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Transposon Mutagenesis, Characterization, and Cloning of Transformation Genes of *Haemophilus influenzae* Rd

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A plasmid library of *Pst*I fragments of *Haemophilus influenzae* Rd genomic DNA was mutagenized in *Escherichia coli* with mini-Tn*10kan*. The mutagenized *Pst*I fragments were introduced by transformation into the *H. influenzae* chromosome, and kanamycin-resistant transformants were screened for the transformation-deficient phenotype by a cyclic AMP-DNA plate method. Fifty-four mutant strains containing 24 unique insertions that mapped to 10 different *Pst*I fragments were isolated. Strains carrying unique insertions were tested individually for DNA uptake, transformation efficiency, UV sensitivity, and growth rate. The transformation frequencies of these mutants were decreased by factors of 10^{-2} to 10^{-6}. Five of the mutants had normal competence-induced DNA uptake, and the rest were variably deficient in competence development. Three strains were moderately UV sensitive. All strains but one had doubling times within 50% of that of the wild type. Mutated genes were cloned into an *H. influenzae-E. coli* shuttle vector, and wild-type loci were recovered by in vivo recombinational exchange. Hybridization of these clones to *Sma*I genomic fragments separated in pulsed-field gels showed that these insertions were not clustered in a particular region of the chromosome.

In the late logarithmic growth phase or in response to environmental stimuli such as a nutritional shift down and/or a decrease in oxygen tension (15, 16), *Haemophilus influenzae* cells undergo a transient physiological change (35) which results in a state of competence (that is, the ability of cells to bind, take up DNA, and transform). Three changes have been documented as being associated with competence development and transformation. The first is modification of the cellular envelope. This includes the synthesis of new polypeptides (11, 12, 42, 49), an alteration in the lipopolysaccharide composition (50), and the appearance at the cell surface of vesicle-like structures, or transformasomes, which presumably are the sites of DNA binding and entry (20). The second is an enhanced recombination capacity of the cells, measured as increased frequencies of phage and plasmid recombination (3, 7, 27). The third is the appearance of single-stranded gaps and tails in the chromosome (24, 29). Although relief of catabolite repression is thought to be an important factor in the regulation of the process (31, 47), the physiological link(s) between the different environmental stimuli and the onset of these changes (48) and the exact nature of the involvement of essential and inhibitory metabolites (31, 33, 34) have yet to be determined.

The genetic and biochemical characterization of transformation-deficient mutants generated by nitrosoguanidine mutagenesis (5, 9, 22, 37) led to the identification of steps involved in DNA uptake and transformation (1, 3, 4). As a result, a model for chromosomal transformation of competent *H. influenzae* cells was proposed (17–19). Despite these efforts, a systematic genetic analysis of the transformation pathway has not been achieved, in part due to the lack of good methods for genetic analysis in *H. influenzae* and the difficulty encountered in cloning the genes of interest.

In this paper, we briefly describe the use of mini-Tn*10kan* transposon mutagenesis (45) for isolation of transformation-deficient mutants and describe the characterization of these mutations and the cloning of the mutated alleles and the corresponding wild-type loci. Since a system for transposon mutagenesis in *H. influenzae* is not available, the mutagenesis was carried out on a plasmid library of *H. influenzae* *Pst*I DNA fragments in *Escherichia coli*. The transposon used has the advantage of being a small 2-kilobase-pair (kb) element carried on phage lambda along with, but separate from, the Tn*10* transposase under *tac* promoter control. On infection of the library clones in a *lacI* background, the transposase is induced and high levels of transposition are achieved. After the transposon mutagenesis had occurred in our library population, the mutated DNA was introduced back into the *Haemophilus* chromosome by additive transformation (40). From among a large number of kanamycin-resistant clones obtained, a number of transformation-deficient (Tfo^-) strains were isolated after screening with a cyclic AMP (cAMP)-DNA plate method (47). These mutants were grouped, their competence-induced DNA uptake and transformation efficiency were measured, and their UV sensitivity and growth rate were determined.

