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Construction of Minitransposons for Constitutive and Inducible Expression of Pertussis Toxin in *bvg*-Negative *Bordetella bronchiseptica*

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Appropriately detoxified pertussis toxin (PT) of *Bordetella pertussis* is considered to be an essential component of new-generation whooping cough vaccines, but the development of a procedure to obtain high levels of purified toxin has been and continues to be a major difficulty. To produce a system enabling the biological separation of PT from other virulence determinants of *B. pertussis* and the attainment of high yields of the toxin, minitransposons containing the PT operon were constructed and stably integrated into the chromosome of *Bordetella* virulence regulatory gene (*bvg*)-negative *Bordetella bronchiseptica* ATCC 10580. Since the minitransposons introduced into *Bordetella* spp. lack the cognate transposase function, they are unable to undergo further transposition events or mediate gene deletions and rearrangements that lead to strain instability. The Tn*PtacPT* minitransposon contains the PT operon under the control of the *tac* promoter and directs IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression of PT in *B. bronchiseptica* ATCC 10580. The level of IPTG-induced PT expression was, however, lower than that found for the wild-type *B. pertussis* Tohama I strain. The Tn*fusPT* minitransposon contains a promoterless PT operon which is only expressed after insertion of the transposon downstream of an appropriately oriented indigenous promoter. After "promoter probing" of *B. bronchiseptica* with the transposon, clones were screened for PT production by immunoblotting with specific monoclonal antibodies. One clone, designated *B. bronchiseptica* 10580:: Tn*fusPT1*, expresses significantly higher levels of PT than does *B. pertussis* Tohama I. The recombinant toxin produced was biologically active in the Chinese hamster ovary cell-clustering assay. High-level expression of PT from a *B. bronchiseptica* host promoter should provide better yields of the toxin from bacteria not producing other *bvg*-regulated pathogenesis factors that may play a role in the undesired side effects of current pertussis vaccine preparations.

Increasing concern about side effects of immunization with whole-cell preparations of *Bordetella pertussis* has, in several countries, led to decreased acceptability of whooping cough vaccines and a consequent increase in the incidence of the disease. It is imperative that an effective, nonreactogenic vaccine of higher acceptability be developed. Potentially protective antigens that are candidates for inclusion in a purified component vaccine include toxins, such as pertussis toxin (PT) and adenylate cyclase, and several cell surface or secreted antigens like filamentous hemagglutinin (FHA) and serotype-specific fimbriae (33, 34). Purification of antigens directly from *B. pertussis* is hampered by phase variation controlled by the *bvg* (*Bordetella* virulence gene) positive regulatory locus (12, 19, 49), the presence of reactogenic contaminants, poor bacterial growth rates, and low yields from this nutritionally fastidious microorganism. To circumvent 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 PT have been separately cloned and expressed in *E. coli* under the control of the lambda p_L promoter (6). The serotype 2 fimbrial subunit has also been expressed from the lambda p_L and p_R promoters (47). In both cases, the recombinant proteins failed to assemble into products that were immunologically identical to the native antigens. Recombinant serotype 2 fimbriae have been produced in *Bordetella parapertussis* and *Bordetella bronchiseptica* strains under the control of the native *B. pertussis* promoter and in *B. bronchiseptica* under the control of the *lac* promoter (46). Pertussis holotoxin has also been produced in recombinant *B. parapertussis* and *B. bronchiseptica* strains under the control of the original *B. pertussis* promoter, although the level of expression obtained was less than that in wild-type *B. pertussis* and such expression remained subject to modulation and *bvg*-controlled phase variation. Recombinant broad-host-range plasmids carrying the PT operon were also extremely unstable, being subject to both plasmid loss and gene deletions (21).

Pertussis toxoid will be a central component of new-generation vaccines, and the development of a system for the stable production of high yields of toxin free of potential reactogenic virulence determinants which may play a role in the observed side effects of pertussis vaccination is a major preoccupation of a number of laboratories. In this report, we describe the construction of minitransposons for the stable expression of native pertussis holotoxin in a *bvg*-negative strain of *B. bronchiseptica*. This strategy, in combination with methods for the genetic detoxification of PT (18, 23, 30), should provide a source for the production of

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a highly purified nonreactogenic acellular vaccine component.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *B. pertussis* Tohama I (serotype 1.2) *fim*$^+$ *fha*$^+$ (40) and *B. bronchiseptica* 5376 *fim*$^+$ *fha*$^+$ were obtained from R. Brownlie; *B. bronchiseptica* ATCC 10580 *fim fha* was obtained from M. Höfle. *E. coli* JM109 *endA1 recA hsdR supE thi gyr lac-pro* F' *tra-36 proAB lacI*^{q}Z M15 was used as the recipient strain in cloning steps not involving pUT derivative plasmids (51); *E. coli* CC118(λ*pir*) was used as the recipient strain for pUT derivatives (14); *E. coli* SM10(λ*pir*) (27) was used to mobilize pUT derivatives (8, 14) to *B. bronchiseptica*. The plasmids used in this work were pRK600 (9), pDSK519 (17), and pUC18 (51). pRMB2 (4, 25) was used as a source of the *bvg* operon. pTX42 (22) was a kind gift from J. Keith and was used as a source of the PT operon, pUC18NotI (14), pUT::miniTn*5*/*Km* (8), and pUT::Tn*Ptac*, which was constructed by V. De Lorenzo.

E. coli was grown on Z agar (45), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) medium (24), and Luria broth (24), and *Bordetella* spp. were grown on BG agar or in SS broth (43). Where appropriate, strains were grown with 0.2% cyclodextrin, 100 μg of ampicillin per ml, 50 μg of cephalexin per ml, or 50 μg of kanamycin per ml. IPTG (isopropyl-β-D-thiogalactopyranoside) induction was carried out by the addition of 1.0 mM IPTG. Bacterial cultures were routinely grown at 37°C. Liquid cultures were aerated by shaking at 300 rpm in a New Brunswick Environmental Incubator Shaker.

