APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1998, p. 4697–4702 Vol. 64, No. 12
0099-2240/98/$04.00+0

In Vivo Transposon Mutagenesis in *Haemophilus influenzae*

ANITA KRAIß, STEFAN SCHLÖR, AND JOACHIM REIDL*

Zentrum für Infektionsforschung, Universität Würzburg, 97070 Würzburg, Germany

Received 8 June 1998/Accepted 22 September 1998

In order to devise an in vivo insertion mutagenesis scheme for *Haemophilus influenzae*, a novel set of transposons has been constructed. These are Tn*10*-based minitransposons carried on pACYC184- and pACYC177-based replicons, which are suitable for in vivo transposition in *H. influenzae*. The transposon delivery system was designed to contain an *H. influenzae*-specific uptake signal sequence which facilitates DNA transformation into *H. influenzae*. The following mini-Tn*10* elements have been made suitable for specific tasks in *H. influenzae*: (i) Tn*10d-cat*, which can be used to generate chloramphenicol-selectable insertion mutations; (ii) Tn*10d-bla*, an ampicillin-selectable translational fusion system allowing the detection of membrane or secreted proteins; and (iii) Tn*10d-lacZcat*, a chloramphenicol-selectable *lacZ* transcriptional fusion system. For the rapid identification of the transposon insertions, a PCR fragment enrichment method was developed. This report demonstrates that this in vivo mutagenesis technique is a convenient tool for the analysis of biochemical and regulatory pathways in the human pathogen *H. influenzae*.

Haemophilus influenzae type b is a gram-negative coccobacillus that is responsible for significant morbidity and mortality in humans (12, 34). In addition to type b, a large group of so-called nontypeable strains are responsible for diseases like sinusitis, otitis media, and pneumonia (22). To understand the physiology of this human pathogen and also to find potential targets for further antimicrobial therapies, it will be necessary to dissect its biochemical and regulatory pathways. Recently, the complete DNA sequence of the genome of *H. influenzae* was determined (11). Nevertheless, there is still a need for suitable techniques allowing genetic manipulations to determine knockout phenotypes and to study gene regulation in this organism.

Different transposon mutagenesis schemes have been applied to *H. influenzae* (14, 28, 32), which address shuttle mutagenesis, gene replacement, and in vitro transposon mutagenesis. A typical shuttle mutagenesis scheme for *H. influenzae* requires the construction of genome plasmid libraries of *H. influenzae*, which have to be transformed into *Escherichia coli* strains containing some type of transposon system allowing a general insertion mutagenesis. Subsequent reisolation and retransformation of mutated plasmids into *H. influenzae* will eventually result in insertions located on the chromosome as a result of gene replacement via homologous recombination (4). Recently, an in vitro mutagenesis procedure was established (13). This system comprises purified Tn*7* transposase, purified transposon DNA, and chromosomal target DNA. In vitro transposition then results in manipulated DNA which can be retransformed into *H. influenzae*, whereupon insertions can be selected. Another in vitro transposition system was also recently developed and applied to *H. influenzae* by Akerley et al. (1). This technique specifically addresses the detection of essential gene products contained on genomic segments which are necessary for bacterial growth and viability.

As reported earlier, transposition of a natural transposon (Tn*916*; 16.4 kbp) of *Enterococcus faecalis* was demonstrated in *H. influenzae* and *Haemophilus parainfluenzae* (17). Tn*916* transposition resulted in tetracycline-selectable insertions located on the chromosome. However, Holland et al. (15) reported that Tn*916* insertions in *E. coli* and *H. influenzae* tend to be unstable under nonselective conditions and that *E. coli* possesses preferred integration sites. Nevertheless, applying Tn*916* transposition to *H. influenzae* led to the identification of two genes which are involved in the expression of transferrin-binding proteins (15). Other transposons, such as Tn*5* and Tn*9*, have not been found to be active in *H. influenzae* (7).

In this report a convenient in vivo insertion mutagenesis system is presented. This technique combined with a fast insertion identification procedure represents a powerful tool for studying (i) gene regulation, (ii) knockout phenotypes, and (iii) protein location and topology in *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue was used as a recipient strain for the construction of pAK*bla*, pAK*cat*, and pAK*lacZcat*. XL1-Blue was grown on Luria broth medium supplemented with tetracycline (12 μg/ml), at 37°C under aerobic conditions. *H. influenzae* Rd was obtained from A. Wright (Department of Microbiology, Tufts Medical School, Boston, Mass.) and was grown on 3.8% brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with NAD (10 μg/ml) (Sigma, Deisenhofen, Germany) and hemin chloride (20 μg/ml) (Sigma) (4). *Haemophilus* strains were grown under anaerobic conditions, using GasPak 150, in a BBL GasPak Plus generator with a catalyst (Baxter Diagnostics Inc.) or aerobically at 37°C. Plasmid pJR207 (24) was used as a donor plasmid for the construction of the minitransposons; pACYC184 and pACYC177 (6, 26) were used as recipient plasmids for the construction of Tn*10d-bla*, *-cat*, and *-lacZcat* derivatives. For *H. influenzae* the following antibiotics were used: ampicillin, 6 μg/ml; chloramphenicol, 2 μg/ml; and kanamycin, 10 μg/ml. *E. coli* strains were grown in the presence of ampicillin at 100 μg/ml, chloramphenicol at 30 μg/ml, and kanamycin at 50 μg/ml.

Genetic methods. Chromosomal DNAs of *H. influenzae* strains were prepared by the method of Barcak et al. (4). Plasmid DNA preparation was carried out by the Qiagen kit protocol (Qiagen, Hilden, Germany). Cloning and restriction analysis were done by procedures described by Maniatis et al. (20).

