Competence for Genetic Transformation in *Streptococcus pneumoniae*: Molecular Cloning of *com*, a Competence Control Locus

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To identify and map genes involved in competence for genetic transformation in *Streptococcus pneumoniae*, we have cloned DNA surrounding an *ermB* insertion mutation that causes a competence factor deficiency. We recovered the insert and approximately 500 base pairs of neighboring pneumococcal DNA in pMB9. Larger pieces of DNA from this region were unstable in pMB9 and pBR325. However, larger pieces were stable in pKK232-8, an *Escherichia coli* vector containing strong transcription terminators. Overlapping pieces of wild-type DNA from this competence control region were cloned and mapped in this vector. Insertion mutations were constructed in vitro throughout the cloned region. When crossed into the pneumococcal chromosome, they showed that the *com* locus is 4.2 to 5.2 kilobases long.

In *Streptococcus pneumoniae* (pneumococcus), competence for genetic transformation is a specialized cell state (28, 42). Cells typically enter this state only in an actively growing culture as it reaches a particular critical cell density (41, 44). The phenomenon is regulated by a secreted protein, competence factor (CF), which serves as a population-measuring feedback device. Competence is induced throughout a culture within a few minutes, once CF has accumulated to a threshold level (41, 45). The cell’s response to CF is complex, involving a global shift in protein synthesis pattern and resulting in several new properties, including an efficient system for processing DNA for recombination (24, 26, 42, 46). Tomasz and Mosser have characterized CF as a basic protein of *M* r about 10,000 (43, 45). A very similar system has also been described for several other streptococcal species (7, 8, 15, 16, 30, 33, 45).

Initial steps in genetic dissection of the mechanisms involved in streptococcal competence have been taken. A variety of mutations have been described which affect the DNA processing steps of transformation, including DNA uptake (10, 12, 13, 27), recombination (12, 27), and overlapping pieces of heteroduplex repair (5, 10). Few mutations have been described, however, which affect the regulation of the competence state itself. Lacks and Greenberg described a trypsin-tolerance mutation, *trt*, which causes constitutive competence (11). There are also reports of nontransformable mutants, including *ntr* (11, 12), and incompetent strains that are responsive to CF (29; A. Tomasz, Genetics 52:480, 1965). Finally, in the group H streptococci, naturally occurring strains are known with defects in CF production or CF responsiveness or both (16, 30). The most thoroughly described of these regulatory mutants is the *com* mutation found in strain CP1415. Studies of this mutant showed a set of properties consistent with the idea that the defect in CP1415 prevented accumulation of CF, but not the competence response to CF, although other more complex regulatory deficiencies could be imagined.

Whereas the other mutations mentioned above were obtained after chemical mutagenesis, the mutation in CP1415 was produced by a gene-disruption method, insertion-duplication mutagenesis. The insertion-duplication mutagenesis used the ordinary processes of homologous recombination active during transformation to insert into the pneumococcal chromosome at random sites the *ermB* drug resistance marker, chosen for its expression in both *S. pneumoniae* and *Escherichia coli* (22). With the aim of identifying genes involved in control of the competence response and of determining the roles played by their products, we have exploited the *com::ermB* marker of CP1415 to identify the wild-type *com* locus and clone it in *E. coli* plasmid vectors.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture media.** The pneumococcal strains used in this work were CP1200, CP1415, and CP1515. CP1200 was obtained from the Rx wild type (34, 38) by transformation to *Str*' with DNA from DP1500 (′SMC′, nov-1 bry str-l ery-2 ery-6) (38) and to *Mal*′ (mal511) with DNA from the R6 strain, C36MK (9), as described previously (27). The *Com*′ mutant CP1315 was backcrossed to CP1200 to produce CP1415 (mal511 str-l com-15::ermB) (29). CP1515 was obtained by insertion-duplication mutagenesis of CP1200 with pXF154. *E. coli* DH1 (17) was used as a plasmid host.

