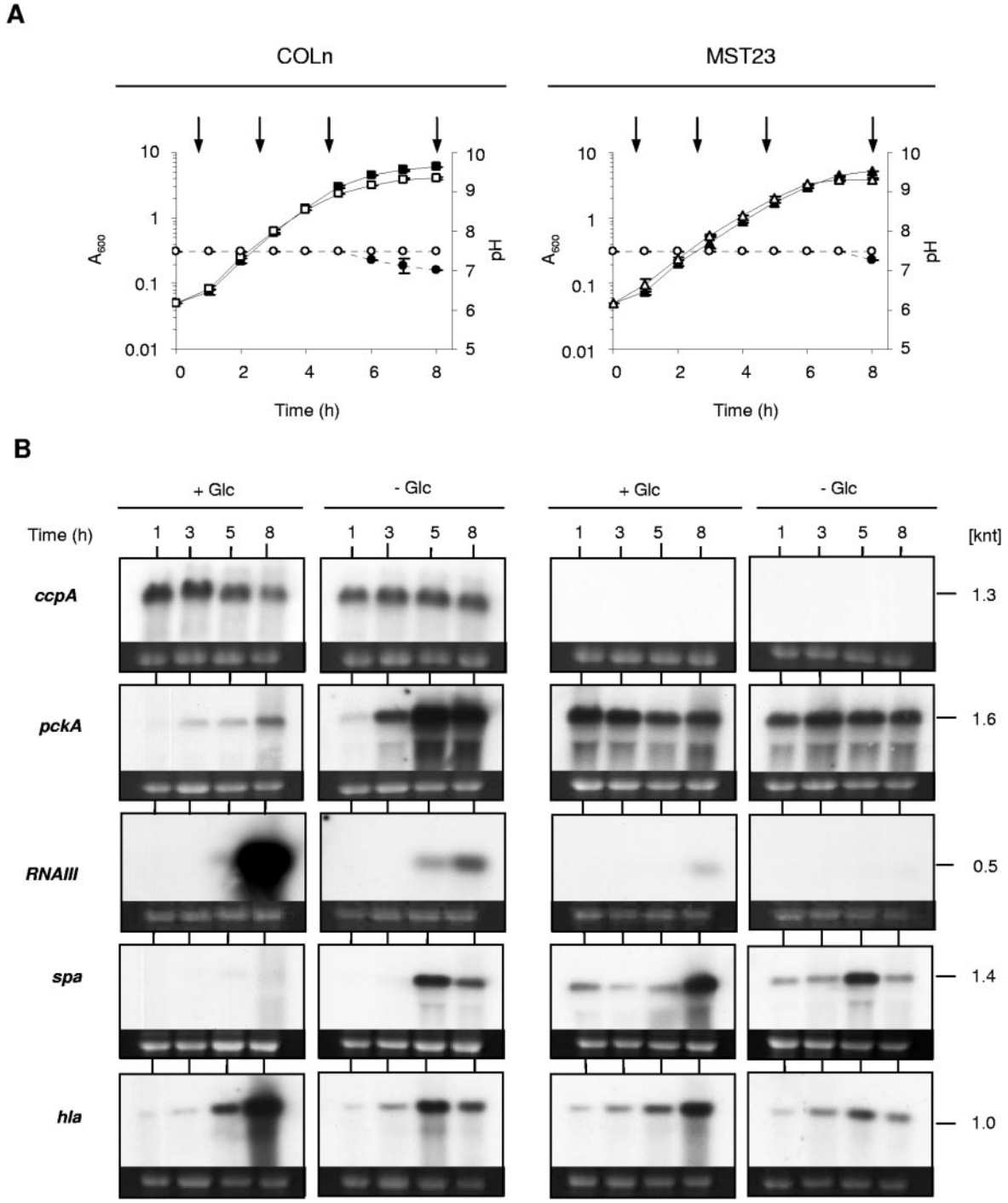


FIG. 4. Northern blot analyses of COLn and its Δ*ccpA* mutant MST23 during growth. (A) Growth characteristics of COLn (squares) and MST23 (triangles) grown in HEPES-buffered LB (open symbols) and HEPES-buffered LB supplemented with 10 mM glucose (closed symbols). At 1-h intervals, an aliquot (2 ml) was removed, the absorbance at 600 nm was measured, and the pH in the culture supernatants (circles) was determined. The results presented are mean values of at least three independent experiments. Time points of sampling for the Northern blot analyses are indicated by arrows. (B) Transcription of *ccpA*, *hla*, *pckA*, *RNAIII*, and *spa* in COLn and MST23 during growth in HEPES-buffered LB (−Glc) and in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Relevant transcript sizes and time points of sampling are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

