INFECTION AND IMMUNITY, Jan. 1987, p. 154–161
0019-9567/87/010154-08$02.00/0

Vol. 55, No. 1

Cloning of the Filamentous Hemagglutinin of *Bordetella pertussis* and Its Expression in *Escherichia coli*

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Received 10 June 1986/Accepted 7 October 1986

Bordetella pertussis **UT25 DNA was cloned into the kanamycin resistance gene of cosmid pCP13 to construct a genomic library in *Escherichia coli* LE392. One clone containing plasmid pDB441 expressed the filamentous hemagglutinin (FHA) as identified by protein immunoblots with the use of rabbit anti-*B. pertussis* antiserum, rabbit anti-FHA antiserum, and a monoclonal antibody to FHA. FHA is a protein of 220 to 210 kilodaltons, but the immunoreactive FHA, as expressed in *E. coli*, was larger than that expressed in *B. pertussis*, suggesting that there was a difference in the processing of this protein between these two bacteria. The *fha* gene was mapped to a 6.5-kilobase pair DNA fragment by the use of various restriction endonucleases. The kanamycin resistance gene of pCP13 was found to provide the promoter function but probably not the translation start signal for the *fha* gene. Conjugative transfer of pDB441 to *B. pertussis* BP353, a transposon Tn*5*-induced FHA mutant, increased the expression of the FHA over that seen with wild-type *B. pertussis*.**

Bordetella pertussis is the noninvasive respiratory tract pathogen which causes whooping cough. With decreasing incidence of this disease, there is increasing concern regarding the reactivity of the current vaccine (15, 22). Research on an improved vaccine has been hampered by both the fastidious growth characteristics of this organism and by its proclivity for rapid genetic and phenotypic variation. Most solid and liquid media inhibit the growth of *B. pertussis*, and it elaborates products that are toxic to itself (22, 26, 27). In addition, the organism undergoes two different types of phenotypic changes which result in the loss of virulence-associated factors. The first, modulation, manifests itself as a reversible, environmentally regulated loss and reacquisition of virulence-associated factors (18). The second type, degradation, appears to be caused by mutation in the *vir* gene which results in the coordinate loss of virulence-associated factors (45). Vaccines produced from organisms which lack virulence-associated factors do not provide immunity to virulent organisms (22, 25).

B. pertussis expresses several virulence-associated factors, the best studied of which are pertussis toxin (PT), filamentous hemagglutinin (FHA), agglutinogens, demonecrotic toxin, and adenylate cyclase (13, 22, 26, 47). Research on an acellular component vaccine has been directed toward the use of PT and FHA (33). Active or passive immunization against PT gives protection against aerosol challenge from the organism, whereas active, but not passive, immunization against FHA protects against aerosol challenge (23, 31). More importantly, simultaneous active or passive immunization against both proteins has a synergistic effect. Both PT and FHA are hemagglutinins and have been reported to act in concert as adhesins to human ciliated epithelial cells. Interest has been shown in the possibility of a vaccine which blocks adherence of *B. pertussis* (42, 43). The agglutinogens, as well as PT and FHA, are implicated as mediators of attachment in assays with tissue culture cells (13, 29).

The FHA is a candidate for inclusion in an improved vaccine. Several laboratories report that purified FHA consists of a heterogeneous population of polypeptides, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2, 5, 16). Arai and Sato suggested that FHA fragmentation might be due to proteolytic cleavage (2). Evidence to support this suggestion was presented by Irons et al. (16). The FHA from *B. pertussis* Tohama 1 showed multiple bands with molecular masses ranging from 160 to 52 kilodaltons (kDa). For strain Wellcome 28, the molecular sizes of FHA bands ranged between 220 and 89 kDa. Preparations of FHA which lacked the 220- and 210-kDa components showed reduced specific hemagglutinating activity (16).

We undertook the cloning of *B. pertussis* DNA because of the interest in developing an acellular vaccine. We report here the cloning and expression of the structural gene for FHA and provide evidence that we have also cloned a gene controlling the expression of FHA in *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. pertussis* UT25 was isolated from a pertussis outbreak in Texas (9). *B. pertussis* BP353, a Tn*5*-induced FHA mutant and its parent, BP338, a nalidixic acid (50 μg/ml)-resistant derivative of strain Tohama 1, the Japanese vaccine strain, were kindly provided by A. Weiss (33, 46). A spontaneous streptomycin-resistant (Sm^r) variant of strain BP353 was isolated in this laboratory on media containing 100 μg of streptomycin per ml. *Escherichia coli* LE392 (40) was used as the cloning recipient. Cosmid pCP13 (7) and plasmids pBR322, pBR328 (37), pMK16 (17), and pRK2013 (10) were used in this study. The *B. pertussis* strains were stored in defibrinated sheep blood (Hazelton Research Products, Inc., Denver, Pa.) at −70°C. *E. coli* LE392 was stored in 15% glycerol in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) at −70°C. All strains were lyophilized in 10% skim milk medium (Difco) for long-term storage.

Media and cultural conditions. *B. pertussis* strains were grown on homemade Bordet-Gengou agar with 20% defibrinated sheep blood (4). *B. pertussis* UT25 was also grown in modified SS broth (38) for 36 h as previously described (35). *E. coli* strains were grown in Luria broth (LB; Difco) (21) or

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on LB agar. Media were supplemented with antibiotics when appropriate: 25 μg of kanamycin per ml for *B. pertussis* and 50 μg of kanamycin per ml for *E. coli*; 40 μg of carbenicillin per ml; 30 μg of chloramphenicol per ml; 15 μg of tetracycline per ml; and 100 μg of streptomycin per ml.