PCR amplification of the DNA fragment containing the *cat* gene was performed with an Extension kit, according to the procedures described by Gibco BRL-Life Technologies (Karlsruhe, Germany), and the MWG thermal DNA cycler protocol, based on that described by Mullis and Faloona (23). The following specific primers, synthesized by MWG-Biotech (Ebersberg, Germany), were used for the amplification of the *cat* gene DNA fragment: Cat5′, 5′-AACTGCAGTACGTAGCACCTCAAAAACACCATCATACAC-3′, and Cat3′, 5′-AATACGTACTGCAGCAGGCGTTTAAGGGCACCAATAACT-3′. These oligonucleotides were designed to anneal to the flanking DNA sequences of the *cat* gene carried on plasmid pACYC184 at bp 495 for Cat5′ and bp 3768 for Cat3′, according to the DNA sequence published by Rose (26). *Pst*I restriction sites were inserted at the 5′ ends of primers Cat5′ and Cat3′, and in addition, a

* Corresponding author. Mailing address: Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, 97070 Würzburg, Germany. Phone: 49-(0)931-312153. Fax: 49-(0)931-312578. E-mail: joachim.reidl@rzroe.uni-wuerzburg.de.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
E. coli XL1-Blue	F′::Tn*10 proA*$^+$ *B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/ *recA1 endA1 gyrA96* (Nal^r) *thi hsdR17*(r_K^- m_K^+) *supE44 relA1 lac*	New England Biolabs
H. influenzae Rd KW20		A. Wright
Plasmids		
pACYC177	Kan^r Ap^r	5
pACYC184	Cm^r Tet^r	25
pJR207	pLG339, Kan^r, Tn*10d-bla*	23
pJRP4	pACYC184, Cm^r, *hel*	24
pAK1	pACYC177, Kan^r Ap^s	This work
pAK2	pAK1, Kan^r Ap^s, Tn*10d-bla*	This work
pAK*bla*	pACYC184, Cm^r, Tn*10d-bla*	This work
pAK*cat*	pACYC177, Kan^r Cm^r Tn*10d-cat*	This work
pAK*lacZcat*	pACYC177, Kan^r Cm^r, Tn*10d-lacZcat*	This work

*Sna*BI site was designed to be contained at the 5′ end of Cat5′ (underlined sequences).

Identification of the mini-Tn*10*-based chromosomal insertions was done by PCR amplification, utilizing the 27-bp IS*10* sites as the amplification primer (IS10, 5′-CTGATGAATCCCCTAATGATTTTGGTA-3′) and isolated chromosomal DNA of *H. influenzae* as the template.

For the fragment enrichment method, based on an uptake signal sequence (USS) and a transposon-specific oligonucleotide, a touchdown programmed PCR (annealing temperature, 56 to 46°C, with the Elongase kit from Gibco Life Technologies) was performed with a series of isolated Tn*10d-cat*, Tn*10d-bla*, and Tn*10d-lacZcat* insertions (see below).

Southern blot analysis (30) was performed as described by the manufacturer (Amersham Life Science). DNA was cut with appropriate restriction enzymes and separated on a 0.7% agarose gel. DNA was then transferred onto a nylon membrane (Amersham Life Science). By using specifically labelled mini-Tn*10* probe DNA, detection of hybridizing fragments was done according to the ECL protocol (Amersham Life Science).

Transformation of plasmid or linear DNA into *H. influenzae* Rd was accomplished by the method described by Tomb et al. (32).

DNA sequencing. The insertion sites of the Tn*10* minitransposon elements were determined by the dideoxynucleotide chain termination method of Sanger et al. (27). The sequence reactions were performed with the PCR cycling reaction according to Amersham Life Science. The sequencing and detection were done with an infrared dye-labeled primer (IRD41) monitored with the automatic sequencing method of the LiCor system (MWG). The sequencing primer used is an antiparallel oligonucleotide (IS10seq, 5′-CAACTGATCTTCAGCATCTTT TAC-3′) of the 5′ end of the *blaM* gene, which can be used to detect fusion joints of Tn*10d-bla*, Tn*10d-cat*, and Tn*10d-lacZcat* insertions.

Western blot analysis. Derivatives of *E. coli* XL1-Blue containing plasmid pACYC177, *H. influenzae* harboring pACYC177, and *H. influenzae* containing *ccmE*::Tn*10d-bla* and *napC*::Tn*10d-bla* were grown in Luria broth medium (*E. coli*) or in BHI medium (*H. influenzae*) at 37°C for 18 h under aerobic and anaerobic conditions. Cells were washed off the agar plates, washed twice, and resuspended in sodium phosphate buffer (100 mM, pH 7.4). Twenty-five-fold-concentrated cell suspensions were dissolved in sample buffer, boiled, and analyzed by electrophoresis in 12% polyacrylamide gels containing sodium dodecyl sulfate (19). Separated proteins were transferred to nylon membranes (33) and subsequently probed with antibody (5′-3′ Inc. Boulder, Colo.) directed against BlaM as described by Reidl and Mekalanos (25). By employing an ECL photoaffinity procedure (Amersham Life Science) with peroxidase-coupled antirabbit antibody, the β-lactamase-specific complexes were detected.

