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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 pneumococcus chromosome, they showed that the *com* locus was 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 (25, 27), and heteroduplex repair (5, 10). Few mutations have been described, however, which affect the regulation of the competent 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 pneumococcus 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^r with DNA from DP1500 ('5MC', *nov-1 bry str-1 ery-2 ery-6*) (38) and to Mal^- (*malM511*) with DNA from the R6 strain, C36MK (9), as described previously (27). The Com^- mutant CP1315 was backcrossed to CP1200 to produce CP1415 (*malM511 str-1 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 pAMβ1 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 *Cla*I *ermB* determinant of pR29 to replace the Tet^r 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 buoyant density 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 $NaH_2PO_4 \cdot H_2O$, 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 ^{32}P in vitro by nick translation as described by Maniatis et al. (18).

Direct selection of the *Hin*dIII *ermB* fragment. The *ermB* determinant inserted in the *com* mutant CP1415 was excised with some neighboring DNA by *Hin*dIII 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^r Tc^r transformants. Analysis of plasmids in the transformants showed that they contained pMB9, *ermB* (truncated at its *Hin*dIII site), and 510 base pairs of pneumococcal DNA (including a smaller *Taq*I 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 (*Eco*RI, *Pst*I, *Bam*HI, *Sal*I, *Apa*I, or *Hin*dIII), ligated, and used to transform *E. coli*. Tetracycline- or erythromycin-resistant colonies were detected for each enzyme tested. 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: *Cla*I, *Asu*II, *Hpa*II, and *Taq*I. After agarose gel electrophoresis, full-length linear plasmid was recovered by using an NA-45 membrane and ligated to the *ermB Cla*I 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^r transformants was examined by restriction analysis. Those plasmids carrying an *ermB* insertion in the full-length plasmid were used to transform CP1200 with selection for Em^r.

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

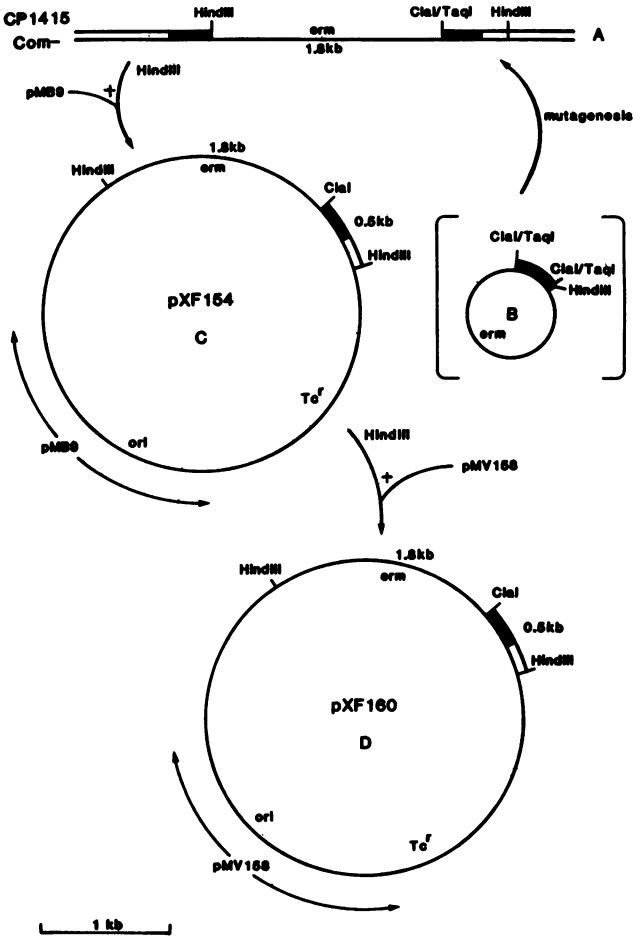

FIG. 1. Direct cloning from the *com*::*erm* mutant. Maps of (A) the putative structure of the *com* mutation in CP1415 and (B) the putative mutagenic circle causing that mutation by insertion-duplication. *Hin*dIII digestion of CP1415 released a 2.3-kb fragment carrying the *ermB* determinant and 500 base pairs of pneumococcal DNA. Insertion of the *Hin*dIII 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

