

## Localization of the Promoter for the *ptl* Genes of *Bordetella pertussis*, Which Encode Proteins Essential for Secretion of Pertussis Toxin

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**The *ptl* locus of *Bordetella pertussis*, which encodes proteins necessary for the secretion of pertussis toxin into the extracellular medium, is located directly downstream from the *ptx* locus, which encodes the structural subunits of the toxin. We have found that the *ptx* promoter is essential for expression of the *ptl* genes. A strain of *B. pertussis* which lacked only the *ptx* promoter region but which retained all other portions of the *ptx-ptl* region did not produce PtlF. Moreover, insertion of a functional *ptx* promoter from *B. pertussis* at the 5' end of the *ptx* region of *Bordetella bronchiseptica* resulted in the production of PtlF in *B. bronchiseptica*, a species which normally does not produce PtlF. These results suggest that the *ptx* operon is larger than originally proposed and contains both the *ptx* and *ptl* genes.**

Pertussis toxin (PT) is an exotoxin produced by *Bordetella pertussis*, the causative agent of the disease pertussis (whooping cough). PT is believed to play an important role in pathogenesis, since mutants of *B. pertussis* that lack PT are less virulent than the wild-type organism in animal models of the disease (22). A vaccine composed solely of inactivated PT was approximately 80% efficacious in protecting children against severe pertussis (1), suggesting that a human immune response to the toxin is capable of attenuating the disease process and thus emphasizing the importance of the toxin in pertussis. Secretion of PT, not simply production of PT, is required for maximum virulence of the organism in an animal model of the disease (21).

PT is composed of six subunits (S1, S2, S3, S4, and S5 found in a 1:1:1:2:1 ratio) and has a molecular weight of 105,000 (13, 15, 20). In order to be secreted, the toxin subunits must traverse both the inner and outer membranes of *B. pertussis*. While the mechanistic details concerning the transport process remain obscure, PT is known to utilize a set of specialized transport proteins, Ptl proteins (10, 23), to cross at least the outer membrane barrier of the bacterium.

As shown in Fig. 1, the *ptl* genes are located directly downstream from the *ptx* genes, which encode the structural subunits of PT. The *ptl* locus contains eight open reading frames (A through H). Previously, it was suggested that the *ptx* genes and *ptl* genes are parts of separate operons (23). In that study, a vector (pPTL7) containing a cloned region spanning from the S5 subunit gene to midway through *ptlC* (Fig. 1) was integrated into the *B. pertussis* chromosome by homologous recombination, resulting in a duplication of this region interrupted by the vector. If the *ptx* genes and *ptl* genes were parts of a single operon, this insertion would have been expected to disrupt the operon such that the bacteria would lose the ability to secrete PT. In contrast, if the *ptx* genes and *ptl* genes were parts of different operons, both *ptx* and *ptl* genes should be expressed and the bacteria should secrete PT. Since this strain, BPPTL7, was found to secrete PT, the *ptx* genes and *ptl* genes were proposed to be parts of different operons. Recently, however,

reexamination of this strain revealed that expression of the *ptl* locus was likely being driven by a foreign promoter, possibly a promoter present on the vector next to which the *ptl* genes were inserted, rather than being driven by its own promoter as originally thought. Therefore, the finding that BPPTL7 secreted PT could not be used as evidence that the *ptx* and *ptl* genes were parts of two separate operons, and the possibility remained that the *ptx* and *ptl* genes might constitute a single operon. Localization of the promoter for the *ptl* genes is critical for a more complete understanding of the regulation of these genes. In this work, we provide evidence that expression of both the *ptx* and *ptl* genes is driven by a single promoter, the *ptx* promoter.

