In Vivo Transposon Mutagenesis in *Haemophilus influenzae*

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In order to devise an in vivo insertion mutagenesis scheme for *Haemophilus influenzae*, a novel set of transposons has been constructed. These are Tn10-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-Tn10 elements have been made suitable for specific tasks in *H. influenzae*: (i) Tn10d-cat, which can be used to generate chloramphenicol-selectable insertion mutations; (ii) Tn10d-bla, an ampicillin-selectable translational fusion system allowing the detection of membrane or secreted proteins; and (iii) Tn10d-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*.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XLI-Blue was used as a recipient strain for the construction of pAKbla, pAKcat, and pAKlacZcat. XLI-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 NaCl (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 BB 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 Tn10d-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 Barak 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 cycle protocol, based on that described by Mullis and Falouma (25). The following specific primers, synthesized by MWG-Biotech (Ebersberg, Germany), were used for the amplification of the cat gene DNA fragment: Cat5′-AAC TGACGTAAGCTAGCCATACATATGATAAAGATTCGAAAGATATACAGCTC-3′ and Cat3′-5′ AATACGGTACCTGAGCGGAGTGCTTAAAGGGCCACCCAAA TA -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). Parrestriction sites were inserted at the 5′ ends of primers Cat5′ and Cat3′, and in addition, a

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SnuBI site was designed to be contained at the 5' end of Cat5' (underlined sequences).

Identification of the mini-Tn10-based chromosomal insertions was done by PCR amplification, utilizing the 27 bp IS10 sites as the amplification primer (IS10, 5'-CTGATGACTCCCTATAGTTTGTGTA-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, 36 to 46°C, with the Elongase kit from Gibco Life Technologies) was performed with a series of isolated Tn10d-cat, Tn10d-bla, and Tn10d-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). 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). 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). 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). 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). 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).

The insertion sites of the Tn10d-bla and ccmE insertions were 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 Tn10 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'TTTCCGTTTTACCAACAGT-3') oriented 5' to the amplified fragment.

Western blot analysis. Derivatives of E. coli XL1-Blue containing plasmid pACYC177, H. influenzae harboring pACYC177, and H. influenzae containing ccmE::Tn10d-bla and nac::Tn10d-bla were grown in Luria broth medium (E. coli) or in BH 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 photodensity 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 pACYC194 and -177, (ii) an H. influenzae specific USS site, and (iii) a functional transposon unit based on Tn10, constituting the transposon and individually constructed defective minitransposons. The various steps of the construction of the mini-Tn10 transposons are outlined in Fig. 1. The Tn10d-bla-containing plasmid pAKb1a (Fig. 1A) was constructed by subcloning a blunt-ended 3.7 kb EcoRI fragment containing the Tn10d-bla element (24) into the SnuBI site of plasmid pACYC184-based plasmid, pJR207 (23), carrying one Tn10d-bla, Kan' and Cm' on both sides of the mini-Tn10d-bla. The resulting plasmid pAKb1a was introduced, resulting in pAK2 (Fig. 1B). Plasmid pAK2 was then cut with PsI, and a PCR-generated 1.1 kb cat-containing DNA fragment with PsI engineered flanking sites was used in the ligation, resulting in plasmid pAKcat. This plasmid confers Kan' and Cm' on both E. coli and H. influenzae. The cat PCR fragment was designed to contain the native constitutively expressed cat promoter. Finally, Tn10d-lacZcat was constructed as follows. A blunt-end-generated promoterless 3.2 kb lacZ BgItI-DraI DNA fragment, originating from plasmid PMD35 (8), was subcloned into a SnuBI-digested pAKb1a plasmid. The resulting plasmid contained the lacZ gene, followed by the cat gene oriented in the same transcriptional direction (Fig. 1C).
TABLE 2. Frequency of Ap<sup>r</sup> cells after mutagenesis with Tn<sub>10d-bla</sub><sup>a</sup>

<table>
<thead>
<tr>
<th>Pool</th>
<th>Cells/ml after growth in:</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BHI medium</td>
<td>BHI medium with ampicillin</td>
</tr>
<tr>
<td>1</td>
<td>1.42 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>6.05 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1.11 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8.47 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>2.11 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>7.35 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>2.60 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>5.75 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>2.23 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.50 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Frequency was determined as described in the text. After overnight growth, colonies were counted.

