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Molecular Characterization and Transcriptional Analysis of Type 8 Capsule Genes in *Staphylococcus aureus*

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A 20.5-kb contiguous DNA fragment from *Staphylococcus aureus* Becker affecting type 8 capsule (CP8) biosynthesis was previously cloned. Sequencing analysis indicated that 16 open reading frames (ORFs) encoded within this fragment might be involved in CP8 synthesis. Using various plasmids containing DNA inserts derived from the 20.5-kb region, we showed by complementation of chemical mutants that 8 of the 16 ORFs were required for CP8 synthesis. To determine the involvement of the remaining eight ORFs, nonpolar gene-specific chromosomal mutations located in each of these ORFs were constructed. We found that three additional ORFs were also involved in the CP8 synthesis. Thus, 11 of the 16 ORFs were shown to affect CP8 synthesis. Complementation analyses of these 11 type 8 capsule (*cap8*) genes affecting CP8 production showed several promoters within the *cap8* gene cluster. However, by Northern hybridization using either the entire *cap8* gene cluster or the internal fragments of individual ORFs as probes, one 17-kb *cap8*-specific transcript was detected. Using *xylE* as the reporter gene, we found that the promoter at the beginning of the *cap8* operon was much stronger than any of the internal promoters. These results suggest that the *cap8* genes are transcribed mainly as a single large transcript. In addition, Southern hybridization analyses showed that *cap8H*, *cap8I*, *cap8J*, and *cap8K*, located in the central region of the *cap8* gene cluster, were CP8 specific.

Staphylococcus aureus strains producing type 5 capsular polysaccharide (CP5) and CP8 account for more than 80% of clinical staphylococcal isolates (2, 3, 14, 37). These strains are referred to as microencapsulated, as they produce a small amount of CP on the cell surface (47). In comparison, rarely isolated type 1 and type 2 strains produce a large amount of CP, which results in a mucoid phenotype when these strains are grown on solid agar plates. CP1 and CP2 have been shown to be antiphagocytic virulent factors (29, 30, 35). However, the role of CP5 and CP8 of microencapsulated strains in virulence has been controversial (1, 4, 16, 45, 48). The controversy may stem from the fact that different systems or animal models were used by different investigators. Nevertheless, most recently, Fattom et al. (10) were able to show that the antibodies against CP5 and CP8 were protective against *S. aureus* infections when immunized mice were challenged intraperitoneally. A recent study also suggested that CP5 and CP8 were adhesins (43).

CP8 is a trisaccharide-repeated polysaccharide with the following structure: →3)-4-*O*-Ac-β-D-ManNAcA*p*(1→3)-α-L-FucNAc*p*(1→3)-β-D-FucNAc*p*(1-. Its structure is almost identical to that of CP5 except for the location of O acetylation and the position of the linking of the monosaccharides (12, 17, 32). Molecular characterization of the genes required for CP expression in *S. aureus* has not been initiated until recently. Our laboratory has reported the cloning and characterization of a cluster of 13 *cap1* genes required for the biosynthesis of CP1 (22, 23, 29). The cloning of a type 5 capsule (*cap5*) gene from *S. aureus* Reynolds has also been reported (28). Most recently, we have reported the cloning of a 20.5-kb contiguous DNA fragment from type 8 strain Becker, using the *cap1* genes as probes under low-stringency conditions, and showed that the type 8 capsule (*cap8*) genes were clustered (41). We have also shown that most *S. aureus* strains contain sequences with extensive homology to the *cap8* gene cluster and that type 1 strain M possesses two capsule gene clusters, including one with extensive homology to the *cap8* gene cluster (41). In this communication, we report the molecular analyses of the *cap8* gene cluster by genetic complementation tests, promoter cloning, and Northern blot analysis. Sequence analysis of the *cap8* gene cluster will be reported elsewhere.

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MATERIALS AND METHODS

Strains, reagents, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were routinely cultivated either in Trypticase soy medium or in Columbia medium (Difco Laboratories, Detroit, Mich.). *Escherichia coli* HB101 and XL1-Blue were grown in Luria-Bertani medium (Difco Laboratories) and were used for plasmid transformation and plasmid preparation. *S. aureus* RN4220 was used as the recipient in electroporation which was carried out by the procedure of Kraemer and Iandolo (19). Phage 52A was used to transduce different plasmids from strain RN4220 to Becker or the Becker Cap8$^-$ mutant (22). Ethyl methanesulfonate (Sigma Chemical Co., St. Louis, Mo.) and *N-methyl-N′*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co.) mutageneses were performed as described by Miller (31). Becker Cap8$^-$ mutants were screened by an immunoblotting procedure and confirmed by Rocket immunoelectrophoresis (RIE) analysis using anti-CP8 rabbit antiserum (11) as described previously (41). Plasmid pCL52.2 was essentially the same as pCL52.1 (29) except that the multiple-cloning site was derived from pUC18. Plasmid pSL24 was derived from pLC4 (38) by replacing the multiple-cloning site with that of pUC18. Plasmid pCL10 was constructed by ligating a 2.3-kb *Pvu*II-*Hin*dIII fragment containing the ampicillin resistance gene and the origin of replication (originally derived from pBR322) from pLI50 (24) together with a 4.2-kb *Pst*I-*Pvu*II fragment containing the chloramphenicol resistance gene and the origin of replication (originally from pE194ts) from pTV1 (34) after Klenow treatment of the DNA fragments.

