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Production of Recombinant *Bordetella pertussis* Serotype 2 Fimbriae in *Bordetella parapertussis* and *Bordetella bronchiseptica*: Utility of *Escherichia coli* Gene Expression Signals

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Serotype-specific fimbriae of *Bordetella pertussis* are considered potential components of new-generation vaccines against whooping cough. Attempts to characterize fimbriae, and indeed other virulence determinants, produced by *B. pertussis* have been frustrated on one hand by low yields from *B. pertussis* itself and on the other by an inability to produce native recombinant products in *Escherichia coli*. In order to try to circumvent this problem, we have examined the expression of *B. pertussis* serotype 2 fimbriae in *Bordetella parapertussis* and *Bordetella bronchiseptica* from native as well as *E. coli* expression signals. These studies revealed that the fimbrial gene product was expressed from the original *B. pertussis* promoter and Shine-Dalgarno sequence in both *B. parapertussis* and *B. bronchiseptica*. The transcriptional start site of the gene was located 146 nucleotides upstream of its ATG start codon. A recombinant fimbrial subunit gene containing P_{LAC} and the *atpE* translation initiation region of *E. coli* was also expressed in *B. bronchiseptica*. In all cases in which gene expression was detected the gene product was expressed as serotype 2-specific fimbriae as determined by enzyme-linked immunosorbent assay (ELISA) and immunoelectron microscopic investigation of the bacterial cell surface.

Bordetella pertussis is the causative agent of whooping cough, a particularly severe disease of young children characterized by repeated bouts of severe paroxysmal coughing. Until recently this disease was controlled through vaccination. However, increasing concern about side effects associated with immunization with whole-cell preparations of *B. pertussis* has led to decreased vaccine acceptability. This in turn has been accompanied by an increase in the incidence of whooping cough (22). It is therefore imperative that an effective, nonreactogenic vaccine of higher acceptability be developed. Virulence-associated gene products that have been suggested for incorporation into a purified-component, acellular vaccine include pertussis toxin, filamentous hemagglutinin, serotype-specific fimbriae, adenylate cyclase, and major outer membrane proteins (21, 22). Expression of the genes encoding all of these antigens is positively controlled by the virulence regulatory determinant, *bvg* (6, 11, 29). Purification of such proteins from *B. pertussis* is hampered by poor bacterial growth rates, low yields, antigenic variation, and the presence of other reactogenic contaminants. To avoid these problems, attempts have been made to obtain expression of recombinant antigens in *Escherichia coli* by using strong transcriptional and translational signals. The five genes encoding pertussis toxin have been expressed in *E. coli* under the control of the lambda p_L promoter (3). The serotype 2 fimbrial subunit gene has also been expressed from the lambda p_L and p_R promoters (28). However, in neither case did the recombinant products assemble to form products, pertussis holotoxin and serotype 2 fimbriae, respectively, that were immunologically identical to the native products. On the other hand, pertussis holotoxin is produced in recombinant *Bordetella parapertussis* and *Bordetella bronchiseptica* and is expressed from its native promoter (13). Nontoxic mutant pertussis toxin has also been expressed in *B. pertussis* (10, 19). In this report, we have examined the usefulness of *E. coli* transcriptional and translational signals for the expression of pertussis components in faster growing and less fastidious *Bordetella* spp. and have constructed broad-host-range expression plasmids containing the serotype 2 fimbrial subunit gene to determine whether the fimbrial subunit expressed in *B. parapertussis* and *B. bronchiseptica* is assembled into native serotype 2 fimbriae.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work and their sources are as follows: *B. pertussis* Tohama, 1.2. serotype Fim$^+$ (24); *B. pertussis* H36, 1.3 serotype Fim$^+$, and *B. parapertussis* M/S180 Fim$^+$, N. Preston; *B. bronchiseptica* 5376 Fim$^+$, R. Brownlie; *B. pertussis* ATCC 9797, 1 serotype Fim$^-$, *B. parapertussis* ATCC 15311 Fim$^+$, and *B. bronchiseptica* ATCC 10580 Fim$^-$ and ATCC 19395 Fim$^-$, M. Höfle; *E. coli* JM109, *endAl recA hsdR supE thi gyr lac-pro* F' *tra-36 proAB lacI^{q}Z* M15 (31); and *E. coli* CAG629 *lon htpR165*-Tn*10*, C. Gross. The plasmids used in this work were pIL22 (15), pMW3 and pMW10 (28), pRK600 (5), and pDSK519 (9).

E. coli was grown on Z agar (27), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium, and Luria broth (16), and *B. pertussis* was grown on BG agar (26). When appropriate, plasmid-bearing strains were grown with 100 μg of ampicillin per ml or 50 μg of chloramphenicol, cephalexin, or kanamycin per ml. Bacterial cultures were routinely grown at 37°C unless otherwise stated. Broth cultures were aerated by shaking at 300 rpm in a New Brunswick Environmental Incubator Shaker. Temperature induction was carried out at 37°C, and isopropyl-β-D-thiogalactopyranoside (IPTG) induction was carried out by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside.

DNA manipulations. Restriction endonucleases, T4 DNA

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ligase, and polynucleotide kinase were used essentially by the method of Maniatis et al. (16). Plasmids were isolated by alkaline lysis (16). Transformation was carried out by the method of Hanahan (7). Agarose gel electrophoresis was as previously described (27). DNA sequencing was carried out by the chain termination method of Sanger et al. (23). Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA synthesizer used in accordance with the manufacturer's instructions.

