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Stable Expression of Pertussis Toxin in *Bordetella bronchiseptica* under the Control of a Tightly Regulated Promoter

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Pertussis toxin (PT) is an essential component of acellular vaccines against whooping cough. However, the industrial production of PT from *Bordetella pertussis* is impaired by slow growth and poor yields. To overcome these problems, we have constructed a minitransposon containing the *tox* operon under the control of a tightly regulated promoter responsive to an aromatic inducer. The expression cassettes have been integrated into the chromosome of *Bordetella bronchiseptica* 5376 and ATCC 10580 *bvg*. Five recombinant clones containing the *tox* operon under the control of the *Psal* promoter, which is activated by the product of *nahR*, were further characterized. The recombinant clones expressed PT after only 3 h of induction with sodium salicylate at levels similar to those of *B. pertussis* grown for 24 h. The stability of the engineered phenotype was 100% after 72 h of growth without selective pressure. The growth pattern was not modified either under noninducing conditions or in the presence of the inducer at low concentrations, suggesting that strain performance would not be affected in bioreactors when uncoupled from gene expression. Recombinant PT, which was localized mainly in the periplasm, was purified by affinity chromatography. The recombinant protein was immunologically indistinguishable from wild-type PT and retained its biological activity as determined by the CHO cell-clustering test. These recombinant clones appear to be useful tools for the cost-effective production of PT under conditions of improved biosafety, as demonstrated by the inducible expression of PT uncoupled from the bacterial biomass in a nonvirulent and fast-growing *B. bronchiseptica* background.

Whooping cough is a human respiratory tract disease caused by *Bordetella pertussis*, and it can be prevented by vaccines prepared with inactivated bacteria (34). However, increasing public concern regarding the side effects associated with whole-cell heat-killed vaccines urged the development of nonreactogenic vaccines composed of well-defined antigens (5, 7). Recent studies aimed at comparing the efficacy of different acellular pertussis vaccines in infants showed that acellular vaccines have higher absolute efficacies and milder adverse reactions than whole-cell vaccines licensed in the United States (7, 9, 11, 12). All acellular vaccines tested in these studies contained inactivated pertussis toxin (PT) as the central component and included other pertussis antigens such as filamentous hemagglutinin, serotype-specific fimbriae, and the 69-kDa outer membrane protein pertactin (9, 11, 12).

PT is a 105-kDa exotoxin composed of five noncovalently linked subunits (S1 to S5), which are encoded by a single operon in the order S1, S2, S4, S5, and S3 (22, 33). The individual subunit precursors appear to be separately processed and translocated across the cytoplasmic membrane into the periplasmic space, where they are assembled as a holotoxin with the characteristic A-B structure (10, 33, 41). The NAD-dependent ADP-ribosyltransferase activity is located in the A subunit (S1), whereas the cell receptor-binding and translocation activities are due to the B oligomer, which comprises two dimers, S2-S4 and S3-S4, joined by the S5 subunit (33, 36, 41).

The industrial production of PT for vaccine purposes is hampered by the slow growth of *B. pertussis* and the poor PT yields. Moreover, the factor(s) responsible for the severe side effects associated with vaccination has not been identified and may contaminate PT batches. Therefore, the economical production of defined PT free of potential reactogenic factors would lead to reduction of manufacturing costs, making the vaccine accessible to bigger sectors of the world population.

To overcome these problems at least in part, different media have been used to increase PT expression by wild-type strains (15). In an attempt to further increase protein yields, PT subunits have been independently expressed in *Escherichia coli* (4, 26); however, the recombinant proteins were not assembled into a product immunologically identical to the wild-type protein (26). Recombinant PT has also been produced in *Bordetella* strains under the control of the homologous and heterologous promoters (17, 19, 23, 25, 36, 42). However, the expression of PT was found to be unstable, poor, subject to phase variation, or resulting in a decreased growth rate of the recombinants compared with wild-type strains. Moreover, the purification of PT from *B. pertussis* is associated per se with the biosafety concerns of handling a human pathogen.