Construction of minitransposons. To introduce the minitransposon elements into *H. influenzae*, we utilized a set of plasmids consisting of (i) *H. influenzae* replicative plasmids pACYC184 and -177, (ii) an *H. influenzae* specific USS site, and (iii) a functional transposon unit based on Tn*10*, consisting of the Tn*10* transposase and individually constructed defective minitransposons. The various steps of the construction of the mini-Tn*10* transposons are outlined in Fig. 1. The Tn*10d-bla*-containing plasmid pAK*bla* (Fig. 1A) was constructed by subcloning a blunt-ended 3.7-kb *Eco*RI fragment containing the Tn*10d-bla* element (24) into the *Sna*BI site of a pACYC184-based plasmid, pJRP4 (Cm^r) (25), carrying one USS site within the *e*(P4) outer membrane protein-encoding *hel* gene of *H. influenzae*. The construction resulted in a chloramphenicol-selectable plasmid, pAK*bla*, with an interrupted *hel* gene. For the construction of the Tn*10d-cat* element, plasmid pACYC177 was used and a 320-bp *Fsp*I-*Hin*cII fragment was deleted to obtain a plasmid, pAK1, conferring Ap^s and Kan^r (data not shown). pAK1 was further cut with *Bam*HI, and a 4.6-kb *Bam*HI fragment containing the *hel* gene and Tn*10* minitransposon Tn*10d-bla* of pAK*bla* was introduced, resulting in pAK2 (Fig. 1B). Plasmid pAK2 was then cut with *Pst*I, and a PCR-generated 1.1-kb *cat*-containing DNA fragment with *Pst*I engineered flanking sites was used in the ligation, resulting in plasmid pAK*cat*. This plasmid confers Kan^r and Cm^r on both *E. coli* and *H. influenzae*. The *cat* PCR fragment was designed to contain the native constitutively expressed *cat* promoter. Finally, Tn*10d-lacZcat* was constructed as follows. A blunt-end-generated promoterless 3.2-kb *lacZ Bgl*II-*Dra*I DNA fragment, originating from plasmid pMD35 (8), was subcloned into a *Sna*BI-digested pAK*cat* plasmid. The resulting plasmid contained the *lacZ* gene, followed by the *cat* gene oriented in the same transcriptional direction (Fig. 1C).

RESULTS

Demonstration of in vivo transposition in *H. influenzae*. Plasmids pAK*bla*, pAK*cat*, and pAK*lacZcat* carry a Tn*10* transposase under the control of the P_{tac} promoter (9, 36), which may act constitutively in *H. influenzae*. Plasmid pAK*bla* cannot confer Ap^r to cells unless translational hybrid fusions have been generated by transposition of Tn*10d-bla* (24). In order to test the transposition activity of the Tn*10d-bla* element, plasmid pAK*bla* was used to transform competent cells of *H. influenzae* Rd (4). After phenotypic expression at 37°C for 90 min, the cells were plated on BHI agar plates containing 2 μg of chloramphenicol per ml. After overnight incubation, the Cm^r transformed cells were pooled and frozen at −80°C. Five independent pools were generated this way. To determine the frequency of Ap^r cells, an aliquot of 1 μl of each pool was inoculated into 1 ml of BHI medium, diluted appropriately, and plated on BHI agar (20 μg of hemin per ml and 10 μg of NAD per ml) with and without ampicillin (6 μg/ml). As shown

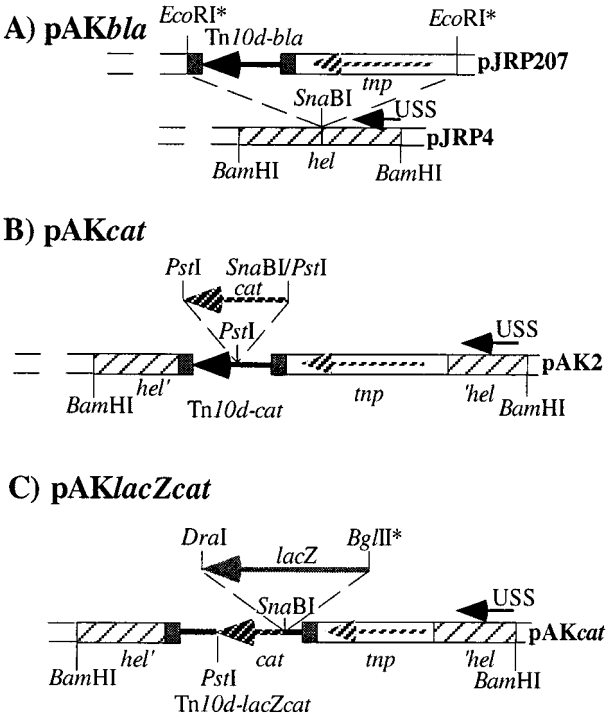

FIG. 1. Construction of plasmids. The asterisks indicate that sticky ends have been turned into blunt ends by the fill-in reaction with the large DNA polymerase fragment Klenow (Gibco Life Technologies). Hatched bars, *hel* gene, encoding the *e*(P4) lipoprotein of *H. influenzae*; small black arrows, USSs; light hatched arrows, *tnp*, encoding the IS*10* transposase; large black arrows, *blaM* part of Tn*10d-bla*, Tn*10d-cat*, and Tn*10d-lacZcat*, which is embedded within the 29 bp of IS*10*R (small black bars); large hatched arrows, *cat* gene, encoding chloramphenicol acetyltransferase; shaded arrow, promoterless *lacZ* gene, encoding β-galactosidase of *E. coli*. pAK*bla*, pAK*cat*, and pAK*lacZcat* were constructed as described in Materials and Methods.

TABLE 2. Frequency of Ap^r cells after mutagenesis with Tn*10d-bla*[a]

Pool	Cells/ml after growth in:		Frequency[b]
	BHI medium	BHI medium with ampicillin	
1	1.42×10^8	6.05×10^4	4.2×10^{-4}
2	1.11×10^9	8.47×10^5	7.6×10^{-4}
3	2.11×10^9	7.35×10^5	3.4×10^{-4}
4	2.60×10^9	5.75×10^5	2.2×10^{-4}
5	2.23×10^9	3.50×10^5	1.5×10^{-4}

[a] Frequency was determined as described in the text. After overnight growth, colonies were counted.
[b] Ratio between Ap^r colonies (BHI medium with ampicillin) and absolute cell numbers (BHI medium).

in Table 2, after overnight growth, calculation of the ratio of Ap^r cells to all viable cells of five independent pools of transformants resulted in an average of about 3.8×10^{-4}, indicating that about 1 of 10,000 to 100,000 transformed cells has obtained an Ap^r phenotype due to a transposition event.