The pneumococcal vector pMV158 is a 5.4-kilobase (kb) plasmid which contains a tetracycline resistance determinant (3, 12a). pR29 contains the noninducible *ermB* determinant of pAMBl on a 1.8-kb *Cla*I fragment (29). The terminator vector pKK232-8 (2) was used to clone DNA fragments with promoter activity. Plasmids pMB9 (35) and pBR325 were also used in cloning experiments. pJDC4, constructed from pMB9 by insertion of the *Clal ermB* determinant of pR29 to replace the Tet′ determinant (J.-D. Chen and D. A. Morrison, Gene, in press), was generously provided by J.-D. Chen.

Casein hydrolysate broth, complete transformation medium, and conditions used for growth and transformation of pneumococci have been previously described (27). For selection in pneumococcus, tetracycline and erythromycin were added to growth media to concentrations of 1 and 2

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μg/ml, respectively. E. coli strains were grown in LB medium (18) supplemented, when required, with 20 μg of chloramphenicol, 25 μg of tetracycline, 100 μg of ampicillin, or 750 μg of erythromycin per ml.

**DNA isolation.** Pneumococcal DNA was isolated as previously described (20). Purified pneumococcal plasmid DNA was prepared by the method of Currier and Nester (6). E. coli plasmid DNA was prepared by the method of Marko et al. (19). All plasmid DNA used for cloning and Southern analysis was purified by CsCl-ethidium bromide band preparative centrifugation. For clone analysis, small-scale plasmid preparations were made by alkaline sodium dodecyl sulfate lysis (1) of overnight cultures of E. coli grown in the presence of the appropriate antibiotic. Frozen competent E. coli cells were prepared and used as described by Morrison (23).

**Preparative agarose fractionation.** Restriction fragments were purified from agarose by electrophoresis onto NA-45 membrane (Schleicher & Schuell Co.) strips inserted into the agarose (48). DNA bound to the membrane was obtained by elution in buffer containing 1 M NaCl at 60°C, followed by dialysis and ethanol precipitation before it was used in cloning experiments.

**Restriction endonuclease mapping and hybridization analysis.** Restriction enzyme digestions were carried out according to the suppliers' recommendations. Comparisons of single, double, triple, and partial digestions with restriction endonucleases were used to identify the relative positions of cleavage sites within the cloned DNA. Horizontal gels containing 0.7 to 2.0% agarose or 5% polyacrylamide were run in a Tris-borate-EDTA buffer system and visualized with ethidium bromide. For hybridization studies, DNA fragments separated in agarose were transferred to nitrocellulose by the method of Southern (39). DNA was transferred to nitrocellulose with 20× SSPE (0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM disodium EDTA, pH 7.4) (18) as the transfer buffer. Prehybridization was done for 4 h in 5× SSPE–5× Denhardt solution (19)–200 μg of denatured salmon sperm DNA per ml at 65°C. Hybridization with nick-translated probe was for 15 h in 5× SSPE–5× Denhardt solution–100 μg of denatured salmon sperm DNA per ml at 65°C. The filters were washed at room temperature twice for 20 min in 2× SSPE–0.1% sodium dodecyl sulfate and then twice for 20 min in 0.1× SSPE–0.1% sodium dodecyl sulfate. Colony hybridizations were carried out under the same conditions. DNA probes were labeled with 32P in vitro by nick translation as described by Maniatis et al. (18).

**Direct selection of the HindIII ermA fragment.** The ermA determinant inserted in the com mutant CP1415 was excited with some neighboring DNA by HindIII digestion and ligated to pMB9. (pMB9 was selected as a vector to avoid introducing a new beta-lactamase gene into pneumococcus in later crosses.) Transformation of E. coli DH1 yielded Em' Tc' transforms. Analysis of plasmids in the transformants showed that they contained pMB9, ermA (truncated at its HindIII site), and 510 base pairs of pneumococcal DNA (including a smaller TaqI fragment, as expected). One was retained for further study and named pXF154 (Fig. 1).

**Cloning by excision of inserted plasmid.** In attempts to excise the DNA surrounding pMB9 in CP1515, samples of purified DNA from CP1515 were digested with various restriction enzymes (EcoRI, PstI, BamHI, SalI, ApaI, or HindIII), ligated, and used to transform E. coli. Tetracycline- or erythromycin-resistant colonies were detected for each enzyme treated. These recombinant plasmids were analyzed by digestion with appropriate restriction enzymes to determine the size of the inserted DNA and to determine whether intact insert-vector junctions were present.