DNA manipulations. DNA manipulations were performed as described by Sambrook et al. (40). Plasmid DNA was purified by the method of Birnboim (6) and further purified by CsCl-ethidium bromide density gradient centrifugation or by using a plasmid kit (Qiagen, Inc., Chatsworth, Calif.). Bulk chromosomal DNA from *S. aureus* was purified by the method of Dyer and Iandolo (8). Rapid small-scale plasmid DNA purification was done by the method of Holmes and Quigley (15). The transfer of DNA to nitrocellulose membranes was by the procedure of Southern (44). The conditions used for Southern analysis have been described before (25). Restriction enzymes and other enzymes used in the study

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference
S. aureus strains		
RN4220	8325-4 (r^-)	20
8324-5	Capsule nontypeable	J. J. Iandolo
80CR3	Capsule typing not done	P. A. Pattee
M	CP1 strain	J. H. Hash
Smith	CP2 strain	ATCC[a]
Mardi	CP3 strain	A. Fattom
7007	CP4 strain	A. Fattom
Reynolds	CP5 strain	J. C. Lee
PS29	CP7 strain	J. J. Iandolo
Becker	CP8 strain	J. C. Lee
CYL5619	*cap8-1*	This study
CYL5620	*cap8-3*	This study
CYL5622	*cap8-5*	This study
CYL5623	*cap8-7*	This study
CYL5624	*cap8-9*	This study
CYL5626	*cap8-11*	This study
CYL5627	*cap8-14*	This study
CYL5628	*cap8-16*	This study
CYL5629	*cap8-18*	41
CYL5630	*cap8-32*	41
CYL5632	*cap8-50*(Ts)	41
CYL5643	*cap8-2*	41
CYL5645	*cap8-10*(Ts)	41
CYL5648	*cap8-19*	41
CYL5649	*cap8-20*	41
CYL5650	*cap8-21*	41
CYL5651	*cap8-23*	41
CYL5653	*cap8-27*(Ts)	41
CYL5656	*cap8-34*	41
CYL5657	*cap8-35*	41
CYL5660	*cap8-38*	41
CYL5664	*cap8-49*	41
CYL5666	*cap8-53*	41
CYL5668	*cap8-55*	41
CYL5669	*cap8-56*	41
CYL5670	*cap8-57*	41
CYL5673	*cap8-61*	This study
CYL5676	*cap8-64*	This study
CYL5952	*cap8K316*	This study
CYL5953	*cap8N368*	This study
CYL5974	*cap8C597*	This study
CYL6001	*cap8P458*	This study
CYL6052	*cap8I482*	This study
CYL6062	*cap8B511*	This study
CYL6132	*cap8A583*	This study
CYL6151	*cap8J615*	This study
Plasmids		
pLI50	Shuttle cloning vector	24
pCL8	Shuttle cloning vector	29
pCL10	Temperature-sensitive cloning vector	This study
pCL52.1	Temperature-sensitive cloning vector	29
pCL52.2	Temperature-sensitive cloning vector	This study
pSL24	*xylE* reporter gene fusion vector	This study

[a] ATCC, American Type Culture Collection.

were purchased from either GIBCO-BRL (Gaithersburg, Md.) or New England Biolab, Inc. (Beverly, Mass.). DNA sequencing was performed by using a sequencing kit from U.S. Biochemical Corp. (Cleveland, Ohio).

Complementation analyses. DNA fragments containing various *cap8* genes were cloned either in pLI50 or pCL8 (Table 1 and Fig. 1). All plasmids shown in Fig. 1 except pCL7941 were constructed by ligating the restriction enzyme-generated fragments to a suitable digested vector. The plasmid pCL7941, which contains a 3.8-kb insert (kb 14.4 to 18.2) (Fig. 1) was generated by deleting the insert of pCL7841 from the left end (at kb 14.0) by using *Bal*31. All the plasmids constructed were electroporated into strain RN4220 and then transduced to Becker Cap8$^-$ mutants by using phage 52A. The transductants were assayed for CP8 production by the immunoblotting procedure and RIE analysis using anti-CP8 rabbit antiserum.

Construction of gene-specific mutants. To construct various mutants with mutations in *cap8A*, *cap8B*, *cap8C*, *cap8I*, *cap8J*, *cap8K*, *cap8N*, and *cap8P*, plasmids pCL7979, pCL7922, pCL7870, pCL7932, pCL8015, pCL7869, pCL7877, and pCL7918 containing mutations located in the respective open reading frames (ORFs) were first constructed as described below. To construct pCL7979, a 2.7-kb *Fsp*I fragment (kb 0.7 to 3.4) (Fig. 1) was first cloned into pCL52.1 (Table 1). The resultant plasmid was digested with *Xcm*I (at kb 1.8 in ORF *cap8A*) and ligated with a *Bgl*II linker. To construct pCL7922, a 5.7-kb *Eco*RI fragment (kb 0 to 5.7) (Fig. 1) was cloned into pCL52.2. The resultant plasmid was digested with *Sal*I and *Pvu*II, treated with Klenow fragment, and ligated with an *Eco*RI linker. As a result, a 32-bp DNA fragment within ORF *cap8B* (*Sal*I and *Pvu*II sites, both at about kb 2.5 in Fig. 1, are located within ORF *cap8B*) was replaced with an *Eco*RI linker in the insert of pCL7922. The plasmid pCL7870 was constructed by ligating, in tandem, a 3.2-kb *Eco*RI-*Bcl*I (kb 0 to 3.2) fragment and a 2.3-kb *Fsp*I-*Eco*RI (kb 3.4 to 5.7) fragment to pCL52.1. The resultant plasmid thus contains an insert in which a 226-bp region between the *Bcl*I and *Fsp*I site within ORF *cap8C* was deleted. To construct pCL7932, a 2.4-kb *Xba*I-*Sma*I DNA fragment (kb 9.0 to 11.4) was cloned into the multiple-cloning site of pCL10 (Table 1). The resultant plasmid was digested with *Eco*RV (within ORF *cap8I* at kb 10.1) and ligated with an *Eco*RI linker. The plasmid pCL8015 was constructed by cloning a 5.7-kb *Eco*RI-*Sal*I DNA fragment (kb 9.2 to 14.9) into pCL52.1. The resultant plasmid was digested with *Sma*I (at kb 11.4 within ORF *cap8J*) and ligated with an *Eco*RI linker. To construct pCL7869, the same plasmid used to construct pCL8015 was digested with *Pvu*II (at kb 12.9 in *cap8K*) and ligated with an *Eco*RI linker. To construct pCL7877, a 6.8-kb *Sma*I-*Eco*RI DNA fragment (kb 11.4 to 18.2) was cloned into pCL52.1. The resultant plasmid was digested with *Sca*I (at kb 15.1 in *cap8N*) and ligated with an *Eco*RI linker after treatment with Klenow fragment. Plasmid pCL7918 was constructed by ligating a 4.4-kb *Sal*I-*Eco*RI DNA fragment (kb 16.1 to 20.5) into pBluescript(KS$^+$), which lacks the *Nsi*I site. The resultant plasmid was digested with *Nsi*I and then religated to generate a deletion of a 69-bp DNA fragment (at about kb 17.7 within *cap8P*). The insert fragment of the resultant plasmid containing the desired deletion was recloned into pCL52.1.

