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Molecular Cloning of Two Linked Loci That Increase the Transformability of Transformation-Deficient Mutants of *Haemophilus influenzae*

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A plasmid containing a 13.3-kb insert (pER194) was isolated from an *Eco*RI genomic library of *Haemophilus influenzae* on the basis of its ability to increase the transformability of the transformation-deficient mutants Com$^-$78 and Com$^-$101. The plasmid failed to increase the transformability of the Rec$^-$1 and Rec$^-$2 mutants, indicating that the mutations producing the Com$^-$78 and Com$^-$101 phenotypes are distinct from those giving rise to the Rec$^-$1 and Rec$^-$2 phenotypes. The physical mapping of the cloned fragment on the *H. influenzae* chromosome was found to be consistent with the genetic mapping of the Com$^-$101 trait. A 2.8-kb *Eco*RI-*Bgl*II subfragment, representing one end of the 13.3-kb clone, was found to increase the transformation frequency of the Com$^-$78 and Com$^-$101 mutants when supplied in *trans*, indicating that the subfragment carries one or more loci required for chromosomal transformation. The corresponding region of the Com$^-$101 chromosome was determined by hybridization analysis to contain a 0.3-kb insertion, suggesting that the Com$^-$101 strain may contain an insertion mutation at this locus. A 3.0-kb *Eco*RI-*Mlu*I subfragment, representing the other end of the 13.3-kb *Eco*RI fragment, was found to increase the transformation frequency of the Com$^-$101 mutant but not of the Com$^-$78 mutant, suggesting that the Com$^-$101 phenotype results from a complex genotype involving mutations at two or more transformation-related loci. This conclusion is consistent with data indicating that the Com$^-$101 trait can be genetically separated into at least two components.

Considerable progress has been made in the analysis of the genes encoding the functions required for genetic transformation in gram-positive organisms such as *Bacillus subtilis* (1, 13, 40), and *Streptococcus pneumoniae* (12). While transformation-deficient mutants have been available for gram-negative *Haemophilus influenzae* for some time (8, 11, 19, 38) and the fate of transforming DNA in these strains has been worked out in considerable detail (7, 19, 20, 28, 30), the genetic analysis of these mutants has lagged behind the biochemical analysis of their phenotypes. Recent progress in the analysis of genes required for transformation in *H. influenzae* has been made through the application of molecular techniques. As examples, Setlow et al. (34) and Stuy (36) have reported cloning the *rec-1* locus by using complementation of the UV-sensitive phenotype, and Barcak et al. have cloned the same locus by using hybridization to probes derived from other *recA*-like genes (5). McCarthy has cloned the *rec-2* locus by using transposon tagging in a heterologous system. Since there are no known *H. influenzae* transposons, the donor DNA was first mutagenized by passage through an *Escherichia coli* host containing the transposon mini-Tn*10km* (25). Tomb and coworkers (39) have used the same technique to mutagenize and map additional genes required for transformation in *H. influenzae*. More recently, Kauc and Goodgal have demonstrated transposon-mediated mutagenesis in *H. influenzae* by using Tn*916* (17) and have been able to mutagenize and map transformation-related genes by using a homologous system (16a).

This report describes the isolation and characterization of a cloned chromosomal fragment capable of restoring the transformability of two competence-deficient mutants. The plasmid bearing the fragment was isolated from a wild-type *H. influenzae* library on the basis of its ability to increase the transformability of a mutant host. The ability to separate the fragment into two nonoverlapping subclones, both of which are able to increase the transformability of one of the mutants, suggests that the original cloned fragment contains at least two transformation-related loci.

MATERIALS AND METHODS

Bacterial strains and plasmids. *H. influenzae* Rd1967 was used in this laboratory in 1967 and originated from an Rd isolate of Alexander and Leidy (2). *H. influenzae* Rd V23 has been described elsewhere (17). The Com$^-$78 and Com$^-$101 strains were constructed in this laboratory from an Rd1967 parent by *N*-methyl-*N*-nitroso-*N'*-nitroguanidine mutagenesis; the isolation and some of the properties of these strains have been described previously (11, 29–31). The multiply marked strain RdSKNDR is resistant to streptomycin, kanamycin, novobiocin (cathomycin), dalacin (streptovaricin), and rifampin. The strain was constructed by transforming an RdSKND strain available in this laboratory with DNA from a spontaneous rifampin-resistant mutant isolated from Rd1967. The multiply marked strain RdSKNDErGNa is resistant to erythromycin, nalidixic acid, and gentamicin in addition to the same antibiotics as the RdSKND strain. Com$^-$ strains resistant to particular antibiotics (streptomycin, erythromycin) were constructed by chromosomal transformation using the MIV procedure (see below) and were then retested for the Com$^-$ phenotype (29). The antibiotic-resistant derivative of the Com$^-$101 strain, Com$^-$101SNDR, was constructed by transformation with RdSKNDR DNA and retested for the presence of the Com$^-$101 phenotype.