Enzymes and chemicals. Enzymes used for digestion of DNA (*Xho*I, *Eco*RI, *Bam*HI, *Bgl*II, *Pst*I, and *Hin*dIII) were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. *Cla*I and T4 DNA ligase were from International Biotechnologies, Inc., New Haven, Conn. Calf intestinal phosphatase was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Unless otherwise indicated, all other chemicals were from Sigma Chemical Co., St. Louis, Mo.

Preparation of DNA. Chromosomal DNA was isolated from *B. pertussis* UT25 by the method of Schleif and Wensink (34) as modified by M. McIntosh (personal communication). Briefly, the cell pellet from 400 ml of SS broth culture was suspended in 9 ml of 150 mM NaCl–10 mM Tris hydrochloride (pH 7.2) and quick frozen in a dry-ice–ethanol bath. An equal volume of 1% sodium dodecyl sulfate–100 mM NaCl–100 mM Tris hydrochloride (pH 8.8) was added, and the pellet was thawed while we continuously inverted this mixture. The mixture was extracted with equal volumes of phenol saturated with 50 mM Tris hydrochloride (pH 8.0), then with phenol-chloroform (1:1), and finally with chloroform. The DNA was precipitated with 1.8 ml of 3 M sodium acetate and 40 ml of ice-cold ethanol. The DNA was wound on a glass rod, dried under nitrogen, and dissolved in sterile H_2O to a final concentration of 1 mg/ml. Plasmids pCP13, pBR322, pBR328, and pMK16 were isolated by scaling up the procedure of Portnoy and White as described elsewhere (6). $CsCl_2$ centrifugation was performed as described by Maniatis et al. (21).

Agarose gel electrophoresis. DNA was electrophoresed on agarose or low-melting-temperature agarose gels (Bio-Rad Laboratories, Richmond, Calif.) containing 2.5 μg of ethidium bromide per ml of gel. The gels were run at room temperature and submerged in the TBE buffer (21) at 20 V direct current for 16 h. The gels were photographed with Polaroid type 52 film. The size of the DNA fragments was determined with lambda DNA digested with *Hin*dIII or φX174 DNA digested with *Hae*III (Bethesda Research Laboratories) as standards. When required, DNA fragments were recovered from low-melting-temperature agarose (21).

Construction of an *Xho*I gene bank. Conditions for the partial *Xho*I digestion of *B. pertussis* DNA and sucrose density gradient ultracentrifugation of the fragments were developed as described (21). DNA fragments of 13 to 27 kilobase pairs (kbp) were used to construct the gene bank. pCP13 was digested with *Xho*I, was treated with calf intestinal phosphatase, and was purified through a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, N.J.) (21). Ligation was conducted at 15°C for 2 h with a 2:1 molar ratio (insert:vector) with 300 μg of DNA per ml and 50 U of T4 DNA ligase per ml in a 20-μl volume. The cosmids were packaged in vitro with a lambda packaging kit and transduced into *E. coli* LE392 as recommended by the manufacturer (Amersham Corp., Arlington Heights, Ill.). The cells were plated on tetracycline-LB agar. Isolated colonies were tested on tetracycline-LB agar and kanamycin-LB agar. A total of 1,152 Km^s colonies were stored in 15% glycerol in brain heart infusion medium in microtiter plates at −70°C.

Conjugation and transformation. Plasmids were conjugated into *B. pertussis* from *E. coli* (44) and were transformed into *E. coli* by a $CaCl_2$ procedure (21).

HA. To determine hemagglutination (HA), we grew *B. pertussis* cells on Bordet-Gengou agar containing appropriate antibiotics for 3 to 4 days. Cells were scraped off and suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 0.25 mM $MgSO_4$, 2.5 mM KH_2PO_4 [pH 7.2]). The suspensions were adjusted to 200 Klett units, representing approximately 3×10^{10} CFU/ml and 3 mg of protein per ml (20). FHA from *B. pertussis* 18334 was purified at the Michigan Department of Health by the procedure of Sato et al. (32), and was a gift of L. Winberry. The purified FHA was used as a positive control at 0.005 mg of protein per ml. A 50-μl volume of each sample was added to the first well of a microtiter plate and twofold serially diluted 11 times. Then, 50 μl of 0.05% sheep erythrocytes in phosphate-buffered saline was added to each well, and the plates were held at room temperature for 3 h for HA to occur. The data were expressed as specific activity, calculated as the inverse of the amount of protein (milligrams) in the well with the highest dilution that gave complete HA.

Antisera. Rabbit anti-*B. pertussis* antiserum was obtained from a female New Zealand White rabbit (Wallenburn Rabbitry, Otterville, Mo.) immunized twice weekly for 4 weeks with 0.5 ml of a *B. pertussis* UT25 vaccine prepared from cells grown in SS broth (11). The antiserum was absorbed twice with *E. coli* LE392(pCP13) cells. Rabbit anti-FHA was made at the Michigan Department of Health and was a gift of L. Winberry. Monoclonal antibody (MAb) 12H3 was produced in this laboratory (11) and shown to be specific for FHA. Goat anti-rabbit immunoglobulin G-peroxidase and goat anti-mouse immunoglobulin G-peroxidase were purchased from Cooper Biomedical, Inc., West Chester, Pa.

