Isolation and Characterization of a Mutant of *Haemophilus influenzae* Type b Deficient in Outer Membrane Protein P1

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The gene for outer membrane protein P1 of *Haemophilus influenzae* type b has been previously cloned and expressed in *Escherichia coli*. To investigate the physiologic role of the P1 protein, the cloned P1 gene was insertional inactivated with the Tn5 derivative TnStacl, and an isogenic P1-deficient *Haemophilus influenzae* mutant was then generated by transformation with linearized plasmid DNA containing the insertionally inactivated gene. The P1-deficient strain grew normally in vitro and induced bacteria in the infant rat model.

Outer membrane protein P1 of *Haemophilus influenzae* type b has been purified (8, 12), and the gene for outer membrane protein P1 has been cloned (5, 12) and sequenced (12). Epitopes of P1 on the surface of intact cells have been demonstrated (5, 12, 15), and antibody against outer membrane protein P1 has been shown to be protective in an infant rat model of bacteremia (5, 8). We sought to determine if P1 is necessary for viability of *H. influenzae* type b or for virulence in this model by isolating and characterizing a P1-deficient mutant. The mutant grew normally in vitro and was virulent in the infant rat model. Similar results have been presented by Hansen and co-workers (M. S. Hanson, L. D. Cope, and E. J. Hansen, Abstr. 28th Intersc. Conf. Antimicrob. Agents Chemother., abstr. no. 1142, p. 312, 1988).

Plasmid pRSM188 (12), a pBR322 derivative containing the outer membrane protein P1 gene (*ompP1*), was transformed into the suppressor-free *Escherichia coli* K-12 strain W3350 (3) by the CaCl$_2$ technique (14). Transformants were selected on L agar supplemented with 50 μg of ampicillin per ml, and a clone was chosen for further study. Dot blot analysis, with polyclonal rabbit anti-P1 (12), indicated that P1 was produced in this strain. The P1 gene in strain W3350(pRSM188) was then insertionally inactivated by infection with λ::TnStacl b221 c1857 Oam29 Pamb80 at a multiplicity of infection of approximately 5 (4). Transductants were isolated on L agar supplemented with 35 μg of kanamycin and 50 μg of ampicillin per ml. Plasmids were prepared from pools of resistant cells. Strain W3350 was transformed with these plasmid preparations, and Amp' Kan' transformants were selected and characterized. Only one transformant from each pool was chosen in order to characterize unique transposition events. Plasmids were isolated and analyzed by restriction analysis on 0.7% agarose gels by standards methods (10, 14). Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. Incubations were carried out as recommended by the vendors. Fifteen plasmids were analyzed. Six contained TnStacl in the 4.2-kilobase-pair *Haemophilus* insert, and two of these contained TnStacl in the P1 gene. Strains containing plasmids in which TnStacl was mapped in or near the P1 gene were further characterized by Western blot (immunoblot) analysis (data not shown). One of the P1-negative constructs was chosen for further study and was designated pRSM188 *ompP1*::TnStacl. The TnStacl insertion was mapped 5' to the *Hind*III site in the P1 gene by restriction analysis and was further localized to approximately base 600 of the P1 gene by DNA sequence analysis (data not shown).

The P1 gene in pRSM188 was derived from *H. influenzae* type b strain MinnA (12). We have been unsuccessful in transforming strain MinnA, possibly because of restriction barriers. Strain 1613 had been successfully employed as a transformation recipient in other experiments, and therefore we chose strain 1613 as a recipient. Strain 1613 was isolated from the blood of a patient in St. Louis who had periorbital cellulitis. This strain has the outer membrane protein subtype 3L (1, 2). The P1 gene from strain 1613 has been sequenced and is more than 90% identical to the MinnA gene (R. S. Munson, Jr., M. Einhorn, S. Grass, and C. Newell, Abstr. 28th ICAAC, abstr. no. 1124, p. 308, 1988). Cells competent for transformation were prepared by the method of Herriot et al. (6) with MIV medium. A 0.25-ml portion containing 4 × 10$^7$ cells was incubated statically with 1 μg of *Sal*I-linearized pRSM188 *ompP1*::TnStacl for 40 min at 37°C. After the 40-min incubation, 2 ml of heat infusion medium supplemented with 10 μg each of NAD and heme per ml was added, and the incubation was continued for 2.5 h. Cells were plated on Mueller-Hinton agar supplemented with 15 μg each of NAD and heme (7) and 20 μg of kanamycin per ml. Kanamycin resistance was observed with a frequency of approximately 0.1%. A typical clone, designated 1613 *ompP1*−, was saved for further analysis.

