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Cloning of *Bordetella pertussis* Outer Membrane Proteins in *Escherichia coli*

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We constructed and screened a gene bank of phase I chromosomal DNA of *Bordetella pertussis* in *Escherichia coli*. A single immunopositive clone was recovered, and the hybrid plasmid obtained, designated pFSH200, had a molecular size of 46.6 kilobases. Smaller derivatives were generated by partial digestion of plasmid pFSH200 and were further characterized. One such derivative, plasmid pFSH201, contained a 4.5-kilobase chromosomal DNA fragment of *B. pertussis* which coded for the synthesis of the two outer membrane proteins of 33 and 30 kilodaltons specific to *B. pertussis*.

Bordetella pertussis, the causative agent of whooping cough, is a fastidious microorganism which displays several determinants of pathogenicity such as lymphocytosis promoting factor (14), protective antigens (18), fimbrial hemagglutinin (19), dermonecrotic toxin (2), and extracellular adenylate cyclase (5). Presently, there is a great deal of interest in these cellular components, some of which are major outer membrane proteins (17), as potential immunogenic proteins to be incorporated into a subcellular vaccine. These antigens, which are involved in the protection of mice against intracerebral challenge with *B. pertussis* and probably in the protection of children against whooping cough, are biochemically characterized, although genetic analysis of their immunological activities has not received much attention. *B. pertussis* strains have shown phase variation (from phase I to phase IV) after in vitro passage (9) and a phenotypic change, similar to the osmoregulation of the expression of the porins of *Escherichia coli* (16) called antigenic modulation (from X mode to C mode), when the organism is grown in medium containing a high concentration of Mg^{2+} (7, 23). Phase IV strains and C-mode cells are characterized by a loss or attenuation of several antigenic determinants and certain outer membrane proteins (3, 15, 17), which are mainly the proteins of 90, 86, 81, 33, and 30 kilodaltons (Kd) (17) and the five subunits of the pertussis toxin (IAP, LPF), which are low-molecular-weight proteins (21). The molecular nature of the antigenic modulation observed in phase I strains of *B. pertussis* is unknown, as is phase variation.

To obviate the difficulties of working with a fastidious organism showing antigenic variation, the genetics of which are poorly understood, we undertook cloning experiments which were aimed at transferring into *E. coli* the genes of *B. pertussis* coding for the synthesis of these protective antigens. We report here the preliminary results on the molecular cloning and expression in *E. coli* of the two major outer membrane proteins of ca. 33 and 30 Kd.

Phase I *B. pertussis* cells were grown in minimal Stainer-Scholte broth (20), and chromosomal DNA was extracted by the method of Marmur (10). A cosmid gene bank was constructed in *E. coli* DH1 by the procedure of Hohn and Collins (6), with plasmid pHC79 as the cloning vector. This procedure enabled us to clone into *E. coli* contiguous stretches of chromosomal DNA (average size, 40 kilobases [kb]) in 2,000 recombinants recovered after in vitro packaging into λ phages and further transduced in *E. coli* DH1. Statistically, this gene bank represented ca. 15 times the total genome of *B. pertussis*. Since the synthesis of outer membrane proteins of *B. pertussis* has shown modulation in the presence of high concentrations of Mg^{2+} (23), 200 Am^r colonies were spot-plated on minimal M9 medium and assayed for expression of *B. pertussis* proteins with *E. coli* DH1-adsorbed mouse immune serum against Formalin-fixed *B. pertussis* cells by the method of Meyer et al. (11). The autoradiogram of the experiment revealed that a single clone was immunopositive. This positive clone was picked up from the master plate, purified twice on the same medium, and tested again with mouse antiserum before further analysis. Plasmid pFSH200 was extracted from the clone, and agarose gel analysis of *Bam*HI restriction fragments (Fig. 1A) enabled us to determine that plasmid pFSH200 consisted of cosmid pHC79 (6.43 kb) and five chromosomal fragments of 15, 12.5, 4.7, 4.3, and 3.7 kb, for a total length of 46.6 kb.

The replicon in plasmid pHC79 is derived from that of plasmid ColE1, which is under relaxed control (ca. 50 copies of plasmid per cell). The large plasmid pFSH200, however, isolated from the positive clone could have a copy number as low as five copies per cell and could be lost by segregation during growth in the absence of antibiotic selection (6). Thus, to stabilize the plasmid DNA by increasing the copy number of plasmid pFSH200 and to localize more precisely the structural gene(s) coding for the synthesis of *B. pertussis*-specific proteins, we attempted to obtain the smallest plasmid derived from plasmid pFSH200 that was still capable of producing these immunoreactive proteins. Digestion of plasmid pFSH200 with restriction endonuclease *Sal*I yielded more than 20 fragments (cosmid pHC79 is cleaved only once by *Sal*I). Thus, plasmid pFSH200 was partially digested with *Sal*I endonuclease and ligated with T4 DNA ligase; smaller derivatives were introduced by transformation into *E. coli* DH1 by the method of Hanahan (4). This procedure yielded many Am^r transformants; most of them were positive when screened with mouse immune serum against *B. pertussis*. Ten positive clones and one negative clone were picked at random, screened for plasmid DNA content by the mini-lysate method of Birnboin (1), and digested with restriction enzyme *Sal*I. In Fig. 1B it is shown that the smallest plasmid obtained, designated pFSH201 (10.3 kb), contained only 3 of 20 *Sal*I restriction fragments found originally in plasmid pFSH200. The fragment of 7.8 kb common to all derivatives consisted of plasmid pHC79 (6.43 kb) and a small chromosomal DNA fragment of 1.6 kb and