**In vitro mutagenesis.** pXF170, pXF180, and pXF190 were partially digested with one of the following restriction endonucleases: ClaI, AsuII, HpaII, and TaqI. After agarose gel electrophoresis, full-length linear plasmid was recovered by using an NA-45 membrane and ligated to the ermA ClaI fragment of pR29, which was also isolated from agarose. This ligation mixture was used to transform E. coli DH1. The plasmid structure of these Em' transformants was examined by restriction analysis. Those plasmids carrying an ermA insertion in the full-length plasmid were used to transform CP1200 with selection for Em'.

**In situ colony competence test.** The transformability of mutant strains was determined, for each of several single-colony isolates, by plating 100 to 2,000 cells in DNA-agar plates for the in situ colony papillation test, as described previously (11, 27). Transformable (Xfo') cells gave rise after 40 h at 37°C to colonies with large Mal' papillations, whereas Xfo' cells developed into tiny, translucent nonpapillated colonies. The fraction of papillated colonies

FIG. 1. Direct cloning from the com::ermB mutant. Maps of (A) the putative structure of the com mutation in CP1415 and (B) the putative mutagenic circle causing that mutation by insertion-duplication. HindIII digestion of CP1415 released a 2.3-kb fragment carrying the ermA determinant and 300 base pairs of pneumococcal DNA. Insertion of the HindIII fragment into pMB9 formed (C) pXF154, subcloning the same fragment into pMV158 yielded (D) pXF160.
TABLE 1. Phenotype of insertion-duplication mutants at the com locus

<table>
<thead>
<tr>
<th>Donor vector*</th>
<th>Boundaries of insert*</th>
<th>Em* yield† (x10⁶)</th>
<th>No. of Em* mutants tested</th>
<th>No. of subclones tested‡</th>
<th>% Papillated† (range)</th>
<th>% Papillated in control cultures§</th>
<th>Phenotype of mutants†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMB9</td>
<td>Clai-HindIII (2.17-2.68)</td>
<td>0.05</td>
<td>18</td>
<td>250</td>
<td>0-5</td>
<td>93 (Xfo⁻)</td>
<td>Xfo⁻</td>
</tr>
<tr>
<td>pJDC4</td>
<td>HindIII (2.68-5.96)</td>
<td>0.6</td>
<td>8</td>
<td>200-2,000</td>
<td>62-94</td>
<td>85 (Xfo⁻)</td>
<td>Xfo⁻</td>
</tr>
<tr>
<td>pJDC4</td>
<td>EcoRI-Dral (0.75-4.7)</td>
<td>0.5</td>
<td>4</td>
<td>200-400</td>
<td>86-93</td>
<td>90 (Xfo⁻)</td>
<td>Xfo⁻</td>
</tr>
</tbody>
</table>

* E. coli plasmid clone carrying a com region fragment, comprising a vector, a pneumococcal insert, and (for pMB9) an additional EmB determinant.
† Restriction sites and map positions (Fig. 2) of the ends of the pneumococcal insert.
‡ For each donor plasmid, the indicated number of Em* transformants were obtained in 1 ml of cells exposed to 1 µg of DNA for 30 min. In each cross, several pneumococcal Em* transformants were selected for transformability evaluation.
§ Each Em* transformant colony tested was suspended in broth, and portions containing 100 to 1,000 cells were embedded in DNA-agar for an in situ colony competence assay.
† Papillated (Xfo⁺) colonies were scored after 40 h at 37°C.
‡ In each experiment, the indicated authentic Com⁺ and Com⁻ strains served as controls.
§ Assigned from the observed level of papillated colonies.

varied from 0 to 5% for leaky Xfo⁻ lines and from 70 to 99% for Xfo⁺ lines in independent experiments.