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TABLE 1. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pER194	pJ1-8Ω(*Eco*RI::*H. influenzae* Rd 13.3kb)	This study
pJ1-8	Ap^r	16
pGJB103	Ap^r Tc^r	39
pHK	Km^r Tc^r	5
pTGL1	pER194(Δ*Mlu*I 2.5kb)	This study
pTGL2	pGJB103Ω(*Bgl*II::pER194 1.9kb)	This study
pTGL3	pGJB103Ω(*Bgl*II::*Pst*I::pER194 7.2kb)	This study
pTGL6	pHKΩ(*Eco*RI-*Mlu*I::pER194 7.8kb)	This study
pTGL7	pHKΩ(*Mlu*I::pER194 2.5kb)	This study
pTGL9	pHKΩ(*Eco*RI-*Mlu*I::pER194 3.0kb)	This study
pTGL10	pER194(Δ*Bgl*II 9.7kb)	This study
pTGL11	pER194(Δ*Bgl*II 7.8kb)	This study
pTGL12	pER194(Δ*Bgl*II 1.9kb)	This study

[a] Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

Strains bearing the *rec-1* and *rec-2* mutations were gifts of J. Setlow; their construction and phenotypes have been described in detail elsewhere (7, 8, 32, 33). The plasmids used in this study are presented in Table 1. The pJ1-8 vector was provided by N. Notani, who has reported its construction and map elsewhere (16). The vectors pGJB103 and pHK were gifts of G. Barcak, and their maps and construction have been reported elsewhere (5, 39). All other plasmids were constructed in this study and will be described below.

Enzymes. Restriction enzymes were obtained either from Bethesda Research Laboratories or from New England BioLabs and were used as prescribed by the manufacturers. Calf intestine alkaline phosphatase was a product of Boehringer Mannheim, and T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, and *E. coli* DNA polymerase I were obtained from Bethesda Research Laboratories. All were used as described by Maniatis et al. (23).

DNA preparation. Chromosomal DNA for general transformation procedures was prepared by using Marmur's method (24). High-molecular-weight chromosomal DNA for measuring the cotransformation frequencies of chromosomal markers was prepared by using Michalka and Goodgal's (26) modification of the Berns and Thomas procedure (9). *H. influenzae* chromosomal DNA immobilized in agarose beads was prepared and digested by restriction enzymes as described by Kauc et al. (18). Large-scale preparations of plasmid DNA were isolated from *H. influenzae* by using the alkaline-lysis technique (10, 23). Small-scale preparations of *H. influenzae* plasmid DNA were made by using the abbreviated alkaline-lysis procedure described by Morelle (27) except that a 5-ml volume from an overnight culture was pelleted and suspended in 200 μl of protoplasting buffer. Purified insert from pER194 was prepared by separating an *Eco*RI digest using agarose gel electrophoresis and electroeluting the desired band with an ISCO model 1750 electrophoretic concentrator. When the fragment was to be used in ligation reactions, it was purified further with a Schleicher & Schuell ELUTIP-d according to the manufacturer's recommendations.

Bacterial growth and competence development. *H. influenzae* cultures were grown in brain heart infusion (Difco) supplemented with 2 μg of NAD and 10 μg of hemin (both from Eastman Organic Chemicals) per ml. Supplemented brain heart infusion (sBHI) containing 1.5% Bacto Agar (Difco) was used for plating. Cultures were preserved as needed by freezing them in sBHI with 15% glycerol at −70°C. Strains containing chromosomal markers were selected and grown in sBHI medium containing 10 μg of dalacin, 10 μg of erythromycin, 10 μg of kanamycin, 3 μg of nalidixic acid, 10 μg of novobiocin, 10 μg of rifampin, or 200 μg of streptomycin per ml. Strains containing plasmids were selected and maintained under antibiotic selection in sBHI containing 2.5 μg of ampicillin, 5 μg of kanamycin, or 5 μg of tetracycline per ml. All transformations were performed with freshly made competent cells. For most transformations, competence was induced by using Cameron's modification (6) of Goodgal and Herriott's aerobic-anaerobic procedure (14). When highly competent cultures were required, competence was induced by using MIV medium as described by Herriott et al. (15) but at 36°C. Cultures were used for transformation after incubation for 120 min in MIV medium. Strains containing plasmids were always made competent under antibiotic selection. Plasmid transformations were performed by the saline-glycerol procedure described by Stuy and Walter (37) with the following modification: 2.5 ml of transformed, glycerol-treated cells were mixed with 2.5 ml of melted sBHI agar held at 45°C and were then poured onto 10 ml of hardened sBHI agar containing the appropriate antibiotic at 1.5 times the usual concentration.

