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# Mobilization of *Haemophilus influenzae* Chromosomal Markers by an *Escherichia coli* F′ Factor

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**Filter matings between *E. coli* K-12 strains carrying an F′::Tn*5*,Tn*9* factor with *H. influenzae* Rd strains gave rise to kanamycin-chloramphenicol-resistant *H. influenzae* strains at a frequency of approximately $10^{-6}$. Transfer of the F′ factor to *H. influenzae* was verified by expression of unselected markers in *H. influenzae* (*lac*$^+$ or cotransfer of the nonselected antibiotic resistance), physical presence of a high-molecular-weight plasmid in recipient *H. influenzae* cells, and detection by Southern hybridization analysis of DNA sequences specific for the F′ factor replication and partition functions in recipient *H. influenzae* cells. *H. influenzae* (F′ Tn*5*,Tn*9*) strains were capable of transferring kanamycin and chloramphenicol resistances to other *H. influenzae* strains and were capable of mobilizing *H. influenzae* chromosomal markers at a low frequency. Insertion of a Tn*5* element in the *H. influenzae* genome near the novobiocin resistance gene increased the frequency of transfer of novobiocin resistance about 30-fold. Transfer of other chromosomal markers also increased, although to a lesser extent, and ordered transfer of chromosomal markers could be demonstrated. Gene transfer was insensitive to DNase I, and transfer of chromosomal (but not F′ factor) markers was dependent on the *H. influenzae rec-1* and *rec-2* gene functions.**

Genetic exchange of chromosomal markers in *Haemophilus influenzae* by transformation has been extensively characterized (for recent reviews, see references 10 and 20). Conjugal transfer of plasmids in *H. influenzae* has also been demonstrated (8, 17, 18, 23). Although some of these plasmids have been shown to integrate into the *H. influenzae* genome (24), mobilization of chromosomal markers has not been observed. Albritton et al. (1) have described a low-level transfer of chromosomal genes between *H. influenzae* during cell contact in the absence of detectable plasmids; however, they failed to detect ordered transfer of chromosomal markers. More recently, Stuy (25) has claimed that this observed transfer of markers was due to a low-level transformation event. In the experiments described in this paper, we did not observe transfer of chromosomal markers between *H. influenzae* in the absence of plasmids.

Several groups have observed that some drug resistance plasmids of *H. influenzae* can be transferred to *Escherichia coli* by filter mating (5, 8, 18, 23). As part of an ongoing study on the genetics of *H. influenzae*, we have attempted to determine whether reciprocal crosses of *E. coli* conjugative plasmids into *H. influenzae* were possible. Such cross-species transfer of plasmids has proven to be a powerful tool in the study of the genetics of a number of gram-negative bacteria, including *Caulobacter* (9), *Rhizobium* (6), and *Vibrio* (15) spp.

In this paper, we demonstrate that an *E. coli* F′ factor can be transferred into *H. influenzae* by filter mating under appropriate conditions. Such crosses are initially unstable, but become stable with continued selection. F$^+$ *H. influenzae* cells can also mobilize chromosomal markers at a low frequency. If the transposable element Tn*5* is used to provide local homology between the F′ factor and the chromosome, transfer of markers near the Tn*5* insertion is greatly enhanced and ordered transfer of chromosomal markers between *H. influenzae* strains can be demonstrated. The order of transfer of the markers used agrees with the order of the *H. influenzae* genetic map determined by genetic transformation (13, 25; J. Bendler, Ph.D. dissertation, Johns Hopkins University, Baltimore, Md., 1968).

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## MATERIALS AND METHODS

**Bacterial strains and growth.** *H. influenzae* cells were grown on brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of heme (Sigma Chemical Co., St. Louis, Mo.) per ml and 2 μg of NAD (Sigma) per ml (BHI-XV medium). Competent *H. influenzae* cells were prepared by the procedure of Herriott et al. (11). *E. coli* cells were grown in Luria broth (LB) (14). Antibiotic concentrations used were as follows: streptomycin, 100 μg/ml; novobiocin, 2.5 μg/ml; rifampin, 50 μg/ml; nalidixic acid, 10 μg/ml; erythromycin, 25 μg/ml; kanamycin, 20 μg/ml; neomycin, 20 μg/ml; spectinomycin, 10 μg/ml; chloramphenicol, 10 μg/ml (*E. coli*) or 5 μg/ml (*H. influenzae*); viomycin, 150 μg/ml. All antibiotics were obtained from Sigma except viomycin, which was obtained from Pfizer, Inc., New York, N.Y.