Donor vector[a]	Boundaries of insert[b]	Em^r yield[c] (x10^6)	No. of Em^r mutants tested	No. of subclones tested[d]	% Papillated[e] (range)	% Papillated in control cultures[f]		Phenotype of mutants[g]
						CP1200 (Xfo$^+$)	CP1415 (Xfo$^-$)	
pMB9	*Cla*I-*Hin*dIII (2.17–2.68)	0.05	18	250	0–5	93	0.4	Xfo$^-$
pJDC4	*Hin*dIII (2.68–5.96)	0.6	8	200–2,000	62–94	85	0.2	Xfo$^+$
pJDC4	*Eco*RI-*Dra*I (0.75–4.7)	0.5	4	200–400	86–93	90	1	Xfo$^+$

[a] *E. coli* plasmid clone carrying a *com* region fragment, comprising a vector, a pneumococcal insert, and (for pMB9) an additional ErmB determinant.
[b] Restriction sites and map positions (Fig. 2) of the ends of the pneumococcal insert.
[c] For each donor plasmid, the indicated number of Em^r transformants were obtained in 1 ml of cells exposed to 1 μg of DNA for 30 min. In each cross, several pneumococcal Em^r transformants were selected for transformability evaluation.
[d] Each Em^r 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.
[e] Papillated (Xfo$^+$) colonies were scored after 40 h at 37°C.
[f] In each experiment, the indicated authentic Com$^+$ and Com$^-$ strains served as controls.
[g] 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 [^{3}H]DNA from Nov^r strains for 45 min, cultures were assayed for DNA degradation and Nov^r 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. ^{14}C-labeled protein size standards were purchased from Amersham Corp.

RESULTS

Cloning from the *com*::*erm* mutant. A 2.3-kb *Hin*dIII 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^r. Two kinds of experiments verified that the pXF154 clone carried DNA from the *com* locus: reinsertion into the pneumococcus chromosome and Southern hybridization.

The reinsertion experiments showed that the homology provided by the pneumococcal fragment in pXF154 permitted it to transform pneumococcus, and that the Em^r 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^r 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 *Hin*dIII and *Dra*I, 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 *rrnB* 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 *Hin*dIII and *Dra*I 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 *Hin*dIII fragment of 3.3 kb. By using the *Hpa*II A fragment (3.25 to 4.65 kb, Fig. 2) of pXF180 as a probe for colony hybridization, this 3.3-kb *Hin*dIII 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 *Eco*RI-*Dra*I fragment of pXF180 and with