Determination of cotransformation frequencies for chromosomal markers in *H. influenzae*. Cotransformation frequencies for *H. influenzae* chromosomal markers were determined by transformation with high-molecular-weight DNA as described by Michalka and Goodgal (26) with recipient strains made competent by incubation in MIV medium. Competent cultures were cooled on ice, and DNA was added to a final concentration of 30 ng/ml to stay within the linear portion of the dose-response curve (14). The uptake reaction mixtures were transferred to a 35°C water bath and gently mixed at 25 rpm. After 30 min, the speed was increased to 100 rpm, and the incubation was continued for an additional 30 min (1 h total of uptake time). The transformed cultures were diluted and pour plated in sBHI agar by standard procedures. After a 2-h expression period at 37°C, the plates were overlaid with sBHI agar containing the required antibiotics. Viable count plates were not overlaid.

Assay for linkage to the Com$^-$101 trait. The presence of the Com$^-$101 trait was evaluated by a plate transformation assay (11, 29). Colonies to be tested were transferred from antibiotic selection plates to a grid on a master plate containing the same antibiotic(s). RdSKNDR and Com$^-$101SNDR were included as controls. The plates were grown overnight and replica plated to a DNA plate (11, 29) that had been prepared by spreading an sBHI agar plate with 50 μg of chromosomal DNA isolated from *H. influenzae* RdSKNDErGNa. The inoculated DNA plate was grown at 37°C for 14 to 16 h and replica plated in triplicate on plain sBHI agar, on sBHI agar containing erythromycin, and on sBHI agar containing nalidixic acid. Colonies that grew on the plate without antibiotics but not on the plates with antibiotics were considered to have the Com$^-$ trait. Transformants that gave patchy colonies on the antibiotic plates were tentatively classified as intermediate phenotypes.

Evaluation of intermediate Com$^-$101 phenotypes. Transformants suspected of having intermediate Com$^-$ phenotypes were verified by testing their transformation frequency by the aerobic-anaerobic method. Strains found to have transformation frequencies intermediate between wild-type and mutant frequencies were considered true intermediate phenotypes and were retained for subsequent analysis.

Spot test for resistance to UV radiation. Cells were grown to stationary phase, spotted on duplicate sBHI plates, and

allowed to dry. One plate from each pair was exposed to 24 J/m^2 of UV radiation, and the plates were incubated overnight at 37°C (34). Cultures that grew on both plates were classified as UV resistant.

Isolation from *H. influenzae* library of plasmids that increase transformability of Com$^-$ hosts. A library of the wild-type *H. influenzae* Rd chromosome was prepared by ligating chromosomal DNA partially digested with *Eco*RI into *Eco*RI-digested, calf intestine alkaline phosphatase-treated pJ1-8 vector, essentially as described by Joshi and Notani (16). The partially digested DNA was size fractionated by agarose gel electrophoresis to obtain inserts spanning a range of 10 to 20 kb. The ligated DNA was transformed into competent *H. influenzae* Rd V23 (14, 15) and selected for ampicillin resistance. Plasmids were extracted and tested for size by agarose gel electrophoresis. A total of 500 isolates containing inserts greater than 8 kb, with an average size of 12 kb, were cultured and combined into 50 pools of 10 isolates each. Plasmid DNA was isolated from each pool and used to transform the Com$^-$78 and Com$^-$101 mutants. Following a 30-min uptake, the cultures were diluted 1:50 into sBHI containing 2.5 μg of ampicillin per ml and grown overnight. The following day, the cultures were made competent by using the MIV procedure under ampicillin selection. To enrich for Com$^+$ cells, the competent cultures were then transformed with chromosomal DNA from a Com$^-$ mutant that was the same as the parental strain but carried a streptomycin resistance marker. Following a 1-h uptake incubation, the cultures were diluted 1:50 into sBHI containing both streptomycin and ampicillin and were grown overnight. The following day, the cultures were again made competent, this time by using the simpler but lower-competency aerobic-anaerobic procedure. The competent cultures were transformed with chromosomal DNA from a Com$^-$ mutant that was the same as the parental strain but carried an erythromycin resistance marker and were then plated. Colonies resistant to both erythromycin and ampicillin were tested for their chromosomal transformation frequencies by using the aerobic-anaerobic method and chromosomal DNA bearing a novobiocin resistance marker; colonies having transformation frequencies significantly increased over those of a mutant control were selected for further analysis. Strains with increased transformation frequencies originating from back mutations or suppressor mutations were eliminated by preparing plasmid from the isolates and testing once more for the ability to increase the transformability of the parental Com$^-$ strain.

As a final test of the biological activity of the cloned fragments, they were tested for their abilities to restore transformability through chromosomal transformation. The Com$^-$78 and Com$^-$101 mutants were transformed with gel-purified fragment by the MIV procedure. When the resulting cultures were found to have increased transformability, they were enriched for Com$^+$ transformants by transformation to streptomycin resistance as described above. The enriched cultures were then transformed to erythromycin resistance as described above and plated to select the Com$^+$ transformants. Colonies were selected at random and tested for chromosomal transformation frequency by the aerobic-anaerobic procedure. Plasmids that passed the tests described above were stabilized by transformation into a Rec$^-$1 host (3, 4) and were retested for the ability to increase the transformability of the Com$^-$ mutants. The Rec$^-$ host strains were the source for the plasmid DNA used in all subsequent experiments.