Strains of *H. influenzae* Rd and *E. coli* used in this work are listed in Table 1. *H. influenzae* KW20 (26) and MAP (13) were used to construct all other strains. A rifampin-resistant mutant of KW20 was obtained by plating $10^{10}$ cells on BHI-XV/rifampin agar. Fourteen Rif$^r$ colonies grew up, and

TABLE 1. Bacterial strains used in this study

| Strain | Relevant characteristics | Source or reference |
|---|---|---|
| *E. coli* C600 | *supE tonA thr leu* | 2 |
| *H. influenzae* Rd | | |
| KW20 | Wild type | 26 |
| MAP | Str$^r$ Nov$^r$ Spc$^r$ Ery$^r$ Vio$^r$ Nal$^r$ | 13 |
| HDG51 | Rif$^r$ | This study |
| HDG80 | Str$^r$ Nal$^r$ | This study |
| HDG81 | Rif$^r$ Spc$^r$ | This study |
| HDG85 | Str$^r$ Nov$^r$ Ery$^r$ Nal$^r$ Vio$^r$ | This study |
| HDG86 | Str$^r$ Ery$^r$ Nal$^r$ Vio$^r$ Nov$^r$::Tn*5* (Kan$^r$) | This study |

TABLE 2. Episomes used in this study

| Plasmid or phage | Relevant characteristics | Source or reference |
|---|---|---|
| F'ts *lac*$^+$ *trp*$^+$ ZZ28::Tn*5* (F'Tn*5*) | Tn*5* (Kan$^r$) *lac*$^+$ | 15 |
| F'ts *lac trp* ZZ28::Tn*5* Tn*9* (F'Tn*5*,Tn*9*) | Tn*5*,Tn*9* (Cam$^r$) *lac*$^+$ | This study |
| pAA118 | pBR325::nov1.8::Tn*5* (Kan$^r$) (Amp$^r$ Tet$^r$) | This study |
| pAA111 | pLG339(Kan$^r$ Tet$^r$)::8.1-kb *Eco*RI F' factor replication origin fragment | This study |
| pLG339 | Kan$^r$ Tet$^r$ | 22 |
| Lambda *b*221 *c*I857::Tn*5* | Tn*5* (Kan$^r$) | 4 |

one of these was saved as HDG51. All other strains used were obtained by stepwise transformation of either KW20 or HDG51 with DNA from strain MAP.

**Plasmids and bacteriophages.** Episomes used in this work are described in Table 2. F'ts *lac*$^+$ *trp*$^+$ ZZf28::Tn*5* (F'Tn*5*) has been previously described (15). A derivative of this plasmid containing the second transposable element Tn*9* (chloramphenicol resistance transposon) (F'Tn*5*,Tn*9*) was constructed by transposition of Tn*9* from phage P1CmClr100 (14) by standard methods. Transposition of Tn*9* to F'Tn*5* was verified by unselected cotransfer of kanamycin and chloramphenicol resistances and P1 sensitivity of Cam$^r$ recipients. Plasmid pAA118 is a derivative of pBR325 containing a Tn*5* element inserted adjacent to the cloned *H. influenzae* novobiocin resistance gene (see Results).

**Filter matings.** Cells (1 ml) grown under appropriate selective conditions were washed, mixed in fresh BHI broth (without heme or NAD), and drawn onto 30-mm-diameter sterile nitrocellulose disks. Disks were then incubated (cell side up) on BHI-XV agar for 4 h at 30°C, and cells were lifted by scraping into BHI broth. The cell suspension was serially diluted in BHI and plated on the appropriate antibiotic agar. Plates were counted after 48 h. Unless otherwise noted, all frequencies are given as recombinants per viable recipient cell.

**DNA isolation.** Chromosomal DNA was isolated by the procedure of Marmur (12). High-molecular-weight (>20-kilobase [kb]) plasmids were analyzed by the method of Eckhardt (7). Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used as specified by the manufacturers. DNA digests and plasmid preparations were analyzed by agarose gel electrophoresis with 50 mM Tris-acetate–1 mM EDTA (pH 7.8) buffer.