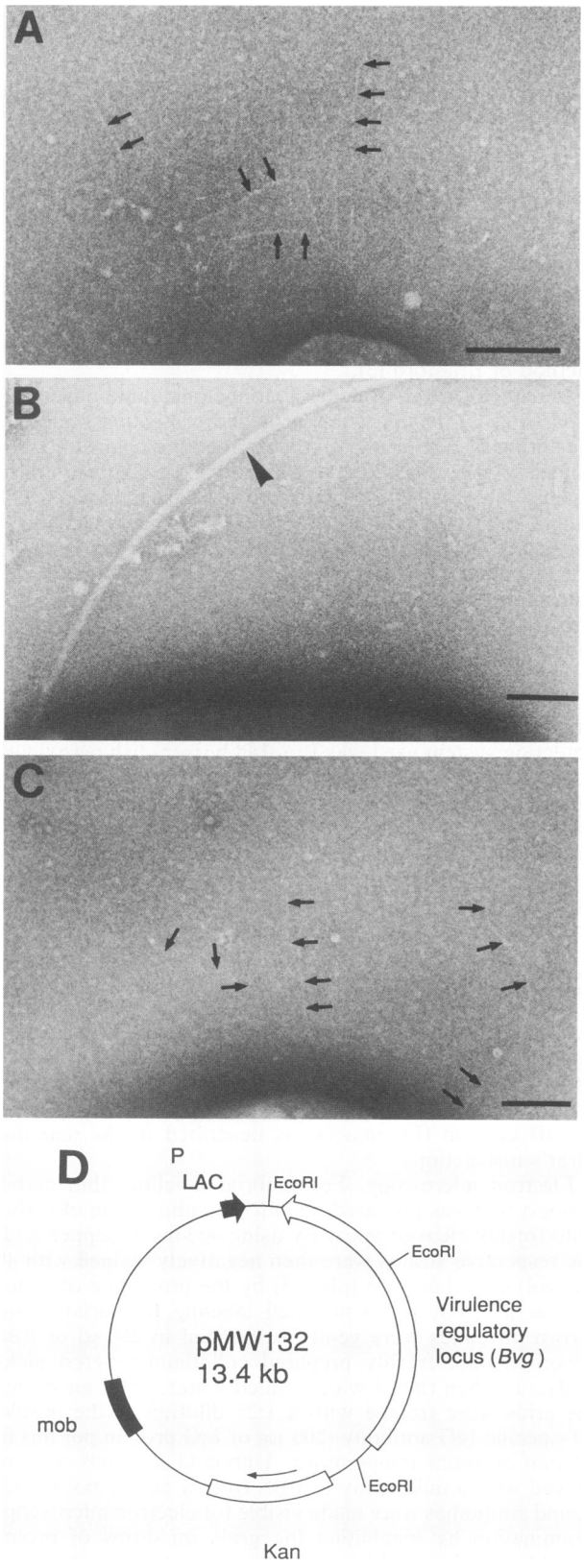
DNA manipulations. Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were used essentially as described by Maniatis et al. (24). Plasmids were isolated by alkaline lysis (24). Transformation was carried out as described by Hanahan (13). Agarose gel electrophoresis was performed as previously described (45). Southern transfer and DNA hybridization with nick-translated DNA as a probe were performed as described by Maniatis et al. (24). Chromosomal DNA was isolated as described previously (32). DNA sequencing was carried out by the chain termination method (37). *Taq* polymerase (Boehringer) was used as described by Scharf (41). The polymerase chain reaction (PCR) primers PT-5'*Xba*I (5'-GGTCTAGAATCAAAACG CAGAGGGGAAGA-3') and PT-3'*Xba*I (5'-CCAGGTCTA GAACGAATA-3') used for cloning the transcriptional start site (31) and S1 subunit cistron of the PT operon as an *Xba*I fragment (*Xba*I sites are underlined) were synthesized by using an Applied Biosystems model 380B DNA synthesizer in accordance with the manufacturer's instructions.

Transfer of plasmids to *B. bronchiseptica*. *E. coli* strains containing both pRK600 and pDSK519 derivatives or *E. coli* SM10(λ*pir*) containing pUT derivatives was grown overnight in nutrient broth with antibiotic selection. A 1.0-ml sample of 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 1-day-old plate cultures of *B. bronchiseptica*, and the plates were incubated for 4 h at 37°C. The mating mixtures were then plated, selecting for *B. bronchiseptica* recombinants with cephalexin (contraselection against the *E. coli* donor strain) and kanamycin (selection for the plasmid or minitransposon).

Protein purification and analysis. Whole-cell extracts of *Bordetella* spp. were prepared by resuspending cells in sample buffer (60 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and boiling for 5 min. Samples were electrophoresed by the method of Laemmli (20) by using a 3.85% acrylamide stacking gel and a 15% acrylamide separating gel. PT isolated from *B. pertussis* was a kind gift from S. Cryz. Recombinant PT from *B. bronchiseptica* ATCC 10580 was partially purified from periplasmic extracts (21) by using heparin Sepharose CL-6B chromatography by the method of Megret and Alouf (26). Chinese hamster ovary (CHO) cell-clustering assays were performed to ascertain the biological activity of recombinant PT as previously described (11, 15). Inhibition of CHO cell clustering with protective monoclonal antibodies was carried out essentially as described by Sato et al. (39). Protein concentration was determined by the method of Bradford (3).

Immunological techniques. Monoclonal antibodies E19, E205, and E251 (48), which provided protection against PT clustering of CHO cells and were reactive against PT subunits S1 (E19), S4 (E205), and S2 and S3 (E251), were raised against detoxified PT (29) by using standard techniques (39). Monoclonal antibody P12H3 to FHA was previously described (10). Western blotting (immunoblotting) was carried out essentially as described by Burnette (5). Proteins were transferred to a nitrocellulose membrane by using 25 mM Tris–192 mM glycine–20% methanol (pH 8.3) as a transfer buffer and a 10% solution of 0.3% low-fat milk in phosphate-buffered saline (PBS) (pH 7.4) as a blocking reagent. A cocktail of appropriately diluted E19, E205, and E251 hybridoma supernatant fluid was used as the first antibody. The detection system used was Bio-Rad horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (IgG) with 4-chloro-1-naphthol as substrate. Colony immunoblotting was carried out by performing colony lifts onto nylon membranes (Biodyne A, Pall), baking the filters at 65°C, and then performing essentially the same procedure as Western blotting after the transfer step. Dot immunoblotting was carried out by heat inactivation of a sample of the bacterial culture (grown at 37°C with shaking in 200 ml of SS-X medium) at 65°C for 15 min, spotting 50 μl of the culture onto a nylon membrane (Biodyne A, Pall), and then performing essentially the same procedure used for Western blotting after the transfer step. For immunoelectron microscopy, antibodies were purified from rabbit antisera raised against pertussis toxoid (29) by using a protein A–Sepharose CL-4B column (Pharmacia) as described in the manufacturer's instructions.