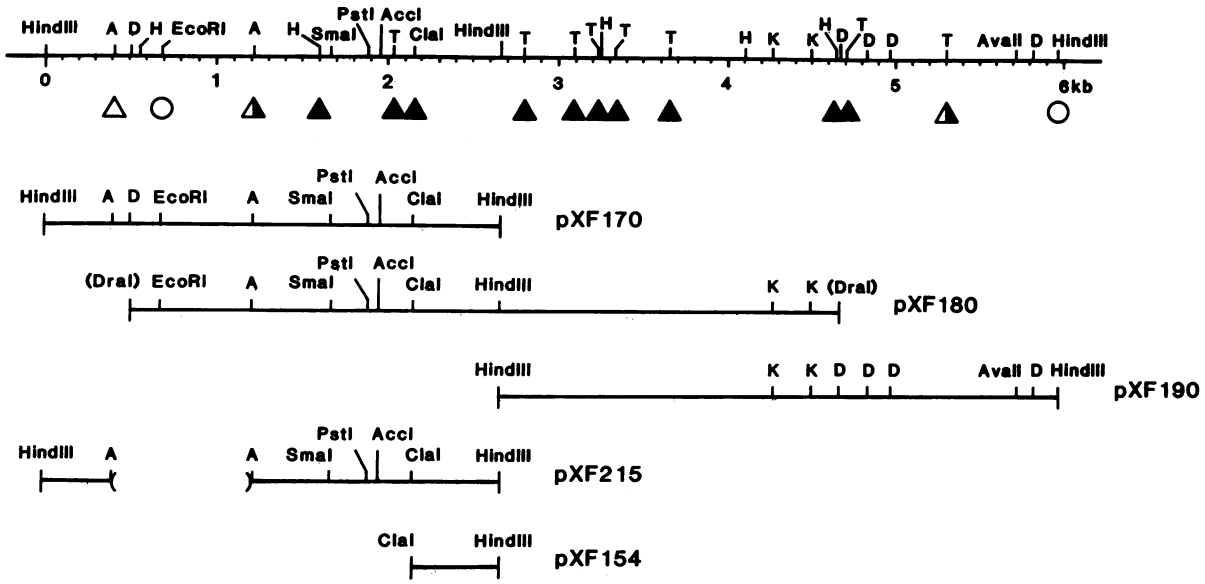

FIG. 2. *com* locus of *S. pneumoniae*. Physical maps of (top) the *com* locus and (bottom) the four independently cloned fragments carried in pXF170, pXF180, pXF190, and pXF154. The structure of pXF215, obtained by *Asu*II digestion of pXF170, is also shown, with the boundaries of the deletion indicated by parentheses. The vector for pXF154 was pMB9; the vector for the other four plasmids was pKK232-8. The locations of *ermB* insertion mutations created in vitro and characterized after transfer into the pneumococcus chromosome are indicated as follows: ▲, Xfo^-; △, Xfo^+; ◮, $Xfo^\pm$. Also shown are the distal endpoints (○) of fragments used for insertion duplication mutagenesis and producing Xfo^+ mutants (Table 1). Restriction enzyme abbreviations: A, *Asu*II; D, *Dra*I; H, *Hpa*II; K, *Kpn*I; T, *Taq*I.

the large cloned *Hin*dIII 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 transformants 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 by the *Eco*RI-*Cla*I (0.69 to 2.15 kb) fragment and larger fragments produced by *Pst*I (8 kb), *Bam*HI (18 kb), *Sal*I (14 kb), and *Apa*I.

Comparison of these negative 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 Eco*RI-*Dra*I fragment of pXF180 was unstable when transferred to pBR325. These results suggest that for this locus the *rrnB* 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 Cla*I fragment, inserted at an *Hpa*II, *Asu*II, *Cla*I, or *Taq*I 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 1.22, 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 *Cla*I site, at position 2.15 kb in Fig. 2. Xfo^+ transformants 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) β-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

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

Plasmid[a]	*ermB* insertion site and orientation[b]	No. of Em^r mutants tested[c]	No. of subclones tested[d]	% Papillated colonies[e] (range)	% Papillated in control cultures[f]		Phenotype of mutant[g]
					CP1200 (Xfo$^+$)	CP1415 (Xfo$^-$)	
pXF211	415::*Asu*II>	2	1,000–1,200	95	97	<1	Xfo$^+$
pXF212	415::<*Asu*II	2	1,100–1,200	95	97	<1	Xfo$^+$
pXF214	1220::*Asu*II>	4	500–1,100	1–21	97	<1	Xfo$^{+/-}$
pXF213	1220::<*Asu*II	4	600–1,400	3–5	97	<1	Xfo$^-$
pXF203	1220:*Asu*II	10	300–1,000	0	97	<1	Xfo^{-h}
pXF207	1600::<*Hpa*II	2	700–900	0	90	0	Xfo$^-$
pXF208	1600::*Hpa*II>	3	550–800	0	90	0	Xfo$^-$
pXF204	2030::*Taq*I>	3	1,100–1,500	0–4	93	2	Xfo$^-$
pXF202	2150::<*Cla*I	6	2,000	1	99	1	Xfo^{-h}
pXF205	2800::<*Taq*I	3	1,000–1,250	3	93	2	Xfo$^-$
pXF221	2800::<*Taq*I	2	1,100–1,200	4–5	95	1	Xfo$^-$
pXF222	3090::<*Taq*I	2	1,100–1,200	3–4	95	1	Xfo$^-$
pXF217	3240::<*Taq*I	2	900–1,000	2	95	1	Xfo$^-$
pXF210	3250::*Hpa*II>	3	180–570	0	90	0	Xfo$^-$
pXF206	3335::*Taq*I>	3	900–1,200	1–2	93	2	Xfo^{-h}
pXF216	3335::*Taq*I>	2	1,000–1,100	2–5	92	3	Xfo$^-$
pXF219	3660::*Taq*I>	2	1,000–1,100	2–4	92	3	Xfo$^-$
pXF209	4645::*Hpa*II>	2	400–600	0	90	0	Xfo$^-$
pXF220	4705::*Taq*I>	2	1,000–1,100	2	92	3	Xfo$^-$
pXF218	5300::<*Taq*I	2	1,000–1,100	17–60	92	3	Xfo$^{+/-}$