expression of *ccpA* was found to be fairly constant at all growth stages analyzed (Fig. 4B). Supplementation of glucose seemed to increase the expression of this gene during the early growth stages (i.e., 1 to 3 h), suggesting that expression of *ccpA* might be positively affected by glucose, a phenomenon that has been observed for *ccpA* in other gram-positive organisms (14). No *ccpA* transcripts were detectable in MST23, confirming that the deletion had occurred as intended. Expression of *pckA*

increased with growth and was clearly higher when COLn cells were grown in the absence of glucose, confirming previous findings indicating that *pckA* expression is negatively affected by glucose (49). MST23, on the other hand, already produced *pckA* transcripts at a rather constant and high level early in growth. No significant differences in *pckA* expression were observed in the presence or absence of glucose, signaling that the effect of glucose on *pckA* expression was mediated via CcpA, as has been suggested by Scovill et al. (49). The idea of CcpA-dependent repression of *pckA* transcription is further supported by the presence of a putative CRE in the promoter region of *pckA* (49) that almost perfectly matched (17/18 nt) the CRE consensus of *B. subtilis* (38), suggesting that the CRE sequences might be similar in the two organisms.

Expression of *RNAIII* from the *agr* locus is known to increase during later growth stages (reference 63 and references within). Accordingly, in COLn cells grown in LB, *RNAIII* transcripts were first detected after 5 h of growth and increased with time. Unexpectedly, in glucose-grown cells, a strong increase in *RNAIII* expression occurred at hour 8, contrary to the findings of Regassa et al. (45), who reported unchanged *RNAIII* transcript levels for *S. aureus* cells grown in a fermentor in either the presence or absence of glucose at a constant pH. However, the discrepancy in *RNAIII* expression might be explained by the differing growth conditions, since Regassa and coworkers kept the glucose concentration at a constant level of 100 mM. It is noteworthy that the increase in *RNAIII* expression was detected at a time point when all glucose was exhausted from the medium, indicating that the depletion of glucose might have triggered a signal that induced *RNAIII* transcription, which was not present under the conditions used by Regassa et al. (45). The slight decrease in pH observed in the later growth stages of the wild-type culture in response to glucose (Fig. 4A) could be excluded as a reason for the strong induction of *RNAIII* transcription, since control experiments performed using a fermenter and an equivalent experimental setup allowed the pH to be kept constant and yielded the same induction pattern (data not shown).

No *RNAIII* transcripts were detectable in MST23 cells grown in LB, and addition of glucose yielded only traces of *RNAIII* transcripts after 8 h of growth. The clear differences in *RNAIII* transcript levels observed for COLn and MST23 suggest that the presence of a functional CcpA had a positive effect on *RNAIII* expression. However, this effect was likely to be indirect, since the screening of the *agr* locus did not reveal any apparent CRE in this genomic region that would fit with the CRE consensus of *B. subtilis* (38). Expression of *spa* was noticeably affected by glucose in the wild type. While *spa* transcripts were clearly detectable in COLn during later growth stages (hours 5 to 8) in the absence of glucose, *spa* transcripts were hardly detectable in the presence of glucose (Fig. 4B). However, in MST23, *spa* transcripts were detectable over the whole growth cycle independently of the presence or absence of glucose, although the *spa* transcription profiles seemed to differ between these two conditions to a certain degree. While *spa* expression in MST23 grown in LB peaked around hour 5, analogous to the situation found for the wild type, in the glucose-supplemented LB, *spa* transcription seemed to be highest at the latest time point monitored (i.e., hour 8). Interestingly, significant amounts of *spa* transcripts were already detectable in MST23 during the early growth stages (hours 1 to 3), irrespective of whether glucose was present in the growth media or not, signaling that CcpA might act as a negative regulator for *spa* expression during these growth stages. Expression of *hla* was found to be less affected by glucose and/or CcpA compared with *RNAIII* and *spa* expression results. In COLn grown in unsupplemented LB, *hla* transcripts were detectable from hour 1 on and peaked around the transition from late logarithmic-growth phase to stationary phase. Supplementation of glucose shifted the peak expression of *hla* to the last growth point monitored and seemed to increase the expression level at this growth stage, probably due to the action of *RNAIII*, which was, as shown above, found to be highly expressed under these conditions. Essentially the same *hla* expression patterns as those identified in the wild type were found for MST23, although the overall amounts of *hla* transcripts seemed to be slightly reduced in the *ccpA* mutant.