**DNA hybridization.** Total DNA from HDG86 and HDG86(F'Tn*5*,Tn*9*) was digested with *Eco*RI and run on a 0.7% agarose gel with 50 mM Tris-acetate (pH 7.8)–1 mM EDTA buffer. DNA was transferred to nitrocellulose sheets and probed by the Southern procedure (21). Probe DNA from plasmid pAA111 was labeled with [$^{35}$S]dCTP (Amersham, Corp., Arlington Heights, Ill.) by nick translation by using an adaption of the procedure of Rigby et al. (16). Unlabeled nucleoside triphosphates and DNase I were obtained from Sigma and *E. coli* DNA polymerase I was from Bethesda Research Laboratories. The activity of the probe was approximately $5 \times 10^6$ cpm/μg, and about $2 \times 10^6$ cpm was used for the hybridization. To enhance sensitivity, filters were dipped in a 1 M sodium salicylate solution and redried as a final step. Filters were exposed on Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −75°C for 3 days.

## RESULTS

To test for conjugal transfer of the *E. coli* fertility factor into *H. influenzae*, two conditions must be met. First, the F factor should have a marker which would allow selection in the *H. influenzae* recipient; and, second, the recipient *H. influenzae* strain should have markers to allow back-selection against the donor *E. coli* strain. The first condition was met by using an F' factor carrying the aminoglycoside resistance factor Tn*5*. The second condition was met by using an *H. influenzae* strain with a high level (2,000 μg/ml) of resistance to streptomycin.

When matings were carried out between *E. coli* C600 carrying F'Tn*5* and *H. influenzae* HDG85, kanamycin (20 μg/ml)-resistant colonies (high-level Kan$^r$) arose with a frequency of $5 \times 10^{-6}$ (average of five crosses). Colonies were verified as *H. influenzae* by colony morphology, inability to grow in the absence of heme or NAD, and retention of unselected drug resistance markers in the recipient strain.

Low-level (7 μg/ml) kanamycin-resistant chromosomal mutations in *H. influenzae* have been reported (Bendler, Ph.D. dissertation). Chromosomal mutations resistant to 20 μg of kanamycin per ml have not been reported. In mock crosses with (F$^-$) *E. coli* C600, *H. influenzae* cells resistant to 20 μg of kanamycin per ml were not found (frequency, less than $10^{-9}$). In addition, all high-level Kan$^r$ *H. influenzae* isolates were also resistant to neomycin (20 μg/ml). These data indicate that kanamycin resistance is due to the Tn*5* aminoglycosidase gene. Further evidence (see below) indicates that this is due to transfer and maintenance of the F'Tn*5* episome in *H. influenzae*.

**Verification of F' factor transfer to *H. influenzae*.** Transfer of F'Tn*5* from *E. coli* to *H. influenzae* was verified genetically by the unselected cotransfer of the *lac*$^+$ genes of F' Tn*5* into *H. influenzae* recipient cells. The Lac$^+$ phenotype was detected by the ability to form blue colonies when plated in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma). When mating mixtures were plated directly on BHI-XV–kanamycin–X-Gal plates, no Lac$^-$ Kan$^r$ colonies were oberved out of more than 1,000 screened. Expression of β-galactosidase activity was at a very low level: colonies required more than 48 h to develop the blue color on X-Gal plates. However, colonies arising from these crosses could easily be distinguished from the wild-type (Lac$^-$) *H. influenzae* strain when streaked on X-Gal plates. No stimulation of β-galactosidase was observed when an inducer of the *lac*$^+$ operon, isopropylthio-β-D-galactopyranoside (Sigma), was added, indicating that *lac*$^+$ expression in *H. influenzae* is not under normal *E. coli* *lac*$^+$ regulation.

As a second test, a second drug resistance marker, Tn*9* (chloramphenicol resistance), was introduced onto F'Tn*5* by transposition and mated into *H. influenzae* from *E. coli*. Transfer of either F' marker occurred at the same frequency, and all *H. influenzae* recipients selected for one marker also expressed the nonselected second marker. The Tn*9* transposon gave resistance to 5 μg of chloramphenicol per ml in *H. influenzae*. Spontaneous mutations resistant to this level of chloramphenicol occur at a frequency of less than $10^{-10}$ in *H. influenzae* (data not shown).