Reverse transcriptase mapping. RNA was isolated by harvesting exponentially grown bacteria by centrifugation (1.5 ml) and resuspending the pellet in 300 μl of a solution containing 6.7 M guanidinium isothiocyanate, 1% (vol/vol) β-mercaptoethanol, 2% (wt/vol) sodium-*N*-laurylsarcosine, and 0.02 M sodium citrate (pH 7.0). The suspension was incubated at 65°C for 10 min. Then, 300 μl of phenol equilibrated with Tris-HCl (0.1 M, pH 8.0) was added, and the suspension was incubated for 5 min at 65°C. A solution (300 μl) of 0.1 M sodium acetate, 0.01 M Tris-HCl (pH 7.5), and 1.0 mM EDTA which was followed by 400 μl of chloroform-isoamylalcohol (24:1) was then added, mixed, and incubated at 65°C for 10 min. After centrifugation at 4°C for 10 min, the aqueous phase was reextracted once with phenol-chloroform and twice with chloroform. After ethanol precipitation, samples were treated with RNase-free DNase I (Boehringer Mannheim) for 30 min at 20°C, reextracted with phenol-chloroform, precipitated with ethanol, and resuspended in sterile water. The RNA concentration was determined by measuring the A_{260}, and the integrity of the RNA was determined by the presence of discrete rRNA bands after electrophoresis in 1.3% agarose gels.

For the primer extension analysis the 20-base primer (3′-GACCGCCGGTAACGCAGGCG-5′; referred to as the fim2RT primer) which is complementary to the noncoding strand of the serotype 2 fimbrial subunit gene was synthesized and labeled at the 5′ end with T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham) as previously described (16). Primer extension was carried out essentially by the method of de Lorenzo et al. (4). Samples were heated at 80°C for 10 min and electrophoresed as described for DNA sequence reactions.

Transfer of broad-host-range plasmids to *Bordetella* spp. *E. coli* strains containing both pRK600 and pDSK519 derivatives were grown overnight in nutrient broth containing antibiotics selective for both plasmids. A 1.0-ml sample of each culture was pelleted by centrifugation at 3,000 × *g* for 15 min and resuspended in 1.0 ml of 0.9% NaCl. The suspension was used to overlay 3-day-old plate cultures of *Bordetella* spp., and the plates were incubated for 4 h at 37°C. The mating mixtures selecting for *Bordetella* spp. recombinants with cephalexin and kanamycin were then plated out.

Protein purification and analysis. Wild-type serotype 2 fimbriae from *B. pertussis* Tohama were dissociated from the bacterial cell surface with 4 M urea at 60°C, partially purified by the method of Mooi et al. (17), and further purified by using CL-6B gel filtration chromatography following the protocol of Irons et al. (8). Methionylated mature recombinant protein was purified from inclusion bodies essentially by the method of Nagai and Thøgersen (18), as modified by Walker et al. (28). Protein samples were mixed 1:1 with sample buffer (60 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and electrophoresed by the method of Laemmli (12) with a 3.85% acrylamide stacking gel and a 10% acrylamide separating gel. Protein concentration was determined by the method of Bradford (1).

Immunological techniques. Mouse antiserum was prepared as previously described (28). Rabbit antiserum was prepared by emulsifying 200 μg of protein at a ratio of 1:1 with Freund incomplete adjuvant in a final volume of 1.0 ml. Groups of two three-month-old chinchilla bastard rabbits were injected subcutaneously and intramuscularly on day 1, day 14, and day 28. After 35 days, the rabbits were sacrificed and the blood was collected and allowed to clot for 3 h at room temperature. Erythrocytes were pelleted at 9,000 × *g*, and the serum was collected, pooled, and stored at −20°C until use. Western immunoblotting was carried out essentially by the method of Burnette (2). Antibodies that react with the serotype 2 fimbrial subunit were raised in rabbits (see Fig. 3A) or mice (see Fig. 3B and C) (antisera were chosen on the basis of least nonspecific reactivity). The detection system used was Bio-Rad horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G with 4-chloro-1-naphthol as a substrate. Serotype-specific agglutination and the preparation of serotype 2-specific antiserum cross-adsorbed against *B. pertussis* H36, *B. parapertussis* M/S180, and *B. bronchiseptica* 5376 were carried out as previously described (20). For enzyme-linked immunosorbent assay (ELISA) and immunoelectron microscopy, cross-adsorbed rabbit antibodies were purified by using a protein A-Sepharose CL-4B column (Pharmacia) by applying the recommended procedure and adjusting the protein concentration to 400 μg of immunoglobulin G per ml. ELISA was performed by adjusting protein concentrations to 10 μg/ml in phosphate-buffered saline (pH 7.4; PBS) and allowing 100-μl triplicate samples and a PBS blank to adsorb to Nunc Maxisorp Immunomodule 96-well plates overnight at 4°C. Protein samples were removed, and wells were blocked with 200 μl of 0.3% gelatin in PBS for 2 h at 37°C. Plates were washed three times with PBS, and a 50-μl sample of 1:200 PBS-diluted serotype 2-specific rabbit antibodies was added to each well. After 15 min at room temperature, the plates were again washed and a 100-μl sample of Bio-Rad horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:1,000 in PBS was added to each well and incubated for 1 h at room temperature. The plates were again washed and then developed by the addition of 200 μl per well of activated substrate solution (consisting of 10 ml of 0.1 M citric acid, 10 ml of 0.2 M Na_2HPO_4, 20 ml of distilled H_2O, 40 μl of 30% H_2O_2, and 40 mg of *o*-phenyldiamine dihydrochloride). After an appropriate time, the reaction was stopped by the addition of 50 μl of 0.25 M H_2SO_4 and the A_{490} was determined with a Bio-Rad model 3550 microplate reader. The standard deviation was calculated for each triplicate sample.