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MATERIALS AND METHODS

Reagents and enzymes. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc., or Pharmacia, Inc. Hemin (equine), β-NAD, DNase I, and all antibiotics were obtained from Sigma Chemical Co. cAMP was obtained from Aldrich Chemical Co., Inc. 5′-[α-^{32}P]dCTP (3,000 Ci/mmol) and [methyl-^{3}H]thymidine (42 Ci/mmol) were obtained from Amersham Corp. Nitrocellulose (BA-85) and nylon (NYTRAN) filters were obtained from Schleicher & Schuell, Inc.

Bacterial strains, phages, and plasmids. All bacterial

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant genotype or phenotype	Source (reference)
H. influenzae Rd		
KW26	Thy$^-$	This laboratory (46)
KW20	Wild-type prototroph	This laboratory (46)
DB117	*rec-1*	J. Setlow (5)
MAP7[a]	Str^r Nov^r	J. Setlow
JG strains[b]	KW20::mini-Tn*10kan* Kan^r Tfo$^-$	This study
E. coli		
MC1060	Δ(*lacI-lacY*)*74 galE15 galK16 relA1 rpsL150 spoT1 hsdR1*	ECGSC[c] (8)
Bacteriophage		
λ1105	*c*I857 *P*am80 *nin*5 Kan^r Ω(*Eco*RI::mini-Tn*10kan*)	N. Kleckner (45)
Plasmids		
pHCV5	Amp^r Tet^r	Danner and Pifer (13)
pHVT1	Amp^r Tet^r	Danner and Pifer (13)
pGJB103	Amp^r Tet^r	This study
pDALK	pGJB103 Ω(*Pst*I::*H. influenzae* DNA::mini-Tn*10kan*)	This study
pDAL	pDALK Δ(mini-Tn*10kan*)	This study
pGB2	Str^r Spc^r	S. Friedman (10)
pUC71K	Amp^r Kan^r	C. apRhys (44)

[a] MAP7 is an erythromycin-sensitive derivative of MAP8.
[b] A total of 116 KW20 strains containing a mini-Tn*10kan* have been isolated after the second cAMP-DNA plate screening (see Materials and Methods and Results). These have been designated JG1 to JG116; 54 of them turned out to be transformation-deficient (Tfo$^-$) mutants.
[c] ECGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

strains, phages, and plasmids are listed in Table 1. Our cloning vector pGJB103 is a smaller version of the 11-kb *E. coli-H. influenzae* shuttle vector pHVT1 (13) with a higher copy number and containing a unique cloning site for DNA fragments with 5′-GATC ends. It was constructed by first preparing a deletion derivative called pGJB101 by cleaving pHVT1 with the restriction endonucleases *Ava*I and *Sph*I and filling in the resulting termini (using the Klenow fragment of DNA polymerase I). The filled-in 8.7-kb fragment was gel purified, self-ligated, and used to transform the *recA E. coli* BW368 to ampicillin resistance. A plasmid with the correct structure was selected and named pGJB101. It should be noted that the deletion giving rise to pGJB101 removed the pBR322 ROP sequences and consequently increased plasmid copy number severalfold (43; G. J. Barcak, unpublished results).

To create a unique cloning site for 5′-GATC DNA fragments in pGJB101, we inserted a *Bgl*II linker into the *Ssp*I site of the untranslated leader region of the plasmid *bla* gene. Because pGJB101 contained several *Ssp*I sites, *Bgl*II linkers were first inserted into the related plasmid pHCV5 (13) at both of its *Ssp*I sites (one of which was in the *bla* gene), giving rise to plasmid pGJB45. Next, the β-lactamase gene region of plasmid pGJB101 was replaced with the modified region from pGJB45, resulting in pGJB103. A map of this plasmid is shown in Fig. 1.