Most of the virulence genes from *Bordetella* spp. are coordinately regulated by the *bvg* locus (*Bordetella* virulence gene [35]). The purification of a recombinant protein from *bvg*-negative strains would be associated with a lower biosafety risk. However, the use of *bvg*-positive bacteria may allow the simultaneous purification of other products encoded by *bvg*-activated genes (e.g., filamentous hemagglutinin). In this paper, we report the construction of a minitransposon containing the PT operon under the control of an inducible promoter. The integration of the resulting expression cassette in both *bvg*-negative and *bvg*-positive *Bordetella bronchiseptica* strains allowed the inducible expression of PT, resulting in improved growth rates and yields respect to wild-type *B. pertussis*.

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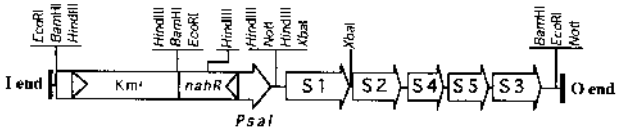

FIG. 1. Restriction map of the expression cassette contained in recombinant plasmid pCG68. The PT operon was cloned as a *Not*I fragment from plasmid pMW125 (37) into the unique *Not*I site of plasmids pCNB4 (8). Open arrows indicate the orientation of transcription of PT genes S1 to S5. The DNA sequences coding for resistance to kanamycin (Km^r) resistance to streptomycin-spectinomycin (Sm^r/Sp^r), regulatory protein (*nahR*), promoter (*Psal*), and relevant restriction sites are indicated. The mobile units are present in the delivery plasmid pUT (13) as *Xba*I-*Eco*RI restriction fragments, the *Xba*I and *Eco*RI sites external to the I and the O end of Tn*5*, respectively (not shown).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work were *B. pertussis* Tohama I (serotype 1.2) (30); *B. bronchiseptica* 5376 (37) and ATCC 10580 *bvg*; and *E. coli* (13) CC118 (λ*pir*) and SM10 (λ*pir*), which were used as the recipient strain for cloning into pCNB4 and to mobilize the pCNB derivative into *B. bronchiseptica* strains, respectively. Plasmid pMW125 (37) was the source of the promoterless PT operon, whereas pCNB4 (8) was the mini-transposon delivery system used for the construction of the mini-Tn*5* derivatives with the PT operon under the control of the *Psal* promoter, which is activated by the product of the *nahR* gene (8).

E. coli strains were grown in Luria-Bertani broth or agar (28), *B. pertussis* strains were grown on Bordet-Gengou agar base (Difco Laboratories, Detroit, Mich.) supplemented with 1% glycerol and 15% (vol/vol) defibrinated horse blood or in Stainer and Scholte medium (32), and *B. bronchiseptica* strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories). For PT purification, recombinant strains were grown in (2,6-*O*-dimethyl) β-cyclodextrin synthetic medium (15). Media were supplemented, where required, with kanamycin (50 μg ml^{-1}) and cephalexin (200 μg ml^{-1}). Bacterial cultures were routinely grown at 37°C, and liquid cultures were aerated by shaking at 200 rpm in a New Brunswick Environmental Incubator Shaker.

DNA manipulations. Plasmid DNA isolation, restriction endonuclease digestion, ligation, transformation, agarose gel electrophoresis, and other standard DNA techniques were carried out as described by Sambrook et al. (28).

Southern blot analysis of chromosomal DNA. Chromosomal DNA was isolated by a simplified lysis procedure with proteinase K-sodium dodecyl sulfate (SDS) (27), digested with *Eco*RI, separated on a 0.7% agarose gel, and transferred to Hybond-N nylon membrane (Amersham, Braunschweig, Germany). The DNA was probed with digoxigenin-labelled oligonucleotides (DIG oligonucleotide 3′-end-labelling kit; Boehringer GmbH Mannheim, Germany) that were complementary to a 22-bp fragment (13) of the mini-Tn*5* I end (5′-GGCCAG ATCTGATCAAGAGACA-3′) and to a 22-bp fragment (1) of the *tnp* gene (5′-GCACCACGAAGCGCTCGTTATG-3′), respectively. After hybridization with the DIG luminescent detection kit for nucleic acids (Boehringer), the membranes were washed and the light emission of bound probes was recorded on Kodak X-ray film.