Colony immunoblots. Cells from the gene bank were transferred to sterile BA85 nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) on tetracycline-LB agar plates and incubated overnight at 37°C. The nitrocellulose filters were removed, air dried, placed in an air-tight container, and exposed to chloroform vapor for 15 min. The filters were removed, allowed to air dry, and then placed in a blocking solution of 3% bovine serum albumin (United States Biochemical Corp., Cleveland, Ohio) in 0.9% NaCl–10 mM Tris hydrochloride (pH 7.4) (TS). They were incubated at 37°C for 1 h with shaking, rinsed briefly in TS, and then placed in 200 ml of fresh blocking solution with 200 μl of rabbit anti-*B. pertussis* antiserum. The filters were kept at 4°C overnight with rocking. The filters were rinsed 10 times with TS and placed in 200 ml of fresh blocking solution with 100 μl of goat anti-rabbit IgG-peroxidase for 4 h at room temperature with rocking. The filters were then rinsed 10 times with TS, and then peroxidase was detected with the 4-chloronapthol reagent (14).

Protein immunoblots (Western blots). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted with a 7.5% separating gel in the presence of 0.5 M urea as described elsewhere (8, 35). Protein immunoblotting was performed as previously described (12, 41). High-molecular-weight markers and all polyacrylamide gel electrophoresis chemicals were purchased from Bio-Rad Laboratories. Purified FHA was used at 0.00011 mg of protein per lane of gel. *B. pertussis* suspensions, prepared for HA, were used at 0.5 mg of protein per lane of gel. The *E. coli* isolates grown in 2 ml of LB were centrifuged and suspended in phosphate-buffered saline; 2 mg of protein was used per lane of gel.

RESULTS AND DISCUSSION

A genomic library of *B. pertussis* UT25 was generated with restriction endonuclease *Xho*I to isolate *B. pertussis*

structural genes which coded for virulence-associated proteins. The genomic library consisted of 1,152 colonies, with an average DNA insert of 20 kbp. There was a 99% probability that any specific sequence was present in the library if one assumed that the *B. pertussis* chromosome was 5,000 kbp in length (21).

Detection of a clone containing the *fha* gene. Colony immunoblots on the members of the library were performed three times, and 37 colonies were found to be reproducibly positive for *B. pertussis* antigens. All 37 immunoblot-positive clones were analyzed by protein immunoblots with rabbit anti-*B. pertussis* antiserum as the primary antibody. We detected high-molecular-weight bands suggestive of FHA in clone 441 (data not shown). The plasmid in this clone was referred to as pDB441 (Fig. 1), and for simplicity, the polypeptides produced by *E. coli* containing pDB441 were referred to as immunoreactive FHA (iFHA). The protein immunoblot was repeated on this clone with MAb 12H3, and high-molecular-mass bands at about 230, 215, 175, and 160 kDa were detected (Fig. 2, pDB441). The apparent molecular masses of these polypeptides were not identical to those of the FHA bands seen with *B. pertussis* UT25, which had molecular masses of about 215 (arrowhead), 210, 208, and 205 kDa (Fig. 2, UT25). Bands were also seen at about 150 and 140 kDa (Fig. 3, UT25).

It was not unreasonable to expect the FHA polypeptides produced by *E. coli* to differ from those produced by *B. pertussis*, since proteases appear to be responsible for the fragmentation of FHA. Evidence supporting the degradation of iFHA by *E. coli* proteases was presented by Reiser et al., who apparently cloned a 1-kbp DNA fragment of the *fha* gene fused to β-galactosidase (30). Products with heterogeneous molecular weights were found, indicating that *E. coli* proteases may have cleaved the cloned portion of FHA. Our largest polypeptide was assumed to be unfragmented and to represent the open reading frame of the *fha* gene. The largest polypeptide seen in *E. coli* appeared to be 15 kDa larger than that seen in *B. pertussis*. This observation could be explained in several ways. If the *fha* gene was located at either end of the *B. pertussis* DNA insert in pDB441, the iFHA could be a fusion protein with a portion of the Km^r protein (Fig. 1). Alternatively, the iFHA could contain sequences that were normally removed by *B. pertussis*, perhaps during secretion.

In *B. pertussis*, FHA is found on the cell surface and as an extracellular protein (2, 11, 28). The first protein immunoblots of *E. coli* had involved cells suspended in their growth medium, so an experiment was conducted to localize the iFHA. Cells were broken with a French pressure cell, and protein immunoblots were prepared from whole cells, spent broth, cytosol, and cell wall plus membranes. Only cytosol and whole cells yielded iFHA (data not shown). These results suggested that the signal sequence for the translocation of FHA across membranes was lacking, or that it was different from *E. coli* signals and thus not processed, or that it was masked by fusion with a portion of the Km^r protein. Maximal expression of iFHA from *E. coli* LE392(pDB441) occurred after 16 h of growth (data not shown). Transformation of *E. coli* LE392 with pDB441 was always followed by iFHA expression.