Immunoelectron microscopy. Bacterial strains grown on plates were gently suspended in 250 μl of PBS, absorbed onto freshly prepared collodium-covered nickel grids, and then rinsed with distilled water. After being air-dried, the grids were treated with a 1:100 dilution of the cross-adsorbed serotype 2-specific antibody for 15 min at room temperature. Unbound antibody was removed by a mild spray of PBS from a plastic bottle. The bound antibodies were made visible for electron microscopic examination by incubating the grids on drops of protein A-gold complexes (10-nm gold particle size, with a concentration (A_{520}) = 0.01) for 10 min at room temperature. The grids were subsequently rinsed with a mild spray of PBS containing 0.01% Tween 20 and then distilled water. After being air-dried the grids were unidirectionally metal shadowed with platinum. Samples

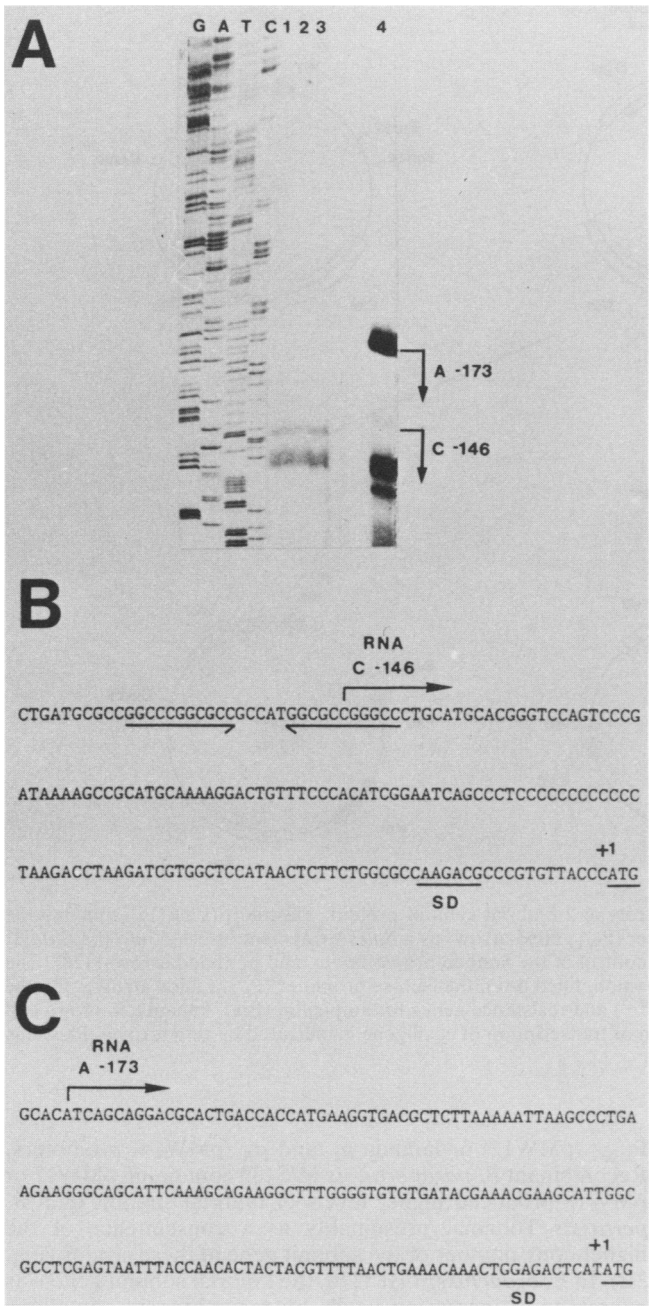

FIG. 1. Reverse transcriptase mapping of the transcriptional start site of the wild-type serotype 2 fimbrial subunit gene of *B. pertussis* Tohama (24) and the recombinant fimbrial subunit carried by pMW10 (28). (A) Lanes G, A, T, and C, DNA sequence reactions of pMW10 with the fim2RT primer; lanes 1 to 3, primer extension analysis with the fim2RT primer of different RNA preparations isolated from *B. pertussis* Tohama; lane 4, primer extension analysis of strain CAG629 carrying pMW10 with the fim2RT primer. The transcriptional start sites and the position of the first base in the RNA message are relative to the adenosine (base +1) of the ATG start codon and are indicated by arrows. For the wild-type fimbrial subunit of *B. pertussis* Tohama, the start site is situated at base C −146 (lower arrow, lanes 1 to 3) and at base A −173 for the recombinant subunit found on pMW10 (upper arrow, lane 4). (B and C) Diagrammatic representations of the upstream regions of the wild-type gene (B) and recombinant fimbrial subunit (C). The transcriptional start sites and their positions relative to the ATG start codons are indicated by arrows. The ATG start codons and consensus Shine-Dalgarno regions (SD) are underlined. The inverted-repeat region of the wild-type gene is underlined with inverted arrows. The DNA sequence of the upstream regions of the wild-type and recombinant genes were taken from those reported for pIL22 (15) and pJLA503 (25), respectively.

were examined with a Zeiss electron microscope 10 B at an acceleration voltage of 80 kV and at calibrated magnifications.