Strains 1613 and 1613 *ompP1*− were grown to mid-log phase in supplemented brain heart infusion medium. The medium for the growth of 1613 *ompP1*− also contained 20 μg of kanamycin per ml. Cells were harvested, and crude sonic extracts, as well as sarcosyl-insoluble fractions enriched in the major outer membrane proteins (2), were prepared and analyzed by Coomassie blue-stained sodium dodecyl sulfate-polycracylamide gel electrophoresis (2, 9) and by Western blot immunoassay (12) as described previously. Strain 1613 *ompP1*− did not produce detectable P1 in Coomassie blue-stained sarcosyl-insoluble preparations (Fig. 1). P1 was also not detectable in Western blots of sonic extracts (data not shown).

To confirm that the P1 deficiency was due to insertional inactivation, we prepared genomic DNA from both strains as described elsewhere (12). Genomic DNA (2 μg) was digested

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with EcoRI and transferred to Hybond-N according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, Ill.). The blot was hybridized with a nick-translated probe consisting of the complete P1 gene. The probe was nick translated with [γ-32P]CTP (New England Nuclear Corp., Boston, Mass.) with the Boehringer kit according to the instructions of the manufacturer. After hybridization, the blot was washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate at 65°C and analyzed by autoradiography. The P1 gene of strain 1613 was localized to an EcoRI fragment of approximately 8.8 kilobase pairs (Fig. 2). Tn5atacl contains EcoRI sites near each end; therefore, if Tn5atacl had been inserted into the P1 gene, cleavage of genomic DNA with EcoRI would yield two fragments totaling approximately 8.8 kilobase pairs (Fig. 2, lane 2).

Strain 1613 ompP1" grew in supplemented brain heart infusion broth with a generation time equal to that of the parental strain. Virulence of this strain was tested in the infant rat model of bacteremia. This model was described elsewhere (13). Litters of 5-day-old infant rats (Sprague-Dawley) were obtained locally. Pups were randomized and infected intraperitoneally with approximately 100 CFU of strain 1613 or strain 1613 ompP1". The next day, rats were bled by cardiac puncture and bacteremia was determined by quantitative culture on chocolate agar. Ten rats were infected with each strain. All rats were bacteremic. The rats challenged with strain 1613 had a geometric mean level of bacteremia of 11,721 CFU/100 μl of blood compared with rats infected with strain 1613 ompP1", which had a geometric mean level of bacteremia of 5,265 CFU/100 μl of blood. The levels of bacteremia were not significantly different (P = 0.44) employing Student's t test for unpaired samples. To rule out the possibility that we were selecting P1-positive revertants in rats infected with the P1-deficient strain, we isolated one clone from each of the 10 rats infected with this strain. Sarcosyl-insoluble fractions were prepared from each clone and characterized by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These clones were P1 negative.

In summary, a P1-deficient mutant of H. influenzae type b 1613 was isolated. This isogenic P1-deficient mutant grew normally in brain heart infusion broth; therefore, P1 is not required for growth in vitro. The P1-deficient strain also caused bacteremia in infant rats. Our experiments clearly demonstrate that P1 is not required for growth of the organism in vivo in infant rats. Furthermore, the levels of bacteremia observed are greater than those thought to be associated with subsequent development of meningitis (11). However, our experiments do not rule out the possibility of subtle differences between the strains in this model.

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LITERATURE CITED


