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Isolation of Transformation-Deficient *Streptococcus pneumoniae* Mutants Defective in Control of Competence, Using Insertion-Duplication Mutagenesis with the Erythromycin Resistance Determinant of pAMβ1

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Several transformation-deficient mutants of *Streptococcus pneumoniae* were isolated after insertion-duplication mutagenesis. Mutagenesis was accomplished by transformation of competent cells with chimeric DNA formed by the ligation of *Taq*I fragments of pneumococcal DNA to the erythromycin resistance determinant of the streptococcal plasmid pAMβ1. The two mutants described were characterized as defective in the control of competence induction, possibly due to a block in the production of the intercellular competence-inducing protein.

Studies of genetic transformation in *Streptococcus pneumoniae* (pneumococcus) have established the outline of DNA processing steps by which double-stranded DNA is bound at the cell surface, single-stranded fragments are extracted during uptake into the cell with concomitant degradation of the complementary strand, and these fragments are inserted into the chromosome at homologous sites (9, 10, 14, 15, 23–25); genetic mismatches in the resulting physical heteroduplex are resolved either by subsequent processing in the chromosome or by replication (3, 7, 30). It has long been known that this process is carried out only by cells in a specialized physiological state, termed competence (19, 33, 34); more recently, it has been found that competence is characterized by a temporary protein synthesis switch to production of a few novel proteins (21, 22, 29). However, very few of the proteins involved in this DNA processing have been identified.

Coordinated induction of competence throughout a culture is mediated by a soluble excreted protein, the competence factor (CF), acting as a population density monitor (28, 33, 34, 36). In strain Rx, the addition of a small amount of CF to sensitive cells elicits the appearance of a high level of CF, detectable in the culture medium; this is followed within a few minutes by the development of competence accompanied by induction of characteristic-specific proteins (21).

Several types of transformation-deficient mutants of *S. pneumoniae* have been characterized, but most of these have been defective in one or another specific DNA processing step (12, 26), rather than in competence control. This reflects both the central role of DNA processing in transformation and the practical advantage of having at least one step of DNA processing available for the assay of the competent state; without such an assay, it is difficult to know whether a mutation blocks transformation specifically or whether it simply changes the conditions appropriate for the development of competence in some nonspecific way.

We now describe a class of mutation causing a defect in the control of competence induction: the mutant could be induced to competence accompanied by normal levels of protein switching, DNA uptake, and recombination by the addition of CF. However, it was never spontaneously competent, it required CF for induction, and it released no detectable CF at competence. It thus appears to be defective in CF production.

Random mutagenesis was accomplished by transformation with chimeric DNA constructed in vitro and comprising the erythromycin resistance (Em^r) determinant of pAMβ1 ligated to small fragments of the pneumococcus chromosome, so as to effect insertional inactivation of chromosomal genes, in a variation of the method described for this transformation system by Mejean et al. (20) and Vasseghi et al. (38) and for the similar transformation system of *Bacillus subtilis* described by Niaudet et al. (27) and Ferrari et al. (8). The chromosomal fragments provide a homologous sequence to target insertion of chimeric circles into the recipient chromosome (Fig. 1). Drug-resistant transformants were then individually screened for their transformation (Xfo) phenotype.

MATERIALS AND METHODS

Strains. Streptococcal strains used are listed in Table 1. Plasmid pR29 contains the noninducible Em^r determinant of pAMβ1 (4), originating from pVA736, and was obtained from pR28 (20) by removal of the *Eco*RI B fragment of *amiA* (J.-P. Claverys, personal communication). It comprises pBR325 with the 1,400-base-pair *Ava*I-*Hin*dIII tetracycline resistance region replaced by the 1,800-base-pair *Ava*I-*Hin*dIII fragment of pVA736 carrying its Em^r determinant. The *Escherichia coli* host was strain 586 (F^- *thy* r_k^- m_k^- *recBC lop-11* Str^r) (2).