Liquid culture competence test. To test susceptibility to CF, cells were grown to an optical density of 0.1 in complete transformation medium (pH 7.0) and stored frozen. After thawing at 0°C, centrifugation, suspension in fresh complete transformation medium (pH 7.9) containing various amounts of CF, and incubation at 37°C with [3H]DNA from Nov⁺ strains for 45 min, cultures were assayed for DNA degradation and Nov⁺ transformants. Wild-type cells usually became competent with the addition of 0 to 1% CF under these conditions. Com⁻ mutants required the addition of 10 to 50% CF to become fully competent (29).

Maxicell analysis. Plasmid-encoded proteins were labeled and analyzed by the method of Sancar et al. (36, 37). After the labeling, the cells were suspended in solubilization buffer, and a portion was analyzed on a 15% sodium dodecyl sulfate–polyacrylamide gel (14). The gel was fixed, treated with 1 M sodium salicylate, dried, and exposed to Kodak XAR-5 film for 1 to 4 days at −70°C. 14C-labeled protein size standards were purchased from Amersham Corp.

RESULTS

Cloning from the com::erm mutant. A 2.3-kb HindIII fragment of the CP1415 chromosome contained approximately 500 base pairs of the mutant com region and the ermB determinant (Fig. 1); this was excised and inserted into pMB9 to form the E. coli plasmid pXF154, identified by selection for Em⁺. Two kinds of experiments verified that the pXF154 clone carried DNA from the com locus: reinserion into the pneumococcus chromosome and Southern hybridization. The reinserter experiments showed that the homology provided by the pneumococcal fragment in pXF154 permitted it to transform pneumococcus, and that the Em⁺ transformants, now carrying an insert of pMB9 and ermB, were defective in transformation (Table 1). Com⁻ mutants have been shown to require a high level of CF for induction of competence and, when so induced, to transform at wild-type levels (29). Four of the Em⁺ Xfo⁻ transformants produced by insertion of pXF154 were tested for CF induction by the same methods. All were induced to competence only by high levels of CF and, after such induction, took up and integrated chromosomal markers as efficiently as wild-type, competent cells (data not shown). Thus, the fragment cloned in pMB9 to form pXF154 directed insertion into the pneumococcus chromosome so as to produce the same phenotype (Com⁻) as the original com::erm mutation.

In the Southern hybridization experiments, the cloned fragment in pXF154 was used as a hybridization probe to compare homologous sequences in com⁺, com::ermB, and com::pMB9::ermB strains. The results (not shown) demonstrated physically that the insertion mutations disrupted DNA at the locus of the cloned 510-base-pair fragment in the pneumococcus chromosome, not at a distant location. They also provided preliminary restriction map data on the region in the wild-type chromosome.

Cloning of wild-type com DNA in the vector pKK232-8. In Southern hybridization experiments with wild-type pneumococcal DNA (data not shown), the pXF154 probe revealed single hybridizing bands of 2.7 and 4.2 kb for the restriction enzymes HindIII and DraI, respectively. These larger fragments from the com region were then cloned from the Com⁺ strain CP1200 in the vector pKK232-8 by using pXF160, a streptococcal plasmid carrying the 500-base-pair com fragment (Fig. 1), as a probe for colony hybridization. (In pKK232-8, a chloramphenicol acetyltransferase gene requires an inserted promoter for expression, and is placed upstream of rnb terminator signals.) Each fragment was cloned after enrichment by agarose gel fractionation of digested chromosomal DNA, selection for fragments expressing chloramphenicol acetyltransferase, and colony hybridization. The HindIII and DraI insert-bearing plasmids were named pXF170 and pXF180, respectively.

pXF180 was then used as the probe in a second Southern hybridization screen of CP1200, revealing an overlapping HindIII fragment of 3.3 kb. By using the HpaII A fragment (3.25 to 4.65 kb, Fig. 2) of pXF180 as a probe for colony hybridization, this 3.3-kb HindIII fragment was cloned, after enrichment by agarose gel fractionation of digested CP1200 chromosomal DNA, in pKK232-8. The resulting plasmid was named pXF190.

Map of the com region. The physical maps of the three overlapping DNA fragments obtained above were determined by comparison of single and multiple restriction enzyme digests of the plasmids pXF170, pXF180, and pXF190, and a map of the com region was deduced (Fig. 2). We observed no rearrangements in the large region (4.2 kb) of overlap among these clones.