Electron microscopy. For fimbrial labeling, thin carbon support film was prepared by indirect sublimation of carbon onto freshly cleaved mica. By using 400-mesh copper grids, the respective strains were then negatively stained with 4% (wt/vol) uranyl acetate (pH 4.5) by the procedure of Valentine et al. (44). For whole-cell labeling, 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 air drying, the grids were treated with a 1:25 dilution of the purified PT-specific IgG antibody (200 μg of IgG protein per ml) for 30 min at room temperature. Unbound antibody was removed with 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 at an optical density at 520 nm of 0.01) for 10 min at room temperature. The protein A-gold complexes were prepared by established procedures (42). The grids were subsequently rinsed with a mild spray of PBS containing 0.01% Tween 20 and then with



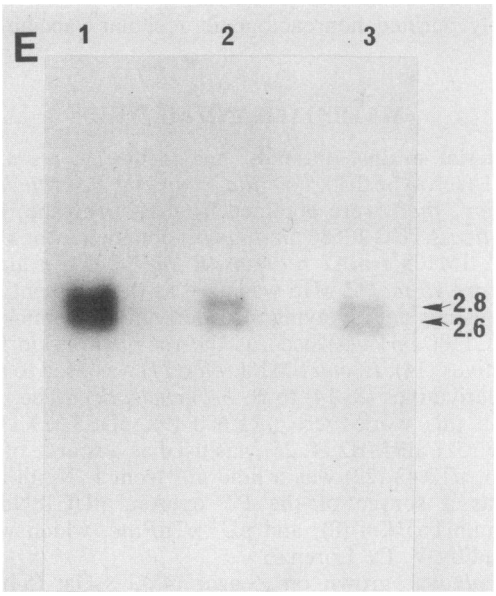


FIG. 1. *bvg* status of *B. bronchiseptica* 5376 and ATCC 10580. Fimbria production by *B. bronchiseptica* 5376 (A), *B. bronchiseptica* ATCC 10580 (B), and *B. bronchiseptica* ATCC 10580(pMW132) (C) is shown. Arrows indicate fimbriae, while the arrowhead indicates a flagellum. Bar, 0.1 μm. (D) Diagrammatic representation of plasmid pMW132, a derivative of pDSK519 (17) containing the *bvg* operon (open arrow indicates the direction of transcription) cloned from pRMB2 (4, 25), *lac* promoter (filled arrow), kanamycin resistance gene (open box; the direction of transcription is indicated by the thin arrow), and *mob* site of pRSF1010 (filled box). (E) Southern hybridization analysis, using the *bvg* operon as a probe, of chromosomal DNA isolated from *B. bronchiseptica* 5376 (lane 1), *B. bronchiseptica* ATCC 10580 (lane 2), and *B. pertussis* Tohama I (lane 3). The sizes of reacting bands are given in kilobases.

distilled water. After air drying, the grids were examined in an electron microscope. For postembedding labeling, cells of the respective strains were fixed directly in the nutrient broth by use of a fixative solution containing 0.5% (vol/vol) formaldehyde and 0.3% (vol/vol) glutaraldehyde in PBS for 1 h on ice. After washing with PBS containing 10 mM glycine, the samples were embedded into 1.5% (wt/vol) agar, and after solidification of the agar, small cubes were cut and embedded by use of a method involving the progressive lowering of the temperature with Lowicryl K4M resin (35). Immunolabeling was done with a 1:25 dilution of the purified PT-specific IgG antibody (200 μg of IgG protein per ml) by incubating ultrathin sections on drops of the diluted antibody for 5 h at room temperature. After being washed with PBS, the bound antibodies were made visible with protein A-gold complexes (10-nm gold particle size; 30-min incubation time). Subsequently, the sections were washed with PBS containing 0.01% Tween 20 and the sections were air dried prior to poststaining with 4% (wt/vol) uranyl acetate for 3 min. Electron micrographs were examined and micrographs were taken with a Zeiss electron microscope EM 10B at an acceleration voltage of 80 kV and at calibrated magnifications.

RESULTS

Characterization of *B. bronchiseptica* 5376 and ATCC 10580. The *Bordetella* virulence regulatory genes (*bvg*) coordinately regulate a number of virulence determinants of

