Genetic Analysis of Type 5 Capsular Polysaccharide Expression by \textit{Staphylococcus aureus}

JEAN C. LEE,* SHILU XU, ANNE ALBUS,† AND PAUL J. LIVOLSI

Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital
and Harvard Medical School, Boston, Massachusetts 02115

Received 27 December 1993/Accepted 9 June 1994

Capsules are produced by over 90\% of \textit{Staphylococcus aureus} strains, and \textasciitilde{}25\% of clinical isolates express type 5 capsular polysaccharide (CP5). We mutagenized the type 5 strain Reynolds with Tn918 to target genes involved in CP5 expression. From a capsule-deficient mutant, we cloned into a cosmid vector an \textasciitilde{}26-kb EcoRI fragment containing the transposon insertion. In the absence of tetracycline selection, Tn918 was spontaneously excised, thereby resulting in a plasmid containing 9.4 kb of \textit{S. aureus} DNA flanking the Tn918 insertion site. The 9.4-kb DNA fragment was used to screen a cosmid library prepared from the wild-type strain. Positive colonies were identified by colony hybridization, and a restriction map of one clone (pJCL19) with an \textasciitilde{}34-kb insert) carrying the putative capsule gene region was constructed. Fragments of pJCL19 were used to probe genomic DNA digests from \textit{S. aureus} strains of different capsular serotypes. Fragments on the ends of the cloned DNA hybridized to fragments of similar sizes in most of the strains examined. Blots hybridized to two fragments flanking the central region of the cloned DNA showed restriction fragment length polymorphism. A centrally located DNA fragment hybridized only to DNA from capsular types 2, 4, and 5. DNA from pJCL19 was subcloned to a shuttle vector for complementation studies. A 6.2-kb EcoRI-ClaI fragment complemented CP5 expression in a capsule-negative derived mutant derived by mutagenesis with ethyl methane sulfonate. These experiments provide the necessary groundwork for identifying genes involved in CP5 expression by \textit{S. aureus}.

Capsule production by staphylococcus was first described in 1931 by Gilbert (9). Because capsule detection methods were crude (India ink negative staining, colony morphology on agar plates and in serum-soft agar, and lack of cell-associated clumping factor), only a few strains of \textit{Staphylococcus aureus} were recognized as capsule positive. These highly encapsulated strains, typified by strains M and Smith diffuse, resisted phagocytosis and were virulent for mice (7, 13, 21, 31).

With more-sensitive serologic methods, capsule polysaccharides can now be detected on approximately 90\% of \textit{S. aureus} strains. Although 11 capsular serotypes have been described, most clinical isolates of \textit{S. aureus} belong to capsular types 5 or 8 (11, 27). Because their capsules are small and not visualized by negative staining, serotype 5 and 8 strains are called microencapsulated, as suggested by Wilkinson (32). This term distinguishes them from the heavily encapsulated serotype 1 and 2 \textit{S. aureus} strains, which are rare.

Capsule expression by \textit{S. aureus} at the molecular level is poorly understood. Smith et al. (26) used plasmid curing experiments to show that plasmid DNA did not play a role in capsule expression. Otomo and Yoshia (23) provided evidence that chromosomal genes encode proteins involved in capsule biosynthesis by using genomic DNA prepared from an encapsulated strain to convert a nonencapsulated \textit{S. aureus} recipient into an encapsulated derivative. More recently, Lee (17) cloned the genes involved in capsule type 1 expression from highly encapsulated \textit{S. aureus} M. He showed that the genes affecting capsular type 1 biosynthesis (\textit{cap}) are clustered and that a cloned 19.4-kb DNA fragment complements capsule expression in a series of capsule-negative mutants that he created from strain M by mutagenesis with ethyl methane sulfonate (EMS). Whether the \textit{cap} genes cloned from the type 1 strain M are present in the more clinically prevalent type 5 or 8 strains was not addressed.