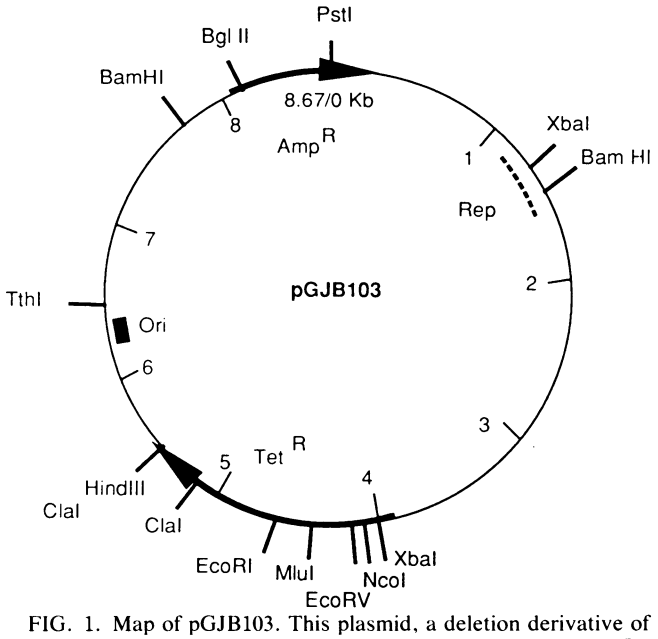

FIG. 1. Map of pGJB103. This plasmid, a deletion derivative of pHVT1 (see text), was used to clone *Pst*I fragments in *H. influenzae*. Arrows indicate direction of transcription. Ori is pBR322 origin of replication, and rep is a region required for plasmid replication in *H. influenzae*.

Bacterial growth and competence development. *H. influenzae* cells were cultured in brain heart infusion broth supplemented with 2 μg of NAD and 10 μg of hemin per ml (sBHI). Competence induction was by the M-IV medium procedure of Herriott et al. (16). Kanamycin was used at 20 or 35 μg/ml, streptomycin was used at 250 or 500 μg/ml, novobiocin was used at 2.5 μg/ml, and tetracycline was used at 5 μg/ml. Plasmid pGB2 was selected for and maintained in *E. coli* by using 50 μg of spectinomycin per ml.

Doubling time and UV sensitivity assays. To determine the doubling time of any particular strain, 1 ml of cells frozen at an optical density at 650 nm (OD_{650}) of 0.3 was thawed, transferred to 35 ml of heart infusion broth supplemented as described above, and shaken at 125 rpm and 37°C. Measurements of OD_{650} were done in plastic cuvettes on a Beckman Spectronic 2000. Doubling times were estimated by using a minimum of eight time points between an OD_{650} of 0.05 and an OD_{650} of 0.35. For UV sensitivity assays, cells were grown to an OD_{650} of 0.3 in sBHI, diluted in saline plus 10% sBHI to 10^{-4} to 10^{-6}, and spread on the surface of sBHI agar plates. Plates were then irradiated on a rotating turntable under a germicidial UV lamp for 0 to 10 s at a UV dose rate of 0.62 J/m^2 per s. The highest dose decreased the survival of KW20 by 10- to 15-fold. Plates were incubated in the dark.

Chromosomal and plasmid DNA preparation. *H. influenzae* chromosomal DNA was prepared from overnight cultures by the method of Silhavy et al. (39) with the omission of lysozyme. [^{3}H]DNA (approximately 10^5 cpm/μg) for uptake studies was prepared from strain KW26 (Thy$^-$) cells grown for several generations in supplemented heart infusion broth containing 25 μCi of [^{3}H]thymidine per ml. Plasmid DNA was prepared from *H. influenzae* essentially as described by Micard et al. (30) with the omission of the column chromatography step and from *E. coli* by the alkaline lysis method of Silhavy et al. (39).

Southern hybridization. Fragments of chromosomal DNA (3 to 5 μg) were separated by electrophoresis through an 0.8

or 1% agarose gel in TAE buffer (26), blotted onto nitrocellulose or NYTRAN filters, and hybridized with the ^{32}P-labeled *Bam*HI fragment of pUC71K containing the *kan* gene as described by Maniatis et al. (26). Probe DNAs were ^{32}P labeled to about 10^8 cpm/μg by the random primer method (14).