Construction of pER194 derivatives. To determine the active regions of pER194 more precisely, two types of derivatives were made: internal deletions and subclones. Diagrams of these derivatives are presented in Fig. 1. Internal deletions of pER194 were generated by removal of internal *Mlu*I and *Bgl*II fragments by standard methods. Subclones of pER194 were prepared by using the gel-purified 13.3-kb *Eco*RI fragment digested either with *Mlu*I, *Bgl*II, or *Bgl*II and *Pst*I. The *Mlu*I and *Eco*RI-*Mlu*I fragments were ligated into similar digests of the pHK vector and transformed into the Rec$^-$1 strain under kanamycin selection. The *Bgl*II and *Bgl*II-*Pst*I fragments were ligated into similar digests of the pGJB103 vector and transformed into the Rec$^-$1 strain under tetracycline selection.

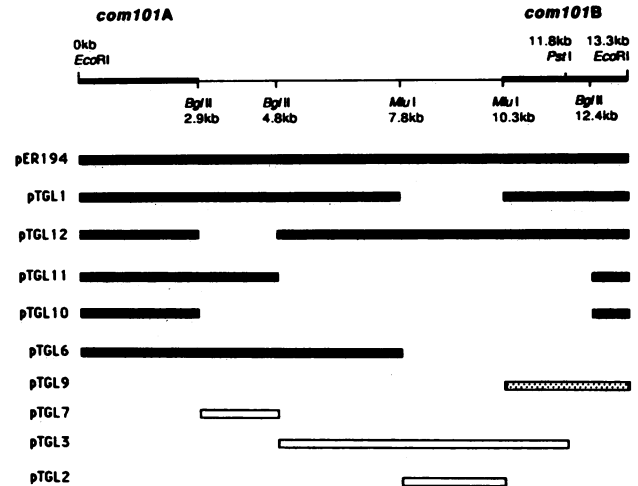

FIG. 1. Ability of the derivatives of the 13.3-kb chromosomal fragment cloned in pER194 to increase the transformability of the Com$^-$78 and Com$^-$101 hosts. The plasmids containing the derivatives are listed at the left. A description of each plasmid is given in Table 1, and actual values for the increases in transformability are given in Table 2. Restoration of transformability to both Com$^-$78 and Com$^-$101 (■), only Com$^-$101 (▨), and neither Com$^-$78 nor Com$^-$101 (□) are indicated.

Assay for increase in transformation frequency of Com$^-$78 and Com$^-$101 strains by pER194 plasmid and its derivatives. Plasmids to be tested for the ability to increase the transformation frequency of the Com$^-$ strains were isolated and amplified in the *H. influenzae* Rec$^-$1 strain. Plasmids were transformed into the Com$^-$78 and Com$^-$101 strains by using the saline-glycerol procedure. Transformants were selected as populations in liquid culture by diluting 0.5 ml of the glycerol-treated, transformed culture into 4.5 ml of sBHI containing the appropriate antibiotic and shaking the mixture overnight at 35°C. The following day, the transformation frequencies of the plasmid-containing cultures were tested by transforming to erythromycin or novobiocin resistance by the aerobic-anaerobic procedure. The transformation frequencies of the mutant strains containing the plasmids were tested immediately after they were constructed, since the transformability of these cultures tended to decrease rapidly after they reached stationary phase. Because of their unstable phenotype, these strains were not preserved. New populations of transformants were always made with plasmid prepared from the Rec$^-$1 strains when the ability of a plasmid to increase the transformability of a Com$^-$ host was to be tested.

The Com$^-$ plasmid transformants were selected as populations because single-colony isolates produced inconsistent

results (data not presented). This phenomenon may be related to the instability of the transformability of the plasmid-bearing Com$^-$ strains noted above or may be due to the tendency of homologous plasmid inserts to recombine with the *H. influenzae* chromosome (3, 4). Experiment-to-experiment variations in the frequency of transformation were minimized by expressing the values obtained as a multiple of (fold increase over) a mutant control frequency or as a percentage of a wild-type control frequency, although some experiment-to-experiment variability was still observed. Plasmid-free strains were used as controls, since the presence of the parental vectors did not appear to affect the transformability of these strains. The chromosomal transformation frequencies of the plasmid-bearing Com$^-$ strains were determined twice from independently prepared populations of plasmid transformants. When an unusually large variation between the two values was found, another determination was made.