<sup>b</sup> Ratio between Ap<sup>r</sup> colonies (BHI medium with ampicillin) and absolute cell numbers (BHI medium).

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

**Determination of mini-Tn10 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-Tn10 insertions and 5<sup>'</sup> 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<sup>'</sup>-N<sub>6</sub>AAAGTGCGGT-3<sup>'</sup>; USS(−), 5<sup>'</sup>-N<sub>7</sub>ACCGCACTT-3<sup>'</sup>]. Another synthetic oligonucleotide, blainv (5<sup>'</sup>-CCGTAAGATGCTTTTCGAGTTGACTGGT-3<sup>'</sup>), was designed, which specifically hybridizes with the complementary 5<sup>'</sup>-oriented Tn<sub>10d-bla</sub>, Tn<sub>10d-cat</sub>, and Tn<sub>10d-lacZcat</sub>-containing DNA strand (Fig. 2A). The production of PCR fragments consisting of a IS10-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<sub>10d-bla</sub> (Fig. 2B, lanes 1 to 4), Tn<sub>10d-cat</sub> (lanes 5 to 10), and Tn<sub>10d-lacZcat</sub> (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).

**Mini-Tn10 insertions analyzed by using Tn10d-cat.** Tn10d-cat insertions were produced after transformation of pAKcat into H. influenzae Rd. Independent Cm<sup>r</sup> (2 μg/ml) transformants were picked randomly and were tested for the loss of the
mosomal DNA of Apr colonies was prepared. PCR analysis of mants were then plated on BHI-ampicillin plates, and chro-
colony transformants were grown overnight in BHI medium, and their chromosomal DNA was isolated and digested with EcoRI, and analyzed by Southern blotting with a 1.7-kb DNA probe specific to Tn10d-bla. Since 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).

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 Tn10d-bla inserts indicates that these putative genes, designated HI0348 and HI1093, are induced under anaerobic conditions.

Production of lacZ fusions by using Tn10d-lacZcat. Tn10d-lacZcat insertions were generated after transformation of pAKlacZcat into H. influenzae, selection for CmR colonies, and testing for KanR and lacZ+ colonies. Those colonies had acquired Tn10d-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 Tn10d-lacZcat had integrated in the transcriptional direction of unknown open reading frames, designated HI0246, HI0219, and a fis gene homologue-encoding gene (HI0348), and a second putative membrane protein-encoding gene (HI0325), with an observed frequency of about 5 to 10%. This frequency can be significantly elevated by pooling pAKcat-transformed cells and subsequently digesting the chromosomal DNA with SmalI (a rare cutter in H. influenzae, with about 17 recognition sites), which cuts in the KanR gene carried on pAKcat. After subsequent retransformation into H. influenzae, mainly chromosomal Tn10d-cat insertions were obtained, leading to CmR and KanR clones. KanR colonies were grown overnight in BHI medium, and their chromosomal DNAs were isolated, digested with EcoRI, and analyzed by Southern blotting with a 1.7-kb DNA probe specific to Tn10d-cat. Since EcoRI cuts once within Tn10d-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 Tn10d-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 Tn10d-bla. Tn10d-bla insertions were produced after transformation of pAKbla into H. influenzae Rd and subsequent isolation of CmR transformants. These transformants were then plated on BHI-ampicillin plates, and chromosomal DNA of ApR colonies was prepared. PCR analysis of this DNA (Fig. 2C, lanes 1 to 4), using specific IS10 oligonucleotides (IS10), produced an 860-bp fragment specific to Tn10d-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 ApR 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 ApR colonies, which showed an ApR growth phenotype (with 6 μg of ampicillin per ml) under aerobic conditions. Two clones which contained Tn10d-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 Tn10d-bla inserts indicates that these putative genes, designated HI0348 and HI1093, are induced under anaerobic conditions.