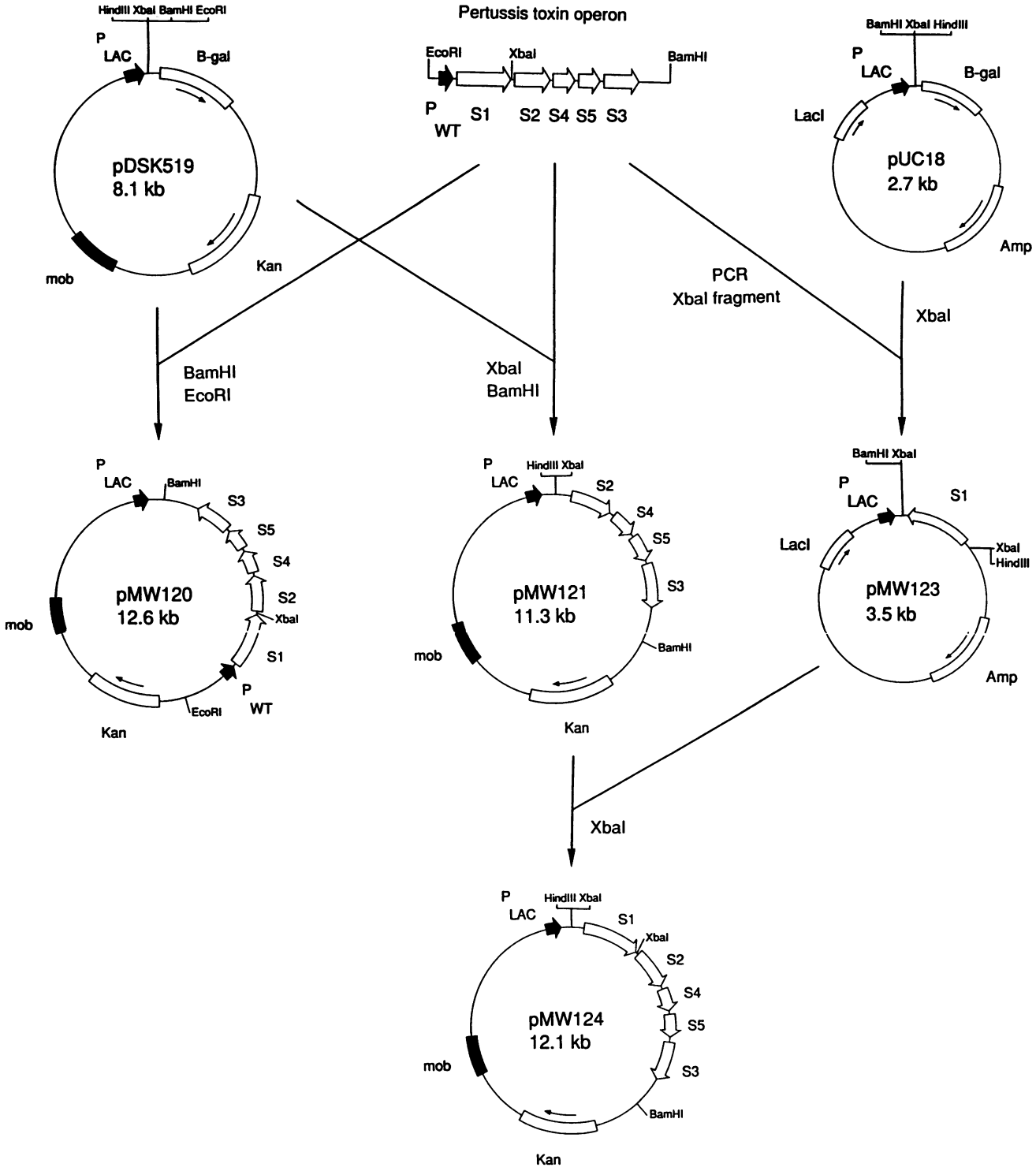


FIG. 2. Construction of broad-host-range hybrid plasmids specifying PT expression controlled by the native *B. pertussis* promoter (pMW120) and *lac* promoter (pMW124). The PT operon was cloned from plasmid pTX42 (22). PT subunit genes S1 to S5 (open arrows) and the PT promoter (filled arrow) are indicated. The broad-host-range plasmid vector pDSK519 contains the *mob* site of pRSF1010 (*mob*; filled box), *lac* promoter (P_{LAC}; filled arrow), β-galactosidase α fragment sequence (β-gal; open box), and kanamycin resistance gene (Kan; open box). Plasmid pUC18 (51) contains the lactose repressor gene (LacI; open box), ampicillin resistance gene (Amp; open box), *lac* promoter, and β-galactosidase α fragment sequence (as described for pDSK519). The direction of transcription is indicated by either thin arrows or the orientation of open arrows. Plasmids are not drawn to scale, and only relevant restriction sites are shown.

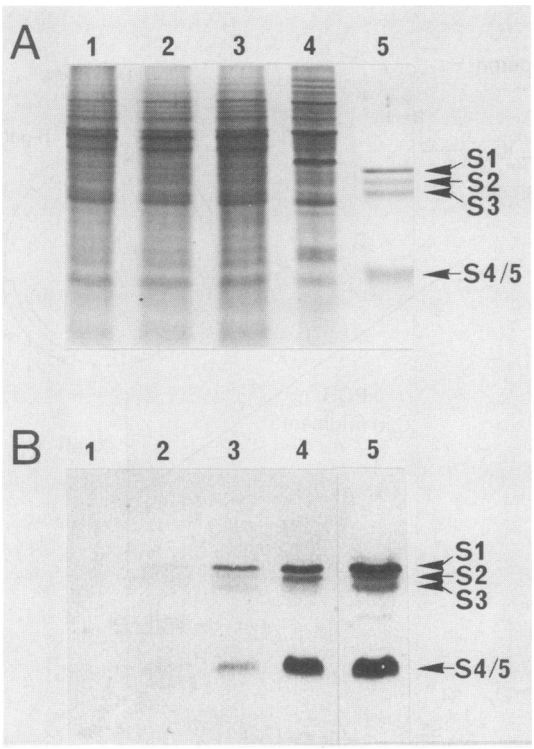


FIG. 3. Analysis of PT expression by *B. bronchiseptica* 5376 recombinants. Whole-cell extracts of bacteria containing pDSK519 (lane 1), pMW120 (lane 2), and pMW124 (lane 3) are shown, as are an extract of *B. pertussis* Tohama I (lane 4) and purified PT (lane 5). (A) Coomassie blue-stained gel of whole-cell extracts; (B) Western blot analysis of whole-cell extracts by using anti-PT monoclonal antibodies E19, E205, and E251. The positions of the PT subunits S1 to S5 are indicated by arrows.

the genus *Bordetella*, including FHA and serotype-specific fimbriae (19, 49). It would be advantageous to produce pertussis vaccine components in *bvg*-negative *Bordetella* spp. and hence achieve the biological separation of vaccine components from other *bvg*-regulated virulence factors that may play a role in the observed side effects of whole-cell vaccines. The *B. bronchiseptica* strains used in this work were studied for the production of FHA and fimbriae to determine the *bvg* status of these strains. By using electron microscopy and the anti-FHA monoclonal antibody P12H3 in Western blots, it was found that strain 5376 produced both fimbriae (Fig. 1A) and FHA (results not shown). On the other hand, strain ATCC 10580 failed to express either of these *bvg*-regulated genes (Fig. 1B and results not shown). However, the latter strain converted to a Fim^+ (Fig. 1C) Fha^+ (results not shown) phenotype through acquisition of pMW132 (Fig. 1D), a plasmid constructed by insertion of the *B. pertussis bvg* operon present in pRMB2 (4, 25) into the broad-host-range vector pDSK519. After transfer of this plasmid to ATCC 10580, transconjugants were found to have a small-colony morphology as previously noted by other workers (25, 28). *B. bronchiseptica bvg*-negative derivatives can arise through spontaneous small deletions or frameshift mutations occurring in the *bvg* locus (28). However, no obvious deletion of the *bvg* operon of ATCC 10580 could be detected by Southern hybridization of *Eco*RI-digested chromosomal DNA by using the cloned *bvg* determinant as a probe (Fig. 1E), indicating that a deletion of less than 100 bp (which would be undetectable by Southern analysis) or a frameshift mutation had occurred.

Broad-host-range plasmid-based expression of PT from the *lac* promoter and native *B. pertussis* promoter. The PT operon was subcloned into the broad-host-range plasmid pDSK519 to assess the utility of the *lac* promoter and the native *B. pertussis* promoter in *B. bronchiseptica*. The complete PT operon was cloned as an *Eco*RI-*Bam*HI fragment to produce pMW120, in which the PT operon is oriented counter to that of the *lac* promoter. Plasmid pMW121 contains the *Xba*I-*Bam*HI fragment containing the S2-, S4-, S5-, and S3-subunit cistron part of the operon inserted into pDSK519, such that these cistrons are read from the *lac* promoter. Primers PT-5′*Xba*I and PT-3′*Xba*I were used in a PCR amplification experiment to isolate a DNA fragment containing the transcriptional start site of the PT operon (31), and the S1 subunit cistron, flanked by *Xba*I sites, was cloned into pUC18 to produce pMW123. This *Xba*I fragment was subsequently ligated to the genes encoding the S2, S4, S5, and S3 subunits contained within pMW121 to construct pMW124, which contains the PT operon under the control of the *lac* promoter (Fig. 2). Plasmids pDSK519, pMW120, and pMW124 were transferred to *B. bronchiseptica* 5376 and analyzed for PT expression. Although the *lac* promoter directed significantly greater levels of PT than the native *B. pertussis bvg*-regulated promoter, these constructions did not attain the level of PT production found in *B. pertussis* Tohama I (Fig. 3). PT expression from pMW124 was not influenced by the presence or absence of 1 mM IPTG, indicating that this strain did not contain a *lac* repressor that could regulate the *lac* promoter (results not shown).