Construction of plasmids carrying the PT operon under the control of the *Psal* promoter. Plasmid pMW125 (37) is a pUC18NotI (13) derivative in which the promoterless PT operon was subcloned as a *Hin*dIII-*Bam*HI fragment to provide flanking *Not*I sites. This plasmid was digested with *Not*I, and the fragment containing the promotorless PT operon was cloned in the unique *Not*I site of plasmid pCNB4 (8), thereby generating the recombinant plasmid pCG68 (Fig. 1).

Mobilization and transposition. Plasmid pCG68 was transferred from the donor strain *E. coli* SM10 (λ*pir*) into *Bordetella* spp. by mobilization via a filter mating technique. In brief, the donor and recipient were grown overnight with antibiotic selection, washed twice with phosphate-buffered saline (PBS; pH 7.4), and mixed in a 1:3 ratio, and the mixture was applied as a 50-μl drop onto an HA45 filter (Millipore, Bedford, Mass.) on the surface of a BHI agar plate. After overnight incubation at 37°C, the cells were resuspended in PBS and 100 μl of the appropriate dilution was plated on BHI plates supplemented with cephalexin and kanamycin.

Analysis of PT expression by *Bordetella* transconjugants. PT expression by transconjugants of *B. bronchiseptica* 5376 and ATCC 10580 containing the mini-Tn*5* derivative was induced by the method of De Lorenzo et al. (8). Briefly, recombinant clones were inoculated in BHI medium supplemented with cephalexin and kanamycin and grown at 37°C for 4 h. Then the *Psal* promoter was activated by the product of the *nahR* gene after induction with sodium salicylate for 24 h. The cells were harvested by centrifugation (5 min at 17,000 × *g*), resuspended, and adjusted to an absorbance at 600 nm (A_{600}) of approximately 0.5 with 2× SDS gel-loading buffer (28)–PBS. Samples were heated at 95°C for 5 min, and after centrifugation (1 min at 17000 × *g*), 10 μl of each supernatant was spotted onto nitrocellulose membranes (Bio-Rad Laboratories, Munich, Germany). The membranes were blocked for 1 h with 10% low-fat milk (0.3%) in PBS and further incubated for 2 h with a cocktail of appropriately diluted monoclonal antibodies E19, E205, and E251 (38), which are reactive with PT subunits. The proteins were detected by using antibody peroxidase-conjugated goat anti-mouse immunoglobulin G as a secondary antibody (Bio-Rad Laboratories). Antigen-antibody complexes were visualized by measuring chemiluminescence with the Amersham ECL system. After three rounds of screening, samples from selected recombinants were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis (18) with a 15% separating gel. Then, the proteins were transferred to nitrocellulose by using a semi-dry-blotting device (Bio-Rad Laboratories) as previously described (3), and the membranes were processed as described above for dot-immunoblotting experiments.

Quantification of recombinant PT production. Overnight cultures of the *Bordetella* strains were diluted to an A_{600} of 0.05, and 25-ml samples were further incubated in 100-ml Erlenmeyer flasks at 37°C with shaking (200 rpm) until the bacterial suspensions reached an A_{600} of 0.4, when PT expression was induced for different times by the addition of sodium salicylate up to final concentrations of 40, 5, and 1 mM, respectively. Then, a 1.5-ml aliquot of each culture was centrifuged (3 min at 17,000 × *g*) and whole cells extracts were prepared by resuspending bacteria in 0.5 volume of lysis buffer (1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 10 μM phenylmethylsulfonylfluoride, 1 nM leupeptin, 0.01 nM aprotinin, and 0.5 mM EDTA in PBS). The suspensions were sonicated for 1 min at 4°C and centrifuged for 5 min at 17,000 × *g*, and the protein concentration in the supernatant was determined with the Micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, Ill.). Approximately 5 to 10 μg of protein was spotted onto a nitrocellulose membrane, and recombinant PT was detected by dot immunoblotting as described above. The X-ray films were analyzed by scanning densitometry with a model DNA35 pdi-Scanner together with the program Quantity One version 2.2 (pdi Inc., New York, N.Y.). The amount of recombinant PT was calculated by using as an internal reference standard purified PT ranging from 1 ng to 50 ng, which was included in each membrane.

