

Promoter of the Pertussis Toxin Operon and Production of Pertussis Toxin

ALFREDO NICOSIA AND RINO RAPPUOLI*

Sclavo Research Center, 53100 Siena, Italy

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Pertussis toxin (PT), the major virulence factor of *Bordetella pertussis*, is composed of five different subunits whose genes are organized as an operon. We report the mapping of the promoter region of the PT operon and show that this promoter is only weakly active in *Escherichia coli*. *Bordetella parapertussis* and *Bordetella bronchiseptica*, which do not produce any PT, are shown to have a weaker promoter sequence for this operon and not to produce any detectable PT mRNA. We show that transcription of the PT operon in *B. pertussis* was constant throughout until the late stationary phase, when transcription significantly decreased. Analysis of the transposon Tn5 mutant BP347 showed that the product of the *vir* locus was required for transcription of the PT operon. Characterization of the Tn5 mutant BP356 showed that subunit S3 was required for the release of PT into the extracellular medium.

Pertussis toxin (PT) is produced by phase I (virulent) *Bordetella pertussis* strains, but not by *Bordetella parapertussis*, *Bordetella bronchiseptica*, phase III *B. pertussis*, or phase I organisms grown in high-magnesium or low-sodium medium (19). PT, an oligomeric protein composed of five different subunits, can be divided into two functionally different moieties: A, which comprises subunit S1, and B, which comprises subunits S2, S3, S4, and S5 arranged in two dimers, D1 (S2 plus S4) and D2 (S3 plus S4), which are held together by S5. The B moiety binds the receptors on the surface of eucaryotic cells and allows the entry of the enzymatically active subunit S1 into the cells (18, 21). The toxicity is due to S1, which is an ADP-ribosyltransferase which ADP-ribosylates a family of GTP-binding proteins involved in the transduction of signals across the membrane of eucaryotic cells (12, 14, 17, 22). The genes coding for the five subunits of PT, which have recently been cloned and sequenced (15, 18), have been shown to be clustered in an operon in the following order: S1, S2, S4, S5, and S3. Subunits S2 and S3 have been shown to have 67% homologous amino acids. A sequence resembling the *Escherichia coli* promoter consensus sequence found upstream from the first gene has been tentatively proposed as the promoter for the PT operon (15, 18). The genes coding for each of the five subunits have been shown to be preceded by a sequence coding for a typical bacterial leader peptide, suggesting that the five subunits are secreted separately into the periplasm of *B. pertussis* cells (15, 18).

Analysis of the transcription of the PT genes and identification of some crucial steps in PT production should provide useful information on the regulation of expression of such a complex bacterial protein.

In this paper we identify the promoter of the PT operon and show that in *E. coli* it is very weak. We confirm that, as previously proposed by Weiss et al. (23), a *trans*-acting factor encoded by the *vir* locus is required for expression of the PT operon in *B. pertussis* and show that regulation is at the transcriptional level. *B. parapertussis* and *B. bronchiseptica* are shown to produce no detectable PT mRNA, most probably because of severe down-mutations in the promoter region which make their promoters very ineffi-

cient. We also show that transcription of the PT operon in *B. pertussis* is constant throughout the different phases of growth until the late stationary phase, when PT mRNA is almost absent. Characterization of the transposon Tn5 mutant of *B. pertussis* BP356, which does not produce any active toxin, allowed us to conclude that PT release into the extracellular medium requires assembly of the five subunits, which are secreted separately into the periplasm.

MATERIALS AND METHODS

Strains and media. *B. pertussis* BP165 was obtained from the Bureau of Biologics, Bethesda, Md. (20). *B. pertussis* Tohama has been described (13). The nonvirulent *B. pertussis* Tn5 mutants BP347 *vir* and the BP356 (which does not produce any active toxin) were obtained from Stanley Falkow (24). The strains of *B. parapertussis* and *B. bronchiseptica* were obtained from the American Type Culture Collection (ATCC 9305 and ATCC 4617, respectively). *E. coli* JM101 has been described (25). *B. pertussis* strains were grown in Bordet-Gengou plates (19) or Vervey liquid medium containing cyclodextrin (1 mg/ml) (13). Vervey medium contains (per liter) 14 g of Casamino Acids, 0.2 g of KCl, 0.5 g of KH₂PO₄, 0.1 g of MgCl₂ · 6H₂O, 20 mg of nicotinic acid, 10 mg of glutathione, and 1 g of starch. *E. coli* strains were grown in LB plates or LB liquid medium (16).