Expression of *fha* in *B. pertussis* strain BP353. Weiss et al. (46) were apparently unable to detect FHA production by strain BP353 with a protein immunoblot involving rabbit anti-FHA. However, MAb 12H3 was able to detect small amounts of the 215- and 210-kDa FHA polypeptides produced by strain BP353; acquisition of pCP13 had no effect on this production (Fig. 3, lanes BP353; see asterisk). The Sm^r variant of strain BP353 produced the same protein immunoblot pattern with MAb 12H3 as was seen with original strain BP353 (data not shown). Weiss et al. (46) suggested that strain BP353 was defective in either the structural gene for FHA or a gene necessary for expression of the FHA. Our findings of reduced amounts of FHA polypeptides of apparently normal size from strain BP353 suggest that Tn*5* exerted a polar effect or that it was inserted into a control gene rather than into the structural gene for FHA.

Attempts to transform pDB441 to *B. pertussis* BP353, the FHA mutant, were unsuccessful, presumably because of the large size of the plasmid and the inefficiency of transformation for *B. pertussis* relative to that for *E. coli* (42). Furthermore, *B. pertussis* could not be isolated from the plate mating procedure when the original strain BP353 was used, because significant numbers of *E. coli* LE392 were able to grow in the presence of nalidixic acid. Conjugal transfer of pDB441 and pCP13 from *E. coli* to *B. pertussis* was successful when a Sm^r variant of BP353 was used as the recipient.

The protein immunoblots performed on transconjugants and control strains showed that *B. pertussis* strains UT25, BP338, and BP353(pDB441) had essentially the same pattern of FHA polypeptides (Fig. 3). Strain UT25 was the DNA donor for the genomic library, and its FHA had polypeptides at 215, 210, 150, and 140 kDa. The data for strain BP338 were in agreement with that reported by Weiss et al. (46). Strain BP338 is isogenic with strain BP353, and conjugal transfer of pDB441 to strain BP353 restored the parental level of expression of FHA. These two strains had the same FHA polypeptides as strain UT25 and a detectable fragment at 160 kDa (Fig. 3). The faint bands at 220 kDa (no arrow-heads) in strains BP338 and BP353(pDB441) may be artifacts, because they were not consistently detected, but they did not affect the interpretation of the results. Since strain BP353 made detectable FHA, the restoration of FHA production by pDB441 was probably due to provision of a *trans*-acting factor necessary for the expression of FHA. The purified FHA from strain 18334 also showed the high-molecular-mass bands of 215 and 210 kDa. Molecular masses for all of the above FHA preparations were in agreement with those reported by Irons et al. (16) for the FHA strain Wellcome 28. These authors reported fragments of 220, 210, 142, 135, and 89 kDa. The molecular masses of the fragments from strain BP338, a Tohama 1 derivative, were much higher than those reported for strain Tohama 1 (2, 16), except for a 160-kDa band reported for Tohama 1 (5). Our data suggested that fragmentation of FHA in our strains occurred by a similar, if not identical, mechanism, since we detected only minor strain differences. Perhaps the differences reported by other workers reflect various cultural conditions or purification protocols used.

HA assays were conducted on the *B. pertussis* strains to determine whether a functional FHA was restored by pDB441 (Table 1). Strains UT25 and BP338 had identical titers, and their specific activities for HA were essentially the same. The data showed that strain BP353 had no detectable ability to hemagglutinate sheep erythrocytes, confirming the earlier report (46). Strain BP353, carrying the cloning vector pCP13, was also unable to hemagglutinate sheep erythrocytes. There was an eightfold increase in the HA titer and a 10-fold increase in the specific activity of HA for strain BP353(pDB441) over that seen with strain BP338. Transcription from multiple copies of the *fha* gene might explain the apparently elevated levels of FHA activity, but this did not explain why we did not see an apparent increase