Structural and biochemical characterizations have been performed on polysaccharides purified from only 4 of the 11 capsule types (Fig. 1). Moreau et al. (22) purified type 5 capsular polysaccharide (CP5) from the prototype \textit{S. aureus} strain, Reynolds, and determined its trisaccharide repeating unit structure. Although the structure of type 4 polysaccharide has not been elucidated, it cross-reacts serologically with CP5 (11, 30). CP5 and type 8 capsular polysaccharide differ only in the linkages between sugars and in the sites of O acetylation of the mannosaminouronic acid residues (22, 30), yet they are serologically distinct. Because of the similarities in their structures, it is likely that the organization of the genes involved in the synthesis of the two capsule types is similar as well. In previous studies, we performed Tn918 mutagenesis on the type 5 strain Reynolds to isolate mutants that were defective in capsule expression (1). Here we describe the cloning of a gene region involved in staphylococcal type 5 capsule expression, an important first step in characterizing the molecular events controlling the biosynthesis, export, and assembly of the \textit{S. aureus} capsule.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. \textit{S. aureus} Reynolds is the prototype strain for production of CP5 (6). Staphylococci were cultivated in tryptic soy medium, and \textit{Escherichia coli} strains were cultivated in Luria-Bertani (LB) medium. Ampicillin (Ap), tetracycline (Te), and chloramphenicol (Cm) were added to culture media at 100, 5, and 15 \textmu g/ml, respectively. Enzyme digestions, Southern hybridization analyses with \textsuperscript{32}P-

* Corresponding author. Mailing address: Channing Laboratory, 180 Longwood Ave., Boston, MA 02115-5899. Phone: (617) 432-2652. Fax: (617) 731-1541. Electronic mail address: jecl@warren.med.harvard.edu.

† Present address: Department of Microbiology, University of Rochester, Rochester, NY 14642.
labeled probes, and pulsed-field gel electrophoresis were performed by standard methods (3). DNA fragments isolated from low-melting-point agarose gels were radiolabeled with \([\alpha-\text{32P}]dCTP\) by a randomly primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). High-molecular-weight chromosomal DNA from \(S. aureus\) was isolated by the gentle protoplast lysis method (28), and DNA was introduced into \(S. aureus\) cells by electroporation (2) or by transduction with phage 80a (12). Transformation of \(E. coli\) was performed by electroporation (3).

Cloning of DNA flanking Tn918 in mutant JL236. Genomic DNA from mutant JL236 was digested to completion with EcoRI and size fractionated on a 10 to 40% sucrose gradient. Fragments of >23 kb were ligated to cosmid pH79 that previously had been digested with EcoRI and dephosphorylated with calf intestinal phosphatase. DNA was packaged with a commercial lambda DNA packaging system (Stratagene Cloning Systems, La Jolla, Calif.), and the mixture was introduced into \(E. coli\) DH1 according to the manufacturer’s instructions. Recombinants were selected for resistance to Ap and Te and carried an EcoRI fragment of \(\sim 26\) kb. In the absence of Te selection, the transposon was excised (see Results), leaving a 9.4-kb EcoRI fragment of cloned \(S. aureus\) DNA (pJCL12).

**Cloning of wild-type capsule genes from strain Reynolds.** A genomic cosmid library of wild-type strain Reynolds was prepared in the cosmid vector pHCT7. CcCl-purified chromosomal DNA was partially cleaved with \(Sau3A\) and size fractionated by centrifugation through a sucrose gradient. Fractions containing DNA in the 30- to 50-kb range were ligated to BamHI-cleaved, alkaline phosphatase-treated pHCT7. DNA was packaged and introduced into \(E. coli\) DH1 as described above. DNA fragments inserted into the BamHI site of the vector pHCT7 were expected to inactivate the Te-resistance gene. Accordingly, recombinant clones were isolated on LB agar plates containing Ap. Replica plating revealed that 70% of the colonies failed to grow on Te-containing media, indicating that they contained cloned segments of chromosomal DNA. The cosmid library was amplified, aliquoted in small portions, and stored in 15% glycerol at \(-70^\circ\)C. Cosmids were colony hybridized with a radiolabeled 9.4-kb EcoRI fragment of DNA (flanking Tn918 in mutant JL236) isolated from pJCL12.