RESULTS

Reverse transcriptase mapping. To localize the native promoter of the gene of the serotype 2 fimbrial subunit, the transcriptional start site was defined by reverse transcriptase mapping of purified *B. pertussis* Tohama RNA. The RNA start site was situated 146 nucleotides upstream of the ATG start codon (Fig. 1). The distance between the promoter and the translational start site was taken into consideration in subsequent experiments designed to clone the native promoter and structural gene into the broad-host-range plasmid pDSK519 (9) as described below. For the recombinant serotype 2 fimbrial subunit expressed in *E. coli*, RNA was isolated from *E. coli* CAG629(pMW10) (28) after induction for 1 h at 42°C. Plasmid pMW10 contains a 0.8-kb fragment carrying the gene of the serotype 2-specific fimbrial subunit downstream of and in the same orientation as the lambda p_L and p_R promoters of the expression vector pJLA503 (25). This plasmid was designed to express an intact fimbrial subunit protein with its original leader peptide. The subunit gene had previously been modified to reduce the RNA secondary structure between the *atpE* translational initiation region (TIR) of the vector and the NH_2-terminal coding region so as to obtain efficient expression in *E. coli* (28). The RNA start site was found to be located 173 nucleotides upstream of the ATG start codon. This is in agreement with the start site reported for the lambda p_L promoter that is incorporated upstream of the ATG start codon in the vector pJLA503 (25). There was no evidence of another start site further upstream correlating with the position of the lambda p_R promoter present in pJLA503 (Fig. 1). This is consistent with the finding that less than 5% of transcripts originate from the p_R promoter in pJLA503 (16a).

Construction of expression plasmids. Broad-host-range plasmids were constructed to transfer the *B. pertussis* serotype 2-specific fimbrial subunit gene, under the control of native and *E. coli* expression signals, to *B. parapertussis* and *B. bronchiseptica*. The modified subunit gene and *atpE* TIR were subcloned from pMW10 into the broad-host-range vector pDSK519 in the same orientation as the P_{LAC} promoter of the vector to produce pMW12. The modified gene, *atpE* TIR, and p_L and p_R promoters were also subcloned into pDSK519 in the opposite orientation to P_{LAC} to produce pMW14. Plasmid pMW11 was identical to pMW12, except that the subunit gene encoding the methionylated mature form of the fimbrial subunit gene (i.e., lacking leader peptide) was cloned from pMW3 (28) into pDSK519. The wild-type *B. pertussis* serotype 2 fimbrial subunit promoter and structural gene was subcloned from plasmid pIL22 (15) into pDSK519 to form pMW17 (in the same orientation as P_{LAC}) and pMW18 (in the opposite orientation) (Fig. 2).

Expression of recombinant fimbrial protein in *E. coli*. Substantial amounts of the 25.0-kDa recombinant fimbrial subunit were detected in *E. coli* CAG629 (*lon* protease-deficient, heat shock protein-deficient) bacteria containing