An insertion duplication mutation is expected to disrupt a locus only if the targeting DNA is an internal fragment (21, 22, 29). To test whether this map encompassed the com unit of transcription, we conducted insertion duplication mutagenesis with the EcoRI-DraI fragment of pXF180 and with...
the large cloned HindIII fragment in pXF190 after transfer to an insertion vector, pJDC4 (Table 1). Since neither caused a transformation deficient phenotype, we conclude that these fragments contained the left and right ends of the locus, respectively, as indicated in Fig. 2.

**Failure of cloning of larger fragments in pMB9.** Strain CP1515 contains the com mutation caused by insertion of pXF154, including the ermB and pMB9 elements, at position 2.15 kb in the com locus (4). CP1515 DNA was digested with appropriate restriction enzymes, ligated, and used to transform E. coli DH1, in attempts to clone fragments of linked DNA directly in pMB9, by the rapid cloning procedure described by Mejean et al. (22). All of the recombinant plasmids obtained from the com region were deleted or rearranged, as indicated by loss of a junction or loss of vector sequences. The only Em\(^r\) transforms obtained with readily interpretable structures were identical to pXF154. The com fragments used for these experiments (as determined from Southern hybridization data, but not shown in Fig. 2) included the EcoRI-ClaI (0.69 to 2.15 kb) fragment and larger fragments produced by PstI (8 kb), BamHI (18 kb), SalI (14 kb), and ApaI.

Comparison of the results with the successful cloning of similar fragments from this region in pKK232-8 (see above) implies that this region contains sequences stable in pKK232-8 but not in pMB9. A direct subcloning experiment confirmed the result: the 4-kb com EcoRI-DraI fragment of pXF180 was unstable when transferred to pBR325. These results suggest that for this locus the rnrB transcription terminators in pKK232-8 may be important for stability with some fragments.

**Extent of the com locus.** To outline the extent of the com locus, new mutations were constructed in vitro within the pneumococcal DNA of pXF170, pXF180, and pXF190 and then purified and characterized in E. coli. Each mutant plasmid carried an ermB ClaI fragment, inserted at an HpaII, AsuII, Clal, or TaqI site. Each insertion mutation was then transferred by transformation into the wild-type (CP1200) pneumococcus chromosome. The determination of the phenotypes of the resulting insertion mutants is presented in Table 2. Plotting the results on the physical map (Fig. 2) shows that in a large section of the map all observed insertions caused a defect in transformability. Several of these mutations (at 1.2, 2.15, and 3.33 kb) were also tested with high levels of CF and found to respond with a normal competence reaction, as does the original Com\(^-\) mutant, CP1415. We conclude that the left end of the com locus lies between 0.7 and 1.2 kb on the map of Fig. 2, and that the right end lies between coordinates 5.3 and 5.9 kb. This entire region was susceptible to mutations causing transformation deficiency, and at the three well-separated sites tested for CF response the mutant phenotype was Com\(^-\).

To show genetically that the DNA in pXF180 included intact com sequences, its transforming activity was tested. We took advantage of the conditional nature of com:ermB mutations, inducing competence in a Com\(^-\) recipient with CF. The recipient was obtained by in vitro mutagenesis with plasmid pXF202, inserting ermB at the ClaI site, at position 2.15 kb in Fig. 2. Xfo\(^+\) transforms were obtained in good yield, with concomitant loss of the ermB marker, as expected for a homologous replacement event (data not shown).