In order to determine whether the promoter for the *ptx* genes is necessary for expression of the *ptl* genes, we constructed *B. pertussis* strains which either contained the entire *ptx-ptl* region or which contained all of this region with the exception of the *ptx* promoter. We then examined these strains for production of PtlF. These constructs, BPRA::pSK06 and BPRA::pSK07, are shown in Fig. 2 and were made in the following manner. First, a plasmid (pSK01) which contained nucleotides 930 to 4569 of the *ptx-ptl* region was constructed (see Fig. 1 for a diagram of the nucleotide numbering system of the *ptx-ptl* region). This plasmid was made by inserting the 3,640-bp *SalI-BamHI* fragment (nucleotides 930 to 4569 of the *ptx-ptl* region) of pUW2036 (provided by Alison Weiss, University of Cincinnati) into the *SalI-BamHI* site of pUC18, using standard techniques (17). We then amplified, using PCR as previously described (10), DNA fragments consisting either of nucleotides 1 to 935 of the *ptx-ptl* region (Fig. 1), which contained the *ptx* promoter (16), or of nucleotides 482 to 935 of the *ptx-ptl* region, which did not contain the *ptx* promoter. Previously, transcription of the *ptx* operon was shown to start at nucleotide 482 (16). The primers used were designed to allow in-frame cloning of the PCR fragments next to the *SalI-BamHI* insertion of pSK01, thus generating contiguous segments consisting of either nucleotides 1 to 4569 or 482 to 4569 of the *ptx-ptl* region. The upstream primer used in the PCR procedure to amplify nucleotides 1 to 935 was 5'-GCGAAGCTTGAATTCGTCGCCTCGCCCTGGTTCG (three nucleotides followed by a *HindIII* site followed by nucleotides 1 to 25 of the *ptx* region). The upstream primer used to amplify nucleotides 482 to 930 was 5'-GCGAAGCTTATCAAACGCAGAGGG

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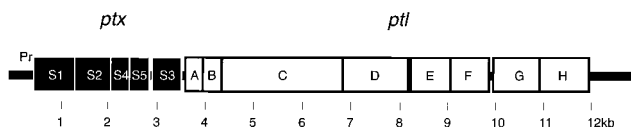


FIG. 1. The *ptx-ptl* region. The structure of the *ptx-ptl* region is shown. Also shown is the nucleotide numbering system used for this region (10, 15). Pr indicates the location of the *ptx* promoter.

GAAGACGGG (three nucleotides followed by a *Hind*III site and nucleotides 482 to 506 of the *ptx* region). The downstream primer used to amplify both fragments was 5'-GGCGGTCGACGTATTCGAAGTACGAGCTG (four nucleotides followed by nucleotides 935 to 911 of the *ptx* region which contains a *Sal*I site). DNA isolated from *B. pertussis*, strain Tohama I (provided by Zhong-Ming Li, Center for Biologics Evaluation and Research, Bethesda, Md.), was used as the template. Each amplified fragment was inserted into the TA cloning vector (Invitrogen, San Diego, Calif.) via the T and A residues added during the PCR. The resulting plasmids were cut with *Hind*III and *Sal*I to remove the cloned fragments consisting of nucleotides 1 to 930 and 482 to 930 of the *ptx* region. These fragments were each inserted into the *Hind*III-*Sal*I site of pSK01, generating pSK04 and pSK05. Restriction of these two plasmids with *Hind*III and *Bam*HI removed two fragments consisting of nucleotides 1 to 4569 and 482 to 4569 of the *ptx-ptl* region. These two fragments were each inserted into the *Hind*III-*Bam*HI site of pSS1129 (19), a vector which confers gentamicin resistance (provided by Scott Stibitz, Center for Biologics Evaluation and Research, Bethesda, Md.), resulting in the formation of two plasmids, pSK06 and pSK07. pSK06 contained nucleotides 1 to 4569 of the *ptx-ptl* region, which consists of the entire *ptx* region including the *ptx* promoter and a portion of the *ptl* region, whereas pSK07 contained the identical region minus the *ptx* promoter (nucleotides 482 to 4569). These plasmids were transformed into *Escherichia coli* SM10 $\lambda$ pir (18) and were then transferred into *B. pertussis* BPRa (provided by Camille Locht, Institut Pasteur, Lille, France) by conjugation as previously described (19). As shown in Fig. 2, BPRa is a mutant strain of *B. pertussis* which has a 2.7-kb deletion in the *ptx* region extending from nucleotides 268 to 3008 or from just upstream of the *ptx* promoter to the beginning of the S3 subunit cistron (2). Since pSK06 and pSK07 cannot replicate in *B. pertussis*, gentamicin resistance is conferred only if homologous recombination occurs between the chromosome and the *B. pertussis* sequences on the plasmid. Such recombination would result in *ptx-ptl* regions, as depicted

in Fig. 2. Exconjugants were selected on Bordet-Gengou agar containing nalidixic acid (50  $\mu$ g/ml) and gentamicin (10  $\mu$ g/ml).