Construction of a plasmid library of *H. influenzae* Rd DNA, transposon mutagenesis, screening for transformation deficiency, and cloning of Tfo$^-$ mutations. A detailed description of the preparation of a library of *Pst*I fragments and of transposon mutagenesis will be published elsewhere (J.-F. Tomb, G. J. Barcak, M. S. Chandler, R. J. Redfield, and H. O. Smith, *in* L. O. Butler, C. Harwood, and B. E. B. Moseley, ed., *Genetic Transformation and Expression*, in press). Briefly, a library of *Pst*I fragments in the low-copy-number vector pGB2 was introduced in *E. coli* MC1060. The spectinomycin-resistant colonies were pooled and infected with λ1105 carrying the mini-Tn*10kan* transposon (45). After overnight selection on drug plates, Spc^r Kan^r transductants were pooled, and plasmid DNA was prepared and cut with *Pst*I. This DNA served as the source of the mutated library and was introduced by transformation into M-IV-competent *H. influenzae* KW20 cells. The initial Kan^r *H. influenzae* colonies were screened for Tfo$^-$ mutants by a cAMP-DNA plate method (47). Mutations were cloned by ligating chromosomal *Pst*I fragments into pGJB103 and transforming DB117 to Kan^r by the glycerol method of Stuy (41); the resulting plasmids were given pDALK numbers. Restriction analysis of these plasmids was used to confirm the presence of *H. influenzae* chromosomal DNA containing a mini-Tn*10kan* insertion. Conversion of pDALK Tfo$^-$ plasmids to the corresponding pDAL Tfo$^+$ was achieved with high efficiency by in vivo recombinational replacement of the mutated allele (38). The resulting plasmids (pDAL numbers) were analyzed to check for the loss of the mini-Tn*10kan* element and replacement by the wild-type *H. influenzae* Rd *Pst*I DNA insert.

DNA-binding, uptake, and transformation assays. A 1-μg sample of [^{3}H]DNA was added to 1 ml of M-IV-treated cells at 37°C (16). After 30 min, a 0.1-ml sample was withdrawn, diluted 1:10 in supplemented heart infusion broth, and incubated at 37°C for 1 h to allow expression before dilution and plating on sBHI agar with or without 250 μg of streptomycin per ml. At the same time, to measure DNA binding (total counts associated with the cells) and DNA uptake (DNase I-resistant counts), a 0.2-ml sample was withdrawn into each of two tubes at 0°C, one of which contained 10 μg of DNaseI. After 5 min, the DNase-treated sample was brought to 0.5 M NaCl, and both samples were centrifuged and washed once with 1 ml of M-IV or M-IV containing 0.5 M NaCl. The pellets were suspended in M-IV, transferred to glass fiber filters, and dried, and the radioactivity was determined.

Distribution of the mutations on the *Sma*I fragments of the *Haemophilus* genome. Pulsed-field gel electrophoresis of *Sma*I-cut *Haemophilus* DNA was done as described previously by Lee and Smith (25). The DNA was then transferred to NYTRAN filters and probed with ^{32}P-labeled plasmid DNA from each of the mutated *Pst*I fragments.

RESULTS

Identification of transformation-deficient mutants. The plasmid library of KW20 chromosomal DNA consisted of about 6,300 Spc^r clones, 70% of which contained recombinant plasmids. These primary transformants were pooled and subjected to mini-transposon mutagenesis (see Materials and Methods), resulting in approximately 300,000 Kan^r transductant colonies. The pooled *Pst*I-cut plasmids from these colonies constituted the mini-Tn*10kan*-mutated library. Approximately 20,000 Kan^r colonies were obtained as a result of transforming M-IV-competent KW20 cells with this library. The transformants were then screened for the Tfo$^-$ phenotype by using the cAMP-DNA replica plate method. About 1,000 potential Tfo$^-$ strains were identified in this first screening. To eliminate false-negatives, these strains were then rescreened, and a total of 116 putative Tfo$^-$ clones were identified and assigned numbers (JG1 to JG116). Twelve of these strains were lost during propagation, and sixteen displayed a "sticky" phenotype (pronounced clumping in broth) and were not included in the study. When the rest were tested for their ability to transform after the M-IV competence induction treatment, 26 proved to be wild type and were not further studied. The insertions in the remaining 62 Tfo$^-$ strains were then characterized by Southern hybridization and molecular cloning.