Transfer of F'Tn*5*,Tn*9* to *H. influenzae* was verified physically by the detection in *H. influenzae* (F'Tn*5*,Tn*9*) of a plasmid of identical apparent mobility as the F'Tn*5*,Tn*9* in

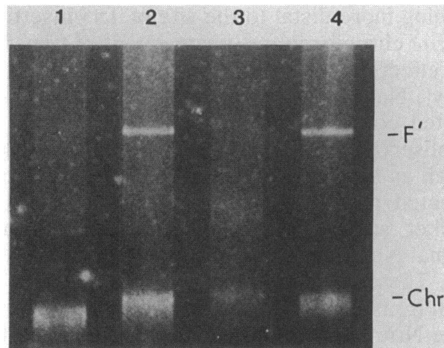


FIG. 1. Agarose gel analysis of plasmids in HDG86(F'Tn5,Tn9). Cells were lysed in the well by the procedure of Eckhardt (7). Lysates were run on 0.8% vertical agarose gels with 90 mM Tris-borate-EDTA (pH 7.8) buffer, and bands were visualized by ethidium bromide-UV fluorescence. Chr indicates chromosomal DNA. Lanes: 1, *E. coli* C600 lysate; 2, *E. coli* C600(F'Tn5,Tn9) lysate; 3, *H. influenzae* HDG86 lysate; 4, *H. influenzae* HDG86(F'Tn5,Tn9) lysate.

*E. coli* by agarose gel electrophoresis (Fig. 1). This result demonstrates that F'Tn5,Tn9 can be maintained as a plasmid in *H. influenzae*.

As a final verification of F'Tn5,Tn9 transfer, total DNA from a *H. influenzae* (F'Tn5,Tn9) strain, HDG86 (F'Tn5,Tn9), was probed by Southern hybridization analysis for F'-factor-specific DNA sequences. An 8.1-kb *Eco*RI fragment of F containing the *rep* and *par* genes from the recombinant phage lambda:585 (3) was subcloned into plasmid pLG339 (22), and the resulting plasmid, pAA111, was used as a DNA probe. Under the conditions used here, no cross-hybridization was observed between the Kan$^r$ marker of pLG339 and Tn5 (Fig. 2). When a total *Eco*RI digest of HDG86 (F'Tn5,Tn9) was hybridized to this probe, three bands reacted strongly, while no specific hybridization to HDG86 (F$^-$) DNA was observed (Fig. 2). The results indicate that F' factor DNA sequences have been transferred

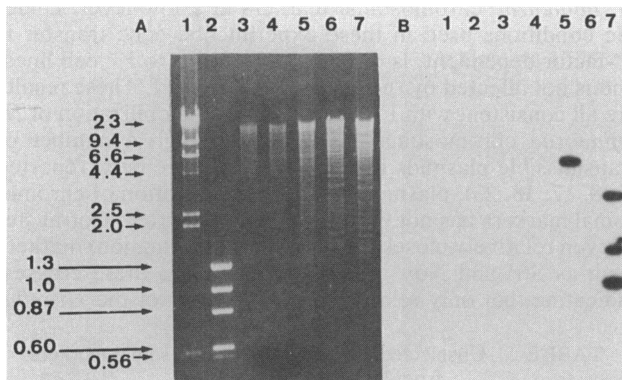


FIG. 2. Southern hybridization analysis of strain HDG86 (F'Tn5,Tn9) total DNA with F'-specific DNA sequences. (A) Ethidium bromide stain of 0.8% agarose gel. Lanes: 1, Lambda DNA (*Hin*dIII cut); 2, ϕX174 replicative-form DNA (*Hae*III cut); 3, *E. coli* C600 DNA (*Eco*RI cut); 4, *E. coli* C600 Lambda *bb*221 cI857::Tn5 lysogen DNA (*Eco*RI cut); 5, *E. coli* C600(F'Tn5,Tn9) DNA (*Eco*RI cut); 6, *H. influenzae* HDG86(F$^-$) DNA (*Eco*RI cut); 7, *H. influenzae* HDG86 (F'Tn5,Tn9) DNA (*Eco*RI cut). (B) Southern transfer of panel A probed with pAA111 DNA, a plasmid containing the F' factor replication origin (see methods). Size standards are given in kilobases.