We found that BPRa::pSK06, which contained the *ptx* promoter, produced PtlF (Fig. 3) as well as PT subunits (data not shown), as visualized by immunoblot analysis using polyclonal antibodies specific for PtlF (10) or a monoclonal antibody reactive with the S1 subunit of PT. However, BPRa::pSK07, which lacked only the *ptx* promoter but had all other sequences present in the *ptx-ptl* region, did not produce PtlF or PT subunits. Neither strain produced PtlF when grown in the presence of MgSO<sub>4</sub> and nicotinic acid, conditions known as modulating conditions which reversibly suppress expression of genes encoding virulence factors which are under the control of products of the *bvg* (stands for *Bordetella* virulence genes) locus (9, 12). The *ptl* genes have previously been shown to be *bvg* regulated (24). Control experiments in which the *ptx-ptl* region extending from nucleotides 2900 to 4700 was amplified by PCR were conducted. With both BPRa::pSK06 and BPRa::pSK07, a 1.8-kb piece of DNA was amplified, indicating that pSK06 and pSK07 integrated into the chromosome as shown in Fig. 2 (data not shown). These results suggest that the *ptx* promoter region is necessary for expression of the *ptl* genes.

In order to provide additional support for this interpretation of the data, we introduced the *ptx* promoter from *B. pertussis* into *B. bronchiseptica*. *B. bronchiseptica* does not produce PT (3, 14), PtlE, or PtlF (10), although most strains of this species contain regions homologous to the *ptx* and *ptl* regions of *B. pertussis* (3, 14). Others have provided evidence which suggests that the *B. bronchiseptica* *ptx* genes are not expressed because of mutations in the *ptx* promoter region (3). Introduction of a functional *ptx* promoter in front of the *ptx-ptl* region of *B. bronchiseptica* would be expected to result in production of Ptl proteins if the *ptl* genes are driven by the *ptx* promoter.

A functional *ptx* promoter was introduced into *B. bronchiseptica* as depicted in Fig. 4. First, the promoter-S1 subunit cistron region (nucleotides 1 to 1311) of *B. pertussis* Tohama I was amplified by PCR. The upstream primer used was 5'-CGC AAGCTTGAATTCGTCGCCTCGCCCTGGTTTCG (three nucleotides followed by a *Hind*III site and nucleotides 1 to 25 of the *ptx* region), and the downstream primer was 5'-CGCGG ATCCACGAATACGCGATGCTTTTCGTAGTA (three nucleotides followed by a *Bam*HI site and nucleotides 1311 to 1287 of the *ptx* region). This fragment was then inserted into the *Hind*III-*Bam*HI site of pSS1129. The resulting plasmid (pSZH8) was transformed into *E. coli* DH5 $\alpha$  and was then introduced via triparental mating (4) into *B. bronchiseptica* Bb55, using *E. coli* DH5 $\alpha$ (pRK2013) (6) as a helper strain.

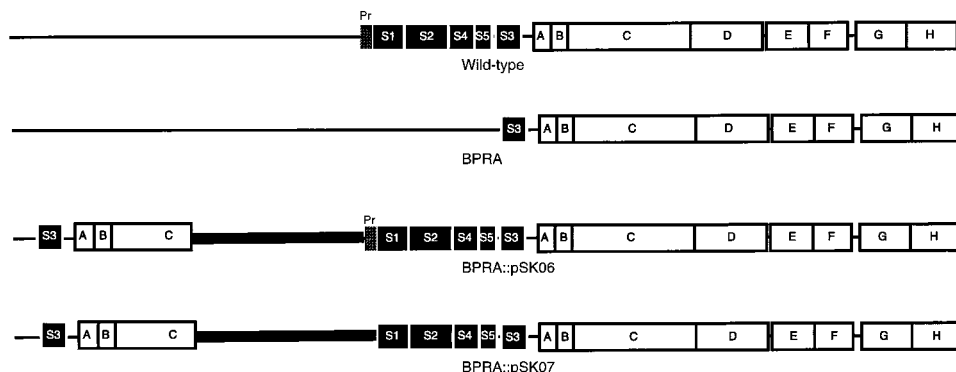


FIG. 2. The *ptx-ptl* region of wild-type and mutant strains of *B. pertussis*. The *ptx-ptl* regions of wild-type *B. pertussis* and strains BPRa, BPRa::pSK06, and BPRa::pSK07 are shown. Pr indicates the *ptx* promoter. Thick solid bars represent integrated vector sequences.