The recombinant plasmids constructed as described above were electroporated into strain RN4220 and then transduced to strain Becker by phage 52A at 30°C. The resultant strains were grown at 42°C with selection of the antibiotic resistance encoded by the plasmid vector to select strains with the plasmid integrated into the chromosome. A single colony was then incubated at 30°C without selection for 24 to 48 h to allow excision of the integrated plasmids. The desired mutant strains, CYL6132, CYL6062, CYL5974, CYL6052, CYL6151, CYL5952, CYL5953, and CYL6001, with mutation sites at *cap8A*, *cap8B*, *cap8C*, *cap8I*, *cap8J*, *cap8K*, *cap8N*, and *cap8P*, respectively, were isolated essentially as described previously (29). These desired mutants were confirmed by Southern hybridization except CYL6001, which was confirmed by PCR amplification (results not shown).

Northern blot analysis. *S. aureus* Becker was grown on Columbia agar for 4 h at 37°C and the total cellular RNAs were isolated from the cells by the procedure of Cheung et al. (7), using a FastRNA kit from BIO 101 (Vista, Calif.). The RNA samples were fractionated on a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane by using a TurboBlotter (Schleicher & Schuell, Keene, N.H.). The conditions for hybridization and washing were as described before (49).

Construction of transcriptional fusions. Transcriptional fusion plasmids were constructed by ligating either the PCR-amplified or the restriction enzyme-generated DNA fragments containing the putative promoter regions at the upstream of the promoterless *xylE* reporter gene of suitably digested pSL24 (see Fig. 3). The PCR-amplified fragments were verified by sequencing. Each fusion plasmid was electroporated into strain RN4220 and then transduced to strain Becker by using phage 52A. The catechol 2,3-dioxygenase (the gene product of *xylE*) activities of the strains containing the fusions were assayed spectrophotometrically as described by Zukowski et al. (50).

Nucleotide sequence accession number. The sequence discussed in this work has been deposited with GenBank under accession number U73374.

RESULTS

Genetic complementations. Previously, we showed that all the 18 Cap8$^-$ mutants that we had isolated were complemented by various plasmids with inserts derived from a 20.5-kb contiguous DNA fragment of *S. aureus* Becker, suggesting that the 20.5-kb fragment contained the majority of the *cap8* genes (41). Sequencing of the 18.5-kb region of the 20.5-kb fragment revealed 17 ORFs. Sixteen of these ORFs were tightly clustered and were transcribed in one orientation. These 16 ORFs were tentatively named *cap8A* through *cap8P* (Fig. 1) and were considered to be involved in CP8 synthesis for the following reasons. First, the ORF upstream of *cap8A* was not clustered