Determination of mini-Tn*10* insertion sites by a PCR fragment enrichment method. To allow rapid identification of the generated insertion sites, a fragment enrichment method was developed. As indicated in Fig. 2A, PCR was used to amplify a junction fragment generated between the mini-Tn*10* insertions and 5′ flanking chromosomal regions. For this method, USS sites were utilized. These are randomly distributed across the chromosome (1,465 copies) and contain the 9-bp core consensus sequence AAGTGCGGT (29). Since the USSs exist in two possible orientations (+ or −), it was necessary to synthesize two 24-mer hemirandom oligonucleotides containing the conserved 9-bp core sequence [USS(+), 5′-N_6AAAGT GCGGT-3′; USS(−), 5′-N_7ACCGCACTT-3′]. Another synthetic oligonucleotide, blainv (5′-CCGTAAGATGCTTTTCT GTGACTGGT-3′), was designed, which specifically hybridizes with the complementary 5′-oriented Tn*10d-bla*-, Tn*10d-cat*-, and Tn*10d-lacZcat*-containing DNA strand (Fig. 2A). The production of PCR fragments consisting of a IS*10*-chromosomal junction fragment was carried out by using the amplification oligonucleotides in a PCR with transposon-mutagenized chromosomal DNAs as templates. PCR fragments ranging in sizes from 0.5 to 4 kb were obtained from insertions generated by Tn*10d-bla* (Fig. 2B, lanes 1 to 4), Tn*10-cat* (lanes 5 to 10), and Tn*10-lacZcat* (lanes 11 to 13). These PCR fragments hybridized specifically to the transposon element (data not shown), indicating that junction fragments had been generated. These PCR DNA fragments were subsequently used for identification of the integration sites by DNA sequence analysis (Table 3). To establish that the DNA sequences indeed represented the junction between the transposon insertion and the chromosome, at least 15 bp of the terminal IS*10* sequences was identified by DNA sequencing, allowing determination of the junction base pair.

Mini-Tn*10* insertions analyzed by using Tn*10d-cat*. Tn*10d-cat* insertions were produced after transformation of pAK*cat* into *H. influenzae* Rd. Independent Cm^r (2 μg/ml) transformants were picked randomly and were tested for the loss of the