Media and culture conditions. Media and culture conditions were as described previously (26), including the basic broth, CAT, and the complete transformation medium, CTM. DNA from strain CP1016 used for testing competence was prepared and labeled with [^{3}H]thymidine at 1.4×10^6 CPM/μg. Plasmid DNA was prepared according to Marko et al. (18). Restriction enzymes were from and used as recom-

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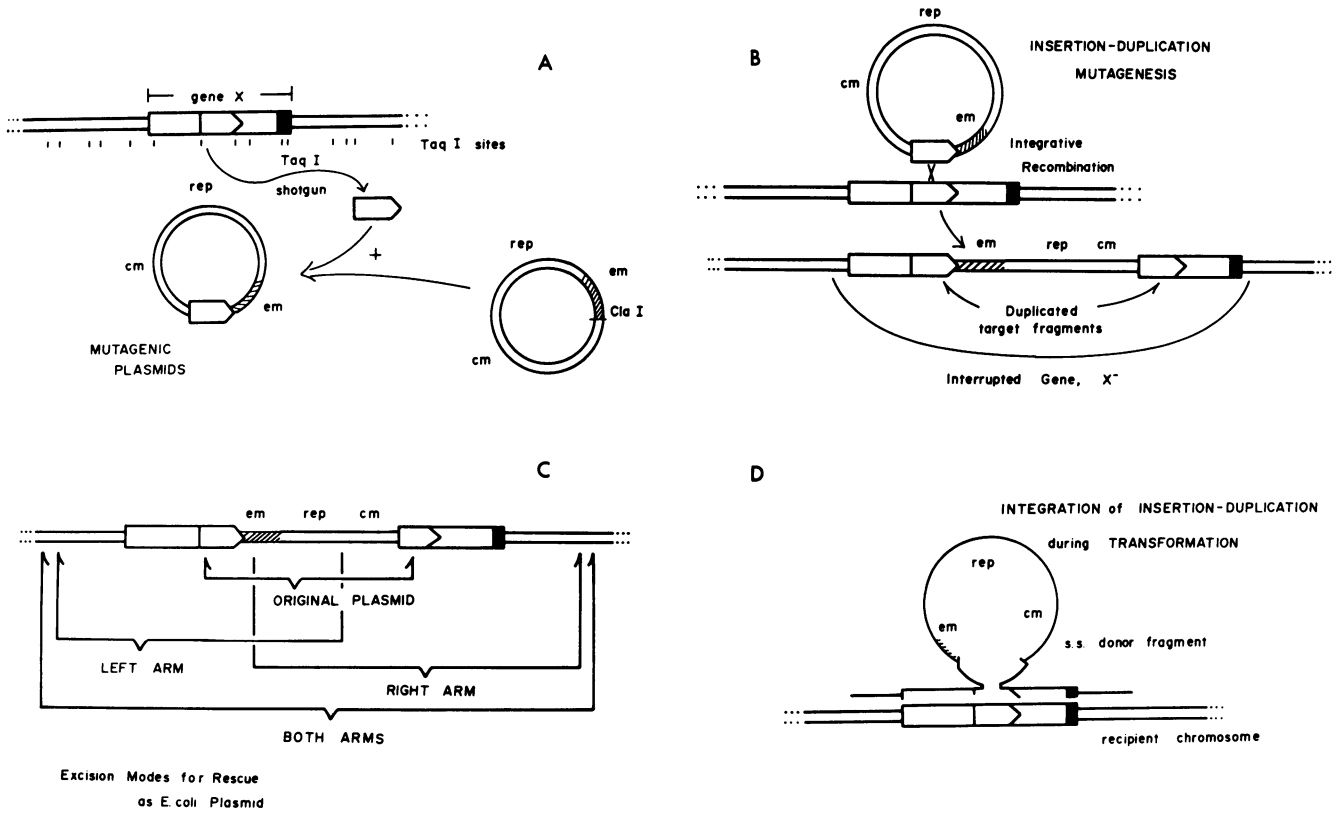

FIG. 1. Insertion-duplication mutagenesis. Illustration of the general case, in which (A) intragenic fragments from a chromosomal digest are inserted in vitro into molecules of a small *E. coli* cloning vector that is not capable of replication in the target species (e.g., *S. pneumoniae*) but carries a selectable marker (e.g., Em^r) expressed in both species. The primary Em^r transformants obtained upon transformation of *S. pneumoniae* with this DNA mixture (B) have the structure which is expected to result from recombinational insertion of the donor DNA circle at the target region of homology specified by each insert. Among the transformants, screening for X^- mutants reveals those produced by a molecule in which the inserted fragment was from gene X. The useful genetic properties of such mutants include precise excision, via reversal of the insertion event shown in (B); (C) rescue of surrounding regions in the form of a plasmid carrying chloramphenicol resistance (Cm^r) or Em^r or both and a replication origin (rep) active in *E. coli*, after restriction, ligation, and transformation of *E. coli*; and (D) transformation into a new host strain of the same species with transfer of the insertion by means of flanking homology.

mended by New England Biolabs. CF was prepared as the supernatant fluid of competent cultures of strain CP1000 in CTM medium and stored frozen, as described previously (21).