Hybridization analysis of *H. influenzae* chromosomal DNA. Rd1967, Com$^-$78, and Com$^-$101 chromosomal DNAs were prepared from cells embedded in agarose beads (18). The immobilized DNA was digested either with *Eco*RI and *Mlu*I or with *Eco*RI and *Bgl*II, and the digests were separated by standard agarose gel electrophoresis. The DNA was depurinated for 10 min in 0.25 M HCl and transferred to a nylon membrane (ZetaProbe; BioRad) by using the alkaline blotting procedure recommended by the manufacturer. The probe was prepared from gel-purified pER194 insert and labeled by nick translation to a specific activity of approximately 2×10^7 cpm/μg by using the conditions described by Maniatis et al. (23). Southern hybridization was carried out under the stringent conditions recommended by the manufacturer of the membrane.

Chromosomal mapping of the pER194 insert. Chromosomal DNA was prepared from the Rd1967 strain in agarose beads and digested with either *Apa*I or *Sma*I (18). The DNA was separated by using field inversion gel electrophoresis under the conditions described for program A by Kauc et al. (18). The DNA was depurinated as described above, but for 15 min, and Southern blotting was performed as described above.

RESULTS

Isolation of a plasmid containing a chromosomal fragment that increases transformability of Com$^-$78 and Com$^-$101 hosts. Two plasmids that were capable of significantly increasing the transformation frequency of the Com$^-$78 and Com$^-$101 hosts were isolated. pER194 was found when the *H. influenzae* library was screened for plasmids that increase the transformability of a Com$^-$78 host, and pER94 was found when the library was screened for plasmids that increase the transformability of a Com$^-$101 host. Both plasmids were found to increase the transformability of both mutant phenotypes, and digestion of the plasmids with *Eco*RI indicated that each contained a 13.3-kb insert. Chromosomal transformation of the Com$^-$78 strain with purified insert from either plasmid produced transformants with essentially wild-type chromosomal transformation frequencies (data not presented). Chromosomal transformation of the Com$^-$101 strain with purified insert from either plasmid produced two classes of transformants: those with essentially wild-type transformation frequencies and those with frequencies approximately 2 orders of magnitude below the wild-type frequency (data not presented). Digestion with additional restriction enzymes and hybridization studies indicated that the plasmids contained independent clones of the same chromosomal fragment (data not presented). For this reason, only pER194 was selected for further study.

TABLE 2. Effect of pER194 and its derivatives on the transformability of Com$^-$78 and Com$^-$101 hosts[a]

Plasmid	% of wild-type (Rd1967) transformation frequency		Fold increase over mutant frequency	
	Com$^-$78	Com$^-$101	Com$^-$78	Com$^-$101
Controls				
No plasmid	0.4	0.03		
pER194	6–9	17–19	15–22	430–630
Derivatives of pER194				
pTGL1	1.6–3.9	11–30	4–10	360–1,000
pTGL2	NT	0.025		0
pTGL3	NT	0.89		30
pTGL6	78	6–15	195	200–500
pTGL7	2.5	0.2	6	7
pTGL9	1	3–4	2	100–130
pTGL10	66	22	165	730
pTGL11	25	29–35	62	970–1,200
pTGL12	14	18	35	600

[a] The aerobic-anaerobic method was used to measure transformation to erythromycin resistance. The region of the pER194 insert contained in each plasmid is shown in Fig. 1. NT, not tested.

Rec$^-$1 and Rec$^-$2 phenotypes are not restored to transformability by chromosomal fragment that restores transformability to Com$^-$78 and Com$^-$101 phenotypes. Both the Rec$^-$1 and the Rec$^-$2 strains have Com$^-$ phenotypes. Further, like Rec$^-$1 (32), Com$^-$78 is sensitive to UV radiation (31), and like Rec$^-$2 (7, 28), Com$^-$101 exhibits depressed nucleoside release following donor DNA binding (unpublished data). The ability of pER194 to restore the transformability of Rec$^-$1 and Rec$^-$2 hosts was tested to confirm that the Com$^-$78 and Com$^-$101 strains are not additional isolates of these mutants. The presence of pER194 slightly decreased the transformability of the Rec$^-$ hosts, indicating that the transformability of these mutants is not restored by the same genes that restore the transformability of the Com$^-$78 and Com$^-$101 mutants (data not presented).

The pER194 plasmid was also tested for its ability to restore UV resistance to the Com$^-$78 and Rec$^-$1 strains but was found not to affect the UV sensitivity of either strain. The inability of the plasmid to restore the UV resistance of the Com$^-$78 mutant is consistent with previous results indicating that the transformation-deficient and UV-sensitive components of the Com$^-$78 phenotype can be genetically separated (31, 31b).