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FIG. 1. Restriction analysis of plasmid pFSH200 and smaller derivatives of pFSH200. Plasmid DNA digested with restriction endonucleases was electrophoresed in agarose gel with Tris-borate buffer by the method of Meyers et al. (12). (A) Lane 1, λ *Hin*dIII molecular weight standards; lane 2, plasmid pHC79 digested with *Bam*HI; lane 3, pFSH200 digested with *Bam*HI. (B) Lane 1, plasmid pFSH200 digested with *Sal*I; lanes 2 to 11, smaller immunopositive derivatives of plasmid pFSH200; lane 4, plasmid pFSH201; lane 12, plasmid pFSH202 isolated from a negative clone. Numbers to the left and right of the gels are in kb.

was needed for the replication and maintenance of all smaller plasmids. The presence of the additional fragments of 1.4 and 1.1 kb present in all positive clones derived from pFSH200 suggest that these three chromosomal DNA fragments are necessary for the synthesis of the immunoreactive proteins of *B. pertussis*. In fact, the clone harboring plasmid pFSH202, which lacked the 1.4-kb *Sal*I fragment, was negative and did not produce any proteins specific to *B. pertussis* (Fig. 1, lane 12). The restriction map of plasmid pFSH201 is shown in Fig. 2. Endonuclease restriction analysis of plasmid pFSH201 revealed that in the process of partial digestion of plasmid pFSH200 with *Sal*I, the small fragment of plasmid pHC79 spanning from the unique *Sal*I site and including the nearest *Bam*HI site was deleted (275 base pairs). Thus, plasmid pFSH201 contains a 4.5-kb fragment of chromosomal DNA of *B. pertussis* that codes for the synthesis of proteins specific to *B. pertussis*.

The immunoreactive proteins expressed by plasmids pFSH200 and smaller derivatives were analyzed by Western blotting of sodium dodecyl sulfate-solubilized total proteins of bacteria harboring these plasmids. The autoradiogram revealed that two proteins encoded by these plasmids reacted with the mouse immune serum against *B. pertussis* and that these proteins had an estimated size of 33 and 30 Kd (data not shown). No protein or truncated immunoreactive protein was synthesized by the clone harboring plasmid pFSH202, which lacked the 1.4-kb *Sal*I fragment found in all positive smaller derivatives of plasmid pFSH200. The 1.4-kb *Sal*I fragment may contain part of the structural genes or transcriptional signals necessary for protein synthesis. The proteins coded by plasmid pFSH201 were localized by analyzing the soluble and envelope fractions of cells harboring this plasmid by the method of Morona and Reeves (13). In Fig. 3, lane 2, it is shown that the two immunoreactive proteins of 33 and 30 Kd are processed into the outer membrane of *E. coli* DH1 as they are in *B. pertussis*.

This is the first report that deals with the molecular cloning and expression in *E. coli* of two major outer membrane proteins of *B. pertussis*. In plasmid pFSH200, we

FIG. 2. Restriction map of plasmid pFSH201. The physical map was constructed by analysis of the molecular weight of restriction fragments generated by single and double digests of plasmid pFSH201 with the position of known restriction sites in cosmid pHC79 used as a reference. The thick line represents the cosmid pHC79, and the thin line represents the 4.5-kb chromosomal DNA fragment of *B. pertussis*. The DNA fragment of 275 base pairs (bp) of cosmid pHC79 spanning from the unique *Sal*I to the *Bam*HI site was deleted in the process of partial digestion of plasmid pFSH200 with endonuclease *Sal*I to produce plasmid pFSH201.

FIG. 3. Immunological detection of proteins by Western blotting (Towbin et al.; 22). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) by the discontinuous system of Laemmli (8). The proteins were transblotted to nitrocellulose for 3 h at 175 mA in 192 mM glycine–25 mM Tris (pH 8.0)–20% methanol. The filters were processed as described by Meyer et al. (11). Prestained proteins (Bethesda Research Laboratories) were run in the gel and used as molecular weight markers during the blotting. The filters were exposed to Kodak X-OMat film with Dupont Cronex intensifier screens for 24 h at −80°C. Lane 1, Whole cells of *E. coli* DH1 harboring plasmid pHC79; lane 2, envelope fraction of cells harboring plasmid pFSH201; lane 3, soluble or cytoplasmic fraction; lane 4, whole cells of phase I *B. pertussis* cells. Numbers to the left and right of the gels are in Kd.

cloned a chromosomal DNA fragment of *B. pertussis* of ca. 40 kb which codes for the synthesis of the two immunoreactive proteins with estimated sizes of 33 and 30 Kd. Furthermore, isolation of plasmid pFSH201 opens a new field of research in the genetically engineered production of these proteins as potential protective antigens. We are currently undertaking studies to determine precisely the organization of these genes with regard to their promoter sequence to increase the synthesis of these proteins by modifying the transcription of their structural genes. However, as shown in Fig. 3, lane 4, at least eight proteins reacted with the mouse immune serum against Formalin-fixed *B. pertussis* cells. The molecular weight of these proteins corresponds to those of the outer membrane proteins extracted from phase I *B. pertussis* (17). Our failure to recover other immunopositive clones that synthesize these proteins, during the screening of the gene bank of *B. pertussis* in *E. coli*, could be explained if we consider the model of the regulation of expression of outer membrane proteins of *B. pertussis* proposed recently by Weiss and Falkow (24). According to this model, a transacting gene product is required as a positive effector for the expression of the structural genes that code for the synthesis of the outer membrane proteins of *B. pertussis*. Studies are in progress to identify in plasmid pFSH200 and in smaller derivatives whether such a gene or gene product is present and necessary for the expression of the outer membrane proteins of *B. pertussis* in *E. coli*.

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