Construction of the minitransposons Tn*PtacPT* and Tn*fusPT* and their integration into the chromosome of *B. bronchiseptica* ATCC 10580. Minitransposons containing the PT operon were constructed to hyperexpress PT in *bvg*-negative *B. bronchiseptica* ATCC 10580. The PT operon was cloned from pMW124 into pUC18NotI to provide flanking *Not*I sites for insertion into the pUT::miniTn*5* chromosomal integration system. The promoterless PT operon was subcloned from pMW125 as a *Not*I fragment in the same orientation as the *tac* promoter of pUT::Tn*Ptac* to form pMW126 (Tn*PtacPT*) and in the opposite orientation in pUT::miniTn*5*/*Km* to produce pMW127 (Tn*fusPT*) (Fig. 4). After mobilization of pMW126 and pMW127 from *E. coli* to *B. bronchiseptica* ATCC 10580, transconjugants were screened for PT production (by colony immunoblotting and Western blotting) and loss of the suicide vector (by kanamycin resistance and ampicillin sensitivity). The levels of PT expression by different Tn*PtacPT* transconjugants (including one transconjugant designated ATCC 10580::Tn*PtacPT1*) were similar and were less than that by *B. pertussis* (Fig. 5); PT expression was dependent upon the presence of IPTG (results not shown). On the other hand, fewer than 10% of transconjugants containing Tn*fusPT* expressed PT, and the level of PT expression in those that did varied greatly (results not shown), as would be expected from "promoter probing" with a transposon designed to produce transcriptional fusions between native promoters and the promoterless PT operon. One selected Tn*fusPT* transconjugant (ATCC 10580::Tn*fusPT1*) directed substantially higher levels of PT expression than did *B. pertussis* (Fig. 5). To confirm the chromosomal location of both Tn*PtacPT1* and Tn*fusPT1* and the loss of the suicide vector, total DNA from ATCC 10580, ATCC 10580::Tn*PtacPT1*, and ATCC 10580::Tn*fusPT1* was digested with *Eco*RI and subjected to Southern hybridization analysis by using pMW126 as a probe (Fig. 6). The loss of the

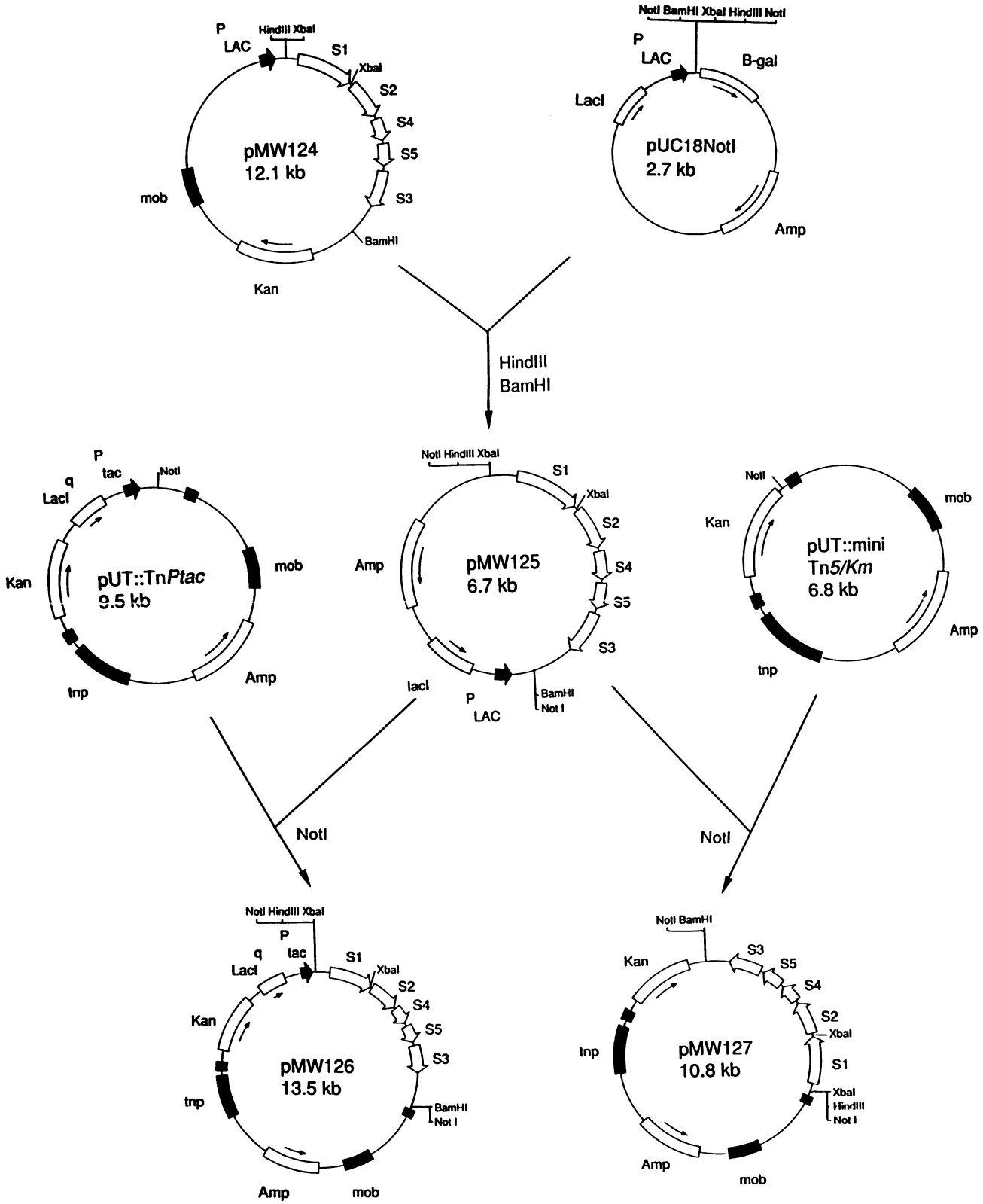


FIG. 4. Construction of suicide vectors containing minitransposons for PT expression under the control of the *tac* promoter (pMW126) and as transcriptional fusions with native promoters (pMW127). Symbols representing the PT subunits S1 to S5, *lac* promoter, lactose repressor gene, β-galactosidase α subunit sequence, *mob* site, kanamycin resistance gene, and ampicillin resistance gene are as described in the legend to Fig. 2. Plasmid pUT::miniTn*5/Km* and pUT::Tn*Ptac* contain the IS*50*$_R$ transposase (*tnp*; filled box) and Tn*5* 19-bp terminal ends (short, filled boxes) flanking the kanamycin resistance gene of pUT::miniTn*5/Km* and flanking the kanamycin resistance gene, lactose repressor gene, and *tac* promoter (P_{tac}; filled arrow) of pUT::Tn*Ptac*. The Tn*5* terminal ends delineate the minitransposons Tn*PtacPT* (pMW126) and Tn*fusPT* (pMW127). Plasmids are not drawn to scale, and only relevant restriction sites are shown.