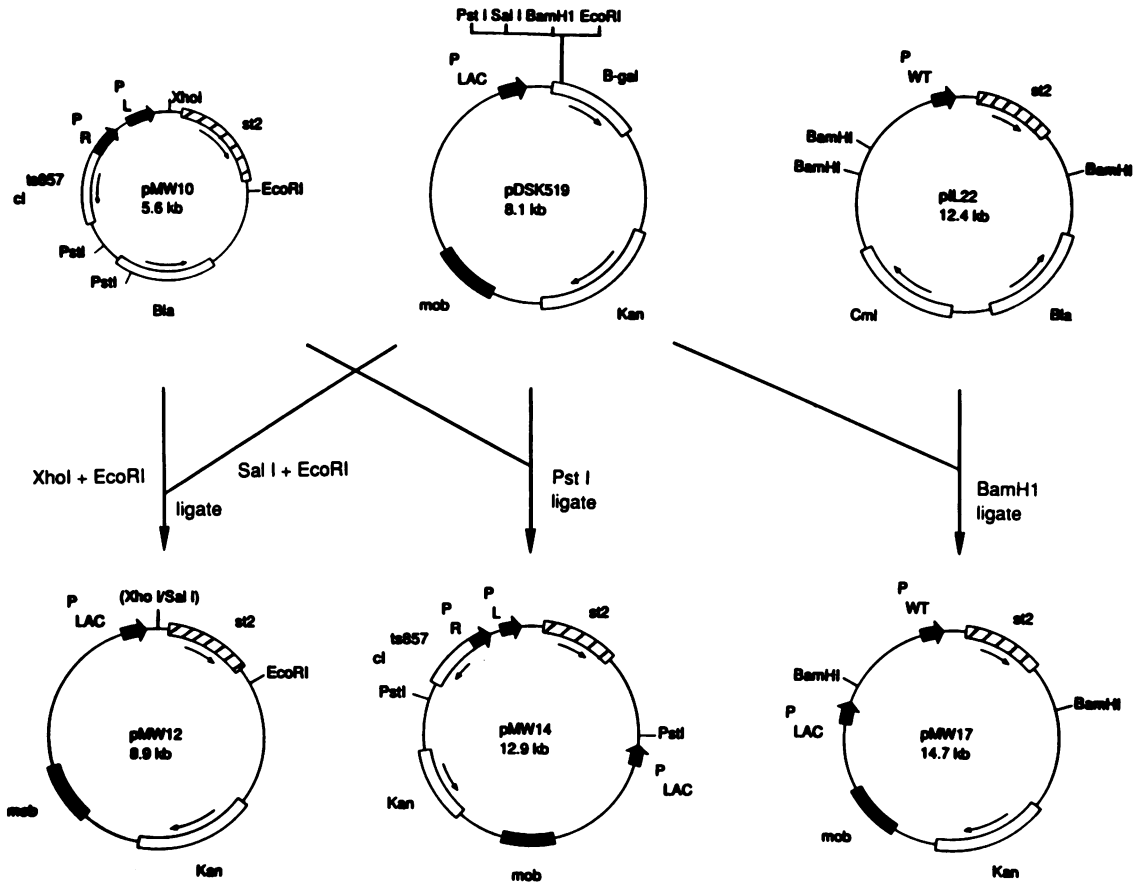

FIG. 2. Construction of broad-host-range plasmids to express the serotype 2 fimbrial subunit protein. Plasmid pIL22 (15) contains the wild-type serotype 2 fimbrial subunit gene (st2, hatched box) and promoter (P_{WT}, filled arrow) as a *Sau*3A fragment inserted into the *Bam*HI site of pBR328. Plasmid pMW10 contains the serotype 2 gene under the control of the lambda promoters p_L and p_R (filled arrows) (28). The broad-host-range vector pDSK519 (9) contains the *mob* site of pRSF1010 (mob, filled box), the lactose promoter (P_{LAC}, filled arrow), and the β-galactosidase α fragment (β-gal, open box). The lambda repressor (cI^{ts857}) and resistance genes for ampicillin (Bla), kanamycin (Kan) and chloramphenicol (Cml) are also represented by open boxes. The direction of transcription of each gene is indicated by thin arrows. Plasmids are not drawn to scale, and only relevant restriction sites are shown.

pMW12 and pMW14 after IPTG or temperature induction, respectively, but not in the same host containing pDSK519, which lacks the fimbrial gene insert; pMW17, which contains the wild-type promoter and gene sequence (Fig. 3A); or pMW18, which contains the same insert as pMW17 in the opposite orientation. Methionylated mature fimbrial subunit was also expressed from *E. coli* CAG629 containing pMW11 after IPTG induction. *E. coli* CAG629 expressing the fimbrial subunit did not produce whole fimbriae as detected by electron microscopic examination of the bacterial cell surface (results not shown).

Expression of recombinant fimbrial protein in *Bordetella* spp. Plasmids pDSK519, pMW11, pMW12, pMW14, pMW17, and pMW18 were conjugally transferred to *B. parapertussis* M/S180, *B. bronchiseptica* 5376, and *B. bronchiseptica* ATCC 10580. Western blot analysis showed that the fimbrial subunit gene was expressed in *B. parapertussis* M/S180 only when under the control of the wild-type *B. pertussis* promoter and Shine-Dalgarno sequence. Similar amounts of fimbrial subunit were produced independently of the orientation of the subunit gene with respect to P_{LAC} (pMW17 and PMW18). No expression was detected from the constructions designed to express the fimbrial subunit from P_{LAC} (pMW12) or lambda p_L and p_R (pMW14) promoters. Recombinant *B. parapertussis* M/S180 containing pMW17 or pMW18 produced higher levels of fimbrial subunit than *B. pertussis* Tohama, presumably as a consequence of the higher copy number of the subunit gene in these strains (Fig. 3B). In *B. bronchiseptica* 5376, the fimbrial subunit gene was expressed at increasing levels by recombinants containing pMW12, pMW18, and pMW17. The higher expression of subunit in pMW17 over that in pMW18 may arise from both P_{LAC} and the native promoter working in tandem, which would increase transcriptional levels of fimbrial subunit gene in *B. bronchiseptica* pMW17. Expression of the subunit protein from pMW12 suggests that P_{LAC} and the *atpE* TIR function in *B. bronchiseptica* but not in *B. parapertussis*. However, no gene expression was detected in recombinants containing pMW14, in which the fimbrial subunit gene is under the control of the lambda promoters (Fig. 3C). No fimbrial subunit expression was detected by Western blot analysis in *B. bronchiseptica* ATCC 10580 containing pMW12, pMW14, pMW17, or pMW18. This strain, in contrast to *B. bronchiseptica* 5376 and *B. parapertussis* M/S180, did not produce native fimbriae (as determined by electron microscopy) or filamentous hemagglutinin (analyzed by