Restriction mapping of the Tfo$^-$ mutations and linkage of the mutant phenotypes to single mini-Tn*10kan* insertions. To determine the physical relationships between insertions in different strains, their chromosomal DNAs were subjected to restriction and Southern blot analysis. We initially analyzed 31 strains and used the information to refine our characterization of the remaining strains. Chromosomal DNAs from mutants of the first group were digested with *Pst*I and with *Eco*RI and then were run on a gel and probed with the *kan* probe. In each digest the probe hybridized to a single band, indicating the presence of a single insertion in each strain (data not shown). To determine whether strains containing comigrating *Pst*I fragments and *Eco*RI fragments carried identical insertions and to map the positions of the insertions, the chromosomal DNAs were digested with *Cla*I, which cleaves once within the mini-Tn*10kan* element. Strains showing apparently identical *Eco*RI, *Pst*I, and *Cla*I bands were assumed to have insertions at the same location. Strains that had comigrating *Pst*I and *Eco*RI bands but had different *Cla*I patterns were considered to have different insertions in possibly identical *Pst*I fragments. The identity was checked by probing clones derived from these strains against each other. All nonidentical insertions were cloned as *Pst*I fragments in pGJB103. Analysis of the first group identified 16 different insertions into 11 different *Pst*I fragments.

Similar analyses of the remaining mutants and cross-hybridization with plasmid clones of the first group yielded 6 unique insertions in four new *Pst*I fragments, 7 new insertions in three *Pst*I fragments that were also found in the first group, and 16 insertions in positions apparently identical to those of insertions identified in the first group. In total, 29 unique insertions in 15 different *Pst*I fragments were identified.

To confirm that the Tfo$^-$ phenotypes were a direct result of the insertion of mini-Tn*10kan* elements, backcross strains were constructed by transforming KW20 with a limiting amount of chromosomal DNA from each of the 29 Tfo$^-$ strains. From each transformation, five Kan^r transformants were tested for the Tfo$^-$ phenotype by the cAMP-DNA plate method, and at least one was tested for DNA binding, uptake, and transformation after M-IV treatment. Exceptions were groups A, B, and C (see below), for which only backcrosses of strains JG56 and JG48 (group A), strain 112 (group B), and strains JG6 and JG40 (group C) were tested by M-IV transformation. In five strains, the transformation defect was not linked to the kanamycin resistance. Southern

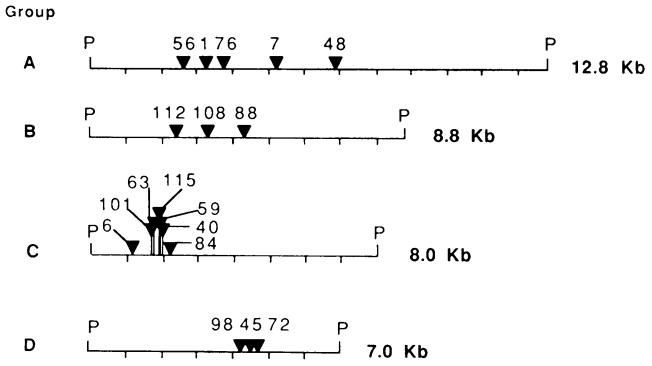

FIG. 2. Positions of unique mini-Tn*10kan* insertions in *Pst*I fragments that received more than one insertion. The numbers refer to JG strains.

blot analysis of chromosomal DNA derived from these backcrossed strains confirmed that the mini-Tn*10kan* insertion in each was identical to that of the parent strain (data not shown). These five strains were then eliminated from further study. In two strains, JG40 and JG58, the backcross derivatives retained the mutant phenotype, but the values for DNA binding and uptake and the transformation frequency (only in JG58) were markedly different. The backcross strains derived from JG40 had about a 20-fold lower DNA binding and about a 40-fold lower DNA uptake than the parent strain. The transformation frequency and DNA uptake of backcross JG58 were about 25-fold higher. The values obtained for the rest of the backcross strains were in good agreement with the values reported in Table 2 for the parent strains. We also tested the UV sensitivity of backcross strains derived from five UV-sensitive parents; two were found to be UV resistant.

In summary, we identified a total of 24 distinct insertions in 10 different *Pst*I fragments. Strains bearing these insertions and their apparent sibs are listed in Table 2. Four insertions were isolated 2 times, four were isolated 3 times, one was isolated 9 times, and one was isolated 11 times. The strains in Table 2 are grouped according to whether their insertions are in the same *Pst*I fragment. Five different insertions were found in a 12.8-kb fragment (group A), three were found in an 8.8-kb fragment (group B), seven were found in an 8.0-kb fragment (group C), and three were found in a 7.0-kb fragment (group D). The relative positions of these insertions are shown in Fig. 2.