**Quantification of CPS production.** Rabbit antiserum raised against formalin-killed \(S. aureus\) was prepared as previously described (33). Sera specific for CPS or teichoic acid were obtained by immunizing rabbits with strain Reynolds or JL252 (a type 8 capsule-negative mutant [4]), respectively. \(S. aureus\) cell lysates were obtained by treating \(\sim 10^{10}\) CFU with recombinant lysostaphin (100 \(\mu\)g/ml) (Applied Microbiology, Inc., New York, N.Y.) for 1 h at 37°C. Cell debris was removed by centrifugation (12,000 \(\times\) g) at 4°C for 15 min. Rocket immunoelectrophoresis was performed as described previously (20).

**RESULTS**

**Pulsed-field gel electrophoresis.** Our prior transposon mutagenesis studies yielded two classes of CPS mutants, each containing a single Tn918 insertion (1). Mutant JL236 expressed a scant capsule (17% of wild-type levels as determined by rocket immunoelectrophoresis), whereas three other mutants (JL231, JL232, and JL234) expressed wild-type levels of an antigenically altered (de-O-acetylated) type 5 capsule. CPS in bacterial extracts from the last three mutants showed lines of partial identity with parental CPS by immunodiffusion against polyclonal CPS-specific antiserum. In addition, monoclonal antibodies reactive with O-acetylated CPS did not react with extracts prepared from mutants JL231, JL232, and JL234. To determine whether these mutations were in the same region of the \(S. aureus\) chromosome, genomic DNA from wild-type strain Reynolds and from each mutant was digested with \(SmaI\) and the fragments were separated by pulsed-field gel electrophoresis. Southern blot analysis revealed that Tn918 was located in the same \(\sim 200\)-kb fragment in all four mutants.
Strains (Fig. 2). This finding suggests that the genes involved in CP5 expression reside on this ~200-kb region of the bacterial chromosome.

Cloning DNA flanking Tn918. We cloned the mutated region of the JL236 chromosome by selecting for the Te resistance conferred by Tn918. EcoRI fragments of JL236 genomic DNA were ligated to pLH79 and introduced into E. coli DH1. Recombinants resistant to Te and Ap carried an ~26-kb EcoRI fragment of cloned DNA. Tn918 segregates at high frequency in E. coli, particularly in the absence of Te selection (8). Therefore, we subcultured five of the Ap' Te' clones three times each either on media containing both Ap and Te or on media containing Ap only. Gels of EcoRI-digested plasmid DNA preparations from each of the five clones showed a common 5-kb EcoRI fragment corresponding to the vector pLH79 (Fig. 3A). When they were grown in the presence of Te, clones carried a large (~26-kb) DNA fragment that was replaced by a 9.4-kb band when Te was omitted from the culture medium. The gel was blotted to a nylon membrane and probed with 32P-labeled Tn916, which cross-hybridizes to Tn918 (6). As shown in Fig. 3B, the transposon hybridized only to fragments (~26 kb) of DNA from clones grown in the presence of Te. Thus, Tn918 was lost in cultures passed in the absence of Te, and the DNA interrupted by the insertion event was presumably reconstituted. Clone 1 (Fig. 3), cultivated in the absence of Te, was designated pJCL12, and it was used as the source of the 9.4-kb EcoRI fragment flanking Tn918 in mutant JL236.

Cloning capsule genes from strain Reynolds. A genomic cosmide library of wild-type strain Reynolds was generated as a source of unmutagenized DNA fragments. By hybridizing colonies from the library with a 32P-labeled 9.4-kb EcoRI fragment from pJCL12, we identified six positive clones. Analyses of restriction digests of plasmid DNA from each of the six revealed common as well as unique fragments, showing that they carried overlapping but distinct cloned DNA fragments ranging from 30 to 35 kb. A restriction map of one of the cosmide clones (pJCL19) carrying putative capsular type 5 genes is shown in Fig. 4.

Mapping the Tn918 insertion sites. To map the sites of transposon insertion in mutant strains, genomic DNA from S. aureus Reynolds and mutants JL232 and JL236 was digested with restriction enzymes and electrophoresed through agarose gels. Southern blots prepared from these gels were hybridized with 32P-labeled fragments from pJCL19 (fragments 1, 2, and 3 shown in Fig. 4). Tn918 has a single HindIII site located 5.3 kb from one end of the transposon but no EcoRV or Clai sites. By analyzing the sizes of the hybridizing HindIII fragments, we were able to demonstrate the approximate sites of the Tn918 insertions shown in the figure. Results indicated that Tn918 was inserted in the same 0.7-kb EcoRV-HindIII fragment in
both JL236 and JL232. However, the insertion sites are distinct, as determined by Southern blot analysis of genomic DNA cut with HindIII and hybridized to a radiolabeled 2.1-kb EcoRV fragment (Fig. 4, fragment 3) of Reynolds DNA (data not shown).