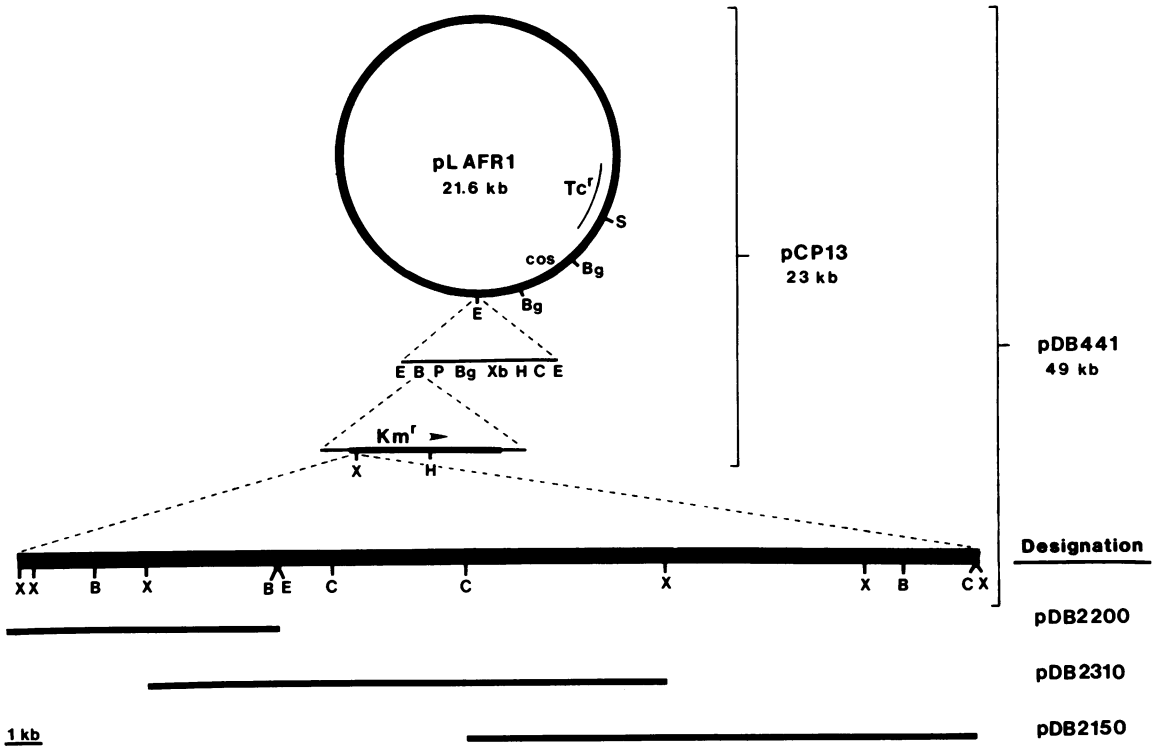


FIG. 1. Restriction endonuclease map of pDB441 and initial subclones. The heavy line represents the 26.5-kbp *B. pertussis* DNA insert in pCP13 and the DNA fragments in the initial subclones are indicated by the medium density lines underneath. The Km^r gene and the polylinker are not drawn to scale. The direction of transcription of the Km^r gene is indicated by the arrowhead. X, *Xho*I; B, *Bam*HI; E, *Eco*RI; C, *Cla*I; P, *Pst*I; Bg, *Bgl*II; Xb, *Xba*I; H, *Hin*dIII.

in the FHA antigen in the protein immunoblots (Fig. 3). However, the HA assay measured activity, whereas immunoblots provided estimates of antigen levels, and the two procedures are not necessarily equivalent. Purified FHA was used as a positive control for HA, and the specific activity was essentially the same as that reported elsewhere (5, 32).

Restriction endonuclease mapping of pDB441 and expression of FHA in *E. coli*. We found (Fig. 1) that the Km^r gene of pCP13 was in the orientation opposite, relative to the polylinker, to that previously reported (7). The *fha* gene was mapped at the left side of the *B. pertussis* DNA insert in pDB441 by the subcloning of three overlapping DNA frag-

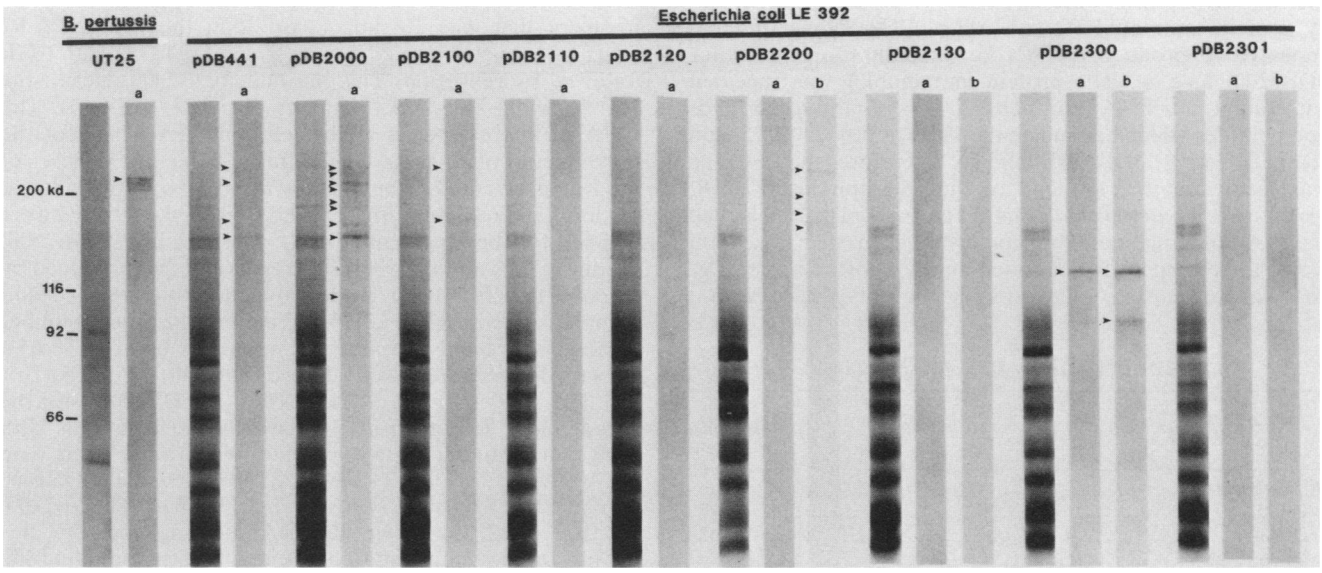


FIG. 2. FHA expression in *B. pertussis* UT25 and *E. coli* LE392 containing various plasmids, as detected by protein immunoblots. Coomassie brilliant blue-stained gel lanes (left sides) are paired with protein immunoblot(s). a, MAb 12H3; b, rabbit anti-FHA. Arrowheads indicate detectable FHA fragments, except for *B. pertussis* UT25, for which only the highest-molecular-size fragment is indicated. Molecular mass markers are indicated.