Purification of recombinant PT and analysis of the biological activity. Recombinant PT was purified from *B. bronchiseptica* ATCC 10580::Tn (*nahR*/*Psal*/*PT*)-*2* periplasmic extracts obtained by fractionation with polymyxin B (19) by using a HiTrap heparin affinity chromatography column and a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) as described by Mégret and Alouf (24). The biological activity of the purified PT was determined by the Chinese hamster ovary (CHO) cell-clustering test as described by Hewlett et al. (14). Neutralization of CHO cell-clustering by antibody E19 (38) was carried out as described by Sato et al. (29). The relative CHO cell-clustering activity was defined as the ratio between the apparent concentration of recombinant PT determined by the CHO cell-clustering assay and the absolute concentration determined by dot-immunoblotting experiments, expressed as a percentage. Two control preparations of PT obtained from IRIS (Siena, Italy) and the Department of Health and Human Services, Bethesda, Md., were used as internal standards.

Stability of the recombinant phenotype. The strains were inoculated in BHI medium without antibiotics, and the cultures were diluted 1:100 in fresh medium every day (24 h of growth corresponds to approximately 25 generations). Aliquots were removed after 24, 48, and 72 h; appropriately diluted; and plated on BHI with and without kanamycin. The number of colonies recovered from plates supplemented with antibiotic was recorded and expressed as a percentage of the number of colonies recovered from antibiotic-free agar plates.

Electron microscopy. Cells were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentration) in PBS (pH 6.9) for 30 min at 4°C. After three washes with PBS containing 10 mM glycine for blocking free aldehyde groups, the cells were immobilized in 1.5% agar. The solidified agar was cut into small blocks and embedded by progressively lowering the temperature, using the polar Lowicryl resin K4M at −35°C as previously described (31). Ultrathin sections (100 to 200 nm thick) were collected on Formvar-coated nickel grids (300 mesh) and incubated at 4°C for 12 h with a protein A-purified polyclonal antibody against PT (immunoglobulin G concentration, 175 μg ml^{-1}). Samples were washed with PBS, incubated with protein A-gold complexes (British Bio-Cell International, Cardiff, United Kingdom) for 1 h at room temperature (gold particle diameters, 10 nm; concentration giving an A_{520} of 0.02), and then carefully washed with PBS containing 0.01% Tween 20 and distilled water. The samples were air dried, poststained with 4% aqueous uranyl acetate (pH 4.5) for 5 min, and examined with a Zeiss EM910 or CEM 902 electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

For the preembedding labelling, 5-ml samples of bacterial cultures were centrifuged, the pellet from each sample was resuspended in 500 μl of PBS, and the suspension was incubated with polyclonal rabbit anti-PT antibodies (immunoglobulin G concentration, 500 μg ml^{-1}) for 2 h at 30°C with occasional shaking. After three washes with PBS, the cells were incubated with 10-nm-diameter protein A-gold complexes (concentration giving an A_{520} of 0.08) for 1 h at 30°C. After being washed with PBS, the samples were fixed with 3% glutaraldehyde, washed with PBS, fixed with 1% aqueous osmium tetroxide for 1 h at room temperature, dehydrated with a graded series of acetone, and subsequently embedded in the epoxy resin formula of Spurr. After heat polymerization at 75°C for 8 h, ultrathin sections were cut with glass knives, collected on Formvar-coated 300-mesh copper grids, and finally counterstained with 4.5% aqueous uranyl acetate (pH 4.5) for 5 min.