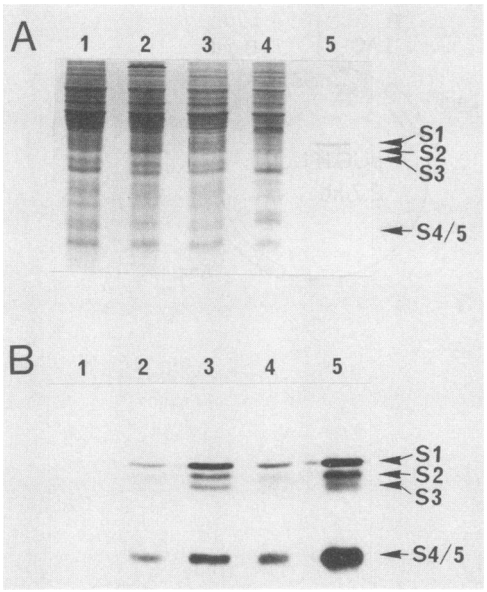


FIG. 5. Analysis of PT expression by minitransposon-containing derivatives of *B. bronchiseptica* ATCC 10580. Whole-cell extracts of bacteria lacking Tn*PtacPT1* and Tn*fusPT1* (lane 1) or containing Tn*PtacPT1* (lane 2) or Tn*fusPT1* (lane 3) are shown, as are extracts of *B. pertussis* Tohama I (lane 4) and purified PT (lane 5). (A) Coomassie blue-stained gel of whole-cell extracts; (B) Western blot analysis of whole-cell extracts, using anti-PT monoclonal antibodies E19, E205, and E251. The positions of the PT subunits S1 to S5 are indicated by arrows.

pUT suicide vector was confirmed. For Tn*fusPT1*, the next available *Eco*RI site upstream of the PT operon was provided by the chromosomal DNA sequence. The cryptic PT operon of *B. bronchiseptica* (1) was also detected.

Characterization of PT expression in *B. bronchiseptica* ATCC 10580::Tn*fusPT1*. The stabilities of plasmid-encoded PT (pMW124) and transposon-encoded PT (Tn*fusPT1*) expressed in ATCC 10580 were compared after growth in SS-X medium (Table 1). Clearly, even after 60 generations without antibiotic selection, transposon-encoded PT appeared to be stable. On the other hand, plasmid-encoded PT was very unstable. PT production was 100% linked to kanamycin resistance in strains containing or having previously contained pMW124, indicating that internal deletions of the PT operon had not occurred. Growth rates and levels of PT production in ATCC 10580, ATCC 10580::Tn*fusPT1*, and *B.*

TABLE 1. Stability of PT expression from *B. bronchiseptica* ATCC 10580(pMW124) and ATCC 10580::Tn*fusPT1* after 60 generations without antibiotic selection

Strain	No. of bacteria/ml[a]		% PT producers[b]	% Linkage of PT to Km[r][c]
	Cp^r	Cp^r Km^r		
ATCC 10580(pMW124)	2.1×10^9	3.0×10^5	<1	100
ATCC 10580::Tn*fusPT1*	3.0×10^9	2.9×10^9	100	100

[a] Strains were cultured for 60 generations without kanamycin selection and then plated with selection for cephalexin (Cp) alone or in combination with kanamycin (Km).

[b] A total of 100 Cp^r colonies were screened for PT production by colony immunoblotting.

[c] A total of 100 Cp^r Km^r colonies were screened for PT production by colony immunoblotting.

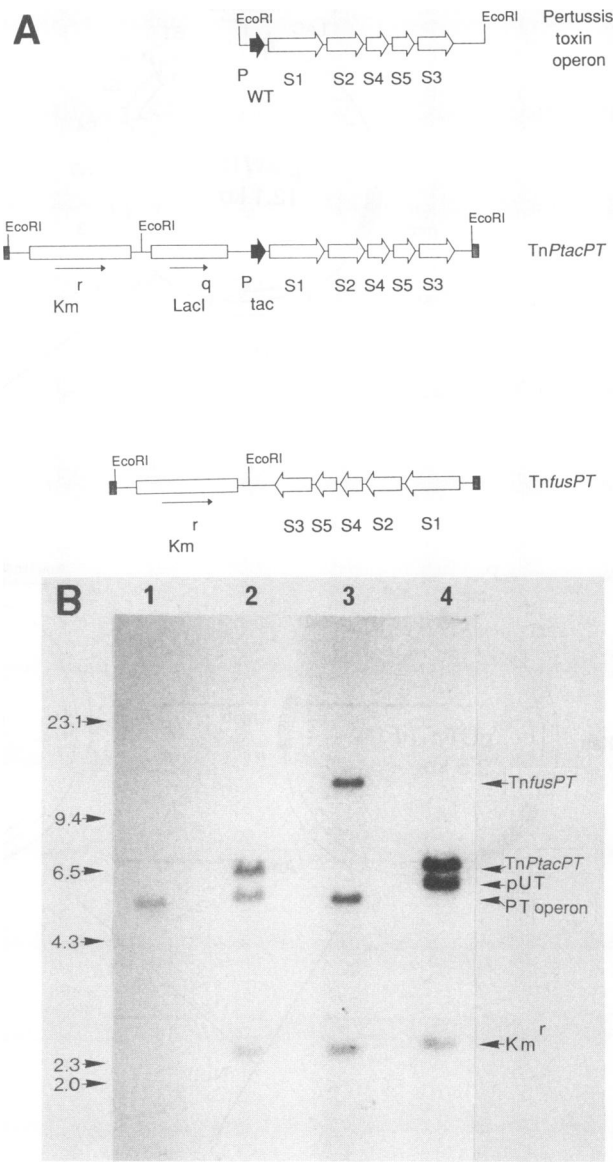


FIG. 6. Southern hybridization analysis of *B. bronchiseptica* ATCC 10580 containing minitransposons. (A) Diagrammatic representation of the PT operon, Tn*PtacPT*, and Tn*fusPT*. Symbols are as described in the legend to Fig. 4; only *Eco*RI restriction sites are shown. (B) Southern blot, using pMW126 as a probe, of *Eco*RI-digested chromosomal DNA isolated from ATCC 10580 (lane 1), ATCC 10580::Tn*PtacPT1* (lane 2), and ATCC 10580::Tn*fusPT1* (lane 3). Plasmid pMW126 DNA digested with *Eco*RI was also included (lane 4). Molecular size markers (given in kilobases) and the origins of reacting bands, as described in panel A, are indicated by arrows.

pertussis Tohama I were analyzed (Fig. 7A and B). Although the growth rate of *B. bronchiseptica* ATCC 10580::Tn*fusPT1* was slower than that of the nonmutant parent strain ATCC 10580, the transposon-containing strain still grew significantly faster than *B. pertussis* (Fig. 7A). Moreover, high levels of surface-expressed recombinant PT produced from *B. bronchiseptica* were detected after 18 h of growth, whereas equivalent amounts of PT were only produced by *B. pertussis* after 36 h of growth (Fig. 7B). Recombinant PT was localized by using immunoelectron microscopic techniques