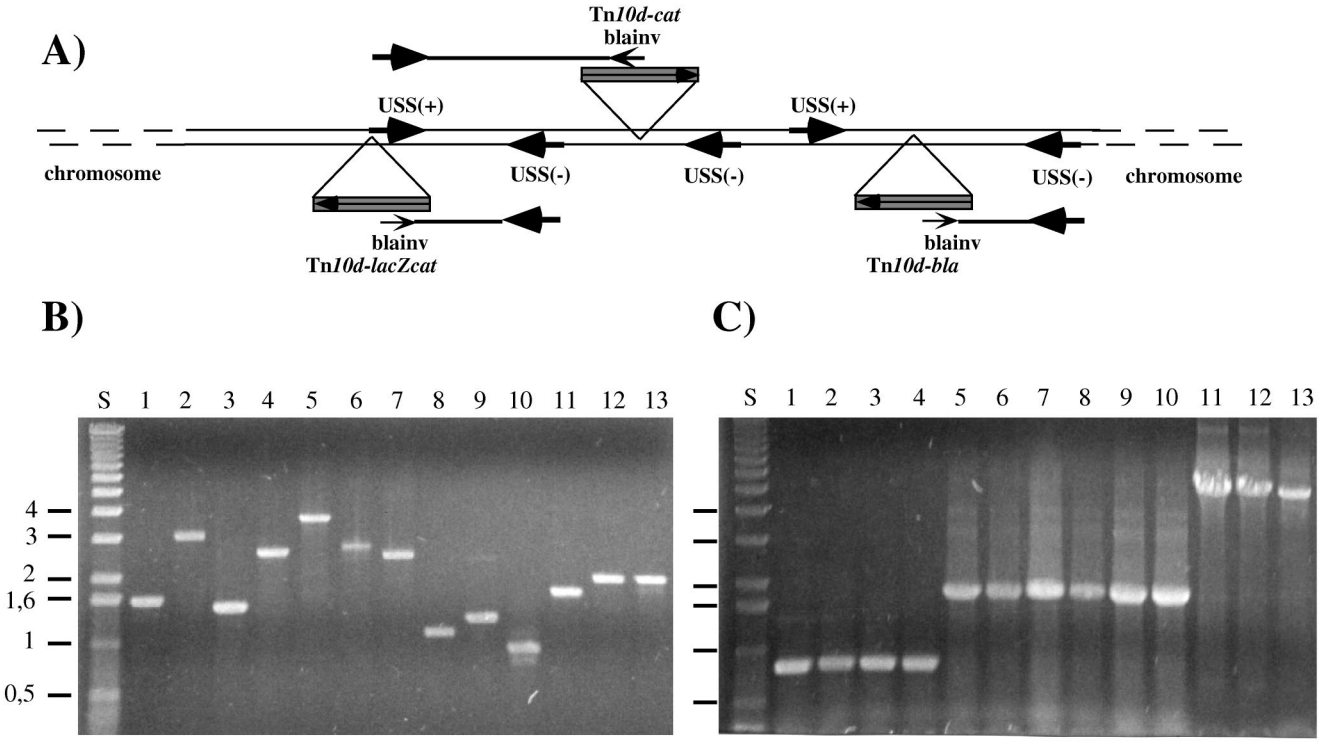

FIG. 2. PCR fragment enrichment method. (A) Generation of PCR products containing Tn*10d* insertions and flanking chromosomal regions, generated by the primer specificity of the USS(−), USS(+), and blainv oligonucleotides. (B) A 0.7% agarose gel with fragments generated by the PCR fragment enrichment method in the range between 0.5 and 4 kbp. These fragments (1 to 13) were used as templates for sequencing (see Table 3). PCR was performed as described in the text. Lane S, 1-kb ladder size standard (Gibco Life Technologies). Junction PCR products were generated with Tn*10d-bla* (lanes 1 to 4)-, Tn*10d-cat* (lanes 5 to 10)-, and Tn*10d-lacZcat* (lanes 11 to 13)-mutagenized chromosomal template DNA and USS(−), USS(+), and blainv amplification oligonucleotides. (C) A 0.7% agarose gel showing the Tn*10d-bla* (860 bp), Tn*10d-cat* (1,700 bp), and Tn*10d-lacZcat* (4,800 bp) elements, identified by PCR with IS*10*-specific oligonucleotides (IS10) and chromosomal DNA of the isolated colonies.

TABLE 3. Mini-Tn*10* insertions on the chromosome of *H. influenzae* Rd[a]

Insertion type and no.	Homologue/ designation	Location (bp)	
		Intragenic	Intergenic
Tn*10d-bla*			
1	—[b]/HI0235	997	
2	*tyrP*/HI0477	701	
3	*napC*/HI0348	211	
4	*ccmE*/HI1093	181	
Tn*10d-cat*			
5	—/HI1339	313	
6	*acoC*/HI0193	508	
7	*potB*/HI1346	402	
8	P1/HI0401	317	
9			247174
10			467293
Tn*10d-catlacZ*			
11	—/HI0246	178	
12	—/HI0219	184	
13	*fis*/HI0980	249	

[a] The base pair locations of characterized insertions and the open reading frame designations (11) are shown. Homology searching was performed with the Blast search engine (2) or with the search program provided by The Institute for Genomic Research (http://www.tigr.org). In all cases the homology on the DNA level was nearly 100% (data not shown).
[b] —, no homologue found.

donor plasmid pAK*cat* (Kan^s), with an observed frequency of about 5 to 10%. This frequency can be significantly elevated by pooling pAK*cat*-transformed cells and subsequently digesting the chromosomal DNA with *Sma*I (a rare cutter in *H. influenzae*, with about 17 recognition sites), which cuts in the Kan^r gene carried on pAK*cat*. After subsequent retransformation into *H. influenzae*, mainly chromosomal Tn*10d-cat* insertions were obtained, leading to Cm^r and Kan^s clones. Kan^s colonies were grown overnight in BHI medium, and their chromosomal DNAs were isolated, digested with *Eco*RI, and analyzed by Southern blotting with a 1.7-kb DNA probe specific to Tn*10d-cat*. Since *Eco*RI cuts once within Tn*10d-cat*, two hybridizing fragments were expected from each insertion into the chromosome. As seen in Fig. 3, all nine clones examined showed specific hybridization with the probe. Most clones appeared to contain single insertions (lanes 1, 2, 3, 6, 7, 8, and 9), but multiple insertions were also detected (lanes 4 and 5). All of the hybridizing bands seen in Fig. 3 are different, indicating that the insertions are unique in each case. Independent single insertions were shown by PCR to harbor the 1.7-kb sequence that is characteristic of Tn*10d-cat* (Fig. 2C, lanes 5 to 10; Table 3). With the PCR DNA fragment enrichment method, clones with a single defined insertion were analyzed by DNA sequencing, which demonstrated that each had been integrated into a different site on the *H. influenzae* chromosome (Table 3).

Characterization of membrane-associated or secreted gene products with Tn*10d-bla*. Tn*10d-bla* insertions were produced after transformation of pAK*bla* into *H. influenzae* Rd and subsequent isolation of Cm^r transformants. These transformants were then plated on BHI-ampicillin plates, and chromosomal DNA of Ap^r colonies was prepared. PCR analysis of this DNA (Fig. 2C, lanes 1 to 4), using specific IS*10* oligonucleotides (IS10), produced an 860-bp fragment specific to Tn*10d-bla*. To investigate whether the predicted fusion between the β-lactamase gene (*blaM*) and exported or membrane protein-encoding genes could be observed, two randomly selected Ap^r colonies were analyzed. Determination of the insertion sites by DNA sequencing of the junction fragment indicated in-frame insertions to membrane protein-encoding genes in each case. One gene (designated HI0325) encodes a putative membrane protein, and the other (HI0477) encodes a tyrosine permease homologue.

With the intention to identify anaerobically induced gene products, we were able to isolate anaerobically induced β-lactamase fusions as Ap^r colonies, which showed an Ap^s growth phenotype (with 6 μg of ampicillin per ml) under aerobic conditions. Two clones which contained Tn*10d-bla* insertions were identified. One insertion was found to be integrated in the *napC* homologue-encoding gene (HI0348), and a second was found in the *ccmE* homologue-encoding gene (HI1093). The corresponding gene products, NapC and CcmE, are known to be involved in nitrite respiration and cytochrome *c*-type biogenesis in *E. coli* (16, 31). With these isolates, cell extracts of aerobically or anaerobically cultivated cells were analyzed by Western blotting with β-lactamase specific antiserum. As shown in Fig. 4, the expression pattern of the Tn*10d-bla* insertions indicates that these putative genes, designated HI0348 and HI1093, are induced under anaerobic conditions.