Biological activity of pER194 and its derivatives in Com$^-$78 and Com$^-$101 hosts. A restriction map of the pER194 insert is presented in Fig. 1 along with a diagram of the derivatives prepared from it. The ability of each construction to increase the transformability of Com$^-$78 and Com$^-$101 hosts is also indicated. The actual transformation frequencies for these plasmids are presented in Table 2. Although the transformation frequencies of both the Com$^-$78 and Com$^-$101 mutants are increased by the pER194 plasmid, the derivatives of the insert display different patterns of activity in each of the mutants. The transformability of the Com$^-$101 strain is increased by both of the *Eco*RI-*Mlu*I end fragments subcloned in plasmids pTGL6 and pTGL9. The transformability of the Com$^-$78 strain, on the other hand, is increased only by

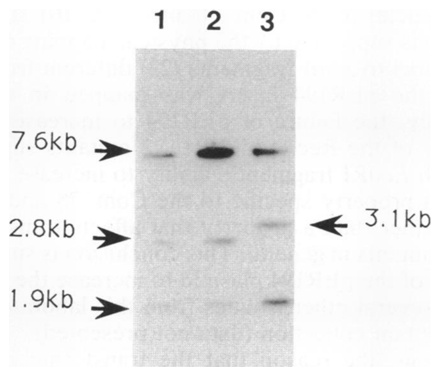

FIG. 2. Hybridization of *Eco*RI-*Bgl*II digests from the Rd1967 (lane 1), Com$^-$78 (lane 2), and Com$^-$101 (lane 3) chromosomes with a probe derived from the pER194 insert. The shift in the size of the 2.8-kb band caused by the 0.3-kb insertion in the Com$^-$101 chromosome is indicated. The 0.9-kb *Eco*RI-*Bgl*II fragment ran off the gel.

the *Eco*RI-*Mlu*I fragment subcloned in pTGL6. These data indicate that two distinct regions of pER194 can increase the transformability of the Com$^-$101 strain, while only one region has activity in the Com$^-$78 strain. The ability of the subfragments carried by plasmid pTGL6 and pTGL10 to increase the transformation frequency of both mutant strains suggests that the active region shared by the two plasmids resides on the 2.8-kb *Eco*RI-*Bgl*II fragment that they have in common.

Origin of the pER194 insert from the *H. influenzae* chromosome. A physical map for the *H. influenzae* Rd chromosome has been constructed by using pulsed-field gel electrophoresis techniques (18, 22). The availability of this map was exploited in determining the origin of the chromosomal fragment cloned in the pER194 plasmid. The plasmid insert was found to produce strong, single bands of hybridization corresponding to the *Apa*I-1 and *Sma*I-1 fragments (data not presented) when mapped according to Kauc et al. (18). These fragments correspond to *Apa*I-A and *Sma*I-A in the nomenclature of Lee et al. (22).

Com$^-$101 chromosome contains 0.3-kb insertion in 2.8-kb *Eco*RI-*Bgl*II subfragment of pER194. Hybridization analysis of chromosomal digests from the Rd1967, Com$^-$78, and Com$^-$101 strains revealed that the Com$^-$101 chromosome contains a 0.3-kb insertion in the restriction fragment corresponding to the 2.8-kb *Eco*RI-*Bgl*II fragment of pER194 (Fig. 2). This result suggests that the Com$^-$101 strain may contain an insertion mutation in the transformation-related locus cloned on the 2.8-kb *Eco*RI-*Bgl*II fragment of pER194. The mobility of the corresponding fragment from the Com$^-$78 chromosome was the same as that of the wild-type fragment, suggesting that the mutation affecting the Com$^-$78 strain is a point mutation or an insertion or deletion too small to be resolved by the gel system used to prepare the digests for hybridization analysis.

Genetic mapping of the Com$^-$101 trait. Preliminary to mapping of the Com$^-$101 trait, the trait's effect on the linkage of chromosomal markers was investigated. When the Com$^-$101 trait was carried by the donor strain, the cotransformation frequencies and map order determined for the antibiotic resistance markers for streptomycin, novobiocin, dalacin, and rifampin (Fig. 3) agreed with those previously determined (8a, 35). These markers were selected on the basis of preliminary experiments suggesting that the Com$^-$101 trait is linked to the novobiocin resistance marker.

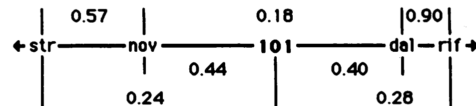

FIG. 3. Proposed map position of the Com$^-$101 trait relative to several widely used chromosomal markers. The frequencies above the map indicate the observed cotransformation frequencies of the markers when an Rd1967 recipient and a Com$^-$101SNDR donor were used. The frequencies below the map indicate the observed cotransformation frequencies of the Com$^-$101 trait with each marker. The abbreviations dal, nov, rif, and str designate chromosomal markers for resistance to dalacin, novobiocin, rifampin, and streptomycin, respectively.

When the Com$^-$101 trait was carried by the recipient strain, however, the cotransformation frequencies of the antibiotic resistance markers were significantly lower than those accepted (data not presented) and gave the map order novobiocin-rifampin-dalacin. For this reason, the map position of the Com$^-$101 trait was determined by using the wild-type Rd1967 strain as the recipient and the multiply marked Com$^-$101SNDR strain as the donor.