Insertion-duplication mutagenesis. The streptococcal Em^r determinant was obtained from pR29 by digestion with *Cla*I, cutting within the pBR325 moiety 6 base pairs from the *Hin*dIII site and within the streptococcal moiety 0.3 kilobases (F. Macrina, personal communication) from the *Ava*I site. The remaining pBR325 portion of this plasmid was inactivated by digestions with *Pst*I, *Eco*RI, and *Pvu*II. The resulting mixture, as a source of 1.5-kilobase *Cla*I fragments carrying the Em^r determinant, was ligated with a *Taq*I digest of pneumococcal chromosomal DNA (mass ratio, 1:5), with incubation at 33 μg/ml for 15 h at 4°C. The minimum amount of T4 ligase (kindly provided by P. Matsumura) required for complete ligation of an equal amount of *Eco*RI-digested lambda DNA was used in ligation buffer containing 66 mM

TABLE 1. Bacterial strains

Strain	Genotype[a]	Isolation no.	Source[b]
CP1000	Wild type	Rx-1	DP1000 (30)
CP1016	*nov-1 str-1 ery-2 vlt*		CP1015 × (CP1014 + CP1500 DNA)
CP1200	*malM511 str-1*		CP1015 × BP272 DNA (26)
CP1302	*malM511 str-1* Xfo^+ ΩEm^r	Ω2	CP1200 × IDM
CP1315	*malM511 str-1 com-15*::Em^r	Ω15	CP1200 × IDM
CP1322	*malM511 str-1 com-22*::Em^r	Ω22	CP1200 × IDM
CP1402	*malM511 str-1* Xfo^+ ΩEm^r	Ω2B	CP1200 × CP1302 DNA
CP1415	*malM511 str-1 com-15*::Em^r	Ω15B	CP1200 × CP1315 DNA
CP1422	*malM511 str-1 com-22*::Em^r	Ω22B	CP1200 × CP1322 DNA

[a] *vlt*, Requirement for 0.5 μg of thymidine per ml.

[b] Each new strain listed was constructed either by transformation with chromosomal DNA from the indicated donor or by insertion-duplication mutagenesis (IDM), transforming with chromosomal fragments ligated to the Em^r determinant, as described in the text.

Tris-hydrochloride (pH 8.0), 33 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 1 mM ATP. The product of this ligation reaction, but not the components, transformed strain CP1200 to Em^r at a frequency of ca. 0.01% with 1 μg of DNA per ml; the transformed cultures were frozen or plated after just 30 min of further incubation, to preclude segregation of sibling mutants.

Selection. Insertional transformants were selected in double-overlay plates with 2 μg of erythromycin per ml in the top layer. Most colonies appeared between 24 and 48 h of incubation and were resistant to at least 100 μg of drug per ml, as expected for this marker (37).

Screening. Twelve hundred individual Em^r clones were picked with a sterile wire, diluted, and immediately plated in maltose-DNA plates for the in situ transformation colony papillation assay of competence described previously (13, 26, 32). A single colony of each putative Xfo^- mutant (i.e., clones producing less than 2% papillated colonies) was picked, restreaked, grown in broth with erythromycin, and then frozen as a stock for further tests. After elimination of strains which were Xfo^+ on further testing, and of slow-growing mutants, 4 Xfo^- remained from this screen among 25 putative Xfo^-; two, named CP1315 and CP1322, are described here.

Testing competence induction. Our wild-type strains attain competence at optical densities (ODs) of ca. 0.03, 0.06, 0.1, and 0.2 (as measured in tubes [diameter, 18 mm] in the Coleman JrII colorimeter) in CTM medium adjusted to initial pH values of 7.9, 7.6, 7.3, and 7.0, respectively. Also, cultures grown in CTM medium (pH 7.0) to an OD of 0.1 can usually be induced to competence earlier by raising the pH (pH jump). Many can also be induced by adding a CF preparation (volume, 0.1 to 1%) to the pH-jump culture. All these conditions were used in testing the insertion mutant strains (see Tables 3 and 4). Induction of competence was routinely measured by assaying the appearance of the DNA-degrading activity that accompanies competence (13, 25). When checked, a peak of DNA-degrading activity was always accompanied by other competence-associated activities, such as DNA uptake and competence-specific protein synthesis shift.