Distribution of the mutations over the genome. To determine whether mutations on different *Pst*I fragments might be near each other on the chromosome, we mapped the *Pst*I fragments to individual *H. influenzae Sma*I fragments (25). Filters containing the *Sma*I fragments of KW20 DNA separated by pulsed-field gel electrophoresis were probed with plasmids representing each *Pst*I-cloned fragment. Table 3 shows that the cloned mutations were distributed over fragments representing 80% of the genome. As an additional check on whether any cloned fragments might be adjacent, we compared their hybridization patterns on filters containing KW20 DNA cut with seven restriction enzymes (*Bgl*I, *Bgl*II, *Eco*RI, *Bst*EII, *Nci*I, *Pvu*II, and *Sac*II, chosen because they cut infrequently, giving fragments mostly between 5 and 50 kb). Any adjacent *Pst*I fragments should have hybridized to common fragments in most digests, but this was not seen in any comparison (data not shown).

Phenotypes of the Tfo$^-$ strains. The transformation phenotypes (DNA binding, DNaseI-resistant DNA uptake, and transformation frequency) associated with each insertion were determined by transformation of M-IV-treated cultures with ^{3}H-labeled KW26 DNA (Table 2). Most strains were defective for DNA binding, DNA uptake (<1% of the wild-type level), and transformation ($<10^{-6}$ to 10^{-2} of the wild-type level). Two mutants, JG37 and JG56, bound and took up DNA at wild-type levels but transformed at $<10^{-5}$ of the wild-type level. The three mutants in group B, JG88, JG108, and JG112, took up normal or near-normal amounts of donor DNA and transformed at about 10^{-2} of the wild-type level.

TABLE 2. Phenotypes of Tfo$^-$ strains

Strain[a]	Radioactivity (cpm) associated with cells[b]		Transformation frequency[b,c]	UV[d] sensitivity	Growth rate[e]
	Without DNase	With DNase			
KW20 Tfo$^+$	15,402	12,797	1.6×10^{-3}	R	N
KW20 zero time		30	8.1×10^{-8}	R	N
JG109 Tfo$^+$	12,757	11,867	1.7×10^{-3}	R	N−
JG56	15,238	13,823	$<10^{-9}$	R	N
JG1	198	48	1.3×10^{-9}	R	N−
JG76 (1)	294	202	$<10^{-9}$	R	N
JG7	342	35	$<10^{-9}$	R	N−
JG48 (2)	116	25	$<10^{-9}$	R	N−
JG112	8,961	7,414	5.6×10^{-6}	R	N−
JG108 (1)	13,469	12,023	7.0×10^{-5}	R	S
JG88 (1)	10,205	10,200	2.9×10^{-5}	R	S
JG6	446	84	2.2×10^{-6}	R	S
JG101	1,062	802	3.0×10^{-5}	R	N−
JG63	4,431	294	5.4×10^{-7}	R	—
JG115	337	150	5.3×10^{-6}	R	N−
JG59	973	204	6.0×10^{-7}	R	S
JG40[f]	14,285	9,180	7.7×10^{-5}	R	S
JG84	186	100	4.1×10^{-6}	R	S
JG98 (2)	354	88	1.8×10^{-9}	S	S
JG45 (2)	205	79	1.6×10^{-7}	S	S
JG72	294	75	3.5×10^{-8}	S	N−
JG16 (8)	1,523	159	2.6×10^{-9}	R	N
JG27	141	96	1.5×10^{-6}	R	S
JG37	12,576	13,847	$<10^{-9}$	R	N
JG49 (10)	647	204	2.4×10^{-6}	R	S
JG58 (2)	941	75	3.8×10^{-9}	R	VS
JG87 (1)	116	8	$<10^{-9}$	R	N+

[a] DNase was added prior to the DNA in the KW20 zero time sample. JG109 contains an insertion which does not affect transformation. Mutant strains whose insertions mapped to the same *Pst*I fragment are grouped together. Number in parentheses is the number of apparently identical isolates.

[b] Radioactivity from a 200-μl sample contained 21,676 cpm; 43 cpm (background) was subtracted. Strains were tested at least twice; the values reported are from experiments with the highest transformation frequency. JG40 gave variable results (see text).