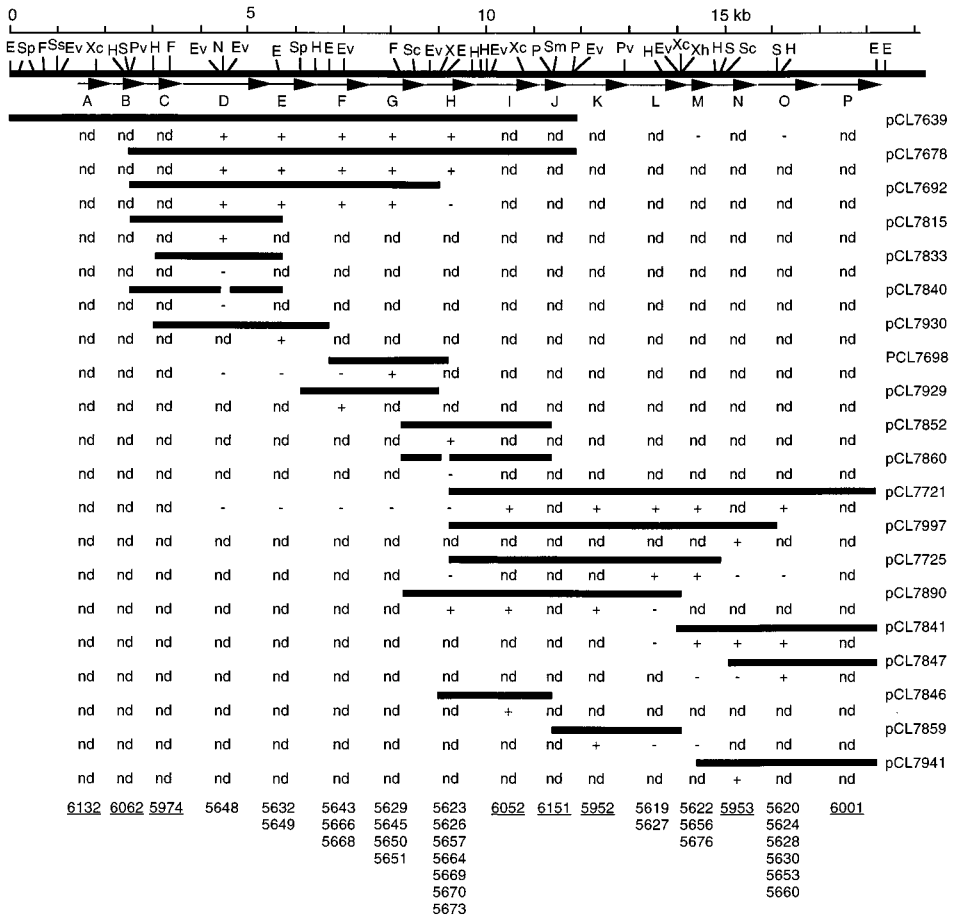

FIG. 1. Characterization of *cap8* genes by complementation tests. ORFs are indicated by arrows. Capital letters under arrows represent the corresponding genes. Solid bars represent the DNA fragments cloned into either pLI50 or pCL8. The correspondent plasmids are shown to the right of the inserts. +, Cap8$^+$ phenotype; −, Cap8$^-$ phenotype; nd, test not done. The mutants used for the test are indicated at the bottom of the figure. Site-specific mutants are underlined. Abbreviations for restriction sites: E, *Eco*RI; Ev, *Eco*RV; F, *Fsp*I; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I; Xc, *Xcm*I; Xh, *Xho*I.

with the rest of ORFs (there were 345 nucleotides between the stop of this ORF and the start of *cap8A*). Second, no Cap8$^-$ mutation was mapped to the region containing this ORF (see results below). Third, although the ORF showed some degree of amino acid homology to alcohol dehydrogenase genes and thus could be involved in carbohydrate metabolism, no homology to genes involved specifically in polysaccharide synthesis was found. Fourth, *cap8A*, *cap8B*, *cap8C*, and *cap8D* showed not only a high degree of homology (60 to 72% identity at the amino acid and nucleotide levels) but also the same organization to *cap1A*, *cap1B*, *cap1C*, and *cap1D*, respectively, the first 4 of the 13 *cap1* genes involved in CP1 synthesis (29).

To determine whether each of the 16 ORFs is involved in CP8 biosynthesis, plasmids with various ORFs were used to complement 28 ethyl methanesulfonate-induced mutants (Table 1), which includes 18 mutants isolated previously (41), by an immunoblotting procedure and by RIE with anti-CP8 specific rabbit antiserum. As shown in Fig. 1, plasmid pCL7639, which contains a 14-kb insert, complemented 17 mutants. Deletion from the left end of the insert of pCL7639 up to the *Sal*I site (kb 2.5) generated plasmid pCL7678, which contains an 11.5-kb insert with intact *cap8C* through *cap8J*. The same 17 mutants complemented by pCL7639 were also complemented by pCL7678, suggesting that these mutations mapped to ORFs *cap8C* to *cap8J*. Deletion from the right end of the insert of pCL7678 up to the *Xba*I site (at kb 9.0) generated pCL7692, which contains intact *cap8C* through *cap8G*. Of the 17 mutants complemented by pCL7678, 10 were also complemented by pCL7692, suggesting that these 10 mutations mapped to ORFs *cap8C* through *cap8G*. Further deletion of the right end of the 6.5-kb insert of the plasmid pCL7692 up to the *Eco*RI site (at kb 5.7) generated pCL7815, which contains intact *cap8C* and *cap8D*. Deletion of 0.5 kb from the left end to the *Hin*dIII site (at kb 3.0) of the 2.2-kb insert fragment from pCL7815 generated pCL7833, which contains intact *cap8D* only. The plasmid pCL7815 complemented mutant CYL5648, whereas the plasmid pCL7833 did not, suggesting that the mutation in 5648 mapped to either *cap8C* or *cap8D* if *cap8D* is cotranscribed with *cap8C*. However, 5648 was not complemented by pCL7840 with a deletion of the 181-bp *Eco*RV fragment located within the ORF *cap8D*, suggesting that the mutation in CYL5648 mapped to *cap8D* and that *cap8D* is transcribed by a promoter upstream of *cap8C*. Plasmid pCL7930 with an insert from kb 3.0 to 6.7 was then constructed; this plasmid contains intact *cap8D* and *cap8E* but does not contain the promoter upstream of *cap8C* which is required for transcribing *cap8D*. Two mutants (CYL5632 and CYL5649) were complemented by the plasmid pCL7930, suggesting that the two mutations they carry mapped to *cap8E*.