Protein shift analysis. Cultures to be examined for competence-specific protein synthesis shifts were induced with CF in CTM medium and then transferred to a semisynthetic labeling medium (26) for pulse-labeling with [^{35}S]methionine during the development of competence. The cells were grown to an OD of 0.1 in CTM medium (pH 7.0), cooled to 0°C, pelleted, and resuspended either in the CF preparation (100%) or in CF diluted 1:10 with CTM medium (pH 7.9) (10%). After 10 min at 37°C, the cells were collected by centrifugation in the cold, resuspended in 1 volume of the labeling medium, and incubated at 37°C for the development of competence. For pulse-labeling, samples were treated with [^{35}S]methionine at 10 μCi/ml for 4 min, at indicated intervals. Labeled cells were lysed and analyzed on 12% sodium dodecyl sulfate-polyacrylamide gels by autoradiography as described previously (16, 22).

CF assay. Strain CP1200 was prepared for use in the CF assay by growth in CTM medium (pH 7.0) to an OD of 0.1 and then frozen with 10% glycerol. To assay CF, these cells were thawed, pelleted in the cold, and resuspended in fresh CTM medium (pH 7.9). During incubation at 37°C with [^{3}H]DNA and CF, these cells achieved half-maximal degradation of DNA at 20, 25, or 60 min when treated with the strain CP1000 CF preparation at 10, 1, or 0%, respectively. Unknown CF preparations were titrated by determining the lowest dilution causing half-maximal DNA degradation by 40 min.

TABLE 2. Linkage of transformation deficiency to erythromycin resistance in transformation backcrosses to CP1200

DNA source	Yield[a] (Em^r colonies)	Em^r clone no.[b]	No. of subclones[c] phenotypically:	
			Xfo^-	Xfo^+
CP1302 (Xfo^+)	1,800	1	0	300
		2	0	290
		3	0	280
CP1315 (Xfo^-)	120	1	200	4
		2	300	6
		3	300	6
CP1322 (Xfo^-)	60	1	9	1
		2	16	0
		3	280	15
		4	290	12
		5	290	6
		6	190	8

[a] Strain CP1200 competent cells (10^8/ml) were incubated for 30 min with 10 μg of DNA per ml, diluted, and plated after 30 min of incubation. A total of 1,000 colonies per plate correspond to 1% transformation.

[b] Several individual Em^r colonies from each cross were sampled at random for examination of the Xfo phenotype.

[c] A sample of cells (10 to 300) from each clone to be tested was plated with mal^+ DNA for the in situ colony papillation assay of transformability (26). In each plate, the smaller class was counted, and the larger was estimated from microscopic counts of ca. 1/5 the area of the plate.

RESULTS

Mutagenesis and screening. Our strategy was a modified form of the general scheme described in Fig. 1. We reduced the mutagenic chimeric plasmid described by Claverys (20, 38) to its simplest form; a fragment bearing a drug marker expressed both in *S. pneumoniae* and *E. coli* was ligated directly to small (*TaqI*) fragments of pneumococcal DNA. Transformation yielded Em^r recombinants at the expected (38) modest rate of about 10^{-4} per recipient, and they exhibited a range of initial growth rates, as described by Claverys et al. (37) for insertion of this segment plus most of pBR325.

Twelve hundred primary transformant colonies were individually screened by the in situ colony transformation test described previously (13, 26). Of four confirmed Xfo^- clones obtained, two displayed the phenotype described here. The other two, which did produce CF, are still under investigation. An Em^r clone with normal transformability (strain CP1302) was selected for use as an Xfo^+ control during further tests.