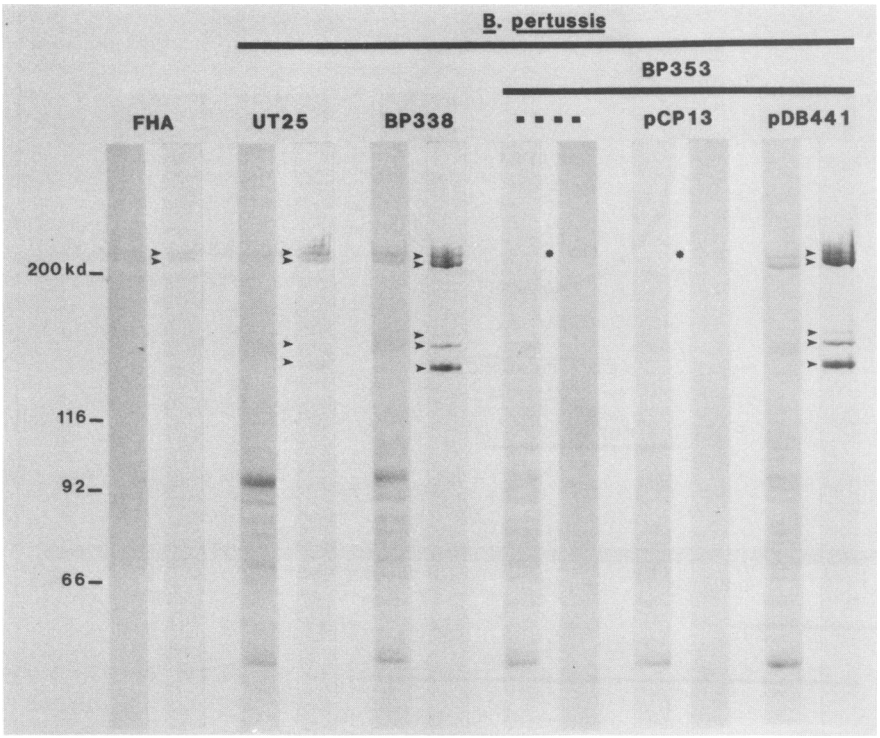


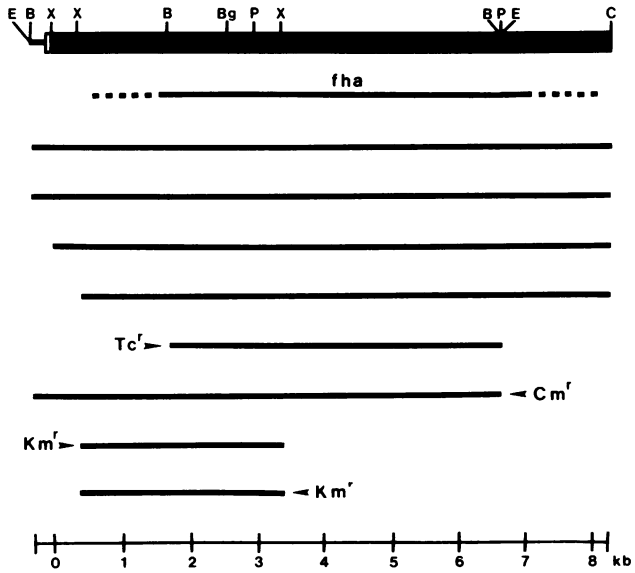
FIG. 3. FHA expression in *B. pertussis* strains as detected by protein immunoblots. Coomassie brilliant blue-stained gel lane (left side) is paired with its protein immunoblot, which received MAb 12H3. Protein immunoblot lanes for BP353 with no plasmid (▪▪▪▪) and pCP13 were developed with 4-chloronaphthol reagent (14) for approximately twice as long as the other blots were to accentuate the faint bands, marked by an asterisk. Arrows indicate detectable FHA fragments. Molecular mass markers are indicated.

ments into various plasmids: pDB2200 consisted of the 7-kbp *Eco*RI fragment cloned into the *Eco*RI site of pBR328; pDB2310 consisted of the 14-kbp *Xho*I fragment cloned into the *Xho*I site of pMK16; and pDB2150 consisted of the 15-kbp *Cla*I fragment cloned into the *Cla*I site of pBR322 (Fig. 1). These plasmids were transformed into *E. coli* LE392, and the cells were analyzed for production of iFHA by protein immunoblots with MAb 12H3. None of them appeared to produce iFHA (Fig. 2; data shown only for pDB2200, lane a). The protein immunoblot was repeated with rabbit anti-FHA, and pDB2200 was found to code for polypeptides which had molecular sizes of 210, 190, 175, and 160 kDa (Fig. 2, pDB2200, lane b). Therefore, the *fha* gene was located at the left end of the 26.5-kbp insert of *B. pertussis* DNA in pDB441. The iFHA from pDB2200 lacked the MAb-binding site. This could be interpreted in several ways: the epitope was not coded on the *Eco*RI fragment, *E. coli* was not capable of expressing the polypeptide with a proper conformation, or subcloning into the *Eco*RI site of the Cm^r gene had led to a fusion polypeptide which had an altered conformation such that only polyclonal rabbit anti-FHA could bind to it.