Production of *lacZ* fusions by using Tn*10d-lacZcat*. Tn*10d-lacZcat* insertions were generated after transformation of pAK*lacZcat* into *H. influenzae*, selection for Cm^r colonies, and testing for Kan^s and $lacZ^+$ colonies. Those colonies had acquired Tn*10d-lacZcat* insertions as demonstrated by Southern blot analysis (data not shown), by PCR (Fig. 2C, lanes 11, 12, and 13), and by DNA sequencing (Table 3). Determination of the insertion sites revealed that Tn*10d-lacZcat* had integrated in the transcriptional direction of unknown open reading frames, designated HI0246, HI0219, and a *fis* gene homologue, HI0980. The *fis* gene product of *E. coli* is a global DNA-binding protein involved in DNA recombination and replication (10). Since the regulation pattern of *fis* has been well characterized for *E. coli* (3), we utilized the *fis*::Tn*10d-lacZcat*

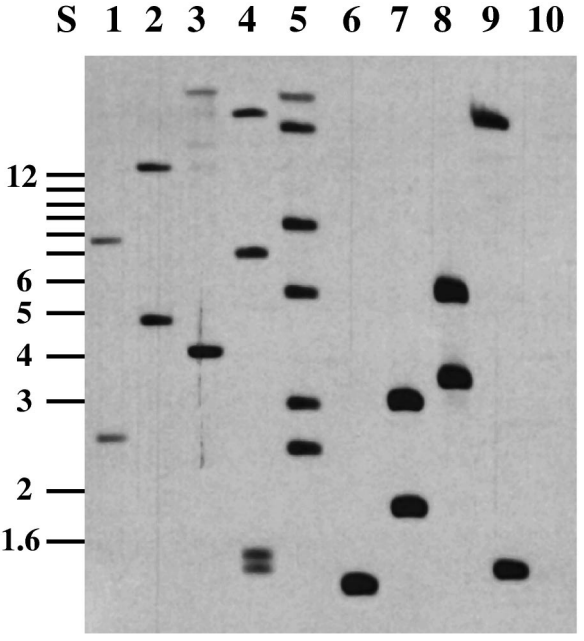

FIG. 3. Southern blot analysis with Tn*10d-cat* insertions. Tn*10d-cat* insertions are shown to be distributed across chromosomal *Eco*RI-digested DNA fragments of mutagenized *H. influenzae* strains. Lanes 1 to 9, mini-Tn*10* hybridizing fragments of *H. influenzae* chromosomal DNA from Cm^r Kan^s colonies. Lane 10, negative control with chromosomal DNA prepared from control strain *H. influenzae* Rd, with no observed hybridization. Lane S, molecular size markers in kilobases.

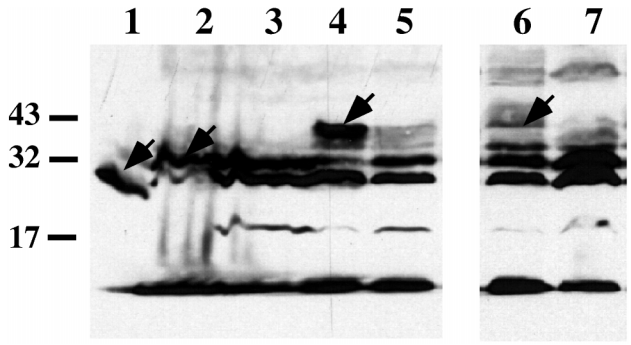

FIG. 4. Western blot analysis with Tn*10d-bla*-mutagenized cells. Whole-cell extracts of cells grown under aerobic (lanes 3, 5, and 7) and anaerobic (lanes 2, 4, and 6) conditions were used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot procedures are described in Materials and Methods. Lane 1, *E. coli*-derived cell extract containing β-lactamase (29 kDa); lanes 2 and 3, *H. influenzae* cells with plasmid pACYC177, encoding β-lactamase; lanes 4 and 5, cell extracts harboring a Tn*10d-bla* insertion in gene *ccmE*; lanes 6 and 7, cell lysates of *H. influenzae* containing a Tn*10d-bla* insertion in *napC*. Positions of prestained protein standards (Gibco Life Technologies) are indicated on the left in kilodaltons. Arrows point to the locations of hybrid proteins.

insertion to determine the kinetics of the expression pattern of *fis* in *H. influenzae*. As shown in Fig. 5, expression of the *fis*::Tn*10d-lacZcat* fusion was maximal in the pre-log phase of cell growth, as previously demonstrated for *fis* expression in *E. coli* (3).

Exchange of Tn*10d-bla* insertions with Tn*10d-cat* or Tn*10d-lacZcat* sequences by transformation and recombination. Since all of the transposons described here contain *blaM* sequences (Fig. 1), we tested whether Tn*10d-bla* insertions could be replaced by Tn*10d-cat* or Tn*10d-lacZcat* due to transformation with linear transposon-carrying DNA fragments. PCR-generated 1.7- or 4.8-kb Tn*10d-cat* or Tn*10d-lacZcat* DNA fragments were used to transform competent *H. influenzae ccmE*::Tn*10d-bla* cells. Cm^r transformants were isolated, and it was confirmed by PCR analysis (data not shown) that the Tn*10d-bla* insertion had been exchanged with Tn*10d-cat* or Tn*10d-lacZcat* by transformation and recombination.

DISCUSSION

H. influenzae was the first organism to be completely characterized in terms of its genomic sequence (11). Genetic manipulation of *H. influenzae* is feasible; however, sophisticated genetic procedures are necessary to produce mutations and to characterize phenotypes. The high efficacy of minitransposons, like the mini-Tn*10*-based systems, and the lack of a convenient transposition mutagenesis scheme for *H. influenzae* prompted us to investigate mini-Tn*10* transposition in this organism. In this report, we demonstrate that mini-Tn*10* transposons can be used for in vivo mutagenesis of *H. influenzae*.