Linkage tests and backcross. To assess the relation between the Em^r and the Xfo phenotypes in these mutants, chromosomal DNA prepared from each mutant was used to transform the wild-type parent (strain (CP1200). All five insertion mutants yielded Em^r transformants, at frequencies from 1 to 100% of that expected for point markers. Cells from each of several Em^r clones from the crosses with strains CP1302, CP1315, and CP1322 were tested for transformability by the in situ colony assay (Table 2). The Em and Xfo traits of strains CP1315 and CP1322 were cotransformed. (Em^r Xfo^- progeny of these backcrosses were named CP1415 and CP1422.) Thus, the entire insertion-

TABLE 3. Spontaneous competence of erythromycin-resistant insertion mutants

Expt	pH of medium[a]	Culture density range tested[b]	Mutant strain											
			CP1302		CP1315		CP1322		CP1402		CP1415		CP1422	
			DNA degraded[c]	Nov[rd]	DNA degraded[c]	Nov[rd]	DNA degraded[c]	Nov[rd]	DNA degraded[c]	Nov[rd]	DNA degraded[c]	Nov[rd]	DNA degraded[c]	Nov[rd]
1	7.9	0.03–0.22	17,000	900	220	<1	40	<1						
	Jump[e]	0.1–0.2	13,800	1,300	34	<1	24	<1						
2	7.0	0.03–0.22	13	<0.1	10	<0.1	19	<0.1						
	7.3	0.03–0.02	1,370	400	25	<0.1	15	<0.1						
	7.6	0.03–0.02	2,130	140	20	<0.1	12	<0.1						
	7.9	0.03–0.02	1,940	240	33	<0.1	18	<0.1						
	Jump[e]	0.1–0.2	2,820	600	39	<0.1	30	<0.1						
3	7.0	0.03–0.33							9	<0.1	9	<0.1	29	<0.1
	7.3	0.03–0.33							1,080	124	13	<0.1	16	<0.1
	7.6	0.03–0.33							925	200	34	<0.1	39	<0.1
	7.9	0.03–0.33							1,007	72	29	<0.1	10	<0.1

[a] The initial pH of CTM medium was adjusted by the addition (per liter) of 3 meq of HCl, nothing, 3 meq of NaOH, or 6 meq of NaOH.
[b] Culture density range during DNA exposure, as OD. DNA was added to 28,000 cpm/ml (experiment 1) or 8,000 cpm/ml (experiment 2 and 3).
[c] DNA degraded to trichloroacetic acid-soluble form (cpm/ml).
[d] Transformants per microliter; diluted samples were plated with erythromycin (2 μg) and novobiocin (10 μg) per ml of top agar.
[e] Cultures grown in CTM medium (pH 7.0) to an OD of 0.1 were mixed with 9 meq of NaOH per liter before treatment with DNA.

duplication structure is apparently readily transferred by transformation, as predicted.

Transformation deficiency. To characterize the phenotypes of these mutants more rigorously, they were exposed to a wide range of conditions normally favorable for competence induction in our strains (Table 3). Competence was monitored both during growth from 1×10^7 to 3×10^8 CFU/ml at pH values from 7 to 8 and after a pH jump. As shown, competence was severely depressed under all these conditions in strains CP1315 and CP1322, by measures of DNA degradation and of transformants. The Xfo^+ Em^r strain CP1302 (Table 3) and wild-type strains (data not shown) regularly displayed normal levels of competence under these conditions. Protein shift assays showed no competence-associated changes of the peptide pattern in strain CP1315 or CP1322 under these conditions (data not shown).

As pointed out elsewhere (26), a potential difficulty in dealing with competence-deficient mutants is that competence for genetic transformation is a temporary property of pneumococcal cultures, often completely absent, and is induced under conditions, and by mechanisms, that are not well understood. One approach to this problem, taken elsewhere, is to restrict attention to those mutations whose phenotype includes at least one of the properties ordinarily concomitant with competence: in such mutants, one can examine competent cultures and describe specific defects in DNA processing in competent mutant cells. In mutants like those described here, whose phenotype is complete absence of any sign of competence under normal conditions, it is more difficult to rule out irrelevant mutations, for example, ones that may make metabolic changes causing subtle alterations in the conditions required for induction of competence, rather than any direct interference in the specific mechanisms of competence or competence induction. However, in this particular case, we show below that competence can be artificially provoked and that such competent cells then do process DNA apparently normally but do not release CF in detectable amounts. This single defect in the induced competent cells is also sufficient to explain the lack of competence under normal conditions.

Repair by CF. Mutants CP1315 and CP1322 responded weakly to the low levels (0.1 to 5%) of CF that we routinely use to provoke competence, showing low levels of DNA degradation and correspondingly low numbers of transformants (Tables 4 and 5). However, their defect could be fully corrected by high levels of CF; when treated with sufficient amounts of CF, they displayed normal levels of DNA-degrading activity and of transformation (Tables 4 to 6), and both CP1415 (Fig. 2) and CP1422 (data not shown) exhibited typical protein shifts at competence. This suggested that the mutations affected specifically the control of competence.