Since *hla* and *spa* expression are known to be under multiple levels of control, including that by *RNAIII*, it was difficult to judge from the results described above whether the effects observed for *hla* and *spa* were the result of direct CcpA-mediated regulation in response to glucose or whether they might represent a secondary effect of *RNAIII* and other regulatory elements that were not part of this study. To better define the impact of glucose and, in particular, of CcpA on the expression of these two virulence factors, we performed a second series of Northern blot experiments, this time monitoring the expression of *ccpA*, *hla*, *pckA*, and *spa* in cells that were grown to midexponential-growth phase (A_{600} = 1). At this time point, glucose was added to one half of the culture, and cells were harvested in 10-min intervals from 0 to 30 min (Fig. 5). This procedure was likely to abolish the effect of *agr* on *hla* and *spa* expression, since *RNAIII* expression was barely detectable at this time point and under these conditions (data not shown). Moreover, by analyzing the gene expression immediately after the addition of glucose, we assumed that we would be more likely able to identify direct CcpA-dependent effects, since secondary effects were expected to occur with a certain delay. Addition of glucose to the exponentially growing cultures did not result in a temporary growth arrest of these cultures, indicating that the glucose addition did not trigger any inhibition of the primary metabolism (data not shown).

In this second series of Northern blot analyses, the level of expression of *spa* in unsupplemented LB appeared to be roughly constant, only slightly increasing with time, in agreement with the previous findings. Addition of glucose, however, resulted in a clear decrease in *spa* transcription that was already visible after 10 to 20 min in the wild type; this effect was not seen with MST23 (Fig. 5A). Moreover, the levels of *spa* transcripts in MST23 seemed clearly to be higher than those found in COLn, supporting the hypothesis that CcpA of *S. aureus* acts as a direct negative regulator of *spa* expression, either in the absence of glucose or, in a stronger way, in the presence of glucose. Further support for CcpA regulating *spa* expression is given by the fact that the *spa* coding region is preceded by a putative CRE consensus sequence (Fig. 5B). Interestingly, a potential CRE element that perfectly matched with the *B. subtilis* CRE consensus was further identified in the genomic region upstream of the open reading frame of *hla*, signaling that α-hemolysin production might be subjected to a