[a] Plasmid clone in *E. coli* carrying pneumococcal DNA interrupted by an *ermB* insertion constructed in vitro.
[b] Restriction site used for *ermB* insertion; distance from the *Hin*dIII 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.
[c] Each mapped insertion was transferred to the pneumococcus chromosome by transformation; several of the pneumococcal Em^r transformants were selected for transformability evaluation.
[d] Each Em^r 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 cells tested for each set of Em^r transformants is given.
[e] Papillated (Xfo$^+$), transformant-containing colonies were scored after 40 h at 37°C. Maximum and minimum results are shown.
[f] In each experiment, authentic Com$^+$ and Com$^-$ strains served as controls. The percentage of papillated colonies among 200 to 2,000 tested is given.
[g] Assigned from the observed level of papillated colonies.
[h] Plasmids for which the transformants were tested with high levels of CF and shown to respond with induction of normal competence.

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 *Eco*RI 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.

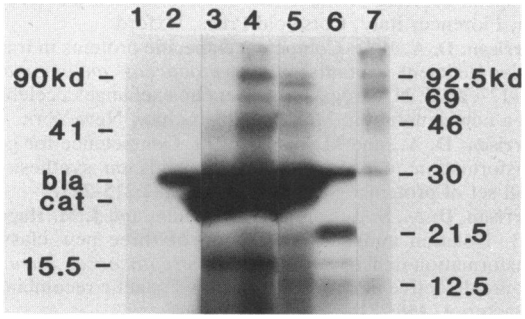

FIG. 3. Analysis of [^{35}S]methionine-labeled proteins produced in maxicells carrying plasmids containing *com* DNA. Proteins were separated in a 15% sodium dodecyl sulfate-polyacrylamide gel followed by fluorography. Lanes: 1, no plasmid; 2, pKK232-8; 3, pXF170; 4, pXF180; 5, pXF215; 6 and 7, standards. 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.