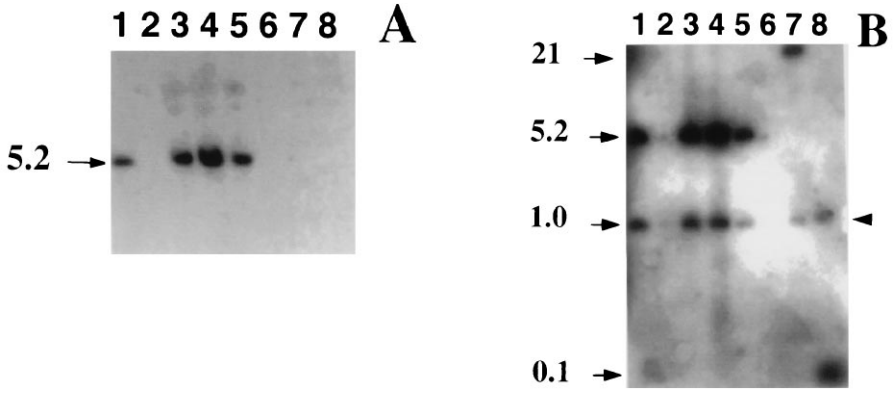

FIG. 2. Southern blot analysis of parental and recombinant *B. bronchiseptica* strains. DNAs were digested with *Eco*RI, and the fragments thereby generated were separated on agarose gels, transferred to nylon membranes, and hybridized with labelled oligonucleotides complementary to either the *tnp* gene (A) or the mini-Tn*5* I end (B). Lanes: 1, pCG68 control DNA; 2, *B. bronchiseptica* ATCC 10580; 3 to 5, *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-1 to -3, 6, *B. bronchiseptica* 5376; 7 and 8, *B. bronchiseptica* 5376::Tn*5* (*nahR*/*Psal*/*PT*)-4 and -5. The sizes of the molecular standards are indicated in kilobases by arrows, whereas a nonspecific band is indicated by an arrowhead.

RESULTS AND DISCUSSION

Stable integration of the PT operon under the control of a tightly regulated promoter in *B. bronchiseptica* strains. The expression of a recombinant protein at high levels may lead to an impaired viability of the carrier. Thus, for several biotechnological applications, the presence of a tightly regulated promoter represents an advantage. This allows optimization of bacterial growth during the fermentation process, inducing expression at the optimal time point and thereby uncoupling biomass production from gene expression. Therefore, to allow the expression of PT in fast-growing *B. bronchiseptica* strains, we decided to use a promoter from the catabolic plasmid NAH of *Pseudomonas putida* (8). This would allow the generation of a conditional phenotype regulated by an aromatic inducer. Furthermore, the stability of the engineered genotype in the absence of selective pressure would be ensured by the loss of the transposase-encoding gene during the delivery of the mini-transposon.

After mobilization of pCG68 into the recipient *B. bronchiseptica* strains, recombinant clones harboring the expected antibiotic resistant phenotype were selected and the expression of PT by transconjugants harboring the mini-Tn*5* (*Psal*/*nahR*/*PT*) cassette was assessed by immunoblotting after overnight induction with 5 mM sodium salicylate. Based on the expression levels and the presence of all PT subunits by Western blotting, three ATCC 10580 derivatives [ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-1 to ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-3] and two 5376 derivatives [5376::Tn*5* (*nahR*/*Psal*/*PT*)-4 and 5376::Tn*5* (*nahR*/*Psal*/*PT*)-5] were selected for further characterization.

Characterization of the recombinant *B. bronchiseptica*::Tn*5* (*nahR*/*Psal*/*PT*). The minitransposon system used in this work was designed with the cognate transposase outside of the inverted repeats on the donor suicide plasmid. Therefore, to discriminate between transposition and cointegration events, recombinant clones were analyzed by Southern blotting with a transposase-specific probe. The results obtained showed that the recombinant clones ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-1 to -3 are the product of a cointegration event whereas 5376::Tn*5* (*nahR*/*Psal*/*PT*)-4 and -5 are true transconjugants (Fig. 2A). The underlying mechanism of the cointegration event in the ATCC 10580 derivatives remains to be elucidated. However, the high proportion of cointegrants suggests the presence of a hot spot for plasmid integration in the chromosome of strain ATCC 10580. The results obtained with the probe specific for the I end of the mini-Tn*5* clearly indicated that the recombinant clones 5376::Tn*5* (*nahR*/*Psal*/*PT*)-4 and -5 are the product of distinct transposition events, since the probe-recognized fragments differed in size (Fig. 2B).

One of the main properties of a strain that will be used in fermentation processes is its growth pattern. To assess whether the random integration event affects any gene involved in this process, the growth of the recombinant clones was compared with that of the parental strain. The growth pattern of the recombinant clones was slightly modified, if at all; however, the presence of the inducer at a high concentration ($\geq$5 mM) resulted in bacterial growth impairment (data not shown). This suggests that the biotechnological performance of the engineered bacteria will not be affected in reactors when uncoupled from gene expression.

The stability of the recombinant phenotype is a second property that should be maintained for biotechnological applications. After 72 h of growth without kanamycin selection (approximately 75 generations), the engineered phenotype was 100% stable in all the recombinant clones (data not shown).