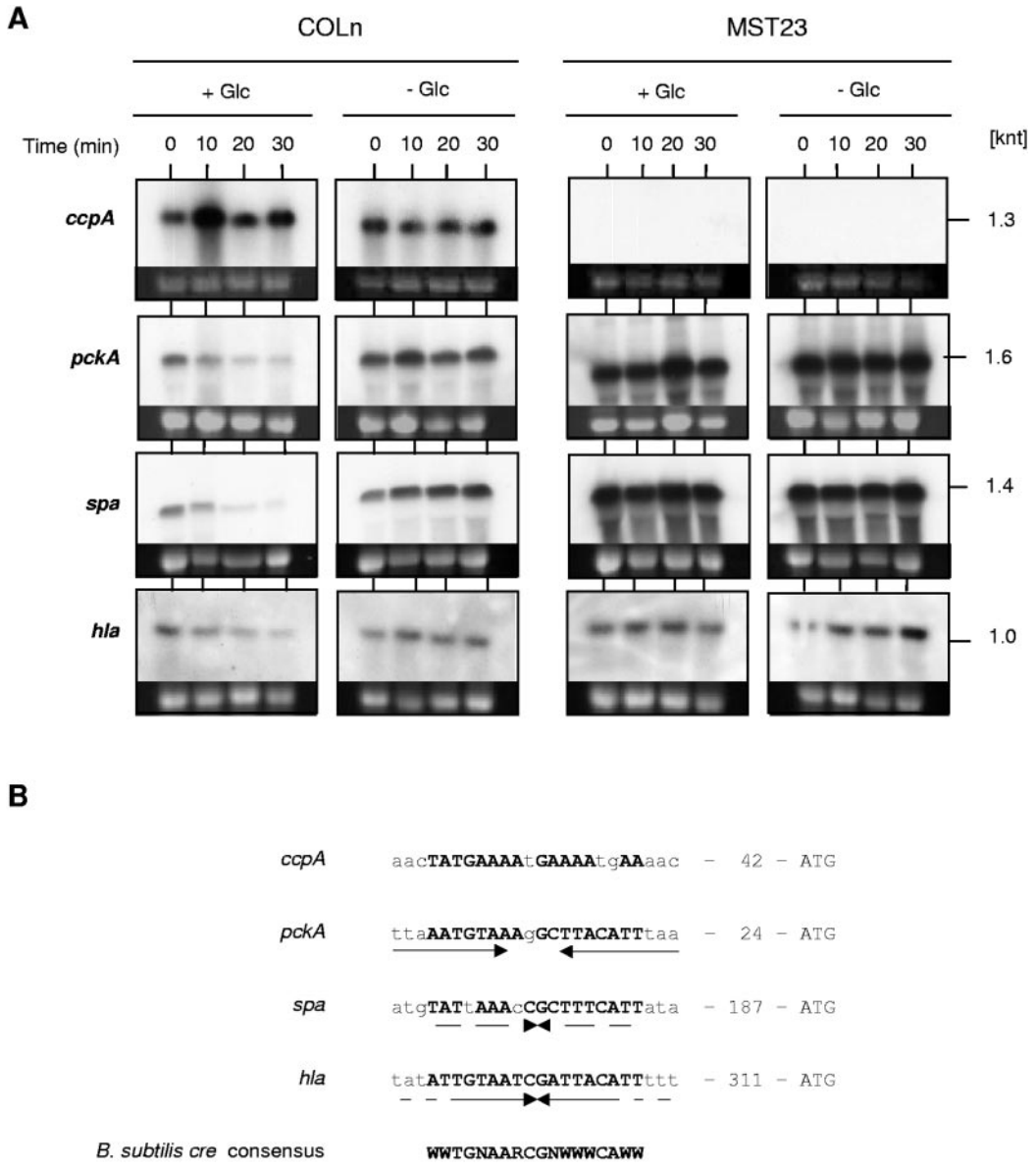


FIG. 5. Effect of glucose addition on the gene expression of COLn and its Δ*ccpA* mutant MST23. (A) Northern blot analyses of *ccpA*, *hla*, *pckA*, and *spa*. Cells were grown in HEPES-buffered LB to midexponential-growth phase (A_{600} = 1), cultures were split in half, and 10 mM glucose was added to one half (+Glc) while the other half was left unchanged (−Glc). Relevant transcript sizes and time points of sampling are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading. (B) Putative CREs identified upstream of *ccpA*, *hla*, *pckA*, and *spa*. Nucleotides fitting with the CRE consensus of *B. subtilis* (38) are highlighted in bold type. Inverted repeats are indicated by arrows. Ambiguity codes are as follows: W denotes A or T, R denotes G or A, and N denotes A, C, G, or T.

CcpA-dependent CCR as well. In line with this assumption, we identified a slight decrease in *hla* transcription in COLn after the addition of glucose that was not detected either in the wild type grown in unsupplemented LB or in the *ccpA* mutant under both sets of conditions (Fig. 5A).