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TABLE 3. Mini-Tn10 insertions on the chromosome of H. influenzae Rd

<table>
<thead>
<tr>
<th>Insertion type and no.</th>
<th>Homologue/ designations</th>
<th>Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intragenic</td>
</tr>
<tr>
<td>Tn10d-bla</td>
<td>—/HI0235</td>
<td>997</td>
</tr>
<tr>
<td>1</td>
<td>—/HI0235</td>
<td>701</td>
</tr>
<tr>
<td>2</td>
<td>napC/ HI0348</td>
<td>211</td>
</tr>
<tr>
<td>3</td>
<td>ccmE/ HI1093</td>
<td>181</td>
</tr>
<tr>
<td>Tn10d-cat</td>
<td>—/HI1339</td>
<td>313</td>
</tr>
<tr>
<td>5</td>
<td>acce/ HI0193</td>
<td>508</td>
</tr>
<tr>
<td>6</td>
<td>potB/ HI1346</td>
<td>402</td>
</tr>
<tr>
<td>7</td>
<td>P1/ HI0401</td>
<td>317</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>247174</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>467293</td>
</tr>
<tr>
<td>10</td>
<td>—/HI0246</td>
<td>178</td>
</tr>
<tr>
<td>11</td>
<td>—/HI0219</td>
<td>184</td>
</tr>
<tr>
<td>12</td>
<td>fis/HI0980</td>
<td>249</td>
</tr>
</tbody>
</table>

* 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).

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FIG. 3. Southern blot analysis with Tn10d-cat insertions. Tn10d-cat insertions are shown to be distributed across chromosomal EcoRI-digested DNA fragments of mutagenized H. influenzae strains. Lanes 1 to 9, mini-Tn10 hybridizing fragments of H. influenzae DNA from CmR KanR 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.
insertion to determine the kinetics of the expression pattern of fis in H. influenzae. As shown in Fig. 5, expression of the fis::Tn10d-lacZcat fusion was maximal in the pre-log phase of cell growth, as previously demonstrated for fis expression in E. coli (3).

Exchange of Tn10d-bla insertions with Tn10d-cat or Tn10d-lacZcat sequences by transformation and recombination. Since all of the transposons described here contain blaM sequences (Fig. 1), we tested whether Tn10d-bla insertions could be replaced by Tn10d-cat or Tn10d-lacZcat due to transformation with linear transposon-carrying DNA fragments. PCR-generated 1.7- or 4.8-kb Tn10d-cat or Tn10d-lacZcat DNA fragments were used to transform competent H. influenzae ccme::Tn10d-bla cells. Cm\(^{r}\) transformants were isolated, and it was confirmed by PCR analysis (data not shown) that the Tn10d-bla insertion had been exchanged with Tn10d-cat or Tn10d-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-Tn10-based systems, and the lack of a convenient transposition mutagenesis scheme for *H. influenzae* prompted us to investigate mini-Tn10 transposition in this organism. In this report, we demonstrate that mini-Tn10 transposons can be used for in vivo mutagenesis of *H. influenzae*.

The mini-Tn10 transposon is the basis for this study. Tn10d-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 Tn10d-bla, Tn10d-cat, and Tn10d-lacZcat to make them suitable for use in *H. influenzae*. Plasmid pAKbla, containing Tn10d-bla, was designed for efficient transformation (pAKbla contains a single USS site which increases transformation efficiency 100- to 500-fold [data not shown]) and replication in *H. influenzae* cells. By using pAKbla, it was possible to test whether the transposase might be active, since selection on ampicillin-containing medium should result in Apr \(^{r}\) *H. influenzae* cells only when Tn10d-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 Tn10d-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 Tn10d-bla can also be used as a gene expression reporter system. Two Tn10d-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 Tn10d-bla element has been modified to contain a constitutively expressed cat gene as a selectable marker. The Tn10d-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 Tn10d-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 Tn10-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 Tn10d-lacZcat element, fis gene expression was characterized by using a generated fis::Tn10d-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::Tn10d-lacZcat fusion proved that the Tn10d-lacZcat element is fully active in *H. influenzae*, thus allowing the identification and

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**FIG. 4.** Western blot analysis with Tn10d-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 Tn10d-bla insertion in gene ccme; lanes 6 and 7, cell lysates of *H. influenzae* containing a Tn10d-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.

**FIG. 5.** LacZ activity of a fis::Tn10d-lacZcat fusion. *H. influenzae* Rd containing a fis::Tn10d-lacZcat fusion was isolated, and Miller 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 Tn10-based mini-transposons exist (18, 35) and are broadly used for mutagenesis in different organisms. However, we want to emphasize that so far no mini-transposon 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 pre-selected 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 mini-transposons, Tn10d-bla, Tn10d-cat, and Tn10d-lacZcat provide some advantages in being defective mini-transposons, 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.

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