Table 4 also shows two unusual features of the CF induction of competence in the insertion mutants. First, the minimum level of CF required for full induction was up to 30 times higher than that for the Xfo^+ control. Second, for borderline levels of CF, induction occurred early or not at all.

Lack of CF release. In the normal sequence of events in Rx

TABLE 4. Competence induction with CF by 35 and 60 min

CF (%)[a]	Competence induced,[b] as DNA degraded[c]					
	by 35 min			by 60 min		
	CP1402	CP1415	CP1422	CP1402	CP1415	CP1422
25	558	457	243	505	655	473
12	659	557	303	405	581	377
6	576	256	178	600	352	292
3	518	48	45	583	84	53
1	457	18	20	582	22	24
0.3	175	19	17	541	18	20
0.1	19	20	—	400	19	21
0.03	20	21	18	16	21	24
0	20	15	17	20	19	22

[a] Percent volume of CF preparation in culture.
[b] Cells were prepared for CF treatment by growth in CTM medium (pH 7.0) from an OD of 0.002 to 0.1 and stored frozen. For CF induction, cells were thawed at 0°C, resuspended in 0.5 volume of supernatant fluid (after the addition of 9 meq of NaOH per liter), and mixed with CTM medium (pH 7.9) containing CF to provide CF at the indicated level. They were incubated at 37°C with Nov^r-[^{3}H]DNA (at 17,000 cpm/ml) and assayed for Nov^r transformants and DNA degradation at the times indicated.
[c] DNA degraded to trichloroacetic acid soluble form (cpm/100 μl). —, Not determined.