In agreement with the results of the first series of Northern blot analyses, we found *pckA* expression to decrease in the wild type in response to the presence of glucose and to be constant in the absence of this sugar. Equivalent to the situation found with *spa*, expression of *pckA* appeared to be clearly increased in MST23, and addition of glucose again failed to exert an effect on *pckA* expression in the mutant, confirming the findings of Scovill et al. (49) showing that the presence of glucose represses *pckA* transcription and supporting the hypothesis raised by these authors that this negative regulatory effect is exerted via CcpA. Interestingly, expression of *ccpA* itself seems to be positively affected by glucose, since transcription of *ccpA* appeared to be increased in response to the addition of glucose, although this increase was only of a transient nature and was only detectable at 10 min after the sugar was added (Fig. 5A). A potential CRE was identifiable in the promoter region of *ccpA* (Fig. 5B), sharing 15 out of 18 nucleotides with the CRE consensus of *B. subtilis* but lacking the palindromic nature of CREs that was detectable in the CRE candidates of *hla*,

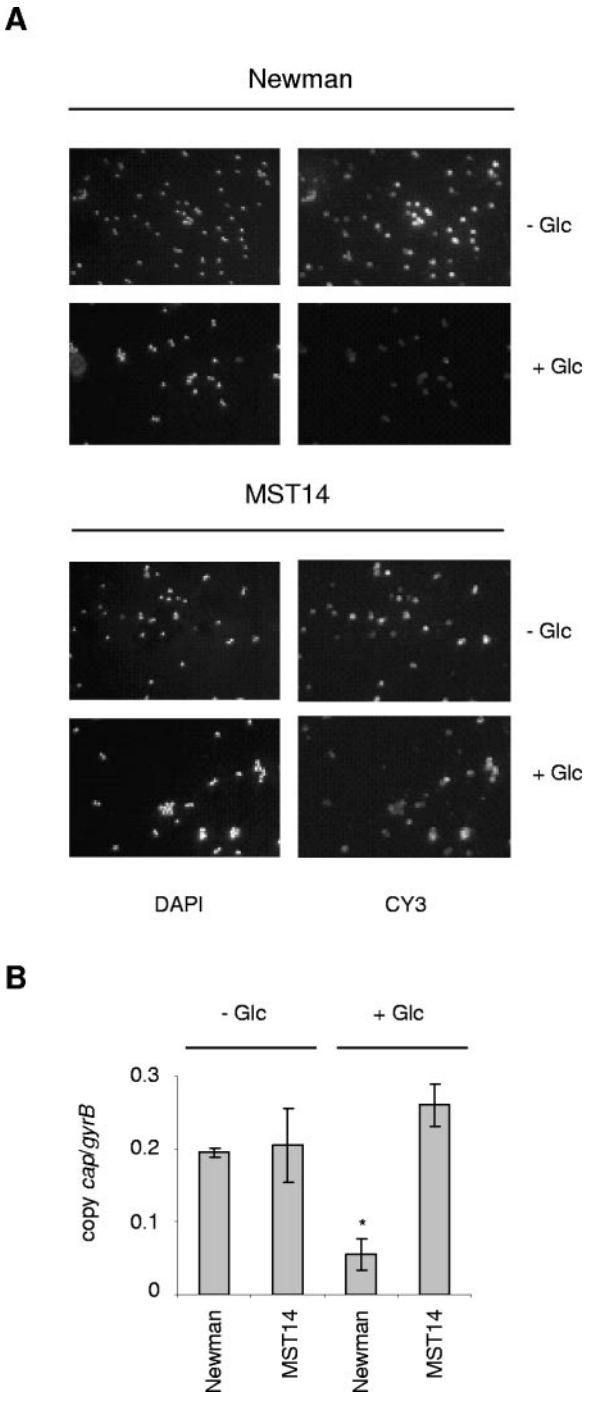


FIG. 6. Capsule production and *cap* expression of Newman and its Δ*ccpA* mutant MST14 in response to glucose. (A) CP5 expression determined by indirect immunofluorescence of strain Newman and its isogenic Δ*ccpA* mutant grown for 24 h at 37°C in HEPES-buffered LB (−Glc), or in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI), and marked with CP5-specific monoclonal antibodies and stained with Cy3-conjugated anti-mouse antibodies (CY3). (B) Quantitative transcript analysis of *capA* by LightCycler RT-PCR of strain Newman and its isogenic Δ*ccpA* mutant grown for 8 h at 37°C in HEPES-buffered LB (−Glc) or in HEPES-buffered LB supplemented with 10 mM glucose (+Glc). Transcripts were quantified in reference to the transcription of gyrase (in copies per copy of *gyrB*). Values from two separate RNA isolations and two independent RT-PCRs each were used to calculate the mean expression (±standard errors of the mean). Glc, glucose; asterisk, $P < 0.05$ for Newman without Glc, MST14 without Glc, and MST14 with Glc.

pckA, and *spa* and leaving the question currently open of whether the observed increase in transcription was mediated via CcpA by itself or not.