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

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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LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27**:151–160.
3. **Burdett, V.** 1980. Identification of tetracycline-resistant R-plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **18**:753–760.
4. **Chandler, M. S., and D. A. Morrison.** 1987. Molecular cloning of a competence control region from *Streptococcus pneumoniae* by use of transcription terminator vectors in *Escherichia coli*, p. 193–196. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
5. **Claverys, J. P., H. Prats, H. Vasseghi, and M. Gherardi.** 1984. Identification of *Streptococcus pneumoniae* mismatch repair genes by an additive transformation approach. Mol. Gen. Genet. **196**:91–96.
6. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
7. **Gaustad, P.** 1979. Genetic transformation in *Streptococcus sanguis*. Distribution of competence and competence factors in a collection of strains. Acta. Pathol. Microbiol. Scand. Sect. B **87**:123–128.
8. **Gaustad, P., J. Eriksen, and S. D. Henriksen.** 1979. Genetic transformation in *Streptococcus sanguis*. Spontaneous and induced competence of selected strains. Acta. Pathol. Microbiol. Scand. Sect. B **87**:117–122.
9. **Lacks, S. A.** 1968. Genetic regulation of maltosaccharide utilization in pneumococcus. Genetics **60**:685–706.
10. **Lacks, S. A.** 1970. Mutants of *Diplococcus pneumoniae* that lack deoxyribonucleases and other activities possibly pertinent to genetic transformation. J. Bacteriol. **101**:373–383.
11. **Lacks, S. A., and B. Greenberg.** 1973. Competence for deoxyribonucleic acid uptake, and deoxyribonuclease action external to cells in the genetic transformation of *Diplococcus pneumoniae*. J. Bacteriol. **114**:152–163.
12. **Lacks, S., B. Greenberg, and M. Neuberger.** 1974. Role of deoxyribonuclease in the genetic transformation of *Diplococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **71**:2305–2309.

12a. **Lacks, S. A., P. Lopez, B. Greenberg, and M. Espinosa.** 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. **192**:753–765.

13. **Lacks, S., and M. Neuberger.** 1975. Membrane location of a deoxyribonuclease implicated in the genetic transformation of *Diplococcus pneumoniae*. J. Bacteriol. **124**:1321–1329.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Leonard, C. G., and R. M. Cole.** 1972. Purification and properties of streptococcal competence factor isolated from chemically defined medium. J. Bacteriol. **110**:273–280.
16. **Leonard, C. G., J. M. Ranhand, and R. M. Cole.** 1970. Competence factor production in chemically defined media by noncompetent cells of group H streptococcal strain challis. J. Bacteriol. **104**:674–683.
17. **Low, B.** 1968. Formation of merodiploids in matings with a class of *rec*$^-$ recipient strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **60**:160–167.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Marko, M. A., R. Chipperfield, and H. C. Birnboim.** 1982. A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. Anal. Biochem. **121**:382–387.
20. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
21. **Martin, B., H. Prats, and J.-P. Claverys.** 1985. Cloning of the *hexA* mismatch-repair gene of *Streptococcus pneumoniae* and identification of the product. Gene **34**:293–303.
22. **Mejean, V., J.-P. Claverys, H. Vasseghi, and A.-M. Sicard.** 1981. Rapid cloning of specific DNA fragments of *Streptococcus pneumoniae* by vector integration into the chromosome followed by endonucleolytic excision. Gene **15**:289–293.
23. **Morrison, D. A.** 1979. Transformation and preservation of competent bacterial cells by freezing. Methods Enzymol. **68**:326–331.
24. **Morrison, D. A.** 1981. Competence-specific protein synthesis in *Streptococcus pneumoniae*, p. 39–53. *In* M. Polsinelli and G. Mazza (ed.), Transformation—1980, Proceedings of the 5th European Meeting on Bacterial Transformation and Transfection, Florence, Italy, Cotswold Press, Oxford.
25. **Morrison, D. A.** 1981. Competence-specific proteins in transformation-deficient mutants of *Streptococcus pneumoniae*, p. 171–177. *In* U. N. Streips (ed.), Genetic exchange: a celebration and a new generation. Marcel Dekker, Inc., New York.
26. **Morrison, D. A., and M. Baker.** 1979. Competence for genetic transformation in pneumococcus depends on synthesis of a small set of proteins. Nature (London) **282**:215–217.
27. **Morrison, D. A., S. A. Lacks, W. R. Guild, and J. M. Hageman.** 1983. Isolation and characterization of three new classes of transformation-deficient mutants of *Streptococcus pneumonie* that are defective in DNA transport and genetic recombination. J. Bacteriol. **156**:281–290.
28. **Morrison, D. A., B. Mannarelli, and M. N. Vijayakumar.** 1982. Competence for transformation in *Streptococcus pneumoniae*: an inducible high-capacity system for genetic exchange, p. 136–138. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
29. **Morrison, D. A., M.-C. Trombe, M. K. Hayden, G. A. Waszak, and J.-D. Chen.** 1984. Isolation of transformation-deficient *Streptococcus pneumoniae* mutants defective in control of