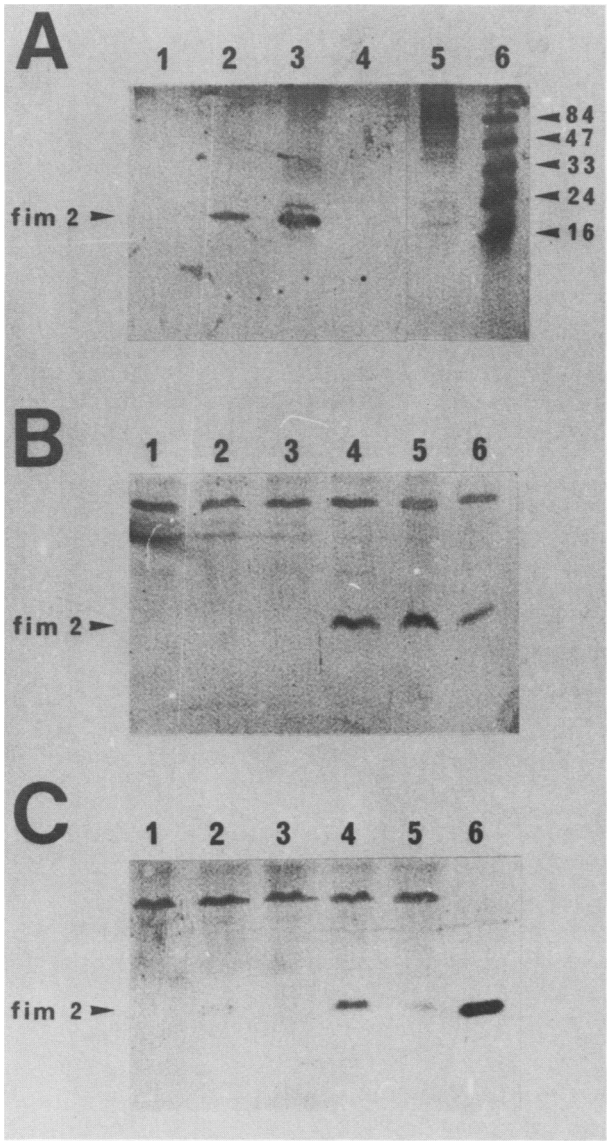

FIG. 3. Western blot analysis with antiserum raised against the recombinant fimbrial subunit purified from *E. coli*. (A) Lanes 1 to 4, *E. coli* CAG629 containing pDSK519, pMW12, pMW14, and pMW17, respectively; lane 5, *B. pertussis* Tohama (serotype 2); lane 6, prestained molecular mass markers (sizes given in kilodaltons). (B) Lanes 1 to 5, *B. parapertussis* M/S180 containing pDSK519, pMW12, pMW14, pMW17, and pMW18, respectively; lane 6, *B. pertussis* Tohama (serotype 2). (C) Lanes 1 to 5, *B. bronchiseptica* 5376 containing pDSK519, pMW12, pMW14, pMW17, and pMW18, respectively; lane 6, purified serotype 2 fimbriae. The fimbrial subunit (fim 2) and molecular mass markers and their sizes (in kilodaltons) are indicated by arrows. High-molecular-weight bands represent proteins that cross-react with the rabbit (A) or mouse (B and C) anti-serotype 2 subunit antiserum.

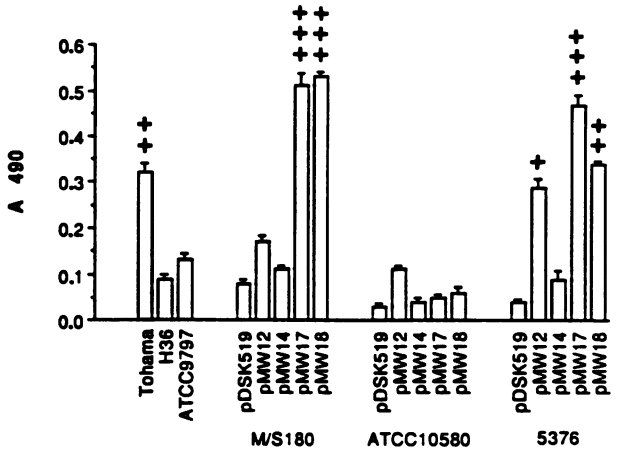

FIG. 4. Serotype 2-specific agglutination and ELISA with anti-serotype 2 fimbrial antibodies cross-adsorbed against *B. pertussis* H36, *B. parapertussis* M/S180, and *B. bronchiseptica* 5376. The strains tested were *B. pertussis* Tohama, *B. pertussis* H36, *B. pertussis* ATCC 9797, and derivatives of *B. parapertussis* M/S180, *B. bronchiseptica* ATCC 10580, and *B. bronchiseptica* 5376 containing pDSK519, pMW12, pMW14, pMW17, or pMW18. Agglutination ranged from slow (+) to medium (++) to fast (+++); those strains that did not agglutinate after 5 min were not designated. ELISA values (open bars) are expressed as A_{490} units per microgram of protein.

Western blotting). However, complementation of this strain with the *B. pertussis bvg* locus resulted in the production of both virulence-regulated phenotypes (results not shown). *B. bronchiseptica* ATCC 10580 was therefore assumed to be a *bvg* mutant. It was expected that pMW12 would still express subunit protein in this strain since P_{LAC} should function as a constitutive promoter; the failure to do so may have arisen from proteolytic degradation of the recombinant product because of the lack of expression of appropriate fimbrial accessory genes in this strain. Similarly, methionylated mature fimbrial subunit was not detected in *B. bronchiseptica* 5376 containing pMW11 (results not shown). The lack of expression of such protein may have resulted from proteolytic degradation of the fimbrial subunit in the cytoplasm of the host.