Analysis of PT production by the recombinant clones. Cultures of the recombinant *B. bronchiseptica* strains were induced by the addition of sodium salicylate (see Materials and Methods), and PT expression in whole-cell extracts and supernatants was analyzed by dot immunoblotting (Table 1). PT was not detected in uninduced overnight cultures of strains ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-1 to -3 or 5376::Tn*5* (*nahR*/*Psal*/*PT*)-4 and -5. Induction for 1 h with sodium salicylate resulted in the detection of PT at similar levels in whole-cell lysates from all the tested clones, whereas very low concentrations were observed in 100-fold-concentrated supernatants (Fig. 3). However, for each clone, the strongest production of PT was reached at different time points after induction and with different concentrations of the inducer (Table 1).

Recombinant clone ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 was selected for further studies because significant levels of the recombinant protein were observed after 3 h of induction in the presence of 1 mM sodium salicylate, which does not affect bacterial growth. The amount of recombinant PT in whole-cell extracts of induced *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 at the 3-h time point was 1.2 mg of bacterial culture liter^{-1}, whereas the content in wild-type *B. pertussis* grown for 24 h was 1.1 mg liter^{-1}. It is important to emphasize that the reported PT yields were obtained under nonoptimized culture

TABLE 1. Production of PT by the *B. bronchiseptica* derivatives containing the mini-Tn*5* (*nahR*/*Psal*/*PT*)

Strain	Concn of sodium salicylate (mM)	PT concn (μg of PT mg of protein^{-1}/mg of PT liter^{-1})[a] at time (h) after induction[b]:				
		1	3	5	7	24
ATCC 10580 mini-Tn*5* (*nahR*/*Psal*/*PT*)-1	0.04	ND[c]	0.7/0.3	0.6/0.4	0.5/0.4	0.5/0.2
	1	0.6/0.2	1.3/0.6	1.5/0.8	1.3/0.9	0.6/0.3
	5	0.5/0.1	0.8/0.3	1.6/0.8	1.3/0.5	0.6/0.3
ATCC 10580 mini-Tn*5* (*nahR*/*Psal*/*PT*)-2	0.04	1.2/0.5	1.2/0.8	1.2/0.7	1.1/0.7	ND
	1	1.2/0.4	1.9/1.2	1.7/1.0	1.3/0.8	1.2/0.6
	5	1.1/0.4	1.4/0.6	1.7/0.9	1.6/1.0	1.0/0.5
ATCC 10580 mini-Tn*5* (*nahR*/*Psal*/*PT*)-3	0.04	ND	1.2/0.6	1.3/0.8	1.1/0.7	0.5/0.3
	1	1.1/0.4	1.5/0.7	1.5/0.8	1.7/1.1	1.6/1.0
	5	ND	1.3/0.5	1.8/0.8	2.0/1.1	1.4/0.6
5376 mini-Tn*5* (*nahR*/*Psal*/*PT*)-4	0.04	1.0/0.3	1.4/1.0	0.9/0.9	0.7/0.6	ND
	1	1.4/0.5	1.7/1.2	1.3/1.1	1.0/0.7	0.9/0.4
	5	1.3/0.6	1.6/1.0	1.6/1.2	1.3/0.9	0.9/0.5
5376 mini-Tn*5* (*nahR*/*Psal*/*PT*)-5	0.04	ND	1.3/0.6	1.4/0.8	1.4/0.9	1.2/0.6
	1	1.3/0.5	1.6/0.7	1.4/0.7	1.6/1.0	1.0/0.5
	5	1.1/0.4	1.3/0.5	1.6/0.6	1.9/1.2	1.3/0.7

[a] PT was detected in whole-cell extracts as described in Materials and Methods by dot-immunoblotting. The PT concentration is expressed both as micrograms of PT per milligram of total protein and as milligrams of PT per liter of bacterial culture. Results are mean values of three independent determinations; standard errors of the mean were lower than 10%.

[b] Bacteria were inoculated in BHI medium supplemented with cephalexin and kanamycin and grown at 37°C until they reached an A_{600} of 0.4, when PT expression was induced by the addition of sodium salicylate.