[c] Ratio of Str^r colonies to total CFU. Between 2×10^8 and 2×10^9 CFU were screened for Str^r.

[d] S, UV sensitive; R, UV resistant (see Fig. 3).

[e] N+, doubling time shorter than KW20; N, doubling time the same as KW20; N−, doubling time <30% longer than KW20; S, doubling time 30 to 50% longer than KW20; VS, doubling time more than 50% longer than KW20. —, Not tested.

TABLE 3. Chromosomal distribution of the Tfo$^-$ mutations

*Sma*I fragment(s)	Size (kb)	No. of *Pst*I fragments containing Tfo$^-$ insertions
A	380	2
B	260	3
C	230	1
D	220	1
E	190	1
G	135	1
I	70	1
F + H + J + K + L + M + N + O + P	393.5	0

Two additional phenotypes were measured, the UV sensitivity and the growth rate. Most strains were at least as UV resistant as KW20 wild type. Of the three strains which were UV sensitive (Fig. 3), none were as sensitive as the *recA*-like mutant *rec-1*. All strains but one had doubling times within 50% of that of the wild type (Table 2).

Cloning of the wild-type alleles by exchange of transposon-mutagenized genes for wild-type genes. Competent KW20 cells were transformed with each of the pDALK plasmids, and the Tet^r clones were scored for kanamycin sensitivity. In each case, 30 to 90% of the Tet^r colonies were Kan^s. Restriction analysis of plasmids from the Kan^s clones showed that they had lost the mini-Tn*10kan* insert without undergoing any other detectable deletions or rearrangements within the *Pst*I fragment (data not shown).

DISCUSSION

We have produced a variety of Tfo$^-$ mutations in *H. influenzae* Rd by using the transposon mini-Tn*10kan*. The capacity of *H. influenzae* to integrate transposon-mutagenized fragments of homologous DNA produced in *E. coli* allowed us to circumvent the lack of a system for transposon mutagenesis in *H. influenzae*. The small size of mini-Tn*10kan* (2 kb) may have been a significant factor, as additive transformation with larger insertions is less efficient (G. J. Barcak, unpublished data). The mutations were easily cloned in recombination-deficient cells by selecting for kanamycin resistance. Wild-type loci were subsequently recovered at a high efficiency by in vivo recombination between the mutant allele on the plasmid and the wild-type chromosomal allele.

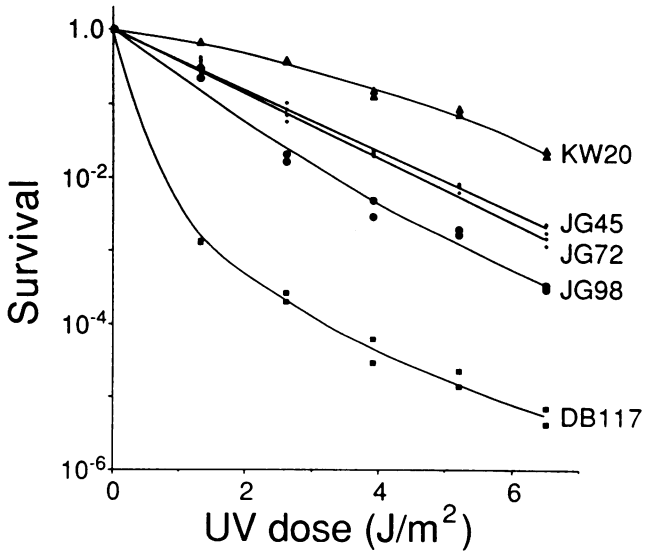

FIG. 3. Survival of UV-sensitive JG strains. Two cultures of each strain were tested in parallel. Each data point is the mean of two separately irradiated plates from a single culture. ▲, KW20; ■, DB117; ●, JG98; •, JG45 and JG72. All other Tfo$^-$ JG strains were at least as UV resistant as wild-type KW20.