TABLE 3. F'-factor-mediated transfer of *H. influenzae* chromosomal markers[a]

| Cross no. | Donor | Recipient | Frequency[b] |
|---|---|---|---|
| 1 | HDG80 | HDG81 | $4 \times 10^{-9}$ |
| 2 | HDG80(F'Tn5,Tn9) | HDG81 | $1.7 \times 10^{-7}$ |
| 3 | HDG81(F'Tn5,Tn9) | HDG80 | $2.2 \times 10^{-7}$ |
| 4[c] | HDG80(F'Tn5,Tn9) | HDG81 | $1.9 \times 10^{-7}$ |

[a] Selection was Rif Str for all cases.
[b] All frequencies are the average of three or more crosses.
[c] DNase I (50 μg/ml) was added to the mating mixture.

from *E. coli* to *H. influenzae*. The hybridization of the probe to multiple *Eco*RI bands from HDG85 (F'Tn5,Tn9) indicates that some physical rearrangement of the F'Tn5,Tn9 DNA has probably occurred during transfer or maintenance in *H. influenzae*, since only a single band (as predicted) was recognized in *E. coli* C600 (F'Tn5,Tn9) DNA (Fig. 2).

**Stability of *H. influenzae* (F'Tn5,Tn9) strains.** The stability of *H. influenzae* (F'Tn5,Tn9) transconjugant strains was tested by allowing cultures to grow in BHI-XV in the absence of selection. Kanamycin resistance was spontaneously lost from new *H. influenzae* (F'Tn5,Tn9) isolates at a high but variable frequency. Retention was at a rate between $10^{-2}$ and $10^{-4}$ Kan$^r$ colonies per viable cell. When colonies retaining Kan$^r$ were retested, no further curing of the Kan$^r$ was observed. Also, when *H. influenzae* (F'Tn5,Tn9) isolates were maintained under selection, the ability to cure Kan$^r$ was lost. These incurable cell lines retained the unselected *lac*$^+$, Cam$^r$ markers, still contained a plasmid with the same apparent mobility as F'Tn5,Tn9, and were still able to act as Kan$^r$ *lac*$^+$ Cam$^r$ donors in subsequent matings (see next section). Unless otherwise noted, incurable strains were used in all subsequent experiments.

**Secondary transfer of F'Tn5,Tn9 from *H. influenzae*.** Since the Kan$^r$ *H. influenzae* transconjugate strains appear to contain an intact F'Tn5,Tn9 episome, we next wished to determine whether these strains could act as F'Tn5,Tn9 donors in subsequent matings. Cultures were filter mated to other *H. influenzae* strains, and transfer of Kan$^r$ was observed at a frequency of $1.8 \times 10^{-5}$ (average of five crosses). These secondary recipients of F'Tn5,Tn9 behaved identically to primary recipients of F'Tn5,Tn9 from *E. coli*; all were *lac*$^+$ Cam$^r$ and could in turn act as donors for subsequent F'Tn5,Tn9 crosses. They did not exhibit the initial instability behavior of primary F' factor crosses. *H. influenzae* (F'Tn5,Tn9) could also transfer Kan$^r$ back to *E. coli* at frequencies of approximately $10^{-6}$. F' factor transfer to *E. coli* was verified by spot testing Kan$^r$ colonies for sensitivity to the male-specific coliphage fd.

**Mobilization of *H. influenzae* chromosomal markers by F'Tn5,Tn9.** We next wished to determine whether F'Tn5,Tn9 could mobilize chromosomal markers in *H. influenzae*. In initial experiments, streptomycin and rifampin resistances were used as test markers, as the spontaneous mutation rate to these resistances in *H. influenzae* is extremely low (about $10^{-9}$) for both (Bendler, Ph.D. dissertation). Results of such an experiment with *H. influenzae* HDG80 (Str$^r$ Nal$^r$) and HDG81 (Rif$^r$ Spc$^r$) are shown in Table 3. The frequency of streptomycin-rifampin double-resistant (Str$^r$ Rif$^r$) colonies in these crosses was $2 \times 10^{-7}$ and was independent of the strain used as a donor. In mock crosses, in which neither strain carried F'Tn5,Tn9, the frequency of Str$^r$ Rif$^r$ colonies was $4 \times 10^{-9}$, which is about the rate predicted by mutation. To determine whether the exchange of markers was directional, Str$^r$ Rif$^r$ recombinants were

TABLE 4. F′-factor mobilization of *H. influenzae* chromosomal markers[a]

| Marker | Mobilization frequency for: | |
|---|---|---|
| | HDG85(F′Tn*5*,Tn*9*) | HDG86(F′Tn*5*,Tn*9*) |
| Nov[r] | $1.1 \times 10^{-7}$ | $3.2 \times 10^{-6}$ |
| Str[r] | $0.8 \times 10^{-7}$ | $1.2 \times 10^{-6}$ |
| Ery[r] | $1.2 \times 10^{-7}$ | $3.8 \times 10^{-7}$ |
| Nal[r] | $2.0 \times 10^{-7}$ | $4.0 \times 10^{-7}$ |
| Vio[r] | $1.2 \times 10^{-7}$ | $1.7 \times 10^{-7}$ |