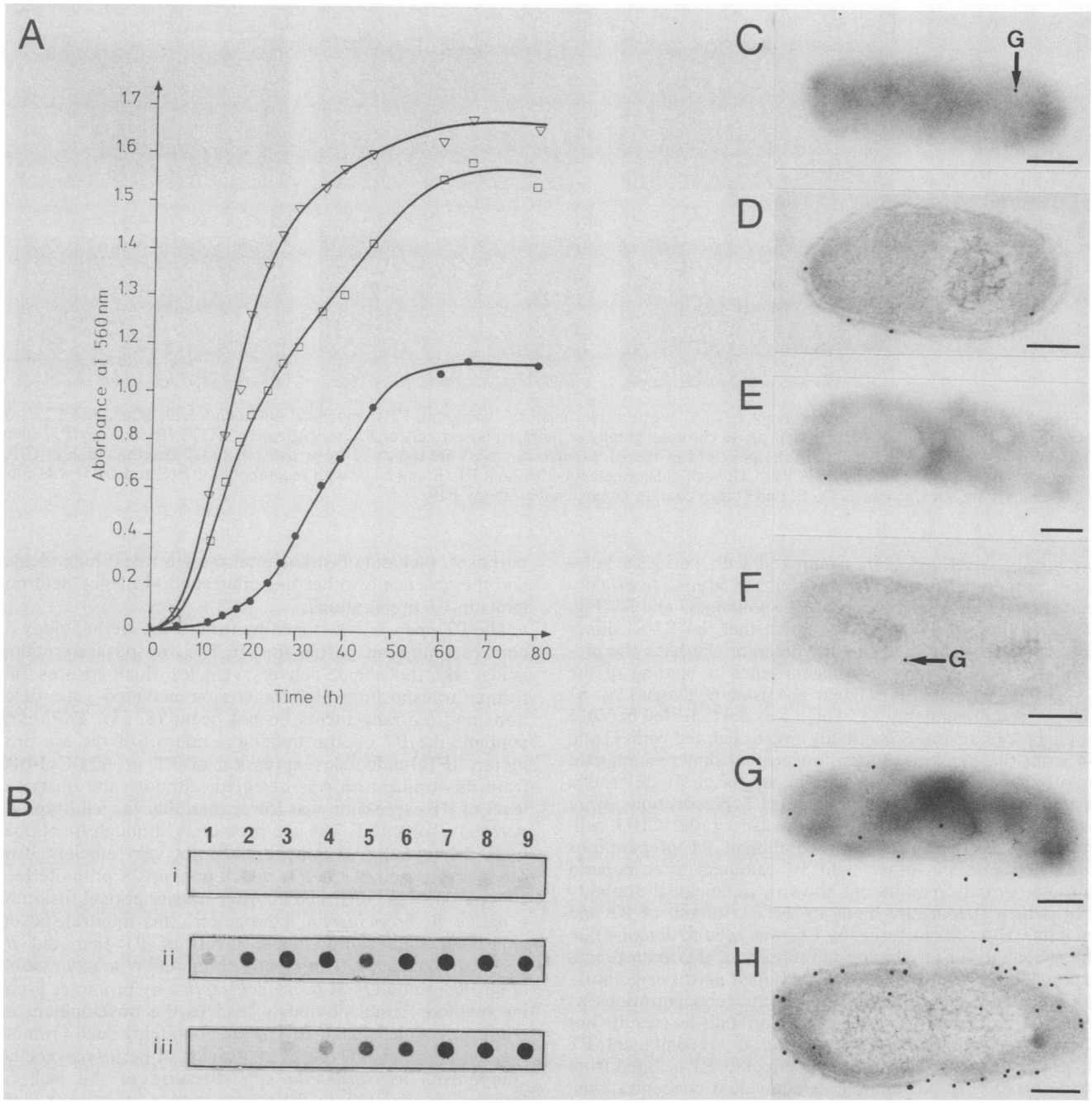


FIG. 7. Characterization of *B. bronchiseptica* ATCC 10580::Tn*fusPT*. (A) Growth curves of *B. pertussis* Tohama I (filled circles), *B. bronchiseptica* ATCC 10580 (open triangles), and ATCC 10580::Tn*fusPT1* (open squares). (B) Dot immunoblots of cultures of (i) ATCC 10580, (ii) ATCC 10580::Tn*fusPT1*, and (iii) *B. pertussis* Tohama I taken from panel A at 12, 18, 24, 30, 36, 42, 54, 63, and 84 h (dots 1 to 9, respectively). The blot was probed with the anti-PT monoclonal antibodies E19, E205, and E251. (C to H) Electron microscopic localization of PT. Whole cells (C, E, and G) and thin sections (D, F, and H) of Tohama I (C and D), ATCC 10580 (E and F), and ATCC 10580::Tn*fusPT1* (G and H) after protein A-gold labeling with anti-PT rabbit polyclonal antibodies are shown. Note in panel H that labeling of ATCC 10580::Tn*fusPT1* thin sections is stronger at the periphery of the cells. Bars, 2 μm. Symbol: G, protein A-gold complexes (indicated by arrows).

in the periplasm and on the cell surface of ATCC 10580::Tn*fusPT1* (Fig. 7C to H). Unlike the situation with *B. pertussis* (16) and recombinant *bvg*-positive *B. parapertussis* and *B. bronchiseptica* (21), which secrete PT into the medium, detectable levels of PT were not found in the supernatant fluid of cultures of ATCC 10580::Tn*fusPT1* even after growth in medium supplemented with 0.2% cyclodextrin (results not shown).

Periplasmic extracts of ATCC 10580::Tn*fusPT1* were subjected to heparin Sepharose CL-6B chromatography as