Hybridization of capsule genes to DNA from S. aureus of different capsular serotypes. To determine which regions of the cloned putative CP5 gene region were unique to strains belonging to specific capsular serotypes, we performed hybridization studies. Since the structures of the type 1, 2, 5, and 8 polysaccharides are known (11, 22, 30), we focused our efforts on strains with these capsular types. DNA fragments A to E (Fig. 4) were radiolabeled and used to probe genomic DNA from the S. aureus strains indicated in Table 2. PvuII-generated fragment E hybridized to a 4-kb fragment in all of the strains, and PvuII-generated fragment A hybridized to an 18.5-kb band in 11 of the 14 strains tested. On blots hybridized to fragments B and D, restriction fragment length polymorphism was observed (Table 2) (Fig. 5). For example, the 9.4-kb EcoRI fragment B hybridized to a 9.4-kb EcoRI fragment of DNA from wild-type strain Reynolds, as expected, and to a DNA fragment of similar size in the majority of S. aureus isolates examined, regardless of capsular serotype. It hybridized to two EcoRI fragments (4.2 and 5.2 kb) of DNA from the serotype 5 strain Clin 200, and the sum of the fragments equaled 9.4 kb. Fragment B hybridized to a 13.3-kb EcoRI fragment in one strain each of serotypes 1 and 2 and in nontypeable strain NT857.

Fragment C (a 3.2-kb ClaI-EcoRI fragment contained within the 9.4-kb EcoRI fragment A) was the most specific DNA probe tested; it hybridized only to DNA from type 2, 4, and 5 strains of S. aureus, as well as DNA from the nontypeable strain RN450 (Table 2) (Fig. 6). The transposon insertions in mutants JL232 and JL236 were located in this region. Because the nontypeable strain RN450 showed the same hybridization patterns with the five fragments (A to E) as did the type 5 isolates, we speculate that RN450 may have a capsular type 5 gene region that is defective in some way for expression.

Complementation studies. E. coli transductants carrying our cosmids clones did not express CP5 by immunoblot (19) analysis (not shown). Therefore, we subcloned the 9.4-kb EcoRI fragment from pJL19 because the Tn918 insertion in mutant

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**TABLE 2. Results of Southern hybridization analysis of chromosomal DNA from S. aureus strains probed with fragments A to E (Fig. 4)**

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>Capsule type</th>
<th>Size (kb) of hybridizing fragment probed with fragmenta:</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
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<tr>
<td>Reynolds</td>
<td>5</td>
<td>18.5 9.4 3.8 10.6 4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>JL232(Tn918)</td>
<td>5 (altered)</td>
<td>18.5 ~26 ~20 &gt;23 4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JL236(Tn918)</td>
<td>5 (deficient)</td>
<td>18.5 ~26 ~20  4.0</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Newman</td>
<td>5</td>
<td>18.5 9.4 3.9 10.6 4.0</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Clin 200</td>
<td>5</td>
<td>18.5 5.2 4.2 10.6 4.0</td>
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<td></td>
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<tr>
<td>Lowenstein</td>
<td>5</td>
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<td></td>
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</tr>
<tr>
<td>7007</td>
<td>4</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Smith diffuse</td>
<td>2</td>
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<td></td>
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<td></td>
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<tr>
<td>K93M</td>
<td>2</td>
<td>~23 13.3 3.8 10.6 4.0</td>
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<td></td>
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<tr>
<td>S1P</td>
<td>8</td>
<td>18.5 9.4 A 12 4.0</td>
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<tr>
<td>Wright</td>
<td>8</td>
<td>18.5 9.4 A 12 4.0</td>
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<td></td>
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<tr>
<td>PS80</td>
<td>8</td>
<td>&gt;23 9.4 ~36 4.0</td>
<td></td>
<td></td>
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<tr>
<td>Becker</td>
<td>8</td>
<td>18.5 9.4 A 12 4.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>JL252(Tn551)</td>
<td>8 (negative)</td>
<td>18.5 9.4 A 12 4.0</td>
<td></td>
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</tr>
<tr>
<td>SA1 mucoid</td>
<td>1</td>
<td>18.5 13.3 10.6 4.0</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>JL24(Tn551)</td>
<td>1 (microcapsule)</td>
<td>18.5 13.3 10.6 4.0</td>
<td></td>
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<tr>
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<tr>
<td>NT857</td>
<td>NT</td>
<td>&gt;23 13.3 ~36 4.0</td>
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<tr>
<td>RN450</td>
<td>NT</td>
<td>18.5 9.4 3.6 10.6 4.0</td>
<td></td>
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</tbody>
</table>