TABLE 1. HA by *B. pertussis* strains

Hemagglutinin source	Sp act (HA U/mg of protein)
B. pertussis	
UT25	27
BP338	38
BP353	<5
BP353(pCP13)	<7
BP353(pDB441)	400
Purified FHA	204,800

The *fha* gene must have extended beyond the 7-kbp *Eco*RI DNA fragment cloned to produce pDB2200, because the encoded iFHA were smaller than that from pDB441. A DNA fragment that was 1.6 kbp larger than this 7-kbp *Eco*RI fragment was constructed by digestion of pDB441 with *Cla*I, followed by religation. This plasmid, pDB2000, lacked the 15-, 4-, and 1-kbp *Cla*I fragments of pDB441 (Fig. 1). The DNA of interest began at the left-hand *Eco*RI site of the polylinker of pDB441, extended through the first 31 bases of the Km^r gene and 8.3 kbp of *B. pertussis* DNA, and ended at the left-hand *Cla*I site (Fig. 1). The *Bgl*II and *Pst*I sites were mapped for this fragment (Fig. 4). *E. coli* LE392(pDB2000) produced high amounts of iFHA relative to that produced by *E. coli* LE392(pDB441), as determined by the greater staining intensity with MAb 12H3 and an increase in the number of detectable bands. Production was maximal after 16 h of growth. Polypeptides were detected at 230, 225, 215, 205, 195, 185, 175, 160, and 117 kDa (Fig. 2, pDB2000). All the polypeptides seen with pDB441 were present in cells with pDB2000, including the polypeptides which were larger than FHA from *B. pertussis* (Fig. 2, UT25). Plasmid pDB2000 contained 1.6 kbp more *B. pertussis* DNA than did pDB2200, and the iFHA contained the MAb-binding site, but this finding was not sufficient to prove that the MAb-binding site was coded on this additional DNA fragment. To ensure that the entire iFHA was coded by pDB2000, the 4-kbp *Cla*I fragment of pDB441 (Fig. 1) was cloned into the *Cla*I site of pDB2000. There was no detectable difference in the pattern



Vector	Designation	Reactivity with 12H3
pCP13	pDB2000	+
pBR322	pDB2100	+
pBR322	pDB2110	−
pBR322	pDB2120	−
pBR322	pDB2130	−
pBR328	pDB2200	−[a]
pMK16	pDB2300	+
pMK16	pDB2301	−

FIG. 4. Restriction endonuclease map of the 8.6-kbp partial *Eco*RI-*Cla*I fragment of pDB2000, which contains the *fha* gene, and its various subclones. The open box, on the left end, represents the portion of the Km^r gene remaining in some of the subclones. The calculated apparent molecular weights of the largest polypeptides which were immunologically detectable for these plasmids were as follows: pDB2000, 230,000; pDB2100, 230,000; pDB2200, 210,000; and pDB2300, 127,000. The structural gene for FHA mapped to the region labeled *fha*. Dashed line represents ambiguity regarding the exact location of the termini. The restriction endonuclease sites are designated as in the legend to Fig. 1. The direction of transcription for the antibiotic resistance gene into which a DNA fragment was cloned is shown, if two orientations are possible. Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance. The map distance is from the left-hand *Xho*I site. Reactivity of the gene products with MAb 12H3 is shown. −[a], Reacts only with rabbit polyclonal anti-FHA.

of iFHA polypeptides, indicating that pDB2000 did contain all of the *fha* gene (data not shown). One end of the *fha* gene was mapped to the region between the *Eco*RI site at 6.7 kbp and the *Cla*I site at 8.3 kbp on the map (Fig. 4).

The 5-kbp *Bam*HI fragment, from 1.7 to 6.7 kbp on the map, was cloned into the *Bam*HI site of pBR322 to construct pDB2130, which aided in mapping the other end of the *fha* gene. A polypeptide coded by this DNA fragment should have been detectable with rabbit anti-FHA only if this fragment contained the promoter for the *fha* gene or if a promoter was supplied by the Tc^r gene into which it was cloned, since one end of the *B. pertussis* DNA would essentially be the same for the cloned fragments in pDB2130 and pDB2200 (Fig. 4). No fragments of iFHA were detected from cells containing pDB2130, which seemed to rule out the possibility that this *Bam*HI fragment contained the promoter for the *fha* gene (Fig. 2, pDB2130, lane b).

Regions to the left and right of the 5-kbp *Bam*HI fragment appeared essential to iFHA expression in *E. coli*. We were able to show that the Km^r gene provided the promoter function for the transcription of the *fha* gene. Plasmids pDB2110 and pDB2120 were constructed by cloning (into *Cla*I- and *Sal*I- digested pBR322) the 8.3-kbp fragment from the *Cla*I to *Xho*I (at 0 kbp) sites or the 7.9-kbp fragment from the *Cla*I to *Xho*I (at 0.4 kbp) sites of pDB2000, respectively (Fig. 4). Neither plasmid expressed iFHA (Fig. 2, pDB2110 and pDB2120). Because all the insert DNA of pDB2000 was present in pDB2110, *fha* was evidently transcribed from left to right and was under the control of the Km^r gene promoter. Further evidence that the Km^r gene promoter was responsible for the synthesis of iFHA was obtained. The 3-kbp *Xho*I fragment, from 0.4 to 3.4 kbp on the map, was cloned in opposite orientations into the *Xho*I site of pMK16 to give pDB2300 and pDB2301 (Fig. 4). Only cells with pDB2300 expressed a peptide of 127 kDa, which was detected by MAb 12H3 as well as by rabbit anti-FHA, which also detected a peptide of 97 kDa (Fig. 2, pDB2300 and pDB2301). Since the MAb-binding site was coded in the 3-kbp *Xho*I fragment, the polypeptide coded by pDB2200 must have had a conformation which prevented the binding of MAb 12H3, because pDB2200 contained this 3-kbp *Xho*I fragment. The possibility of a repeating sequence for MAb binding cannot be ruled out, as MAb 12H3 apparently binds to many polypeptides of FHA. Irons et al. (16) reported similar results with their MAbs to FHA.