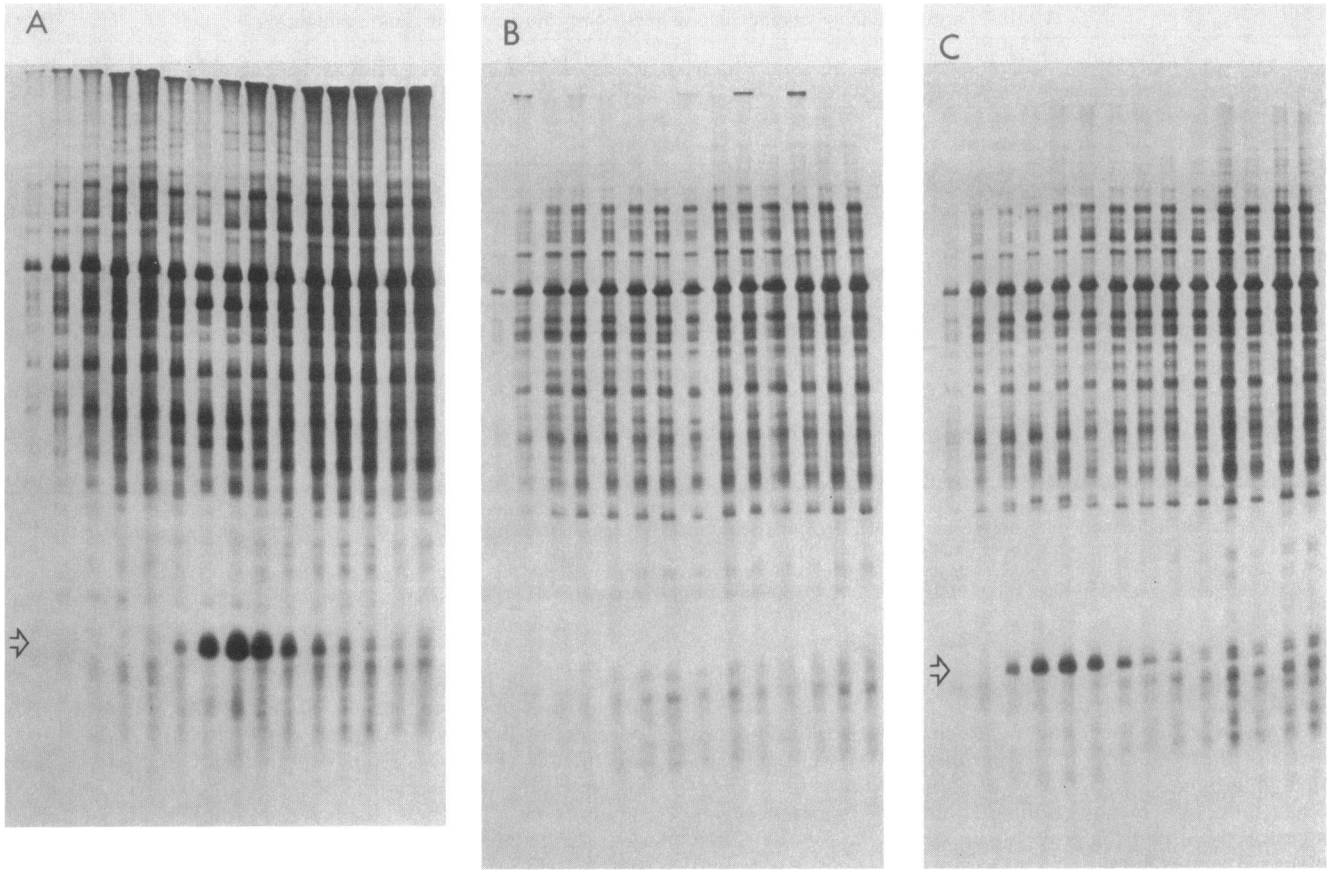

FIG. 2. Protein synthesis shift induced in erythromycin-resistant insertion mutants by competence factor. Cultures were pulse-labeled with [^{35}S]methionine at 4-min intervals starting 1 min after treatment with competence factor and were then analyzed on sodium dodecyl sulfate-polyacrylamide gels. (A) strain CP1402, 10% CF; strain CP1415, 10% CF; (C) strain CP1415, 100% CF. Sequential pulses are shown from left to right. Arrows show position of the major competence-specific band (molecular weight, 19,500).

cultures (21), a low level of CF added to a culture acts indirectly by stimulating the accumulation of CF in the culture medium to higher levels, which directly and quickly induce competence. Since unusually high levels of CF were required for induction of these mutants, it seemed possible either that their sensitivity to CF was reduced or that they were incapable of the burst of CF release usually preceding competence, so that CF must be added initially at fully inducing levels.

To distinguish these possibilities, CF release was assayed in the mutant cultures after induction by high levels of CF (Tables 6 and 7). At the time of competence, high levels of CF were detected in the culture medium of strain CP1402 controls, but no such increase occurred with mutants CP1415 or CP1422. Thus the defect appears to be in CF synthesis, release, or activity, rather than simply in sensitivity to CF.

DISCUSSION

In this paper we describe the first application of the recently developed method of insertion-duplication mutagenesis (8, 20, 27, 31, 38) (Fig. 1) to add to the broad class of transformation-deficient mutants. On the basis of this experience we expect that it will be of further use in this context and that it could be successfully exploited for the study of nonessential genes in any of the naturally transformable species. Also, the two central features of this method (viz., the mutagenic effect of insertion of a circular molecule containing a small homologous fragment and the selection for integrating homologous recombination by using a nonreplicative DNA circle) have been applied in a wide variety of genetic contexts, including *Synechoccus* R2 (39), *B. subtilis* (6), *Agrobacterium* Ti plasmid (5), *Saccharomyces* spp. (11), and *Dictyostelium discoideum* (1).

The molecular mechanism of the transformation event leading to insertion-duplication mutagenesis is not understood; the intracellular circular molecule drawn in Fig. 1B is

TABLE 5. Competence induction with CF by 40 min

CF (%)[a]	Competence induced by 40 min[b] in strain:					
	CP1302		CP1315		CP1322	
	DNA degraded[c]	Novrd	DNA degraded[c]	Novrd	DNA degraded[c]	Novrd
100	575	3,600	456	2,000	275	1,300
30	412	2,600	224	2,300	264	1,800
10	487	2,100	53	400	38	200
3.3	537	1,500	14	40	28	20

[a] See Table 4, footnote *a*.
[b] Cells were prepared as in Table 4, suspended in CTM medium (pH 7.9) with DNA at 16,000 cpm/ml and CF at the indicated levels, and assayed after 40 min at 37°C.
[c] See Table 4, footnote *c*.
[d] Number of novobiocin-resistant transformants per microliter, assayed in double-overlay plates with erythromycin and novobiocin.