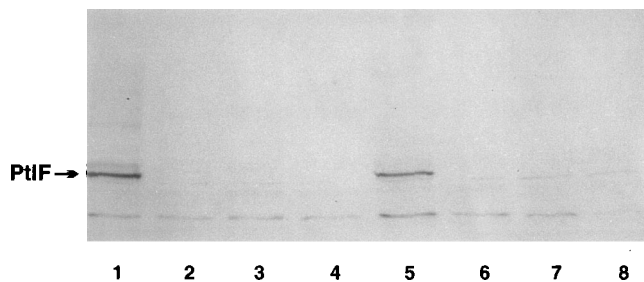


FIG. 3. Immunoblot analysis of cell lysates of wild-type and mutant strains of *B. pertussis* probed with anti-PtIF antibody. Cell extracts (7  $\mu$ l of a culture with  $A_{550} = 1.7$ ) of the virulent form (lanes 1, 3, 5, and 7) or the modulated form (lanes 2, 4, 6, and 8) of *B. pertussis* strains 338 (lanes 1 and 2), BPRA (lanes 3 and 4), BPRA::pSK06 (lanes 5 and 6), and BPRA::pSK07 (lanes 7 and 8) were subjected to immunoblot analysis using antibodies specific for PtIF to probe the extracts for the presence of PtIF. (Coreldraw 3.0 was used to generate this figure from a scanned image.)

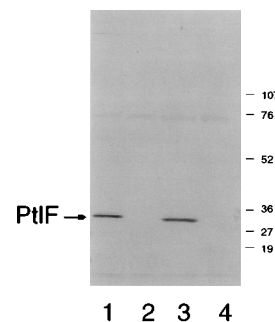


FIG. 5. Immunoblot analysis of cell extracts of *B. pertussis* and *B. bronchiseptica*. Cell extracts (35  $\mu$ l of a culture with  $A_{600} = 2.0$  which had been precipitated with an equal volume of 20% trichloroacetic acid) of the virulent form (lanes 1 and 3) or the modulated form (lanes 2 and 4) of *B. pertussis* 338 (lanes 1 and 2) or *B. bronchiseptica* Bb55::pSZH8-2 were subjected to immunoblot analysis using antibodies specific for PtIF to probe the extracts for the presence of PtIF. (Coreldraw 3.0 was used to generate this figure from a scanned image.) Positions of molecular weight markers are indicated on the right (in thousands).

Homologous recombination between the vector and the chromosome which occurs in the region between the *ptx* promoter and the end of the S1 subunit cistron would result in the placement of the *B. pertussis* promoter and a portion of the pertussis S1 cistron at the position where the corresponding region from *B. bronchiseptica* had been originally. Exconjugants were selected on Bordet-Gengou agar containing ampicillin (100  $\mu$ g/ml), gentamicin (5  $\mu$ g/ml), and cephalixin (50  $\mu$ g/ml), with the vector providing resistance to ampicillin and gentamicin. *B. bronchiseptica* is naturally resistant to cephalixin. Drug-resistant exconjugant colonies were isolated.

The results obtained using one of the exconjugants are shown in Fig. 5. PtIF was produced by the virulent form of this strain; however, it was not produced when the bacteria were grown under modulating conditions. These results indicate that the *B. bronchiseptica* PtIF was expressed from a *bvg*-regulated promoter, as would be expected if it were expressed from the *B. pertussis* *ptx* promoter, since the *B. pertussis* promoter was previously shown to be modulated normally in *B. bronchiseptica* (7). Thus, a functional *ptx* promoter is sufficient for expression of PtIF in *B. bronchiseptica*. Interestingly, our findings indicate that *ptIF* of *B. bronchiseptica* encodes a protein which appears to be stable, is similar in size to PtIF of *B. pertussis*, and is reactive with polyclonal antibodies raised to the *B. pertussis* protein. Thus, no mutation has been introduced in *B. bronchiseptica* which terminates the protein prematurely

or which results in misfolding or destabilization of the protein such that PtIF is readily degraded.

These results suggest that the *ptx* promoter is necessary for expression of the *ptl* genes. The control exerted by the *ptx* promoter could be direct, i.e., the *ptx* and *ptl* genes may be organized as a single operon and are expressed from the *ptx* promoter. Alternatively, this effect could be indirect, i.e., the production of PT might regulate expression of the *ptl* genes. In order to determine whether the latter possibility was the case, PT was expressed in *trans* in the mutant BPRA which contains the entire *ptl* region but which lacks most of the *ptx* region. If PT regulates expression of the *ptl* genes in *trans*, then one would predict that the *ptl* genes present on the chromosome would be expressed in this strain. In order to test this possibility, a plasmid in which the entire *ptx-ptl* region was first inserted into the broad-host-range vector pUFR047 was constructed (5). The plasmid was then digested with *Bam*HI to remove nucleotides 4570 to 11584 and religated, resulting in the formation of a new plasmid, pSZH6, which is capable of replicating in *Bordetella* spp. and which contains the *ptx* promoter and *ptx* genes but lacks most of the *ptl* region. pSZH6 was introduced into *B. pertussis* BPRA via conjugation.

