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Molecular characterization of an operon required for pertussis toxin secretion

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ABSTRACT Mutants of *Bordetella pertussis* which are defective in secretion of pertussis toxin were isolated and characterized. The region of the *B. pertussis* chromosome identified by mutagenesis as playing a role in transport of pertussis toxin was sequenced. Analysis of this region revealed eight open reading frames, seven of which predict a protein exhibiting homology with one of the VirB proteins of *Agrobacterium tumefaciens*, which are involved in the transport of the T-DNA molecule across bacterial and plant membranes. Thus a set of accessory proteins are most likely involved in the secretion of pertussis toxin, and these proteins appear to be members of a family of proteins involved in the secretion of macromolecules from bacteria.

Gram-negative bacteria have a unique feature that distinguishes them from all other living cells, including Gram-positive bacteria. They possess two membranes separated by a cellular compartment called the periplasmic space. The outer membrane presents a formidable barrier to compounds entering and exiting the bacteria. Gram-negative bacteria that are pathogens often produce protein toxins which are secreted from the bacterial cell and subsequently interact with and induce damage to eukaryotic cells. After synthesis, the protein toxin must cross the inner membrane, the periplasmic space, and the outer membrane. We have examined the export of one particular toxin, pertussis toxin (PT), from the Gram-negative organism *Bordetella pertussis*. Since inactivated PT is a major component of new-generation pertussis vaccines, knowledge of the mechanism of secretion of PT may lead to improved and less expensive methods for vaccine production that would allow for expanded use of these vaccines worldwide.

PT is composed of five types of subunits, S1–S5, found in a 1:1:1:2:1 ratio (1). After PT is secreted from the bacterial cell, it interacts with mammalian cells and interrupts signal transduction in those cells by ADP-ribosylating a family of GTP-binding regulatory proteins (2, 3). Little is known concerning the mechanism by which PT is transported across the inner and outer membranes of *B. pertussis*. Each PT subunit is synthesized with a signal sequence (4, 5), suggesting that each subunit may begin the secretion process by entering the general export pathway as exemplified by the *sec* pathway (6) of *Escherichia coli*. To our knowledge, the existence of accessory proteins involved in the secretion of PT has not been demonstrated previously.

In this study, we identified by mutagenesis a region of the *B. pertussis* chromosome located directly downstream from the PT structural genes which appears to encode proteins necessary for transport of PT. Nucleotide sequence analysis[§] of this region reveals eight open reading frames (orfs). Seven of the predicted proteins show striking homology with certain of the VirB proteins of *Agrobacterium tumefaciens* which have been proposed to be involved in transport of the T-DNA molecule across bacterial and plant membranes (7–9).

MATERIALS AND METHODS

Cloning and Mapping of the *ptl* Operon. A cosmid (pUW11-11) containing pHC79 (10) and a fragment of BP338 chromosomal DNA encoding all of PT as well as 20 kb of downstream sequences was subcloned into pBluescript vectors (Stratagene) containing a gene cassette encoding the P-incompatibility group origin of transfer and a gentamicin-resistance determinant. These plasmids were introduced into *B. pertussis* by triparental matings (11). Southern hybridization verified that all transconjugants contained the plasmid sequences integrated by homologous recombination (data not shown).

Secretion of PT. Two methods were used to identify mutants defective in secretion of PT: quantitation of PT activity and a colony blot technique.

PT activity was quantified as follows. *B. pertussis* strains were grown in Stainer–Scholte medium (static culture) at 37°C with appropriate antibiotics included in the medium. For example, the medium for BPM3171 was supplemented with kanamycin (25 μg/ml). Samples (10 ml) were taken at various times to monitor PT secretion. Cells were separated from culture medium by centrifugation (12,000 × *g* for 10 min). The supernatant was filtered through sterile Millex-GV filters (0.22-μm nominal pore size; Millipore). The pellet was suspended in 1 ml of 50 mM Tris·HCl (pH 8.0) containing lysozyme (2 mg/ml) and ethylenediaminetetraacetic acid (50 mM). After incubation for 30 min at 35°C, 9 ml of phosphate-buffered saline (pH 7.2) containing Tween 20 (0.05%) was added. The preparation containing the cell lysate was then centrifuged (12,000 × *g* for 10 min) and the supernatant was collected and filtered through a sterile Millex-GV filter. Biologically active PT in the culture medium and in the cell lysate was quantified by measuring the ability of these fractions to alter the morphology of Chinese hamster ovary cells (12). Purified PT obtained from Institut Merieux, Lyon, France, was used as the standard for this assay.