The linkage of the Com$^-$101 trait with single markers was measured by using the desired antibiotic to select transformants and using the plate transformation assay to test 200 resistant isolates for the Com$^-$ trait. The cotransformation frequencies for the Com$^-$ trait with the markers for resistance to streptomycin, novobiocin, dalacin, and rifampin are presented in Fig. 3. The Com$^-$101 trait appears to be linked more tightly to the novobiocin and dalacin resistance markers than to the streptomycin and rifampin resistance markers, suggesting that the trait maps between the novobiocin and dalacin resistance markers, as illustrated in Fig. 3. The small size of the samples prevents assigning the trait to an exact position on the map, although the linkage values suggest that it is located approximately midway between the novobiocin and dalacin resistance markers.

To corroborate the assigned map position, the values for the segregation of the Com$^-$101 trait with double markers were determined. These values were measured by selecting for transformants resistant to the desired double-antibiotic combination and then testing 100 resistant isolates for the presence of the Com$^-$ trait. The observed frequencies of cotransformation for the Com$^-$101 trait with streptomycin-novobiocin, novobiocin-dalacin, and dalacin-rifampin pairs were, respectively, 0.16, 0.52, and 0.22. The values obtained are consistent with the placement of the Com$^-$101 trait between the novobiocin-dalacin resistance pair in that the frequency of cotransformation with this pair is significantly higher than that with either of the other pairs. The cotransformation of the wild-type trait with the double antibiotic resistance markers in the Com$^-$101 recipient was investigated also. The absolute values obtained for the cotransformation of the wild-type trait with each marker pair were significantly different from the values obtained for the mutant trait when the wild-type recipient was used; however, the relative values obtained with the mutant recipient were still consistent with the assigned map position for the Com$^-$101 trait (data not presented).

Com$^-$101 phenotype results from a complex genotype. Chromosomal transformation of the Com$^-$101 mutant with purified pER194 insert gave two classes of transformants, as described above, suggesting that the Com$^-$101 phenotype results from a complex genotype. When the plate-transformation assay was used to evaluate the segregation of the

TABLE 3. Transformability of intermediate Com$^-$ phenotypes and presence of the 0.3-kb duplication[a]

Strain	Transformation frequency	0.3-kb insertion
Rd1967 (wild type)	1×10^{-3}	−
Com$^-$101	1×10^{-7}	+
Com$^-$101(B1)	7×10^{-5}	−
Com$^-$101(B2)	1×10^{-4}	−

[a] The Com$^-$101(B) strains were isolated when the cotransformation frequency of the Com$^-$101 trait was tested with the novobiocin-dalacin resistance double outside markers. The transformation frequencies were evaluated by transforming cells made competent by the aerobic-anaerobic technique to erythromycin resistance. The presence of the 0.3-kb duplication found in the Com$^-$101 chromosome was evaluated by DNA hybridization.

Com$^-$101 trait with other chromosomal markers, two isolates with phenotypes intermediate to those of the wild-type and Com$^-$101 controls were observed. The transformation frequencies of these two strains were tested by using the aerobic-anaerobic technique and were found to be intermediate to the frequencies of the Rd1967 recipient and Com$^-$101SNDR donor, as shown in Table 3. The isolation of these strains supports the hypothesis that the Com$^-$101 phenotype results from a complex genotype. These strains were designated Com$^-$101(B1) and Com$^-$101(B2) and were saved for further analysis.

Since the 0.3-kb insertion may be a convenient physical marker for an apparent insertion mutation in the Com$^-$101 strain, the Com$^-$101(B1) and Com$^-$101(B2) isolates were tested for its presence. As indicated in Table 3, the 0.3-kb insertion is absent in both of these strains.

DISCUSSION

This report presents evidence that two genes required for genetic transformation were isolated by screening a library for clones able to increase the transformability of two transformation-deficient (Com$^-$) mutants of *H. influenzae* when supplied in *trans* on a plasmid. Mutant cultures were transformed with pools of plasmids isolated from a wild-type genomic library and then enriched for transformable cells. This process eliminated the tedious and time-consuming step of evaluating individual plasmids. Although the method is useful only for mutant strains retaining some residual level of transformability, it may be possible to extend this procedure to mutants that do not transform by using electroporation (16a) for the initial transformation with the pooled library isolates.

Two plasmids that are able to restore the transformation frequencies of both the Com$^-$78 and Com$^-$101 strains were isolated from the collection of transformation-deficient mutants previously isolated in this laboratory by *N*-methyl-*N*-nitroso-*N′*-nitroguanidine mutagenesis (11). Both plasmids were able to increase the transformability of the Com$^-$ mutants after reisolation from Com$^-$ hosts, and purified inserts from both plasmids were able to transform the mutant strains to wild-type in a chromosomal transformation system, suggesting that transformability is increased through *trans* complementation. Data presented in the accompanying paper confirm a *trans*-complementation mechanism in the case of the *com*101A locus (21).