To further map the mutations, more plasmids were con-

structed. The plasmid pCL7698 (with a 2.5-kb *Eco*RI insert from kb 6.7 to 9.2), which contains only intact *cap8G*, complemented four mutants (CYL5629, CYL5645, CYL5650, and CYL5651), indicating that the four mutations they carry mapped to *cap8G*. Plasmid pCL7929 (with a 2.9-kb *Sph*I-*Xba*I insert from kb 6.1 to 9.0), which contains intact *cap8F* and *cap8G*, was then constructed. Three mutants, CYL5643, CYL5666, and CYL5668, were complemented by pCL7929 but not by plasmid pCL7698, which contains only intact *cap8G*, suggesting that the three mutations they carry mapped to *cap8F*. To map the remaining seven mutations (carried by CYL5623, CYL5626, CYL5657, CYL5664, CYL5669, CYL5670, and CYL5673) complemented by pCL7639, two plasmids, pCL7852, which contains a 3.2-kb *Fsp*I-*Sma*I insert (kb 8.2 to 11.4) with intact *cap8H* and *cap8I*, and pCL7860, which contains the same insert as pCL7852 except that the *cap8H* was partially deleted between the *Xba*I and *Eco*RI sites (kb 9.0 to 9.2), were constructed. All seven mutants were complemented by pCL7852; however, none of the mutants were complemented by pCL7860, indicating that all seven mutations they carry mapped to *cap8H* but not *cap8I.*

The remaining 11 mutants not complemented by pCL7639 were all complemented by the plasmid pCL7721 (with a 9-kb *Eco*RI insert from kb 9.2 to 18.2), which contains intact *cap8I* through *cap8O* (note that *cap8P* is truncated at the C-terminal end). Since the overlapping region of the inserts in pCL7721 and pCL7639 contains intact *cap8I* and *cap8J* and the 11 mutants were only complemented by pCL7721, the mutations they carry must be located in ORFs *cap8K* through *cap8O*. To map these 11 mutations more precisely, additional plasmids were constructed. Plasmid pCL7847, which contains a 3.1-kb *Sca*I-*Eco*RI fragment (kb 15.1 to 18.2) with only intact *cap8O*, complemented six mutants (CYL5620, CYL5624, CYL5628, CYL5630, CYL5653, and CYL5660), indicating that the six mutations they carry mapped to *cap8O*. We then constructed plasmid pCL7841 (which contains an *Eco*RV-*Eco*RI fragment from kb 14.0 to 18.2) and pCL7725 (which contains an *Eco*RI-*Sal*I fragment from kb 9.2 to 14.9). Three mutants, CYL5622, CYL5656, and CYL5676, were complemented by both plasmids. Since the overlapping region of the inserts of these two plasmids contains only one intact ORF, *cap8M*, we concluded that the three mutations they carry mapped to *cap8M*. To map the remaining two mutations (carried by CYL5619 and CYL5627) that were complemented by pCL7721, we constructed pCL7890, which contains intact *cap8H* through *cap8K* but truncated *cap8L* (with a 5.9-kb *Fsp*I-*Xho*I insert from kb 8.2 to 14.1). The plasmid pCL7890 was unable to complement the two mutants (CYL5619 and CYL5627 indicating that the mutations they carry mapped to *cap8L.*

The above complementation results showed that 8 of the 16 ORFs were represented by at least one mutant, indicating that these 8 ORFs are involved in the CP8 synthesis. To determine whether the rest of the ORFs are also involved in the CP8 synthesis, we isolated an additional seven mutants by *N-methyl-N′*-nitro-*N*-nitrosoguanidine, as described in Materials and Methods. However, none of these new mutations mapped outside the eight ORFs which were already represented by at least one mutant (results not shown).

Gene-specific mutants. Since no mutation was mapped to the eight remaining ORFs, strains CYL6132, CYL6062, CYL5974, CYL6052, CYL6151, CYL5952, CYL5953, and CYL6001, with gene-specific chromosomal mutations located in *cap8A*, *cap8B*, *cap8C*, *cap8I*, *cap8J*, *cap8K*, *cap8N*, and *cap8P*, respectively, were generated as described in Materials and Methods to determine whether these remaining ORFs are involved in CP8 biosynthesis. The production of CP8 in these strains was tested by immunoblotting and RIE procedures using anti-CP8 rabbit antiserum (data not shown). The results from the immunoblotting showed that strains CYL6052, CYL5952, and CYL5953 were $Cap8^-$ whereas CYL6062, CYL6151, CYL5952, CYL5953, and CYL6001 were $Cap8^+$. The RIE analysis showed that CYL6052 (*cap8I* mutant) produced nondetectable CP8, whereas strains CYL5952 (*cap8K* mutant) and CYL5953 (*cap8N* mutant) produced a small amount of CP8 (about 100- and 25-fold less capsule, respectively, than the wild-type strain Becker) and the rest of the strains produced about the same amount of capsule as the wild-type strain. These results imply that *cap8I*, *cap8K*, and *cap8N* are involved in CP8 biosynthesis, although CYL5952 and CYL5953 still produced a small amount of CP8. On the contrary, *cap8A*, *cap8B*, *cap8C*, *cap8J*, and *cap8P* may not be involved in CP8 synthesis.

To confirm that strains CYL6052, CYL5952, and CYL5953 contain mutations mapped to *cap8I*, *cap8K*, and *cap8N*, respectively, and that the mutations in these mutant strains are not polar, we performed additional complementation analyses. The results (Fig. 1) showed that strain CYL6052 was complemented both by pCL7890, which contains intact *cap8H*, *cap8I*, and *cap8K*, and by pCL7846, which contains the 2.4-kb *Xba*I-*Sma*I (kb 9.0 to 11.4) (Fig. 1) fragment with only intact *cap8I*. Similarly, strain CYL5952 was complemented by pCL7890 and also by plasmid pCL7859, with the 2.7-kb *Sma*I-*Xho*I (11.4- to 14.1-kb coordinates) insert that contains intact *cap8K* only (note that *cap8L* is truncated at the C-terminal end). These results confirmed that the mutant strains CYL6052 and CYL5952 indeed contained nonpolar *cap8I* and *cap8K* mutations, respectively. To confirm that the mutation in CYL5953 is in *cap8N*, pCL7725, which contains intact *cap8I* through *cap8M* (*Eco*RI-*Sal*I from kb 9.2 to 14.9), and pCL7997, which contains intact *cap8I* through *cap8N* (*Eco*RI-*Sal*I from kb 9.2 to 16.1), were used to complement CYL5953. We found that the mutant was complemented by pCL7997 but not by pCL7725, indicating that the mutation site in CYL5953 is in *cap8N* and that the mutation is not polar.