We identified 24 distinct Tfo$^-$ mutations distributed among 10 *Pst*I DNA fragments in this initial application of the method. We have also isolated a number of apparently identical insertions, presumably because the insertion-bearing plasmids were propagated in *E. coli* and pooled before transformation into *H. influenzae*. Five additional Tfo$^-$ mutants were discarded because on backcross their mutant phenotypes were found not to be linked to the mini-Tn*10kan* insertion. The reason for this high frequency of unrelated mutations is being investigated. It may be that the background mutation rate was elevated by the DNA alterations associated with competence development (29), by SOS induction by DNA uptake (36), or by integration of partially homologous *E. coli* DNA that contaminated the mutagenized library.

The distribution of the mutations around the chromosome implies that the genes required for transformation are not arranged in a single operon. The insertions in each of groups B, C, and D are close to each other and produce similar phenotypes. Thus, they are likely to define single genes in each DNA segment. However, the five insertions in group A produce two different phenotypes and so could be in two genes.

We anticipated that Tfo$^-$ mutants could be defective in competence induction, in DNA binding, uptake, or translocation, or in homologous recombination. The mutants that we obtained can be divided in two categories: those that have normal or close-to-normal competence-induced DNA binding and uptake but are deficient in transformation and those that are deficient in competence-induced DNA binding and uptake. In both categories there were mutants that grew more slowly than the wild type, and it is conceivable that some of these could carry insertions that only indirectly affect the development of competence. However, during the course of this work we have isolated some slow-growing strains that show wild-type levels of transformation, suggesting that a slow growth rate by itself is not sufficient to cause the transformation-deficient phenotype.

Mutants with normal or near-normal DNA binding and uptake (JG56, JG37, and the three group B mutants) could have mutations affecting either the translocation or the integration machinery. Two previously described mutants were found to have normal DNA binding and uptake and very low frequencies of transformation: *rec-2*, in which the transforming DNA fails to translocate from the transformasome to the cytoplasm, and *rec-1*, which is recombination deficient and UV sensitive (3). However, Southern analysis using cloned *rec-1* and *rec-2* probes (2, 28) showed that none of the new Tfo$^-$ insertions is in the *rec-1* or the *rec-2* gene (data not shown). A biochemical study of the fate of transforming DNA in these mutants should be helpful in determining the step(s) at which the block has occurred. Strain JG40, which was reported to bind and take up near-normal levels of DNA (Table 2), was not included in this category because we obtained highly variable uptake values (50-fold differences) and because the reported values (the highest of three determinations) did not agree with the ones obtained for two backcross strains (see Results).

Mutations belonging to the second category, which includes 18 different insertions into at least eight genes, do not have normal competence-induced DNA binding and uptake. Two classes of defects could cause this phenotype: defects in the regulatory pathway that leads to competence and defects in the DNA transport machinery. For both, the defects could be in genes specific to transformation or in genes involved in cellular processes that affect transformation. It is important at this stage to distinguish regulatory mutants from others. Toward this end, single-stranded DNA transformation, competence-induced phage recombination, and competence-associated membrane and chromosomal alterations can be investigated. Included in this category are the three moderately UV-sensitive strains of group D. A deficiency in competence-inducible DNA binding has not been seen in other UV-sensitive mutants, *rec-1* (3), com$^-$60 (9), *ird* (23), and HM5 (21). The UV sensitivity in group D strains may or may not be related to the transformation defect.

One of the expected mutant phenotypes, binding proficient and uptake deficient, was not observed in our collection of mutants. Because of limitations inherent to the approach, this mutation and perhaps others as well might not be obtainable. By preparing the *H. influenzae Pst*I fragment library in *E. coli*, we introduced the possibility that certain genes would be selected against and possibly lost. To minimize this we used a low-copy-number vector, pGB2. By using a complete *Pst*I library, we selected against those genes that have *Pst*I sites within them. Because transposon mutations are rarely leaky, insertions in essential genes would not be represented. Moreover, insertions occurring within *Pst*I fragments smaller than 3 kb have a lower probability of being represented. This is due to the fact that on average, 1.5 kb of the 3′ end of transforming DNA is degraded, presumably during the translocation from the transformasome and prior to integration into the chromosome (32). In fact, the smallest fragment encountered in our screen was 3.5 kb in length. A marked improvement on the method would be the development of a transposon mutagenesis system applicable to *H. influenzae*.

Currently, we are locating the transformation-related genes in each cloned fragment and determining their modes of regulation, the functions they encode, and whether or not they complement previously isolated mutations.

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