competence, using insertion-duplication mutagenesis with the erythromycin resistance determinant of pAMβ1. J. Bacteriol. **159**:870–876.
30. **Pakula, R., and W. Walczak.** 1963. On the nature of competence of transformable streptococci. J. Gen. Microbiol. **31**:125–133.
31. **Pozzi, G., and W. R. Guild.** 1985. Modes of integration of heterologous plasmid DNA into the chromosome of *Streptococcus pneumoniae*. J. Bacteriol. **161**:909–912.
32. **Prats, H., B. Martin, and J.-P. Claverys.** 1985. The *hexB* mismatch repair gene of *Streptococcus pneumoniae*: characterisation, cloning, and identification of the product. Mol. Gen. Genet. **200**:482–489.
33. **Raina, J. L., and A. W. Ravin.** 1980. Switches in macromolecular synthesis during induction of competence for transformation of *Streptococcus sanguis*. Proc. Natl. Acad. Sci. USA **77**:6062–6066.
34. **Ravin, A. W.** 1959. Reciprocal capsular transformations of pneumococci. J. Bacteriol. **77**:296–309.
35. **Rodriguez, R. L., F. Bolivar, H. M. Goodman, H. W. Boyer, and M. C. Betlach.** 1976. Construction and characterization of cloning vehicles, p. 471–478 *In* D. P. Nierlich, W. J. Rutter, and C. F. Fox (ed.), Molecular mechanisms in the control of gene expression. Academic Press, Inc., New York.
36. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.
37. **Sancar, A., R. P. Wharton, S. Seltzer, B. M. Kacinski, N. D. Clarke, and W. D. Rupp.** 1981. Identification of the *uvrA* gene product. J. Mol. Biol. **148**:45–62.
38. **Shoemaker, N. B., and W. R. Guild.** 1974. Destruction of low efficiency markers is a slow process occurring at a heteroduplex stage of transformation. Mol. Gen. Genet. **128**:283–290.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
40. **Stassi, D. L., and S. A. Lacks.** 1982. Effect of strong promoters on the cloning in *Escherichia coli* of DNA fragments from *Streptococcus pneumoniae*. Gene **18**:319–328.
41. **Tomasz, A.** 1966. Model for the mechanism controlling the expression of competent state in pneumococcus cultures. J. Bacteriol. **91**:1050–1061.
42. **Tomasz, A.** 1971. Cell physiological aspects of DNA uptake during genetic transformation in bacteria, p. 4–18. *In* L. Ledoux (ed.), Informative molecules in biological systems. North-Holland Publishing Co., Amsterdam.
43. **Tomasz, A.** 1972. Interactions between cell surface components and DNA during genetic transformation of bacteria, p. 311–334. *In* C. F. Fox (ed.), Membrane research. Academic Press, Inc., New York.
44. **Tomasz, A., and R. Hotchkiss.** 1964. Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc. Natl. Acad. Sci. USA **51**:480–487.
45. **Tomasz, A., and J. L. Mosser.** 1966. On the nature of the pneumococcal activator substance. Proc. Natl. Acad. Sci. USA **55**:58–66.
46. **Vijayakumar, M. N., and D. A. Morrison.** 1986. Localization of competence-induced proteins in *Streptococcus pneumoniae*. J. Bacteriol. **165**:689–695.
47. **Vijayakumar, M. N., S. D. Priebe, G. Pozzi, J. M. Hageman, and W. R. Guild.** 1986. Cloning and physical characterization of chromosomal conjugative elements in streptococci. J. Bacteriol. **166**:972–977.
48. **Winberg, G., and M.-L. Hammarskjöld.** 1980. Isolation of DNA from agarose gels using DEAE-paper. Application to restriction site mapping of adenovirus type 16 DNA. Nucleic Acids Res. **8**:253–264.