In some experiments, the secretion phenotype was determined by colony blots. Bacterial strains were grown on Bordet–Gengou agar for 3 days. Nitrocellulose filters (BA85; Schleicher & Schuell) were placed on the colonies, and extracellular material was allowed to bind to the filters overnight at room temperature. The filters were blocked, washed, and processed by standard immunoblot techniques (13). Monoclonal antibody 3CX4 (14), directed against a conformational epitope on the S1 subunit of PT, was used. Secretion mutants could be clearly distinguished from the wild-type strains.

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Abbreviations: PT, pertussis toxin; orf, open reading frame.
‡To whom reprint requests should be addressed.
§The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. L10720).

Nucleotide Sequence Analysis. The DNA sequence of the *ptl* operon was determined by Lark Sequencing Technologies (Houston, TX) using clones pPTL2, pPTL4, and pPTL9. Sequence analysis was performed with Genetics Computer Group programs (15).

RESULTS AND DISCUSSION

Identification of an Operon Required for PT Secretion. Previously (13), mutant BPM3171 was generated by insertion of Tn*5 lac* transposon into BP338 ≈3.2 kb downstream from the end of the PT structural genes (Fig. 1). While growth of BPM3171 was similar to that of the parent strain (data not shown), the level of PT in the culture supernatant of the mutant strain was much lower than that found in the culture supernatant of the parent strain (Fig. 2). PT levels were lower in the culture supernatant of the mutant strain as compared with that of the parent strain when either biologically active PT was measured (Fig. 2) or when the amount of the S1 subunit of PT present in the supernatants was determined by immunoblot analysis (data not shown). In contrast, levels of cell-associated PT were slightly higher in the mutant strain than in the parent (Fig. 2). Preliminary results indicate that the PT which associates with both BP338 and BP3171 cells is membrane-associated (S. Hausman and D.L.B., unpublished observations). Levels of two other exported proteins, filamentous hemagglutinin, which is normally secreted from the bacterium (16), and pertactin, which is normally exported to the outer membrane (17, 18), were found to be similar in the parent and mutant strains as determined by immunoblot analysis (ref. 13; data not shown). Moreover, the mutant strain was hemolytic, suggesting that adenylate cyclase toxin is secreted from the mutant. Thus the defect in BPM3171 appears to affect only the export of PT and not other virulence factors. The gene defined by BPM3171 was originally designated *ptl* for pertussis toxin-linked, but considering its role in secretion, we propose pertussis toxin liberation.

Genetic Mapping of the *ptl* Operon. Mutagenesis was used to define the region essential for PT secretion. Suicide vectors were constructed which contained a gentamicin-resistance determinant and fragments of *B. pertussis* DNA which either included the end of the operon encoding the PT subunits or were from immediately downstream of that operon. Vectors containing a 3652-bp *Bgl* II fragment (pPTL7), a 4523-bp *Bgl* II–*Sal* I fragment (pPTL1), a 5914-bp *Eco*RI fragment (pPTL2), a 1125-bp *Eco*RI–*Kpn* I fragment (pPTL4), a 1062-bp *Kpn* I–*Pst* I fragment (pPTL9), a 1.6-kbp *Kpn* I–*Bam*HI fragment (pPTL8), a 2.6-kbp *Kpn* I–*Sph* I fragment (pPTL5), or a 7-kbp *Kpn* I–*Sst* I fragment (pPTL6) were introduced into BP338. Since these plasmids cannot replicate in *B. pertussis*, gentamicin resistance is conferred only if homologous recombination occurs between the chromosome and the *B. pertussis* sequences on the vector so that the entire plasmid is integrated into the chromosome. The cloned sequences are present twice in the genome but are interrupted by the vector. Mutations can result if the cloned region is internal to an operon, in which case the vector will