[a] HDG81 was used as the recipient strain with rifampin counterselection. All results are the average of three or more experiments.

screened for the unselected markers of HDG80 (nalidixic acid resistance) and HDG81 (spectinomycin resistance). In 199 of 200 colonies screened (100 from each cross), the unselected marker of the recipient ($F^-$) strain was present and the unselected marker of the donor ($F^+$) strain was not present, indicating that transfer of markers is directional from $F^+$ to $F^-$ cells, as predicted if F′Tn*5*,Tn*9* is acting to mobilize the *H. influenzae* genome. When the multiply drug-resistant *H. influenzae* HDG85 was used as a donor, transfer of all individual markers was observed at about the same frequency (Table 4). Cotransfer of nonselected markers was not observed (less than $10^{-9}$). Exchange of markers was shown to be unaffected by the addition of DNase I (50 μg/ml) to the mating mixture (Table 3). Transfer of chromosomal markers was also shown to depend on the *H. influenzae rec-1* and *rec-2* functions (19), since no transfer was detected in *rec-1* and *rec-2* recipient strains. In contrast, transfer of kanamycin resistance was not significantly affected in either *rec-1* or *rec-2* recipients (data not shown).

It has been observed that in other bacterial systems, introducing a portable region of homology between a conjugative plasmid and the host chromosome increases the frequency of transfer of chromosomal markers, presumably by acting as a site of interaction between the episome and the chromosome. To test this in *H. influenzae*, we introduced a Tn*5* element in the *H. influenzae* genome near the novobiocin resistance gene.

Introduction of the Tn*5* element into *H. influenzae* was done in the following manner. A 1.8-kb *Eco*RI fragment of *H. influenzae* DNA containing the *nov* gene was cloned into pBR325 and grown in *E. coli* C600 (unpublished data). These cells were then infected with lambda *b*221 *c*I857::Tn*5* (4), and Amp[r] Kan[r] Tet[r] survivors were screened for insertion of Tn*5* into the plasmid by agarose gel analysis. Plasmids containing Tn*5* insert were isolated, and transpositions into the cloned 1.8-kb *nov* fragment were determined by restriction mapping. One plasmid, pAA118, containing such a Tn*5* insertion was saved. Plasmid pAA118 DNA was digested with *Eco*RI and used to transform *H. influenzae* HDG85 to kanamycin resistance. One Kan[r] colony, strain HDG86, was saved.

F′Tn*5*,Tn*9* was crossed into HDG86, and the resulting strain [HDG86(F′Tn*5*,Tn*9*)] was used as a donor in *H. influenzae* matings. If the chromosomal Tn*5* can act as a portable homology region with the F′Tn*5*,Tn*9*, one would predict that the frequency of transfer of the Tn*5*-proximal *nov* marker would be enhanced. In fact, HDG86(F′Tn*5*,Tn*9*) was about 30-fold more efficient a donor of *nov* than was HDG85(F′Tn*5*,Tn*9*). Enhanced transfer of the Str[r], Ery[r], Nal[r], and Vio[r] markers was also observed in HDG86 (F′Tn*5*,Tn*9*) crosses, although to a lesser extent than was the Nov[r] marker (Table 4). This result is consistent with these markers being more distal to the site of Tn*5* insertion in the *H. influenzae* chromosome. Enhancement of transfer shows a gradient effect, decreasing in the following order: Nov[r] > Str[r] > (Ery[r], Nal[r]) > Vio[r].

Unselected cotransfer of HDG86 chromosomal markers was also observed in these crosses. When a Tn*5*-proximal marker such as Nov[r] was selected, a gradient of transfer of the unselected proximal markers was observed. When a distal marker such as Ery[r] was selected, cotransfer of Tn*5*-proximal Nov[r] and Str[r] markers was observed at a high frequency. These results (summarized in Table 5) are consistent with transfer of the HDG86 chromosome initiating at or near the Nov[r] marker and proceeding through the Nov[r] marker to the other markers. The results are consistent with a marker order of Nov[r]-Str[r]-Ery[r]-Nal[r]-Vio[r], which is in agreement with the order of these markers as determined by genetic transformation (13, 25; Bendler, Ph.D. dissertation).