CAT assay. Cell extracts for the chloramphenicol acetyltransferase (CAT) assay were prepared by sonicating cells from 1 ml of culture in 150 μ l of 0.25 M Tris, pH 8. The lysate was then centrifuged for 10 min in an Eppendorf centrifuge, and the supernatant was heated for 7 min at 60°C. After 10 min of centrifugation, the supernatant was used for the CAT assays, which were performed as described previously (8).

DNA procedures. Manipulations of DNA were performed by standard procedures (16). Oligonucleotide synthesis was performed with the Applied Biosystems model 380A synthesizer. Oligonucleotide-directed mutagenesis was performed as described previously (27).

RNA procedures. To prepare RNA, samples were taken at intervals from a culture of *B. pertussis* growing in liquid medium and centrifuged, and the pellet was frozen in a dry ice-ethanol bath. The pellets were then suspended in 1 ml of cold 10 mM Tris-1 mM EDTA, pH 7.5, containing 500 μ g of proteinase K per ml, vortexed, and mixed with 1 ml of 0.2%

* Corresponding author.

considered severe downmutations for *E. coli* promoters (26). SP (strong promoter), a *Salmonella typhimurium* promoter whose efficiency in *E. coli* has been described (4, 5), was used as a positive control.

The wild-type PT promoter gave 19 times more CAT activity than the control plasmid without any promoter (Fig. 2). As expected, BP17, which has optimal spacing between the -35 and -10 regions, was more efficient than the wild-type BP promoter. The BPP promoter, which contained severe down-mutations in the -10 and the -35 regions, was almost four times less efficient than the wild-type BP promoter. The SP promoter was about 1,000 times more efficient than the BP promoter. By primer extension of the CAT mRNA, we confirmed that transcripts from the above constructions started at the expected base downstream from the -10 region and therefore that the CAT activity we measured was in fact due to the promoters that we were analyzing.

These results showed that the PT promoter, although weak compared with the strong promoter, behaved as predicted in *E. coli*: when the spacing between the -35 and the -10 regions was optimized, it became stronger; when down-mutations were introduced, it became weaker. If the requirements for a good promoter are the same in *B. pertussis* and *E. coli*, the PT promoter should also be rather inefficient in *B. pertussis*, and therefore the efficient expression of this gene which is normally observed in phase I *B. pertussis* would imply an additional regulatory mechanism. The positive regulatory element encoded by the *vir* locus which has been proposed to explain the phenomenon of phase variation in *B. pertussis* (23) is probably required for efficient expression of the PT operon. On the other hand, the low efficiency of the *B. paraptentis* promoter is in agreement with the observation that *B. paraptentis* and *B. bronchiseptica* do not produce any PT (19).

Transcription of PT operon and production of PT. The production of PT is influenced by several factors, such as phase change of *B. pertussis* and the presence or absence of ions such as magnesium and sodium (19). We investigated whether transcription of the PT operon and the production of PT vary during different phases of growth.

B. pertussis Tohama was grown in liquid medium, and at intervals, a sample of the culture was taken, centrifuged, and used to prepare RNA. The PT content in the culture supernatant was determined by ELISA. The RNA was used in a dot-blot hybridization to determine the amount of PT mRNA. The results (Fig. 3) show that PT mRNA was produced at the same rate throughout growth until the late stationary phase, when there was a sharp decrease. PT was not detected in the supernatant until the late log phase and then rapidly accumulated, reaching a maximum in the early stationary phase, after which there was a sharp decrease.

It was also of interest to study in the same system three other strains of *Bordetella* which have been reported not to produce PT. Therefore, we analyzed the mutant strain *B. pertussis* BP347 *vir*, which contains a Tn5 insertion in the chromosome that abolishes the expression of all the virulence factors of *B. pertussis* (23, 24), one strain of *B. paraptentis*, and one strain of *B. bronchiseptica*. None of them produced detectable levels of PT mRNA or PT (Fig. 3). These findings show that the Tn5 mutation of BP347 (possibly affecting the production of the positive regulatory element encoded by the *vir* locus) abolished transcription of the PT operon and therefore that the PT promoter is inactive in *B. pertussis* unless it is activated by the positive regulatory element. Preliminary data (not shown) indicate that the alternative explanation, mRNA instability, is unlikely.