The mini-Tn*10* transposon is the basis for this study. Tn*10d-bla* was originally constructed for use as a translational fusion system to detect exported gene products encoded on bacteriophages (24). We reconstructed the minitransposon elements Tn*10d-bla*, Tn*10d-cat*, and Tn*10d-lacZcat* to make them suitable for use in *H. influenzae*. Plasmid pAK*bla*, containing Tn*10d-bla*, was designed for efficient transformation (pAK*bla* contains a single USS site which increases transformation efficiency 100- to 500-fold [data not shown]) and replication in *H. influenzae* cells. By using pAK*bla*, it was possible to test whether the transposase might be active, since selection on ampicillin-containing medium should result in Ap^r *H. influenzae* cells only when Tn*10d-bla* transposes into suitable target genes encoding some type of exported gene products. This assumption was proven to be correct with the identification of in-frame fusions between Tn*10d-bla* and the reading frames designated HI0325 and HI0477, whose products have significant homology with membrane proteins (11). Furthermore, a limited survey for anaerobically induced genes revealed that Tn*10d-bla* can also be used as a gene expression reporter system. Two Tn*10d-bla* insertions were identified in which *bla* was fused to open reading frames HI0348 and HI1093, whose products correspond to NapC and CcmE, located in the periplasm of *E. coli*. An oxygen-dependent regulation for the corresponding homologous components has also recently been reported for the tetra-hemin-binding protein NapC, involved in nitrite respiration (16), and the putative heme lyase CcmE, involved in *c*-type cytochrome biosynthesis in *E. coli* (31).

For more general insertion mutagenesis, the Tn*10d-bla* element has been modified to contain a constitutively expressed *cat* gene as a selectable marker. The Tn*10d-cat* element was designed to be utilized for randomized insertion mutagenesis, which is not restricted to expression of the target genes or their cellular location. Analysis of nine randomly picked clones containing Tn*10d-cat* insertions indicated different chromosomal locations for the insertions in each case. This result suggests that there are no dominant hot spots for insertion of Tn*10*-based minitransposons in *H. influenzae*. Moreover, the use of a mutant transposase with altered target specificity (5) could essentially exclude this possibility.

To verify the activity of the Tn*10d-lacZcat* element, *fis* gene expression was characterized by using a generated *fis*::Tn*10d-lacZcat* fusion. The Fis gene product was characterized in *E. coli* as a basic 11.2-kDa global DNA-binding protein involved in recombination, phage integration, excision, and initiation of OriC replication (for a review, see reference 10). It was shown that *fis* expression is under the control of early pre-log-phase regulation in *E. coli* (3), and our analysis indicates a similar result for *fis* expression in *H. influenzae*. Determination of β-galactosidase activity at different points of the growth curve shows that *fis* expression is induced in the pre-log phase, while log-phase expression of the *fis* promoter seems to be significantly reduced. The characterization of the *fis*::Tn*10d-lacZcat* fusion proved that the Tn*10d-lacZcat* element is fully active in *H. influenzae*, thus allowing the identification and

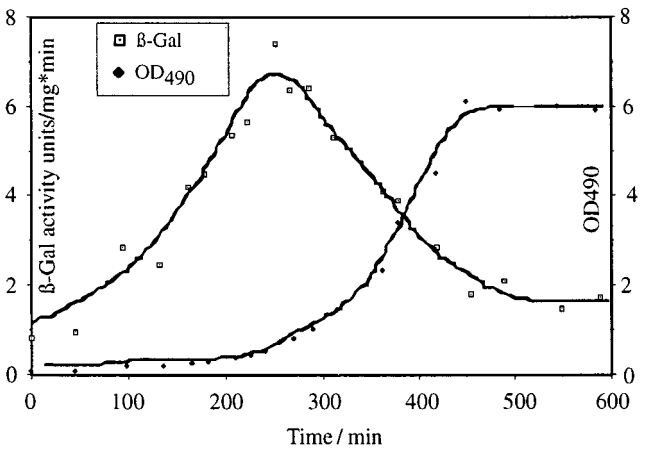

FIG. 5. LacZ activity of a *fis*::Tn*10d-lacZcat* fusion. *H. influenzae* Rd containing a *fis*::Tn*10d-lacZcat* fusion was isolated, and specific β-galactosidase (β-Gal) activity was determined by the method of Miller (21) in units per milligram of protein per minute. Growth was monitored as the optical density at 490 nm (OD_{490}). Cells were grown at 37°C under aeration.

characterization of transcriptional regulation patterns in *H. influenzae*.

In conclusion, an efficient transposon system which is capable of in vivo insertion mutagenesis in *H. influenzae* has been designed. Additionally, sites of transposon insertions can be rapidly identified by using a powerful PCR fragment enrichment method in combination with DNA sequencing. Many versions of Tn*10*-based minitransposons exist (18, 35) and are broadly used for mutagenesis in different organisms. However, we want to emphasize that so far no minitransposon system has been used or was suitable to be used for in vivo mutagenesis in *H. influenzae*. One major advantage of the in vivo mutagenesis is that no genetic manipulation other than transposition itself is necessary to produce targeted mutagenesis. Therefore, no shuttle mutagenesis is necessary to produce insertions on preselected plasmid libraries, and no subsequent transformation barrier or preferred DNA uptake signal can limit the efficacy of mutagenesis. Thus, these elements should find general use, especially in the further characterization of regulatory and biochemical pathways of the human pathogen *H. influenzae*. As shown for other minitransposons, Tn*10d-bla*, Tn*10d-cat*, and Tn*10d-lacZcat* provide some advantages in being defective minitransposons, i.e., (i) their relatively small sizes (0.8, 1.7, 4.8 kb, respectively) and (ii) their transposition only under the influence of an unlinked gene encoding a transposase, thus offering advantages in terms of genetic stability and frequency of transposition.