Northern blot analysis. To study the transcription of the *cap8* genes in *S. aureus* Becker, Northern hybridizations were carried out. By using the 17.2-kb DNA fragment (kb 1.0 to 18.2) containing almost the entire *cap8* gene cluster as a probe, one large transcript of about 17 kb could be readily detected, although it was somewhat smeared (lane 1, Fig. 2). Since the size of the 17-kb band corresponds well to the size of the whole *cap8* locus containing the 16 ORFs, it is likely that all the *cap8* genes are transcribed as a single transcript and that the smearing could be due to degradation of this fragment. To test this, we performed Northern analyses using DNA fragments within the coding regions of *cap8A*, *cap8D*, *cap8I*, and *cap8P* as the probes. The Northern blot patterns were essentially the same as the one using the entire *cap8* gene cluster as the probe; i.e., the 17-kb band was readily detected in each blot (Fig. 2). In addition, by using different regions within the *cap8* gene cluster as the probes the same patterns were detected (not shown). Since the probes of the first and the last ORFs of the gene cluster, *cap8A* and *cap8P*, respectively, detected the same 17-kb band as those probes from the middle of the cluster, these results suggest that all the 16 *cap8* genes are cotranscribed mainly as a single polycistronic message from a promoter upstream of *cap8A.*

Strength of *cap8* promoters. As shown in Fig. 1, the mutations mapped to *cap8F*, *cap8G*, *cap8H*, *cap8I*, *cap8K*, *cap8M*, and *cap8O* were complemented by various plasmids with inserts containing no intact upstream genes (pCL7929, pCL7698, pCL7852, pCL7846, pCL7859, pCL7841, and pCL7847, respectively), suggesting that these seven genes are transcribed

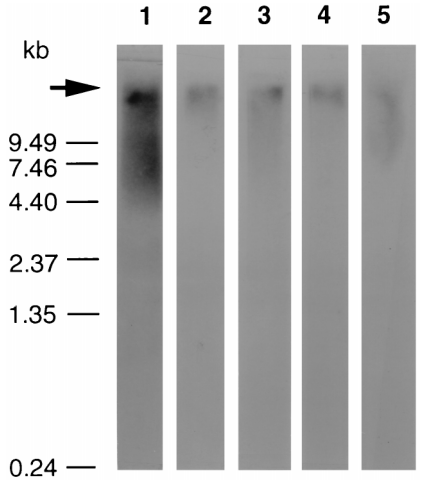

FIG. 2. Northern hybridization analysis of the *cap8* mRNA. Total RNA isolated from strain Becker was resolved by agarose gel electrophoresis and transferred to membranes. The membranes were hybridized with probes made from the 17.2-kb fragment (1.0- to 18.2-kb coordinates in Fig. 1), which contains all 16 ORFs (lane 1), the internal fragment of *cap8A* (lane 2), the internal fragment of *cap8D* (lane 3), the internal fragment of *cap8I* (lane 4), and the internal fragment of *cap8P* (lane 5). The arrow indicates the 17-kb transcript estimated by extrapolation of the size markers shown to the left of the figure.

by their own promoters. In addition, the fact that the *cap8E* mutants were complemented by pCL7930, which contains intact *cap8D* and *cap8E* but lacks the promoter required for *cap8D*, indicates that *cap8E* is transcribed by its own promoter. Furthermore, the mutant CYL5953 mapped in *cap8N* was complemented by pCL7941 (which contains intact *cap8N* and *cap8O*) (see Materials and Methods for the construction of pCL7941), suggesting that *cap8N* is also transcribed by the nearby upstream promoter. Thus, with the exception of *cap8D* and possible exception of *cap8L*, all genes affecting CP8 synthesis were transcribed by the nearby promoters upstream of the respective genes. These data imply that there are several internal promoters within the *cap8* locus which could initiate the transcription of their respective downstream genes. However, the Northern analyses shown in Fig. 2 did not detect any discrete band smaller than the 17-kb band, although a smeared region below the 17-kb band was found. One possible explanation for failing to detect the smaller transcripts could be that these internal promoters are very weak. To test this possibility, we fused several DNA fragments containing the potential promoter regions detected by the complementation tests shown in Fig. 1 to the promoterless *xylE* reporter gene in plasmid pSL24. The resultant plasmids were electroporated to strain RN4220 and then transduced to strain Becker by using phage 52A, and the *xylE* activities of these strains were measured spectrophotometrically as described in Materials and Methods. As shown in Fig. 3, we detected 5.504 mU of the *xylE* activity per mg of protein from strains with fusions containing the upstream region of *cap8A* whereas much lower activities (10- to 25-fold lower) were detected in strains with other fusions. These results suggest that *cap8* genes are transcribed primarily by the promoter upstream of *cap8A* and that the internal promoters detected by complementation are all very weak.