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*a* Blots of DNA cut with PvuII were probed with fragments A, D, and E; EcoRI- and ClaI-digested DNA was probed with fragments B and C, respectively.

*b* — no band hybridized to the probe.

*c* NT, nontypeable.
FIG. 5. (A) Genomic DNA was digested with EcoRI and electrophoresed on a 0.8% agarose gel. (B) The gel was blotted to nylon and probed with fragment B (Fig. 4), which flanked Tn918 in strain JL236.

JL236 was within this fragment. The DNA fragment was purified from low-melting-point agarose and recovered in pLI50. The construct was verified by restriction analyses and named pJCL24 (Fig. 4).

pJCL24 prepared in E. coli was electroporated into RN4220, an S. aureus strain that is defective in one or more restriction systems (14). Cm' transformants, like the host strain RN4220, were negative for CP5 expression by colony immunoblotting (19). Extracts and lysates of the transformants were CP5 negative by immunodiffusion or rocket immunoelectrophoresis.

To show that the region of DNA that we cloned is involved in CP5 gene expression, we performed experiments to complement the gene defects in our mutant strains. Complementation occurs if wild-type CP5 genes on a recombinant plasmid function in trans with mutant chromosomal CP5 gene(s), whether they were inactivated by transposition or by chemical means. pJCL24 transduced into the Tn918 mutant JL293 did not complement capsule expression, but it did restore capsule expression to EMS mutants JL240 and JL243 as determined by immunodiffusion of bacterial extracts with CP5-specific antiserum (data not shown). Rocket immunoelectrophoresis was used to quantify CP5 in bacterial extracts of the parent strain Reynolds, mutants JL240 and JL243, and transductants of JL240 and JL243 carrying pJCL24. Reynolds, JL240, and JL243 produced 9, 0.2, and <0.2 μg of CP5 per 10^{10} CFU, respectively. JL240(pJCL24) and JL243(pJCL24) produced 14 and 10 μg of CP5 per 10^{10} CFU, respectively. When three of the individual JL240 transductants were subcultured in the absence of Cm, they lost pJCL24 and tested negative for CP5.

Antiserum specific for teichoic acid, a cell wall antigen common to both capsule-positive and -negative strains, reacted by immunodiffusion with extracts from wild-type and mutant S. aureus isolates independent of the presence of pJCL24 (data not shown). A subclone of pJCL24 with a 3.3-kb ClaI fragment deleted (pJCL34) (Fig. 4) restored CP5 expression to JL240 but not to mutants JL243 and JL293. No other pJCL24 subclones tested showed any complementation activity.

**CP5 defects in mutant strains.** We know from colony immunoblot experiments and electron microscopic analysis that mutants JL236 and JL240 express scant and no CP5, respectively, on their bacterial surfaces (1). To determine whether the mutants produced CP5 but did not export it to the cell surface or whether they were defective in linking CP5 to the bacterial surface, we analyzed both cell lysates and culture supernatants of these strains. Lysates and concentrated supernatants were examined qualitatively by dot blots incubated with polyclonal and monoclonal antibodies specific for CP5.
CP5 was also quantitated in each sample by rocket immunoelectrophoresis. Results from both assays were similar. By rocket immunoelectrophoresis, cell lysates of S. aureus Reynolds had ∼24 μg of CP5 per 10^{10} CFU, whereas both mutants had <5 μg of CP5 per 10^{10} CFU. We detected 5.6, 1.2, and <0.8 μg of CP5 per ml in the culture supernatants of strains Reynolds, JL236, and JL240, respectively. This experiment indicated that the mutants tested were neither accumulating CP5 intracellularly nor leeching the mature polysaccharide into the culture medium.