**Gene products.** To obtain an initial indication of the com region gene products, plasmid-encoded proteins of pXF170, pXF180, and pXF215 (a derivative of pXF170 with a deletion near the left end) were labeled by the maxicell technique (Fig. 3). Vector gene products visible on the fluorograph were the 28-kilodalton (kDa) \(\beta\)-lactamase and the 26-kDa chloramphenicol acetyltransferase protein (in plasmids carrying a promoter insert). In the clone with the longest insert, pXF180, a 15.5-kDa protein, a 41-kDa protein, and a 90-kDa protein were synthesized in addition to the vector proteins. The next shorter insert, in pXF170, produced a 15.5-kDa protein and a small amount of a 41-kDa protein. With
pXF215 no detectable proteins other than the two proteins of the vector were labeled. As the deletion in pXF215 included one end of the com locus (Fig. 2), the loss in pXF215 of both proteins encoded by pXF170 suggests that the deletion removed the site acting as a promoter. The proteins encoded by the overlapping fragments in pXF170 and pXF180 are also consistent with an organization in which transcription is left to right, with genes for the 15.5-kDa and the 41-kDa proteins followed by one for the larger product, which could be truncated in pXF180. Since approximately 4.0 kb of DNA would be required to code for the 146 kDa of proteins seen with pXF180, and the com locus does not extend to the left beyond the EcoRI site at 0.6 kb, the entire length of pXF180 to the right of that site would be involved in coding for these proteins. Further studies will be required to complete the identification of gene products and gene locations at this locus.

**DISCUSSION**

com is the first locus involved in the control of competence for genetic transformation to be cloned. It is 4.2 to 5.2 kb long and apparently codes for at least three proteins. Since ermb insertions were readily obtained throughout the locus, we conclude that these genes are not essential for viability. Since the mutations studied at this locus so far are all insertions, and thus possibly polar, it remains possible that some of the proteins coded for by this region are not required for normal competence in the pneumococcus. This locus is more complex than would be expected for a gene which codes for the small protein CF. The size of the locus may reflect the complexity of events leading to competence. This complexity is suggested by a consideration of the minimum number of steps we already know are involved (41). First, CF is made at a low constitutive rate. Second, a specific CF receptor and signalling system is presumably responsible for eliciting the high-level CF synthesis that precedes competence. Third, another CF receptor and cognate signalling system may then be responsible for mediating the protein switch seen at competence induction. Mutations

**FIG. 3.** Analysis of [*35S*]methionine-labeled proteins produced in maxicells carrying plasmids containing com DNA. Proteins were separated in a 15% sodium dodecyl sulfate-polyacrylamide gel followed by fluorography. Lanes: 1, no plasmid; 2, pKK222-8; 3, pXF170; 4, pXF180; 5, pXF215; 6 and 7, standards. Sizes and positions of molecular size standards (kDa) are indicated on the right; gene assignments and new protein sizes (kDa) are indicated on the left.

**TABLE 2.** Transformability of insertion mutants obtained by in vitro mutagenesis at the com locus

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th><em>ermB</em> insertion site and orientation*</th>
<th>No. of Em' mutants tested*</th>
<th>No. of subclones tested*</th>
<th>% Papillated colonies* (range)</th>
<th>% Papillated in control cultures*</th>
<th>Phenotype of mutant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pXF211</td>
<td>415::&lt;AsuII&gt;</td>
<td>2</td>
<td>1,000–1,200</td>
<td>95</td>
<td>97</td>
<td>&lt;1</td>
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<tr>
<td>pXF212</td>
<td>415::&lt;AsuII&gt;</td>
<td>2</td>
<td>1,100–1,200</td>
<td>95</td>
<td>97</td>
<td>Xfo*</td>
</tr>
<tr>
<td>pXF214</td>
<td>1220::&lt;AsuII&gt;</td>
<td>4</td>
<td>500–1,100</td>
<td>1-21</td>
<td>97</td>
<td>Xfo-</td>
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<tr>
<td>pXF213</td>
<td>1220::&lt;AsuII&gt;</td>
<td>4</td>
<td>600–1,400</td>
<td>3-5</td>
<td>97</td>
<td>Xfo-</td>
</tr>
<tr>
<td>pXF203</td>
<td>1220::&lt;AsuII&gt;</td>
<td>10</td>
<td>300–1,000</td>
<td>0</td>
<td>97</td>
<td>Xfo-</td>
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<tr>
<td>pXF207</td>
<td>1600::&lt;HpaII&gt;</td>
<td>2</td>
<td>700–900</td>
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<td>550–800</td>
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<td>3</td>
<td>1,100–1,500</td>
<td>0-4</td>
<td>93</td>
<td>Xfo-</td>
</tr>
<tr>
<td>pXF202</td>
<td>2150::&lt;TaqI&gt;</td>
<td>6</td>
<td>2,000</td>
<td>1</td>
<td>99</td>
<td>Xfo-</td>
</tr>
<tr>
<td>pXF205</td>
<td>2800::&lt;TaqI&gt;</td>
<td>3</td>
<td>1,000–1,250</td>
<td>3</td>
<td>93</td>
<td>Xfo-</td>
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<tr>
<td>pXF221</td>
<td>2800::&lt;TaqI&gt;</td>
<td>2</td>
<td>1,100–1,200</td>
<td>4-5</td>
<td>95</td>
<td>Xfo-</td>
</tr>
<tr>
<td>pXF222</td>
<td>3090::&lt;TaqI&gt;</td>
<td>2</td>
<td>1,100–1,200</td>
<td>3-4</td>
<td>95</td>
<td>Xfo-</td>
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<td>pXF217</td>
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<td>2</td>
<td>95</td>
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<tr>
<td>pXF210</td>
<td>3250::&lt;HpaII&gt;</td>
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<td>180–570</td>
<td>0</td>
<td>90</td>
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<tr>
<td>pXF206</td>
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<td>3</td>
<td>900–1,200</td>
<td>1-2</td>
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<td>1,000–1,100</td>
<td>2-5</td>
<td>92</td>
<td>Xfo-</td>
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<tr>
<td>pXF219</td>
<td>3660::&lt;TaqI&gt;</td>
<td>2</td>
<td>1,000–1,100</td>
<td>2-4</td>
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<td>5300::&lt;TaqI&gt;</td>
<td>2</td>
<td>1,000–1,100</td>
<td>17-60</td>
<td>92</td>
<td>Xfo-</td>
</tr>
</tbody>
</table>