As shown in Fig. 6, the S1 subunit of PT is produced in the strain containing pSZH6; however, PtIF is not produced. In

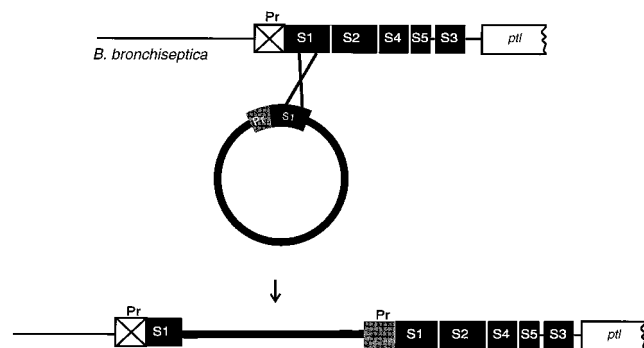


FIG. 4. Introduction of *B. pertussis* *ptx* promoter into *B. bronchiseptica*. The *ptx* promoter (Pr) and S1 subunit cistron of *B. pertussis* were introduced into the *B. bronchiseptica* chromosome by homologous recombination, as described in the text.

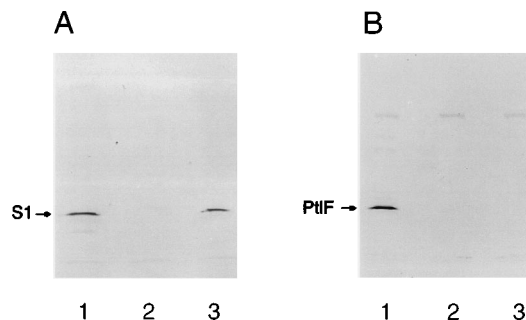


FIG. 6. Immunoblot analysis of cell extracts of *B. pertussis* strains producing PT. Cell extracts (20  $\mu$ l of a culture with  $A_{600} = 2.0$  which had been precipitated with an equal volume of 20% trichloroacetic acid) of *B. pertussis* 338 (lane 1), BPRA (lane 2), and BPRA(pSZH6) (lane 3) were subjected to immunoblot analysis using monoclonal antibody X2X5 (11) to visualize the S1 subunit of PT (A) or polyclonal antibody specific for PtIF (B). (Coreldraw 3.0 was used to generate this figure from a scanned image.)

order to verify that this strain produces all of the PT subunits, we assayed whole-cell extracts for PT activity as measured by toxin-induced clustering of Chinese hamster ovary cells (8). PT activity was detected (data not shown), indicating that holotoxin is produced. Thus, PT does not appear to act in *trans* to induce expression of the *ptl* genes.

These results suggest that both the *ptx* and *ptl* genes are expressed from a single promoter. An additional line of evidence supports this conclusion. *B. pertussis* 356, which contains a Tn5 transposon insertion in the S3 cistron, does not produce PtlE or PtlF (10). If *ptx* and *ptl* genes constitute a single operon, a transposon in the coding region for S3 would be expected to affect the expression of all downstream genes, including *ptlE* and *ptlF*.

These results suggest that the *ptx* operon is larger than originally proposed (15) and contains genes necessary for the production of both the structural subunits of PT and the proteins involved in secretion of the toxin. Coordinate regulation of the *ptx* and *ptl* genes would ensure that the PT transport proteins are produced only if PT is also produced, thereby conserving energy.

Localization of the promoter for *ptl* genes may be useful in the design of strains which are capable of secreting large quantities of PT. Inactivated PT is a component of all acellular pertussis vaccines which have been developed to date. PT is produced in limiting quantities and therefore adds significantly to the expense of the vaccine. Development of a strain of *B. pertussis* which produces large quantities of the toxin and secretes this toxin into the culture supernatant might significantly reduce production costs and, in turn, reduce the cost of the vaccine, allowing for expanded use of these vaccines worldwide.

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