## DISCUSSION

We have demonstrated that under suitable conditions, an *E. coli* F′ factor (F′ *lac*[+] Tn*5*,Tn*9*) can be transferred from *E. coli* to *H. influenzae* by filter mating. Kanamycin (Tn*5*) and chloramphenicol (Tn*9*) resistances on the F′ factor are expressed in *H. influenzae* cells, and selection of either marker gives unselected cotransfer of the second marker at frequencies of greater than 99%. The F′ *lac*[+] marker is also expressed in recipient *H. influenzae* cells. However, *lac*[+] expression is at very low levels and is apparently not under normal *E. coli lac*[+] regulation. F′Tn*5*,Tn*9* is not initially stable in recipient *H. influenzae* cells, but stable lines arise with a low frequency. The nature of the conversion to stability is not clear. Southern hybridization data suggest that physical rearrangement of the F′-factor DNA may be involved.

F′Tn*5*,Tn*9* may also be transferred between *H. influenzae* cells by filter mating. Transfer is DNase independent and does not require the *H. influenzae rec-1* or *rec-2* genes. This agrees with observations on the transfer of naturally occurring mobile resistance elements in *H. influenzae* (23). Of more interest is the observation that F′Tn*5*,Tn*9* mobilized *H. influenzae* chromosomal markers at a low level. Under the conditions used in these experiments, gene transfer is F′-factor dependent, is directional from $F^+$ to $F^-$ cell lines, and is not affected by the addition of DNase I. These results are all consistent with F′-factor-mediated mobilization of *H. influenzae* chromosomal markers. Although a number of transmissible plasmids in *H. influenzae* have been reported (5, 8, 17, 18, 23), plasmid-mediated mobilization of chromosomal markers has not been previously reported. Cotransfer of even relatively closely linked (by transformation) markers such as Str[r] and Nov[r] was not observed in these crosses, indicating that only relatively small regions of the *H. influ-*

TABLE 5. Unselected cotransfer of chromosomal markers from HDG86(F′Tn*5*,Tn*9*)[a]

| Selected marker[b] (no.)[c] | % Cotransfer of unselected marker | | | | |
|---|---|---|---|---|---|
| | Nov[r] | Str[r] | Ery[r] | Nal[r] | Vio[r] |
| Nov[r] (1,000) | | 45.8 | 12.3 | 10.1 | 7.3 |
| Str[r] (452) | 92.0 | | 26.7 | 23.5 | 15.4 |
| Ery[r] (523) | 93.2 | 94.4 | | 81.7 | 61.0 |
| Nal[r] (427) | 86.0 | 91.0 | 92.1 | | 71.5 |

[a] HDG81 recipient was used with rifampin counterselection.
[b] Recombinants were selected from three independent matings.
[c] Number of primary recombinants tested by replica plating.

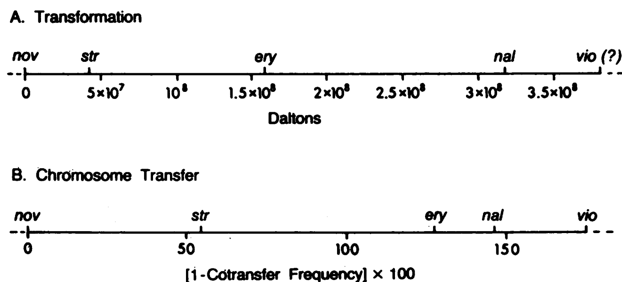


FIG. 3. Partial map of the *H. influenzae* genome. (A) Transformation map. Distances are in kilobases, based on the data on Bendler (Ph.D. dissertation) and Michalka and Goodgal (13). The position of the VioR marker was estimated from the data of Stuy (25). (B) Gene transfer map. Distances are (100 − % cotransfer) and are based on the data in Table 5.

*enzae* chromosome are mobilized in any given cross. All markers tested in these crosses were mobilized to about the same degree, indicating that F′-factor mobilization is apparently a random process.