Immunological characterization of recombinant fimbriae expressed in *Bordetella* spp. Serotype 2-specific agglutination of the recombinant *Bordetella* spp. described above demonstrated that the fimbrial accessory genes of both *B. parapertussis* and *B. bronchiseptica* recognize and assemble *B. pertussis* fimbrial subunit (Fig. 4). Furthermore, the rapidity of agglutination paralleled the amount of fimbrial subunit expressed, as seen in Western blots (Fig. 3). This result was confirmed in ELISA experiments (Fig. 4). The highest levels of expression were measured for *B. bronchiseptica* 5376 (pMW17) and *B. parapertussis* M/S180(pMW17)(pMW18), which were followed by *B. bronchiseptica* 5376(pMW18) and *B. pertussis* Tohama and then finally by *B. bronchiseptica* 5376(pMW12). The values obtained for strains containing pMW12 were slightly higher than in those strains containing the vector alone (pDSK519) or the other recombinant plasmids, as can be most clearly seen for *B. bronchiseptica* ATCC 10580. This may be due to a nonspecific response. The reason for this nonspecific reaction was not investigated.

Immunoelectron microscopic examination of recombinant *Bordetella* spp. confirmed the fimbrial nature of the product. Those strains that did not express the serotype 2 fimbrial subunit, as represented by both *B. parapertussis* M/S180 and *B. bronchiseptica* 5376 harboring pDSK519 (Fig. 5B and D) were not labeled by protein A-gold complexes after incubation with serotype 2-specific antibody, even though wild-type fimbriae were present. On the other hand, when sero-

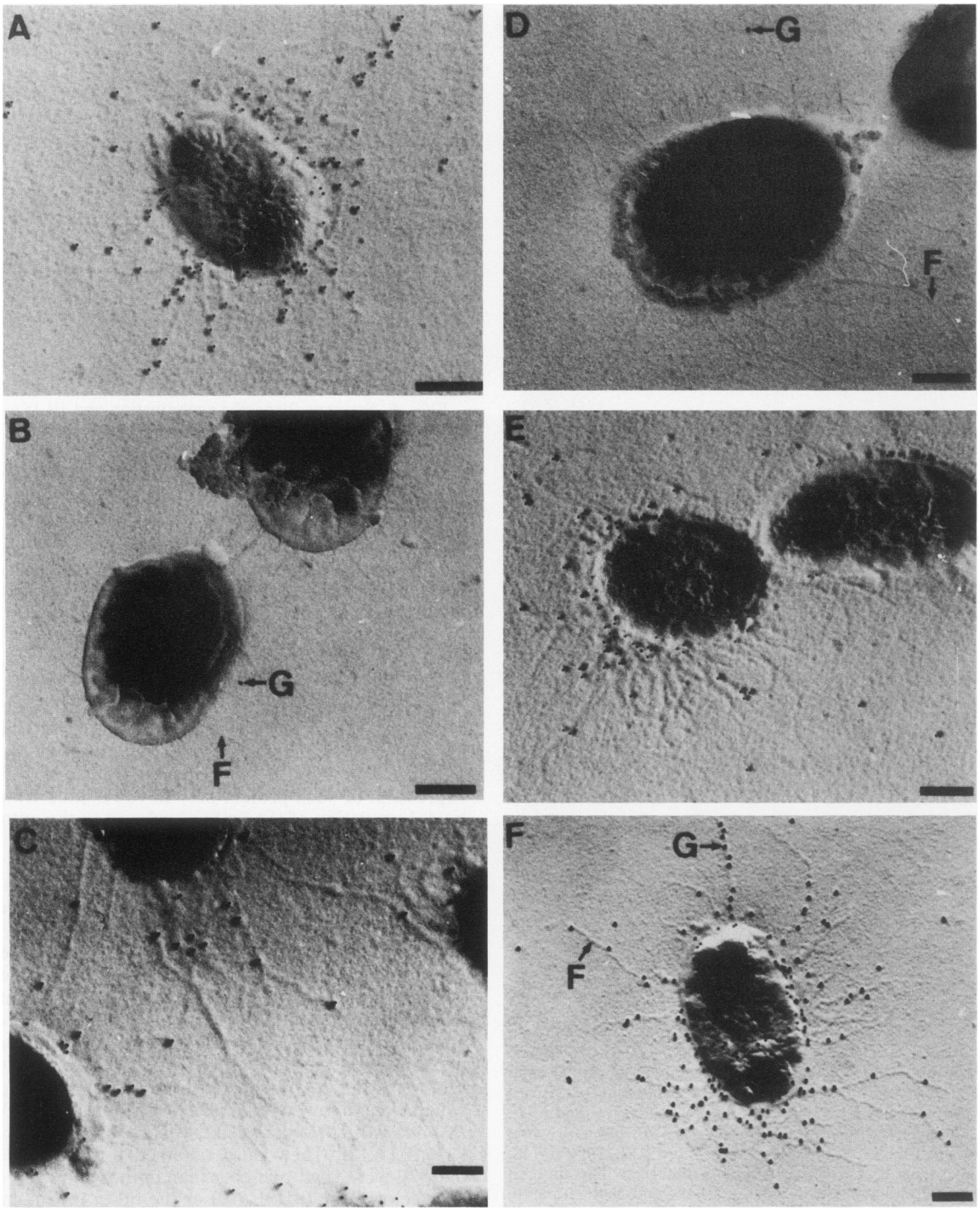

FIG. 5. Immunoelectron microscopic localization of serotype 2 fimbrial protein by using the protein A-gold procedure. Cells were adsorbed to collodium films, air-dried, and incubated with serotype 2 fimbrial antiserum that had been cross-adsorbed with *B. pertussis* H36, *B. parapertussis* M/S180, and *B. bronchiseptica* 5376 bacteria. The bound antibodies were visualized by incubation with protein A-gold complexes (10 nm). Shown are *B. pertussis* Tohama (A); *B. parapertussis* M/S180 containing pDSK519 (B) and pMW17 (C); and *B. bronchiseptica* 5376 containing pDSK519 (D), pMW12 (E), and pMW17 (F). G, Gold particle; F, fimbriae. Bars, 0.25 μm.