ACKNOWLEDGMENTS

We thank Julia Blaß for expert help in DNA sequence analysis, and we thank Inge Mühldorfer, Justin Daniels, and Ute Hentschel for their careful reading of the manuscript and suggestions.

This work was funded by BMBF grant 01KI8906.

REFERENCES

1. **Akerley, B. J., E. J. Rubin, A. Camilli, D. J. Lampe, H. M. Robertson, and J. J. Mekalanos.** 1998. Systematic identification of essential genes by *in vitro* mariner mutagenesis. Proc. Natl. Acad. Sci. USA **95:**8927–8932.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Ball, C. A., R. Osuna, K. C. Ferguson, and R. C. Johnson.** 1992. Dramatic changes in *fis* levels upon nutrient upshift in *Escherichia coli*. J. Bacteriol. **174:**8043–8056.
4. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol. **204:**321–342.
5. **Bender, J., and N. Kleckner.** 1992. IS*10* transposase mutations that specifically alter target site recognition. EMBO J. **11:**741–750.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
7. **Deich, R. A., and B. A. Green.** 1987. Mobilization of *Haemophilus influenzae* chromosomal markers by an *Escherichia coli* F′ factor. J. Bacteriol. **169:** 1905–1910.
8. **Dziejman, M., and J. J. Mekalanos.** 1994. Analysis of membrane protein interaction: ToxR can dimerize the amino terminus of phage lambda repressor. Mol. Microbiol. **13:**485–494.
9. **Elliott, T., and J. R. Roth.** 1988. Characterization of Tn*10d-Cam*: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213:**332–338.
10. **Finkel, S. E., and R. C. Johnston.** 1992. The Fis protein: it's not just for DNA inversion anymore. Mol. Microbiol. **6:**3257–3265.
11. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Frichman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
12. **Funkhouser, A., M. C. Steinhoff, and J. Ward.** 1991. *Haemophilus influenzae* disease and immunization in developing countries. Rev. Infect. Dis. **13:**542–554.
13. **Gwinn, M. L., A. E. Stellwagen, N. L. Craig, J. F. Tomb, and H. O. Smith.** 1997. In vitro Tn*7* mutagenesis of *Haemophilus influenzae* Rd and characterization of the role of *atpA* in transformation. J. Bacteriol. **179:**7315–7320.
14. **Heidecker, G. J., J. M. Pozsgay, and T. L. Stull.** 1994. Construction of an *ori* cassette for adapting shuttle vectors for use in *Haemophilus influenzae*. Gene **150:**141–144.
15. **Holland, J., K. J. Towner, and P. Williams.** 1992. Tn*916* insertion mutagenesis in *Escherichia coli* and *Haemophilus influenzae* type b following conjugative transfer. J. Gen. Microbiol. **138:**509–515.
16. **Iobbi-Nivol, C., H. Crooke, L. Griffiths, J. Grove, H. Hussain, J. Pommier, V. Mejean, and J. A. Cole.** 1994. A reassessment of the range of c-type cytochromes synthesized by *Escherichia coli* K-12. FEMS Microbiol. Lett. **119:** 89–94.
17. **Kauc, L., and S. H. Goodgal.** 1989. Introduction of transposon Tn*916* DNA into *Haemophilus influenzae* and *Haemophilus parainfluenzae*. J. Bacteriol. **171:**6625–6628.
18. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Moxon, R. E.** 1995. *Haemophilus influenzae*, p. 2039–2045. *In* G. L. Mandel, J. E. Bennet, and R. Dolin (ed.) Principles and practice of infectious diseases, 4th ed. Churchill Livingstone Inc., New York, N.Y.
23. **Mullis, K. B., and F. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase chain reaction. Methods Enzymol. **155:**335–340.
24. **Reidl, J., and J. J. Mekalanos.** 1995. Characterization of *Vibrio cholerae* bacteriophage K139 and use of a novel mini transposon to identify a phage-encoded virulence factor. Mol. Microbiol. **18:**685–701.
25. **Reidl, J., and J. J. Mekalanos.** 1996. Lipoprotein *e*(P4) is essential for hemin uptake by *Haemophilus influenzae*. J. Exp. Med. **183:**621–629.
26. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Sharetzky, C., T. D. Edlin, J. J. LiPuma, and T. L. Stull.** 1991. A novel approach to insertional mutagenesis of *Haemophilus influenzae*. J. Bacteriol. **173:**1561–1564.
29. **Smith, H. O., J. F. Tomb, B. A. Dougherty, R. D. Fleischmann, and J. G. Venter.** 1995. Frequency and distribution of DNA uptake signal sequences in the *Haemophilus influenzae* Rd genome. Science **269:**538–540.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **51:**503–517.
31. **Thony-Meyer, L., F. Fischer, P. Kunzler, D. Ritz, and H. Hennecke.** 1995. *Escherichia coli* genes required for cytochrome *c* maturation. J. Bacteriol. **177:**4321–4326.
32. **Tomb, J. F., G. J. Barack, M. S. Chandler, R. J. Redfield, and H. O. Smith.** 1989. Transposon mutagenesis, characterization, and cloning of transformation genes of *Haemophilus influenzae* Rd. J. Bacteriol. **171:**3796–3802.
33. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
34. **Truk, D. C.** 1984. The pathogenicity of *Haemophilus influenzae*. J. Med. Microbiol. **18:**1–16.
35. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1982. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:**369–379.
36. **Way, J. C., and N. Kleckner.** 1984. Essential sites at transposon Tn*10* termini. Proc. Natl. Acad. Sci. USA **81:**3452–3456.