DISCUSSION

To our knowledge, this report is the first to analyze a region of the S. aureus chromosome that is involved in expression of CP5. The capsule gene region was targeted by Tn918 mutagenesis, and DNA flanking the transposon was used to probe a cosmid library of chromosomal DNA from unmutagenized wild-type S. aureus Reynolds. We have characterized one cosmid clone (pJCL19) that carries a contiguous ∼34-kb segment of the S. aureus chromosome.

To determine whether segments of the cloned capsule gene region were specific for S. aureus strains belonging to different capsular serotypes, we probed genomic DNA digests from a variety of S. aureus strains with DNA fragments from pJCL19. Fragments on the ends of the capsule gene region hybridized to fragments of similar sizes in most of the strains examined; whether genes in this region are required for capsule synthesis remains to be determined. DNA sequences within these overlapping segments might encode common proteins involved in transport or secretion of capsular polysaccharide, constituting pathways in capsule biosynthesis that are shared by different capsular serotypes. In E. coli, Neisseria meningitidis, and Haemophilus influenzae, genes directing the surface expression or export of the polysaccharide (referred to as regions 1 and 3) flank region 2, which directs the polymerization of individual sugar precursors (15, 25, 29). A similar organization of genes in the capsule gene region of S. aureus might exist. Although DNA from two nontypeable isolates also hybridized to fragments A and E, it is not known whether these strains are actually capsule negative or whether they express a previously unrecognized capsular type. If they are capsule negative, we do not know the genetic basis for their lack of capsule production.

Blots hybridized to two DNA fragments flanking the central region showed restriction fragment length polymorphism among strains of different serotypes. However, all the type 5 strains yielded similar hybridization patterns. A small centrally located DNA fragment hybridized only to capsular types 2, 4, and 5. This central fragment may be similar to region 2 genes in E. coli and H. influenzae that encode enzymes necessary for the synthesis and polymerization of the serotype-specific polysaccharide (15, 25, 29). The Tn918 insertions in mutants JL232 and JL236 were both located within this central region. Because the ends of the strain RN450 showed the same hybridization patterns to each of the five probes as did the type 5 isolates, we speculate that RN450 may have a capsular type 5 gene region that is defective or that the necessary regulatory mechanisms are lacking. Strain RN450 is known to be deficient in exoprotein production, presumably because of an undefined alteration in its agr gene (24).

To show that the region of DNA that we cloned is involved in CP5 gene expression, we performed complementation studies. pJCL24 (carrying a 9.4-kb DNA insert) and pJCL34 (a subclone of pJCL24 with a 3.3-kb ClaI fragment deleted) both complemented CP5 expression in the EMS capsule-negative mutant JL240. However, when recombinant plasmids were introduced into the Tn918 mutant JL293, they did not restore capsule expression. This observation suggests that the gene interrupted by Tn918 is not present in its entirety on the clone, a possibility consistent with the fact that the Tn918 insertion in JL293 is within 1 kb of the end of the cloned EcoRII fragment. Alternatively, the transposon may exert polar effects distal to its insertion site, and the affected region is not included in DNA used for complementation. Complementation of capsule gene expression in Tn918-induced mutants may be difficult even with larger DNA fragments if the capsule biosynthesis genes are part of a single operon and are cotranscribed from a single promoter, as was recently described for Pseudomonas aeruginosa (5). A similar circumstance may exist in S. aureus since transposon mutants created from a capsular type 1 strain were not complemented by cloned DNA from wild-type strain M (17). In contrast, a 19.4-kb DNA fragment was effective in complementing type 1 capsule expression in EMS mutants.

This study provides the necessary groundwork for identifying genes involved in CP5 expression. Our investigation will lead toward a better understanding of expression and regulation of the capsular polysaccharide antigens that are expressed by most S. aureus strains.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-29040 from the National Institute of Allergy and Infectious Diseases.

We thank Robert Arbet for performing pulsed-field gel electrophoresis and Chioma Nwankwo for providing technical assistance.

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