Effect of *ccpA* and glucose on capsular polysaccharide production. The majority of clinical isolates produce capsular polysaccharides of serotype 5 (CP5) or serotype 8 (CP8), which protect *S. aureus* against opsonophagocytic killing by polymorphonuclear leukocytes (28, 29, 34, 54, 59) and have been shown in a number of animal models of infection to enhance its virulence (40, 43, 54, 56, 61). Expression of CPs is influenced by various environmental signals in vitro and in vivo (reviewed in references 41 and 59), and transcription of the *cap* operon was shown to be affected by regulatory elements such as *agr*, *mgr*, *sae*, *sarA*, and the alternative σ-factor σ^B (2, 10, 33, 34, 35, 42, 51, 57). Both biosynthetic pathways for CP5 and CP8 production utilize precursors of the cell wall, such as UDP-*N*-acetylglucosamine, suggesting that CP-producing proteins might compete with cell-wall-producing enzymes for the availability of UDP-*N*-acetylglucosamine, thereby affecting the carbon flux of *S. aureus*. We therefore analyzed whether glucose, and specifically *ccpA*, might affect CP synthesis, as has been shown recently for the low G+C-content gram-positive pathogen *Clostridium perfringens* (58).

Since the CP serotype 5 strain COLn was found to produce only a little CP under the conditions tested (C. Wolz, unpublished data), the CP5 prototypic strain Newman and its Δ*ccpA* derivative MST14 were used to investigate the effect of glucose and CcpA on CP formation (Fig. 6). Newman wild-type and MST14 cells were grown for 24 h in LB medium in the presence or absence of 10 mM glucose, and the CP5 production was determined by indirect immunofluorescence using monoclonal antibodies raised against CP5 (Fig. 6A). In the absence of glucose, most of the wild-type cells produced CP5. However, in the presence of glucose, CP5 was abolished, indicating that glucose repressed CP formation. Interestingly, CP5 production in the *ccpA* mutant MST14 was not affected and was present irrespective of the presence or absence of glucose in the growth medium. The immunofluorescence data were confirmed by real-time PCR (Fig. 6B). The expression of the *cap* operon was almost indistinguishable between the wild type and mutant in the absence of glucose, whereas in the presence of glucose, strain Newman produced significantly fewer *cap* transcripts ($P < 0.05$) than MST14, which expressed *cap* in roughly the same amounts as in the absence of glucose. Both findings strongly suggested that the presence of glucose repressed CP formation and that this effect was, at least in part, mediated via CcpA on the transcriptional level, adding a further regulator to the complex network of regulatory elements and environmental conditions that control *cap* operon expression. However, since no apparent CRE was identifiable within the genomic region encoding the *cap* operon, it is again likely that the CcpA effect on *cap* transcription was of an indirect nature and might be mediated by downstream regulators.

Concluding remarks. Deletion of *ccpA* had a clear impact on the expression of *RNAIII* and on virulence factors of *S. aureus*, some of which have previously been shown to be affected by glucose. Interestingly, the deletion of *ccpA* produced an effect on gene expression not only in the presence but also in the absence of glucose, indicating that the function of CcpA might not be restricted to CCR. Our findings that CcpA of *S. aureus* influenced the transcription of at least five genes and operons, with most of them being involved in virulence of this pathogen, suggests that CcpA might represent an important global regulator of gene expression in *S. aureus* that, like that of its homologue in *B. subtilis*, may not be limited to regulating carbon uptake and metabolization. A preliminary computational screening of the *S. aureus* COL genome with the CRE consensus of *B. subtilis* (38) indeed indicated more than 110 CREs to be present in the promoter or N-terminal coding regions of genes and operons encoded by *S. aureus*, if allowing one mismatch to occur. Whole genome and proteomic analyses are currently ongoing to identify the CcpA regulon in *S. aureus*.

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