* Plasmid clone in E. coli carrying pneumococcal DNA interrupted by an ermb insertion constructed in vitro.
* Restriction site used for ermb insertion; distance from the HindIII site, in base pairs, as indicated by the scale in Fig. 2; insert orientation indicating, if known, whether ermb transcription is to the right (>), or the left (<), on the map in Fig. 2.
* Each mapped insertion was transferred to the pneumococcal chromosome by transformation; several of the pneumococcal Em' transformants were selected for transformability evaluation.
* Each Em' transformant colony tested was suspended separately in broth. Portions containing from 100 to 1,000 cells were embedded in DNA agar for the in situ colony competence assay. The range of the number of colony transformants for each set of Em' transformants is given.
* Papillated (Xfo'), transformant-containing colonies were scored after 40 h at 37°C. Maximum and minimum results are shown.
* In each experiment, authentic Com*' and Com' strains served as controls. The percentage of papillated colonies among 200 to 2,000 tested is given.
* Assigned from the observed level of papillated colonies.
* Plasmids for which the transformants were tested with high levels of CF and shown to respond with induction of normal competence.

*a* 90kd - 12.5k - 41 - 21.5 - 15.5 - 46 - 2 

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with the Com− phenotype may be expected to affect any component before the second CF receptor. Analysis of the individual roles of the products of the com locus in competence regulation should provide a new path to understanding this complex process.

The com locus presented cloning problems. Specifically, there were at least two areas within the region which could not be obtained as pMB9 clones, but were recovered intact in the terminator vector pKK232-8. Furthermore, these fragments promoted high levels of expression of the pKK232-8 chloramphenicol acetyltransferase gene. Our initial interpretation of these observations, paralleling that of Stassi and Lacks (40) for the mal locus, is that the region contains sequences recognized as strong promoters in E. coli. Apparently similar observations have been reported for several other pneumococcal genes among the few cloned to date, suggesting that this effect may be particularly widespread in pneumococcal DNA (21, 32).

The results described here constitute a further demonstration of several recently developed techniques for this species that were discussed in the report (29) of the isolation of com insertion duplication mutants of pneumococcus (22, 31, 32, 47). Thus, homologous exchange in transformation provides a reliable mechanism both for insertion duplication mutagenesis and for introducing specific insertion mutations made in vitro. Also, with an appropriate choice of the inserted marker, DNA neighboring an insertion duplication mutation can be selectively recovered with the inserted marker.

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