Although several *B. pertussis* genes have been cloned, adequate expression in *E. coli* is not always realized. Shareck and Cameron (36) reported expression of two *B. pertussis* outer membrane proteins in *E. coli*, presumably from their own promoters. Other workers have not been able to achieve efficient expression of PT from its promoter or from the hybrid *lac-trp* promoters (19; W. J. Black and S. Falkow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-10, p. 26). Accurate mapping of *fha* has proved difficult because iFHA is not expressed in the absence of the Km^r promoter in *E. coli*. Subclone pDB2200 provided the best evidence for the location of *fha*. This plasmid apparently coded for a fusion protein of 210 kDa. The direction of transcription of the *fha* was opposite to that of the Cm^r gene, so any fusion polypeptide must have come from the sequences of the negative strand of the Cm^r gene (Fig. 4). The sequence of the Cm^r gene indicates that there are four stop codons within 84 bases of the *Eco*RI site on the negative strand of pBR328, with at least one in each possible reading frame (1, 37). Therefore, the maximum-size polypeptide that could be added to the iFHA polypeptide was 3.1 kDa, since 1 kbp of DNA can code for a 36.6-kDa polypeptide. Assuming 207 of the 210 kDa of iFHA from pDB2200 was derived from the *fha* gene, the site of the translation start signal for iFHA would map 5.7 kbp to the left of the right-hand *Eco*RI

site, or at map distance 1.0 kbp (Fig. 4). Further evidence supporting this location for the site of the translation start signal came from a similar analysis of pDB2300 (24). The translation start signal for iFHA was thus calculated to be at 0.7 kbp, which agreed well with results calculated for pDB2200 (Fig. 4). There is a precedent for a Km^r promoter controlling distal gene expression. Berg et al. (3) have shown the Km^r promoter of Tn*5-112*, when inserted in the proper orientation, can stimulate basal level expression of *lacZ*. We suggest that a similar mechanism functions here.

The sequences that coded for the carboxyl terminal of the iFHA were determined from the polypeptides produced from pDB2000 and pDB2100. These plasmids contained additional DNA at the 3′ end of the *fha* gene and coded for a polypeptide 17 kDa larger than the *fha* gene-derived portion of the iFHA, as seen with pDB2200. The stop codon for *fha* was apparently located no more than 0.5 kbp to the right of the right-hand *Eco*RI site, or about map distance 7.2 kbp (Fig. 4).

As evaluated by immunoblots, *B. pertussis* produced more FHA on a protein basis than *E. coli* produced iFHA. Efficient production of iFHA in *E. coli* might allow purification of an antigen useful for a pertussis vaccine and thereby might avoid problems with potentially toxic *B. pertussis* products. In an effort to increase iFHA production in *E. coli*, the *fha* gene was moved into *Cla*I- and *Eco*RI-digested pBR322, a multicopy plasmid, as a *Cla*I-partial *Eco*RI digestion fragment of pDB2000 to give pDB2100 (Fig. 4). This fragment maintained the Km^r gene promoter, but expression of iFHA was poor, since only iFHA polypeptides of 230 and 175 kDa were detected with MAb 12H3 (Fig. 2, pDB2100). Maximal expression of iFHA occurred at 8 h of growth, with no iFHA detected at 16 h.

The FHA, PT, and the agglutinogens have been reported to be responsible for adherence of *B. pertussis* to cell surfaces (13, 42). Prevention of adherence (and therefore initiation of infection) is a major goal in the development of an acellular vaccine (42). Active or passive immunization of mice against PT toxoid protects them from aerosol challenge with *B. pertussis*. However, PT has been shown to be required for the occurrence of vaccine-associated encephalopathy in mice (39). It has been suggested that PT may cause this same syndrome in certain susceptible individuals. Although immunization with FHA or administration of antisera to FHA may not protect mice against aerosol challenge with *B. pertussis*, simultaneous administration with PT or antisera to PT, respectively, greatly decreased the dose for PT or anti-PT needed to protect 50% of the mice. The synergistic immune response, when both PT and FHA were given, made FHA a prime candidate for inclusion in an acellular vaccine.

We have shown the *fha* gene was 6.2 to 6.5 kbp long and extended from about 0.7 or 1.0 to 7.2 kbp on our map (Fig. 4). We have evidence that the Km^r gene provided the promoter function for iFHA but that the translation start signal may have been as much as 1.0 kbp from this promoter. This evidence, taken with the elevated expression level of FHA in *B. pertussis* BP353(pDB441), suggested that pDB2000 probably contained the promoter for the *fha* gene, which did not function in *E. coli* in the absence of the *vir* gene (S. Stibitz and S. Falkow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D-80, p. 79). We suggest that pDB441 might code for a *trans*-acting factor which is required for proper expression of FHA in *B. pertussis* but is not required for expression of iFHA in *E. coli*. The intracellular location of iFHA and the inability of a multicopy plasmid to increase production of iFHA in *E. coli* may complicate purification of this protein from *E. coli*. However, this report on cloning and expression of FHA in both *B. pertussis* and *E. coli* should provide useful recombinant techniques for the study of *B. pertussis* antigens in *E. coli*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 17682 to C.D.P. from the National Institutes of Health.

We thank Mark A. McIntosh for reading this manuscript and his suggestions during this research. We also thank Allyson Cole for her technical assistance.

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