The pER194 plasmid slightly decreased the transformation frequency of Rec$^-$1 and Rec$^-$2 hosts, indicating that the transformation frequencies of these mutants are not increased by the same gene(s) that increases the transformation frequencies of the Com$^-$78 and Com$^-$101 strains. This conclusion is supported by the physical mapping of the *rec-1* and *rec-2* loci to *Apa*I fragments (22) different from the one to which the pER194 insert was mapped in this study. Additionally, the failure of pER194 to increase the transformability of the Rec$^-$1 and Rec$^-$2 mutants suggests that the 13.3-kb *Eco*RI fragment's ability to increase transformability is a property specific to the Com$^-$78 and Com$^-$101 mutants rather than a property that affects transformation-deficient mutants in general. This conclusion is supported by the failure of the pER194 plasmid to increase the transformability of several other isolates from this laboratory's competence mutant collection (data not presented).

At present, the reason that the transformability of the mutants is not restored to wild-type levels by pER194 and its derivatives is unknown. One possible explanation is that competition between wild-type and mutant gene products leads to semidominance of the cloned wild-type allele with the host's mutant allele. A second possibility is that the apparent instability of the plasmids in the Com$^-$78 and Com$^-$101 hosts noted above leads to a significant population of defective plasmids in a culture. Preparation of plasmid DNA from stationary-phase cultures of the mutant hosts shows a significant amount of plasmid that is smaller than the original input plasmid, suggesting deletions of the insert. The instability of *H. influenzae* plasmids in *rec*$^+$ hosts has been documented by other workers (3, 4).

For the genetic mapping of the Com$^-$101 trait, the wild-type strain was chosen for the recipient, since the Com$^-$101 strain produces aberrant linkage values for chromosomal markers when used as a recipient. The available evidence suggests that donor DNA processing is blocked in the Com$^-$101 mutant and that this blockage may be responsible for the altered linkage values (20a, 30). The linkage values obtained for chromosomal markers when the multiply marked Com$^-$101SNDR strain was used as the DNA donor (Fig. 3) appear to agree reasonably well with accepted values, indicating that reliable mapping data can be obtained by using a Com$^-$101 donor and a wild-type recipient. The cotransformation frequencies of the Com$^-$101 trait with the streptomycin, novobiocin, dalacin, and rifampin resistance markers suggest that the trait maps approximately midway between the novobiocin resistance and dalacin resistance markers, producing the genetic map presented in Fig. 3. This map position for the Com$^-$101 trait was confirmed by examining the cotransformation of the trait with double antibiotic resistance markers. A similar result was obtained when the cotransformation of the Com$^-$78 trait with double antibiotic resistance markers was examined (14a), suggesting that the Com$^-$78 trait maps to the same region of the chromosome as the Com$^-$101 trait.

The location of the *Eco*RI fragment cloned in the pER194 plasmid was physically mapped on the *H. influenzae* chromosome by using field inversion gel electrophoresis (18) to determine whether or not the physical mapping of this fragment is consistent with the genetic mapping of the Com$^-$101 and Com$^-$78 traits. The pER194 insert was found to hybridize to the *Apa*I-1 and *Sma*I-1 fragments, which define a region that is approximately 300 kb long, or about 15% of the chromosome, and that has been shown to carry the markers for resistance to novobiocin and dalacin (18, 22). Since this region is quite large, the physical mapping of the fragment cloned in the pER194 plasmid to this region does not establish that the fragment originated from the precise region that carries the two Com$^-$ traits; however, the physical mapping of the cloned fragment to the region of the

chromosome that carries the markers for resistance to novobiocin and dalacin is consistent with the genetic mapping of the Com$^-$ traits. Additionally, Tomb and his colleagues have used transposon tagging to locate transformation-related loci and have found that at least four such loci are located in the chromosomal region defined by the *Apa*I-1 and *Sma*I-1 fragments (31a, 39). This result lends additional support to the assertion that transformation-related loci may be carried by a clone derived from the chromosomal region bounded by the *Apa*I-1 and *Sma*I-1 fragments.

When the Com$^-$101 mutant is chromosomally transformed with purified pER194 insert, two classes of transformants are produced, suggesting that the mutant phenotype results from a complex genotype involving two or more mutations. Such a hypothesis is plausible, considering that the strain was derived by chemical mutagenesis. The ability of two nonoverlapping subclones of the pER194 insert to each increase the transformability of a Com$^-$101 host corroborates this hypothesis and further suggests that the mutations may be at separate loci. The presence of a 0.3-kb insertion in the region of the Com$^-$101 chromosome corresponding to one of the pER194 subclones suggests that one of the mutations may have originated from an insertion event. The apparent loss of the mutant allele containing the insertion in the Com$^-$101(B1) and Com$^-$101(B1) strains, which have intermediate phenotypes, provides additional evidence consistent with a complex genotype for the Com$^-$101 phenotype. In keeping with this multigene hypothesis, the two transformation-related loci believed to be cloned on the pER194 insert will tentatively be designated *com*101A and *com*101B, where the *com*101A locus bears the insertion in the Com$^-$101 mutant. The *com*101A locus is examined in greater detail in the accompanying paper (21).

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