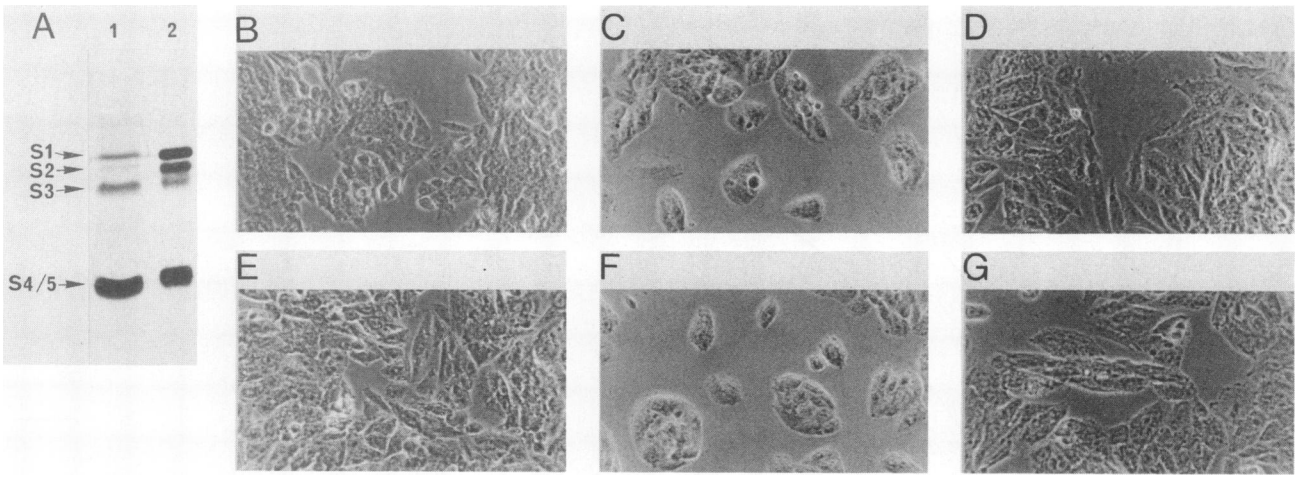


FIG. 8. Characterization of recombinant PT. (A) Western blot analysis, using anti-PT monoclonal antibodies E19, E205, and E251, of recombinant PT eluted after heparin Sepharose chromatography of periplasmic extracts of *B. bronchiseptica* ATCC 10580::Tn*fusPT1* (lane 1) and purified wild-type PT (lane 2). The positions of the PT subunits S1 to S5 are indicated by arrows. (B to G) Determination of CHO cell-clustering activity of recombinant PT. CHO cell-clustering assays without PT (B and E) or with recombinant PT (C and D) or wild-type PT (F and G) are shown. Panels D, E, and G also contain monoclonal antibody E19.

previously described (26). Compared with pertussis holotoxin prepared from *B. pertussis*, these preparations contained relatively higher levels of PT subunits S3 and S4 (Fig. 8A). Witvliet et al. (50) have shown that the S3-S4 dimer contains a carbohydrate binding receptor. Perhaps the preponderance of S3 and S4 reflects efficient binding of the S3-S4 dimer to heparin, a linear glycosaminoglycan (26). S1 and S2 (and presumably S5, which was not detected because of the lack of an anti-S5 antibody) were coeluted with S3 and S4 from the heparin column, suggesting that recombinant holotoxin is formed in the bacterial periplasm. To determine if this was the case, recombinant PT preparations were assayed for biological activity by use of the CHO cell-clustering assay. Since our recombinant PT preparations contained proteins other than PT subunits after heparin chromatography (results not shown), monoclonal antibody E19, which specifically binds to the S1 subunit of PT and inhibits CHO cell clustering by PT, was used to demonstrate the specificity of any biological activity of the toxin preparation. Tested wild-type and recombinant pertussis holotoxins were adjusted to similar S1 subunit concentrations as assessed by comparative Western blot analysis (results not shown). As shown in Fig. 8B and C, recombinant PT produces the same morphological effect as PT isolated from *B. pertussis* (Fig. 8E and F), at equivalent concentrations, and this activity was specifically inhibited by monoclonal antibody E19 (Fig. 8D and G).

DISCUSSION

Previous efforts to produce recombinant PT include expression of separate PT subunits in *E. coli* (6) and *Bacillus subtillus* (36, 38) and pertussis holotoxin in *B. parapertussis* and *B. bronchiseptica* (21). PT subunits expressed separately in *E. coli* were nonprotective as assessed in a standard animal model (6). Plasmid-specified PT expression in *Bordetella* spp. was found to be unstable, gave low product yields, and was subject to phase variation (21). In this report we describe the stable, high-level expression of PT in *bvg*-negative *B. bronchiseptica*. The use of such strains offers several advantages for the production of PT for vaccine purposes, including fast bacterial growth rates, high yields, and the absence of other *bvg*-controlled virulence determinants in PT preparations.

The PT operon was cloned by using PCR methodology to construct minitransposons (8, 14). The transposase function is lost with the suicide delivery vehicle, which ensures that further transposition and transposon-mediated gene deletions and rearrangements do not occur (8, 14). Tn*PtacPT* contains the PT operon under the control of the *tac* promoter. IPTG-inducible expression of PT in ATCC 10580 from this transposon was detected, although the observed level of PT expression was lower than that for wild-type *B. pertussis* Tohama I. The *tac* promoter, although functional in *B. bronchiseptica*, is apparently not very efficient. Another transposon, Tn*fusPT*, which contains a promoterless PT operon, was constructed. After transcriptional fusion to a native *B. bronchiseptica* promoter, this minitransposon expressed significantly higher levels of PT than did *B. pertussis* Tohama I. The identification and characterization of strong promoters of *B. bronchiseptica* by promoter probing methods could obviously lead to the development of minitransposons similar to Tn*Ptac* containing such promoters that direct high levels of expression of pertussis vaccine components in *Bordetella* spp. Because of the lack of transposon immunity in the minitransposon system (8, 14), the use of minitransposons containing different antibiotic resistance markers (8, 14) could yield *bvg*-negative strains containing multiple insertions, thereby creating vaccine production strains capable of yielding multiple antigens. In a study of the stability of plasmid-encoded PT (pMW124) and minitransposon-encoded PT (Tn*fusPT1*), it was found that after 60 generations without antibiotic selection, less than 1% of tested bacteria contained the plasmid (pMW124) or produced PT, whereas 100% of bacteria still contained Tn*fusPT1* and expressed PT. Other workers have also shown that plasmids derived from pLAFR2 or pRSF1010 and encoding the native PT operon are unstable (21).

PT active in the CHO cell-clustering assay was produced by *B. bronchiseptica* ATCC 10580::Tn*fusPT1*. Burns et al. (7) have shown that this morphological response requires

association of the S1 subunit with the B oligomer (S2 to S5). The induction of low-level CHO cell clustering by individual components that has been observed was most likely due to cross-contamination with the S1 subunit or the B oligomer, since recombinant S1 subunit purified from *E. coli* fails to exhibit any CHO cell-clustering activity yet restores purified B oligomer to full toxic activity (2). Periplasmic extracts prepared from *B. bronchiseptica* ATCC 10580::Tn*fusPT1* bacteria contained recombinant PT exhibiting CHO cell-clustering activity, demonstrating that pertussis holotoxin is formed in the periplasm of the *bvg*-negative production strain. Periplasmic extracts contain both recombinant pertussis holotoxin and PT dimers (S3-S4), indicating that holotoxin and dimer formation are not dependent on *bvg*-regulated gene products. However, unlike the case with *B. pertussis* (16) and recombinant *bvg*-positive *B. parapertussis* and *B. bronchiseptica* (21), holotoxin was not secreted from growing bacteria. To determine whether PT secretion is controlled by a *bvg*-regulated secretion system, we are currently constructing isogenic *bvg*-positive and *bvg*-negative strains of *Bordetella* spp. which express PT. The identification and cloning of genes involved in PT secretion and their expression in *B. bronchiseptica* would further facilitate the purification of recombinant PT.

Genetically detoxified analogs of PT, expressed in either *B. pertussis* or *B. parapertussis*, have been developed by several groups (18, 23, 30). We expect that the production of detoxified PT in *bvg*-negative *B. bronchiseptica* would be accompanied by the same benefits (i.e., increased bacterial growth rates, stable expression, high yields, the absence of other *bvg*-controlled virulence determinants in PT preparations, and the lack of phase variation) as those described here for recombinant PT. Experiments designed to express genetically detoxified PT in *bvg*-negative *B. bronchiseptica* are currently in progress.

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