CP8-specific genes. We previously reported that each of the 17 *S. aureus* strains with various microcapsule serotypes contained two regions of sequence with extensive homology to the *cap8* gene cluster flanking a central nonhomologous region (41). The homologous regions could contain common genes for various serotypes, whereas the central unique regions could contain type-specific genes. The CP8-specific sequence was previously localized in a 2.2-kb *Eco*RI-*Sma*I DNA fragment (at kb 9.2 to 11.4) (Fig. 1). Sequencing of the region revealed that the 2.2-kb fragment contained intact *cap8I* and truncated *cap8H* and *cap8J*. To determine more precisely which genes are CP8 specific, we generated gene-specific probes internal to *cap8G*, *cap8H*, *cap8I*, *cap8J*, *cap8K*, and *cap8L*. These probes were then used in a Southern hybridization using *Eco*RI-digested DNAs prepared from strains with various serotypes. As shown in Fig. 4, the *cap8H*-, *cap8I*-, *cap8J*-, and *cap8K*-specific probes hybridized only to strains Becker, PS29, 80CR3, and M, with bands as predicted from the restriction map. In contrast, the *cap8G*- and *cap8L*-specific probes hybridized to all strains (data not shown). These results imply that these four ORFs,

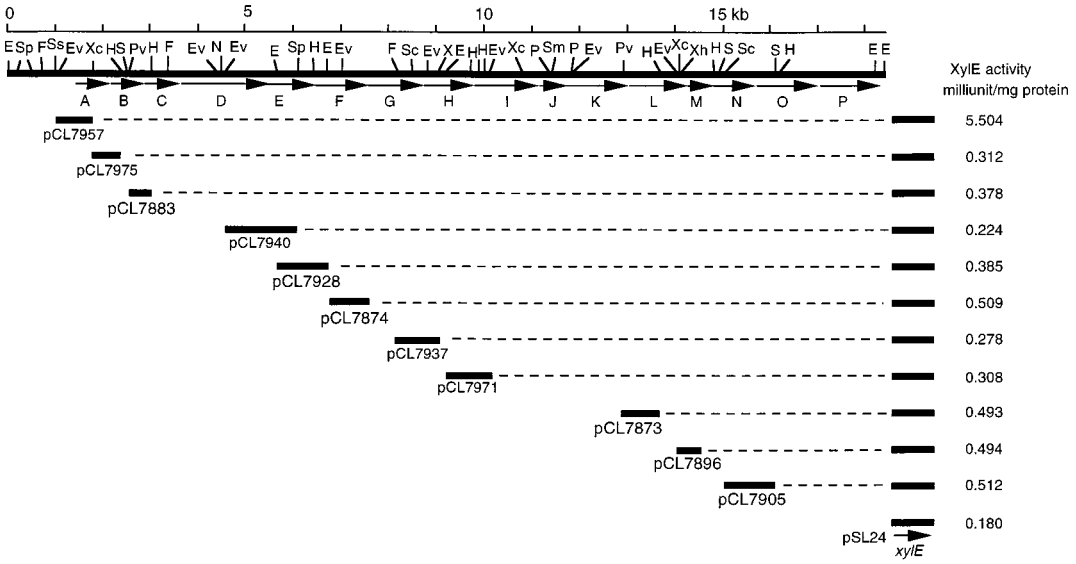

FIG. 3. XylE activities of the fusion plasmids. Various potential promoter regions from the *cap8* gene cluster were fused to the promoterless *xylE* reporter gene in pSL24. Symbols and abbreviations are the same as those described in the legend to Fig. 1.

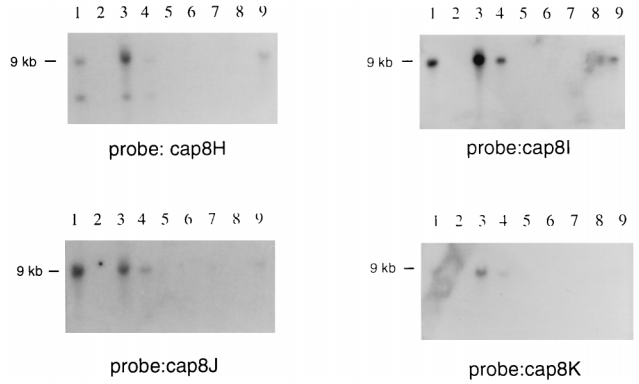

FIG. 4. Southern analysis of CP8-specific genes under high-stringency washing conditions. Chromosomal DNAs from various strains were digested with *Eco*RI and hybridized with the gene-specific probes indicated under each panel. Lanes contain chromosomal DNA from the indicated strains as follows: lane 1, 80CR3; lane 2, 8325-4; lane 3, Becker; lane 4, PS29; lane 5, Reynolds; lane 6, 7007; lane 7, Mardi; lane 8, Smith; lane 9, M. Note that the panel probed with the *cap8K*-specific gene was slightly underexposed; overexposed film showed exact the same pattern as those shown by panels probed with *cap8I* and *cap8J*.

cap8H, *cap8I*, *cap8J*, and *cap8K*, are CP8 specific. Previously, we showed that DNAs from strains 80CR3, PS29, and an M hybridized to the 2.2-kb CP8-specific sequence and that strains 80CR3, PS29, and an M strain with a *cap1* deletion (CYL5556) did not react with anti-CP8 antisera (41). The result in Fig. 4 indeed shows that these strains contain all the CP8-specific genes. This result therefore suggests that CP7, the structure of which has not been determined, produced by PS29 and the capsules produced by 80CR3 and CYL5556 could be closely related to CP8.