[c] ND, not detected.

conditions whereas the specific product formation rate for *B. pertussis* growing in a chemostat, as reported by Licari et al., was 6.8 μg of PT mg of dry cell weight^{-1} (21). This suggests that the observed yields could be significantly improved by growing the recombinant strain in reactors.

Induced bacterial cells were fractioned and the presence of PT was analyzed by dot immunoblotting and Western blotting. All five subunits were detected in cytoplasmic and periplasmic fractions of ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2; however, the bulk of the recombinant PT was localized in the periplasmic fraction (Fig. 3). This data was further confirmed by immunoelectron microscopy studies, which demonstrated that most of the recombinant PT was present in the periplasm or associated with the outer membrane (Fig. 4). Interestingly, in approximately 20% of the bacterial cells, the PT-specific antibodies have access to the protein from the outside, as demonstrated by the preembedding labelling (Fig. 4D and E).

The export of PT into the medium requires accessory transport proteins (6, 16, 39). The expression of these proteins is transcriptionally linked to the PT operon and regulated by the product of the *bvg* locus (2, 6). Therefore, it would be possible to introduce these genes into the recombinant clones to achieve PT export. However, the recombinant PT is released into the periplasmic space, which is characterized by a minor protein content. Thus, it may represent an advantage for large-scale purification, when the concentrated periplasmic PT bulk could be released in a small volume.

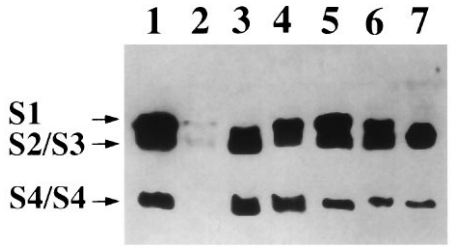

FIG. 3. Expression of PT by recombinant *B. bronchiseptica* strains. Bacterial proteins of subcellular fractions from *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 (lanes 2 to 4) and *B. pertussis* (lanes 5 to 7) strains were separated by SDS-polyacrylamide gel electrophoresis and PT was detected by Western blotting as described in Materials and Methods. Lanes: 1, purified PT (150 ng); 2 and 5, supernatants concentrated 100-fold; 3 and 6; periplasmic fractions; 4 and 7, cytoplasmic fractions. A 10-μg portion of protein was loaded per lane; the main protein products are indicated by arrows.

PT was purified from the periplasmic fraction of clone ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 and from supernatants of *B. pertussis* by affinity chromatography (see Materials and Methods). The stoichiometry and assemblage of PT subunits in recombinant and wild-type PT were confirmed by Western blot analysis (Fig. 5). The coelution of all subunits from the column confirmed that the holotoxin was assembled in the periplasm of the recombinant strain, as the S3-S4 dimer contains the carbohydrate binding site that mediates binding to heparin (40).

The biological activities of the wild-type and recombinant proteins were compared by performing the CHO cell-clustering test. The relative CHO cell-clustering activity of purified PT from *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 was 93%, whereas that of the PT purified from *B. pertussis* was 100%. This result showed that the purified protein retained most of the biological activity and confirmed that adequate folding of the holotoxin was achieved in the periplasm of the recombinant clone. The specificity of the reported effect was confirmed by the specific neutralization with the neutralizing monoclonal antibody E19 (data not shown).

Although the minimal number of antigens required to achieve protective immunity in acellular pertussis vaccines remains to be established, there is general agreement that inactivated PT is an essential component (9). Strains engineered to achieve overproduction of PT must meet a number of requirements such as the stable maintenance of the engineered phenotype and adequate levels of expression in the fermentation