type 2-specific fimbriae were produced, as represented by *B. pertussis* Tohama, *B. parapertussis* M/S180(pMW17), and *B. bronchiseptica* 5376 containing pMW12 or pMW17, the fimbriae were specifically labeled (Fig. 5A, C, E, and F). The fate of the original fimbrial subunit in these recombinant strains is unknown since most of the fimbriae produced in strains expressing the serotype 2 subunit are antibody labeled. In *B. bronchiseptica* 5376(pMW12), in which serotype

2 subunit expression is least, the intensity of fimbrial immunolabeling was decreased compared with *B. pertussis* Tohama and *B. bronchiseptica* 5376(pMW17) fimbriae. This suggests that the fimbriae are of a mixed nature, containing both wild-type and recombinant fimbrial subunits. Fimbriae were not detected on every bacterium for all the strains tested which were taken from BG plates (Fig. 5D and E). It was found that vigorous resuspension of cells resulted in loss through shearing of almost all fimbriae and that gentle cell suspension was a critical step in fimbrial detection. However, fimbrial phase variation may also play a role in this process (30).

DISCUSSION

It has been demonstrated that the serotype 2 fimbrial subunit protein is immunologically distinct from whole fimbriae (14). In an earlier communication, we reported expression of the *B. pertussis* serotype 2 fimbrial subunit in *E. coli*. After purification and renaturation, the subunit assembled to form fimbriallike structures. However, although the recombinant subunit polymers and wild-type fimbriae were composed of the same protein subunit, they exhibit only limited cross-reactivity (28). In this report we demonstrate that recombinant serotype 2 fimbrial subunit is produced in *Bordetella* spp. expressing fimbrial accessory genes as fimbriae that are indistinguishable from native fimbriae. As was previously shown for the pertussis toxin promoter (13), the serotype 2 fimbrial subunit promoter was found to function in both *B. parapertussis* and *B. bronchiseptica*. Although the regions upstream of both the pertussis toxin operon and the serotype 2 fimbrial subunit gene show some degree of homology (30), the distances between the start sites of transcription and translation are very different, namely, 25 nucleotides in the case of the pertussis toxin S1 subunit (19a) and 146 nucleotides in the case of the serotype 2 fimbrial subunit gene. Expression of fimbriae in *B. pertussis* is controlled by the *bvg* virulence regulatory locus and is subject to fimbrial phase variation. Recently, Willems et al. (30) reported that fimbrial phase variation may be due to insertion-deletion events within a C-rich region located some 57 nucleotides upstream of the ATG start site of the serotype 2 subunit gene. However, further investigation is required to determine whether or not other factors, including the inverted repeat region that overlaps the wild-type transcriptional start site (Fig. 1B), are involved in the control of fimbrial expression.

In contrast to *B. parapertussis*, in which only the native *B. pertussis* promoter was functional, the heterologous expression signals P_{LAC} and the *atpE* TIR functioned in *B. bronchiseptica* 5376 to express the fimbrial subunit. Although this construction was expected to function constitutively, the fimbrial subunit was not detected by Western blot analysis in nonfimbriated *B. bronchiseptica* ATCC 10580. The lack of fimbrial accessory gene expression as a result of the *bvg* mutant genotype of this strain may result in proteolytic degradation of the fimbrial subunit. Similarly, another construction designed to express the methionylated mature form of the fimbrial subunit did not produce detectable levels of subunit protein in *B. bronchiseptica* 5376. These experiments highlight the importance of fimbrial accessory genes and correct processing and transport of the fimbrial subunit to the periplasm: loss of either function results in a total absence of product accumulation. We are currently trying to isolate the *B. pertussis* fimbrial accessory genes, with the expectation that constitutive expression of such genes may facilitate the expression of *B. pertussis* fimbriae in *B. bronchiseptica* and *E. coli* strains.

For the preparation of a recombinant acellular whooping cough vaccine, purified antigens that are immunologically similar to the wild-type form must be produced. It has been shown that both recombinant pertussis toxin and serotype 2 fimbrial subunit are antigenically distinct when prepared from *E. coli* (3, 28) but that recombinant pertussis toxin subunits produced in *B. parapertussis* and *B. bronchiseptica* assemble as native holotoxin (13). As shown here, recombinant serotype 2 fimbrial subunit is also produced as whole fimbriae in *Bordetella* spp. expressing fimbrial accessory genes. Such bacteria offer several advantages for antigen production over *B. pertussis* with regard to growth rates and requirements (13). Furthermore, the fimbrial subunit was expressed in *B. bronchiseptica* under the control of P_{LAC} and the *atpE* TIR. The constitutive expression of vaccine components in *B. bronchiseptica* may offer considerable advantages in terms of product yields.

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