DISCUSSION

In this report, we showed by complementation of chemical Cap8$^-$ mutants and by gene-specific mutagenesis that 11 of the 16 ORFs were involved in CP8 synthesis in *S. aureus* Becker. Interestingly, gene-specific mutations in *cap8A*, *cap8B*, *cap8C*, *cap8J*, and *cap8P* resulted in no change in the amount of CP8 production compared to the wild-type strain. Based on these data, we therefore cannot conclude that these five ORFs are involved in CP8 synthesis. However, as discussed below, these five ORFs, with the exception of *cap8C*, could still be involved in the synthesis of CP8. (i) In the case of the *cap8A*-specific mutant, further examination by sequencing showed that the mutation was a five-codon in-frame insertion (not shown). This result raises the possibility that the mutation is a silent mutation which did not affect the CP8 production. (ii) Recently, we found that the *cap8B*-specific mutant, CYL6062, produced CP8 with a lower molecular weight than that produced by the wild-type strain Becker (42). This result indicates that *cap8B* could be a chain length regulator which, when inactivated, may not affect the amount of CP8 production. (iii) Cap8J showed some degree of homology, especially in the region containing the proposed active site, with various bacterial acetyltransferases, including Cap1G and Cap5H (26, 29). We have shown that Cap1G is required for CP1 synthesis. Most recently, Cap5H has been shown to be required for O acetylation in CP5 produced by *S. aureus* Reynolds (26). Since CP8 and CP5 are almost identical, it is likely that *cap8J* is also involved in the O acetylation in CP8 synthesis. The reason that the *cap8J* mutant produced the same amount of CP8 as the wild-type strain could be that the antibody that we used was able to recognize both the native CP8 and de-O-acetylated CP8. However, further experiments are required to determine whether *cap8J* is involved in O acetylation. (iv) Since Cap8P showed homology to several gene products proposed to be involved in polysaccharide synthesis in other organisms, it is possible that *cap8P* could also be involved in CP8 synthesis under certain conditions. However, it is also possible that the in-frame mutation of the 69-bp deletion in the *cap8P* mutant CYL6001 did not result in loss of Cap8P function.

By reporter gene fusion, we showed that the internal promoters detected by complementation tests were all very weak compared to the primary promoter upstream of *cap8A*. In fact, the activities of these internal promoters were barely detectable by *xylE* reporter gene fusion. It is likely that these internal promoters do not contribute significantly to the transcription of the *cap8* genes. Surprisingly, however, these promoters were strong enough to drive sufficient expression for positive complementation of the respective genes. We reason that the positive complementation results were due to the following. (i) These complementation tests were performed with multiple-copy plasmid vectors so that the complementing gene dose was artificially increased. (ii) The Becker strain, like other microencapsulated strains of *S. aureus*, produces a small amount of capsule. It is likely that only a small quantity of gene products (therefore only a small amount of *cap8* message) is needed for CP8 synthesis. (iii) The complementation test under our experimental conditions doesn't require full complementation to be scored as positive. Indeed, many of the results of the complementation tests were only partial based on our RIE results.

The transcription of the *cap8* genes was further studied by Northern analysis. In order to detect *cap8*-specific RNA, we grew the cells in solid agar to increase the yield of *cap8* message, as others had reported that the production of microcapsules was much higher on solid medium than in liquid broth (27). Our result showed a distinct band of about 17 kb, with smearing below the 17-kb band. The smearing pattern could be due to degradation of the large transcript or to numerous transcripts with various transcriptional start points. The fact that the internal promoters were very weak and hence were unlikely to produce enough messages to be detected argues for the possibility that the smearing is due to degradation of the large transcript. Taking the results from the Northern analysis and the reporter fusion study together, we propose that the *cap8* gene cluster is transcribed as one single primary transcript.

We have also studied the transcriptional organization of *cap1* genes required for CP1 synthesis in *S. aureus* M (33). Unlike the gene clusters required for the synthesis of various microcapsules, the *cap1* gene cluster does not share extensive homology with the *cap8* gene cluster, though the first four genes between the two gene clusters have moderate homologies (41). Interestingly, we found that the tightly clustered 13 *cap1* genes were transcribed mainly as a 14-kb primary message with five weak internal promoters (33). Thus, the *cap1* and *cap8* genes and possibly the *cap* genes of other serotypes in *S. aureus* are all transcribed similarly, i.e., by a primary promoter located at the beginning of the operon. Similar transcription organization of polysaccharide genes has also been reported in other bacterial systems (5, 18), indicating that this feature could be common. Although the *cap8* operon and the *cap1* operon are transcribed similarly, the promoter activity of the primary *cap1* promoter was about 60-fold stronger than that of the primary *cap8* promoter and the internal promoters of the *cap1* gene cluster were 10- to 50-fold stronger than those in the *cap8* gene cluster (33). In fact, the internal promoter of the *cap1* locus is about the same strength as the *cap8* primary

promoter under the same conditions in the same genetic background (data not shown). Since CP1 is produced by strain M in a much larger quantity than CP8 is produced by strain Becker, we propose that the promoter activity at the beginning of the operon is the major, if not the only, factor determining the amount of capsules produced in different strains of *S. aureus.*

By Southern hybridization using gene-specific probes, we showed that *cap8H*, *cap8I*, *cap8J*, and *cap8K*, located in the central region of the *cap8* gene cluster, were specific to the CP8-producing strain Becker. Interestingly, this arrangement of genes is similar to that of the polysaccharide genes of many gram-negative bacteria (13, 21, 39). However, it has been reported recently that in the *cap* locus of *Haemophilus influenzae*, the *kps* locus of *E. coli* K5, and the *cps* locus of *Neisseria meningitidis*, the genes in the central region were transcribed as one or more transcriptional units independent from the genes in the common regions (9, 36, 46). Thus, the transcriptional organizations of the capsule genes of *S. aureus* Becker and of these gram-negative bacteria may be different.

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