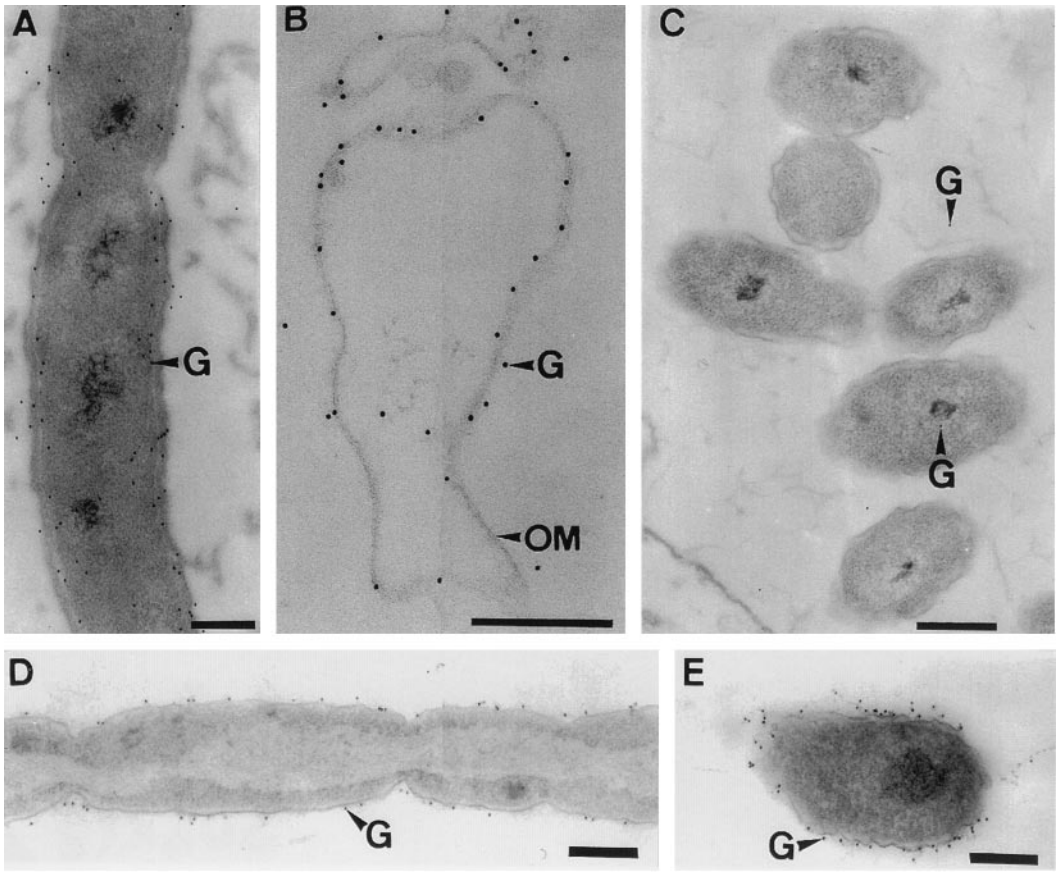

FIG. 4. Immunoelectron microscopic localization of recombinant PT. *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2 after (A, B, D, and E) and before (C) 3 h of induction with 1 mM sodium salicylate. Noninduced cells exhibit no labelling (C), whereas induced cells show the label at the cell periphery (A) and at the occasionally observed outer membrane fragments (B) when analyzed by postembedding labelling. The preembedding labelling demonstrates that PT is transported through the cytoplasmic membrane and is also accessible from the outside of the cells for the gold markers (D and E). Abbreviations: OM; outer membrane; G, gold particles. Bars, 0.25 μm (A, B, D, and E) and 0.5 μm (C).

reactor under controlled conditions. The application of recent advances in high-cell-density culture technology (20) to obtaining inducible expression of recombinant PT in fast-growing *Bordetella* spp. provides a solution to these problems. In fact, the high-level inducible expression of PT observed in the *B. bronchiseptica* derivatives containing the mini-Tn*5* (*nahR*/*Psal*/*PT*) after 3 h of induction and the lack of effects on bacterial growth suggested that they are valid alternatives for the cost-effective production of PT in bioreactors. Finally, the use of *bvg*-negative derivatives may constitute an additional advantage for the biological safety of the industrial production process.

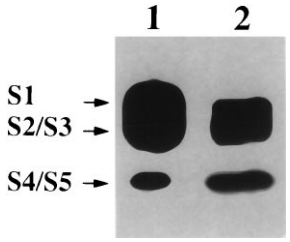

FIG. 5. Western blot analysis of the recombinant PT purified from *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2. PT has been purified from periplasmic fractions by the procedure described by Mégret and Alouf (24). Lanes: 1, PT produced by *B. pertussis*; 2, recombinant PT produced by *B. bronchiseptica* ATCC 10580::Tn*5* (*nahR*/*Psal*/*PT*)-2. A 10-μg portion of protein was loaded per lane; the main protein products are indicated by arrows.

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