TABLE 6. Genetic transformation in induced cultures of erythromycin-resistant insertion mutants

Time[a] at 37°C after CF + DNA	[^{3}H]DNA degraded[b]			Transformants[c]		
	CP1402	CP1415	CP1422	CP1402	CP1415	CP1422
5	31	36	32	20	<1	<1
10	37	34	33	—	—	—
15	228	183	169	—	—	—
20	1,150	723	625	—	—	—
25	2,234	1,362	1,332	—	—	—
30	2,318	1,754	1,410	2,500	1,300	1,300
40	3,082	1,941	1,990	2,800	1,400	1,900
50	2,980	2,431	2,259	—	—	—

[a] Cells were prepared for CF treatment by growth in CTM medium (pH 7.0) from an OD of 0.002 to 0.05, concentrated threefold, and stored frozen. For CF induction, cells were thawed at 0°C, resuspended in fresh CTM medium (pH 7.9), with 15% CF and [^{3}H]DNA at 3,000 cpm/0.01 ml, and then incubated at 37°C.

[b] DNA degraded to trichloroacetic acid soluble form (cpm/150 μl).

[c] Number of Nov^r transformants per microliter. Transformation was determined by diluting samples in CAT broth plus DNase, incubating for 60 min at 37°C, and plating with an overlay of novobiocin and erythromycin. —, Not determined.

used only as a convenient shorthand to represent the types of genetic results observed, but the results are the same as would be expected from a double-strand circular intermediate. In any case, it has now been reported repeatedly that the main product is a tandem duplication of the homologous fragment bracketing the rest of the mutagenic circle (8, 27, 37, 38). We suggest the term insertion-duplication mutagenesis for this procedure to reflect and emphasize the two significant features of the mutation it produces. The phenomenon allows easy mutagenesis formally analogous to that described in yeasts by Shortle et al. (31). Mejean et al. (20) have used this genetic phenomenon for the rapid cloning of many alleles from the chromosomal gene, *amiA*, and have also reported induction of mutations at other loci (M. Gherardi and J. P. Claverys, Proc. European Mtg. Bacterial Transformation Transfection, 6th, Lisbon, Portugal, abstr. no. 14, p. 35, 1982). They have shown that inactivation of the target gene produces a duplication and insertion and that insertion occurs at the target gene (20, 38).

The insertion-duplication mutants described here are apparently the first mutants clearly characterized as defective in achieving competence spontaneously; thus we designate them Com$^-$ (Table 1). These mutants are inducible at high CF levels (up to 30-fold higher than that required for wild type). Once induced, they process DNA normally and are fully transformable but do not appear to release active CF as do the Com$^+$ strains.

TABLE 7. CF activity in culture medium of induced cultures of erythromycin-resistant insertion mutants

Time[a] at 37°C after CF + DNA	[^{3}H]DNA degraded[b]			Relative CF titer[c]		
	CP1402	CP1415	CP1422	CP1402	CP1415	CP1422
5	31	24	19	4.5	4.0	3.8
15	146	82	48	13	1.3	1.1
20	821	551	429	23	0.9	0.9
25	1,010	883	833	27	0.8	0.7
30	1,278	1,084	1,055	50	—	—
35	1,245	1,150	1,156	—	—	—
45	1,070	1,148	1,125	—	—	—

[a] Cells were prepared for CF treatment by growth in CTM medium (pH 7.0) from an OD of 0.002 to 0.05, concentrated threefold, and stored frozen. For CF induction, cells were thawed at 0°C, resuspended in fresh CTM medium (pH 7.9), with 15% CF and [^{3}H]DNA at 5,000 cpm/0.1 ml, and then incubated at 37°C.

[b] DNA degraded to trichloroacetic acid soluble form (cpm/40 μl).

[c] The titer of CF in culture supernatant samples taken at the indicated times is expressed relative to that of the inducing CF preparation and is determined by end-point dilution assay, as described in the text. —, Not determined.

The nature of the defect in these Com$^-$ strains is not yet clear. The phenotype is consistent with the Com$^-$ mutants producing no CF or inactive or unstable CF or with failure in CF export. However, since so little is known of the details of the action of CF in competence induction, it is impossible to rule out other, less direct, causes of the Com$^-$ phenotype. For example, a competence inhibitor has been described in competent cultures of *S. pneumoniae* (35) and might play the controlling role in these mutants. Also, the characterization of CF and the demonstration of its activity (17, 34, 36) did not eliminate the possibility of other intercellular effectors important in the competence response, although no such effectors have yet been described.

Whatever the molecular nature of their defect, these mutants do give access to a chromosome locus involved in the control of competence for genetic transformation and will allow a better understanding of that physiological state.

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