When a local region of homology was created by introducing a Tn*5* element into the *H. influenzae* chromosome near the Nov$^r$ marker, two effects were observed. First, transfer of Nov$^r$ increased 30-fold. Transfer of other markers was also increased, although to a lesser extent. Second, unselected cotransfer of chromosomal markers was also observed. The results are consistent with ordered transfer of the *H. influenzae* chromosome starting from a point near the Nov$^r$ marker and proceeding through the Nov$^r$ marker to the other, more distal, markers. This conclusion is supported by the further observation that if a distal marker (such as Str$^r$ or Ery$^r$) is initially selected, unselected cotransfer of the proximal Nov$^r$ marker is seen at a high frequency. By testing unselected cotransfer of the markers used, it was possible to establish a gene order, Nov$^r$-Str$^r$-Ery$^r$-Nal$^r$-Vio$^r$, which agrees with the order of these markers as obtained by genetic transformation (13, 25; Bendler, Ph.D. dissertation). In addition, since a gradient of transfer of markers was observed, it is possible to assign distances between markers and to generate a partial conjugative map of the HDG86 chromosome (Fig. 3). The map obtained is similar to the transformation-derived map of *H. influenzae* MPA, the major difference being the relatively greater distance from the Nov$^r$ to the Str$^r$ marker by conjugal transfer. Further studies on the genetic organization of the *H. influenzae* chromosomes are planned.

One major unresolved problem remains about the transfer of the F′ factor into *H. influenzae aemophilus* cells. It is apparent that the F′-factor *rep-par* DNA probe binds to three distinct *Eco*RI fragments (Fig. 3) and not to the single 8.1-kb *Eco*RI fragment expected. This banding pattern is not present in the parental strain, *E. coli* C600, which shows the predicted 8.1-kb *rep-par* fragment. In addition, we have observed other rearrangements of this region in different *Haemophilus* F′-factor isolates (data not shown). Since this region of the F′ factor is involved in plasmid replication and stability, it is possible that these rearrangements are involved in the conversion of the F′ factor to a form which is stably maintained in *H. influenzae*.

Another unexpected observation from these studies was the high cotransfer frequency of distant proximal markers when a distal marker was selected. One would normally expect this frequency to drop to about 50% for widely separated markers. However, when a distal marker (such as Nal$^r$) was selected, all markers proximal to the selected marker cotransferred at very high frequencies (>85%) (Table 5), even though some of the markers are apparently widely separated. These data suggest either that the recombination frequency in *H. influenzae* is low (at least under conditions used in these experiments) or that there is a crossover hot spot at or near the presumed initiation point of chromosome transfer (i.e., the Tn*5* insertion site).

The F′-factor-mediated chromosome transfer system has several advantages and disadvantages relative to the *H. influenzae* transformation system. One major advantage is simplicity: transformation requires purification of transforming DNA and, for best results, use of a complex medium and multiple cell washes to induce high levels of competence in cells (11). A second advantage is that gene linkage by mating can be demonstrated over a much longer region of the *H. influenzae* chromosome. For example, in this study, the Nov$^r$ and Vio$^r$ markers cotransferred at a frequency of about 0.07, while linked cotransformation of these markers has never been reported. This occurs primarily because linked cotransformation is limited by the length of transforming DNA in solution (normally less than 50 kb, unless special precautions are taken).

One major limitation of this transfer system is the relative difficulty of generating local homology regions between the F′Tn*5*,Tn*9* and the *H. influenzae* chromosome. In this regard, a random transposon mutagenesis system for *H. influenzae* would be of great value. We have tested several transposon suicide delivery systems but have been unable to demonstrate transfer of transposon-encoded drug resistances to the *H. influenzae* chromosome (unpublished observations). We have also attempted to cure the F′Tn*5*,Tn*9* from *H. influenzae* by use of the temperature-sensitive replication of this episome. However, *H. influenzae* Rd is not viable above 40°C, and growth at lower temperatures has not resulted in any measurable curing of the F′ factor. Another limitation is the relative inefficiency of gene transfer in *H. influenzae*: nearly 4 orders of magnitude less than transformation of markers.

It is evident that F′-factor-mediated gene transfer and genetic transformation can be used as complementary methods for studying the genetics of *H. influenzae*. In this study, we have used genetic transformation to generate the local homology regions used in the gene transfer system, and chromosome transfer could, for example, be a useful tool for studying genes involved in transformation. It has also been reported for other bacterial pathogens (15) that different clinical isolates can have major rearrangements of chromosomal markers. This mating system should allow us to determine whether such rearrangements also occur in clinical *H. influenzae* strains and if the rearrangements correlate with other strain-typing systems (i.e., biotype or outer membrane protein profile).

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