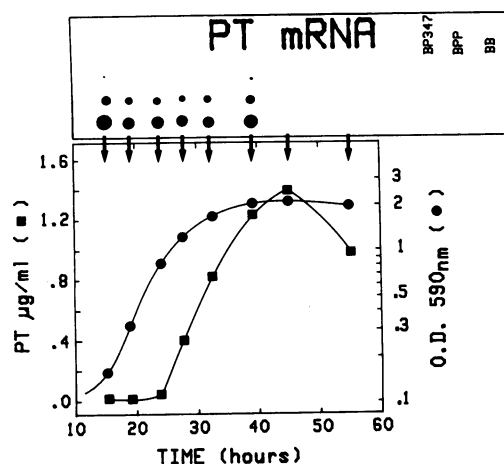


FIG. 3. Transcription of the PT operon and production of PT. The optical density of the culture (●), the amount of PT in the supernatant (■), and the amount of PT mRNA for *B. pertussis* Tohama are shown. For BP347, *B. paraptentis*, and *B. bronchiseptica*, we show only the dot hybridization of the RNA extracted at mid-log phase; the other points of the growth curve were also negative by ELISA and dot-blot hybridization.

For *B. paraptentis* and *B. bronchiseptica*, we can conclude that transcription of the PT operon is affected by the low efficiency of the promoter (as shown in Fig. 2) and possibly by alteration of a positive regulatory element, because these strains are in phase I and therefore are likely to produce a regulatory element analogous to that encoded by the *vir* locus of *B. pertussis*.

Subunit S3 required for extracellular release of PT. Weiss et al. (24) described a Tn5 mutant of *B. pertussis* (BP356) which does not produce any active toxin. By nucleotide sequencing of the region containing the Tn5 insertion in the chromosome of BP356, we found that the Tn5 element is located within the gene coding for subunit S3, at nucleotide position 3436 (18), and that the sequence GCGCCAGCCC is repeated at both ends of the Tn5 insertion. Since S3 is the last of the PT genes, strain BP356 should produce the S1, S2, S4, and S5 peptides and the first 136 amino acids of subunit S3. Tamura et al. (21) have shown that at least in vitro, both dimers D1 and D2 are required to assemble the toxin. If this is also true in vivo, the incomplete sets of subunits produced by BP356 should not be assembled, and this might be the reason for the lack of toxicity of BP356 supernatants seen in the CHO cell assay (24).

To test this assumption, we performed the following experiment. Cultures of the wild-type *B. pertussis* BP165 and of the BP356 mutant were grown in parallel. Samples were taken at intervals and centrifuged. Each pellet was then suspended and divided into two samples; one was incubated in Tris buffer, and the other was incubated in the same buffer containing 2 mg of polymyxin B per ml, which releases the periplasmic content of the cell (7). After centrifugation, the toxin content in the Tris extract, the polymyxin B extract, and the culture supernatant was determined by ELISA. The results (Fig. 4) show that the BP356 mutant produced some of the subunits of PT but did not release them into the supernatant. Once released from the periplasm by polymyxin B treatment, the toxin subunits produced by BP356 were still active in the CHO cell assay. Other laboratories have confirmed by Western blotting that the BP356 mutant is able to produce subunits S1, S2, S4, and S5 but not

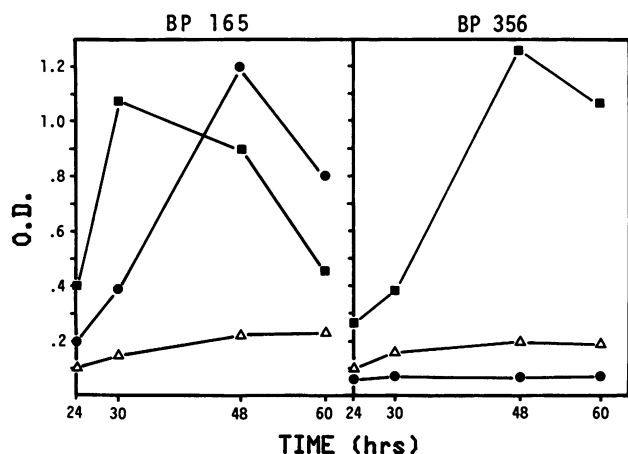


FIG. 4. PT (as determined by ELISA) in the supernatants (●), the Tris extract (Δ), and the polymyxin B extract (■) of strains BP165 and BP356. The amount of toxin is reported in arbitrary optical density units at 405 nm.

S3 (S. Falkow, personal communication). This experiment showed that the BP356 strain of *B. pertussis* is defective for the synthesis of the S3 subunit, which is essential for the release of PT into the culture medium, and implies that in *B. pertussis* the five subunits are collected separately in the periplasm of the cell, where they are assembled and released. In the absence of the S3 subunit, PT is not released into the extracellular medium, suggesting that this protein is necessary for complete assembly of the toxin. This would be similar to the situation described for *Vibrio cholerae*, in which the two subunits of the cloned *E. coli* heat-labile enterotoxin are secreted in the periplasm of the cell and released into the culture medium only after assembly (11).

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