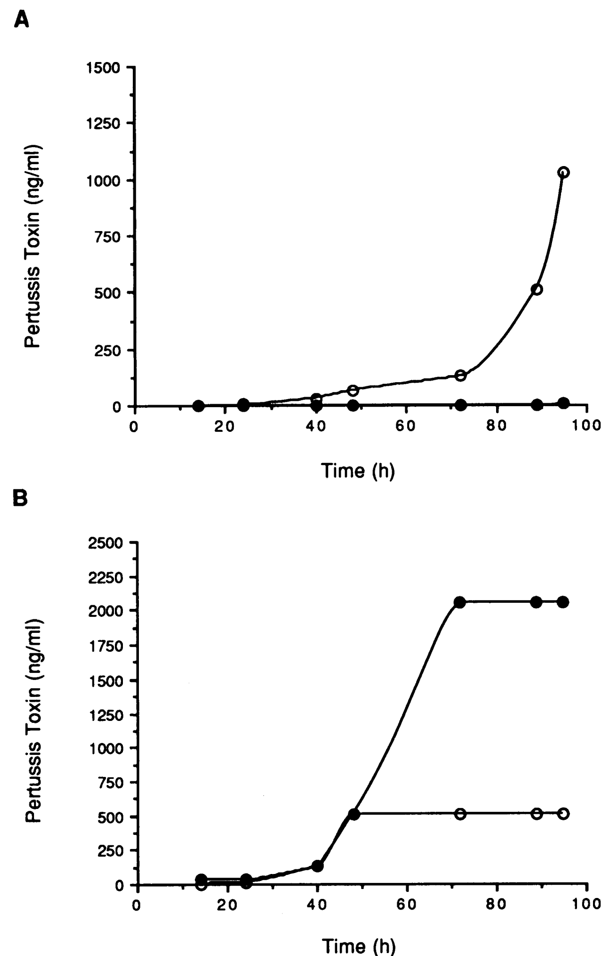

FIG. 2. Comparison of the amounts of active PT produced by BP338 (parent) and BPM3171 (mutant) cells. Bacteria were grown for the indicated times. Cells which had been separated from culture supernatant were lysed and the concentration of active PT in the culture supernatant (*A*) and cell lysate (*B*) was determined for both BP338 (○) and BPM3171 (●).

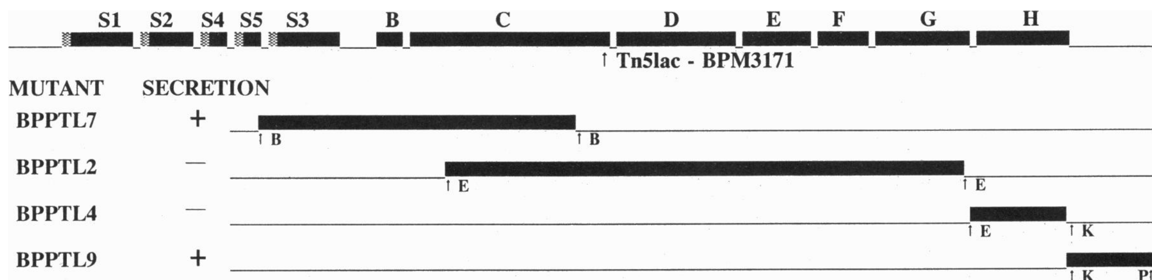

FIG. 1. Map of the *ptl* operon. (*Upper*) Regions encoding the structural subunits (S1–S5) of PT in the *ptx* operon and potential orfs (B–H) in the *ptl* operon are indicated by solid blocks. Stippled blocks indicate regions encoding the signal sequences of the PT structural subunits. The approximate location of the Tn*5 lac* insertion of BPM3171 is indicated. (*Lower*) Schematic diagram of *ptl* region of mutant strains. BPPTL7, BPPTL2, BPPTL4, and BPPTL9 are strains in which pPTL7, pPTL2, pPTL4, and pPTL9, respectively, have integrated into the chromosome. When the plasmid integrates into the chromosome, the cloned sequences are present twice in the genome but interrupted by the vector (see text). Solid bars represent the integrated plasmid and the homologous sequences on the chromosome. BPPTL7 and BPPTL9 were positive for PT secretion. BPPTL2 and BPPTL4 were negative for PT secretion as measured by both PT activity assays and the colony blot technique. Restriction sites are indicated: B, *Bgl* II; E, *Eco*RI; K, *Kpn* I; P, *Pst* I.

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                                                                                                       M  G  H  P  G  H
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 R  P  R  H  A  R  R  L  R  P   E  R  A  A  R  L  A  V  S  R   W  Q  A  P  D  S  H  Q  H  R   R  P  G  A  R  I  V  M  P  D
CCCGCGCCCCTTGACGCCTGACCAGACGCA TGGCGCGCGGGCATGCCGAGGCCGCGGTGGA CTGGGAAGCGTCCCGCCTGTACCGGCTGGC GCAGTCGGAACGCCGTGCATGGACGGTGGC   8483
 P  R  P  L  T  P  D  Q  T  H   G  R  G  H  A  E  A  A  V  D   W  E  A  S  R  L  Y  R  L  A   Q  S  E  R  R  A  W  T  V  A
GTGGGCGGCGCTCGCCGTGACCGCGCTGTC ATTGATCGCCATCGCGACGATGCTTCCGCT CAAGACCACCATTCCTTACCTGATCGAGGT CGAAAAGAGCAGCGGCGCGGCCTCGGTCGT   8603
 W  A  A  L  A  V  T  A  L  S   L  I  A  I  A  T  M  L  P  L   K  T  T  I  P  Y  L  I  E  V   E  K  S  S  G  A  A  S  V  V
CACGCAATTCGAACCCCGAGATTTCACCCC GGACACCTTGATGAACCAGTACTGGCTGAC GCGCTATGTCGCGGCGCGCGAGCGCTACGA TTGGCACACGATCCAGCACGATTACGACTA   8723
 T  Q  F  E  P  R  D  F  T  P   D  T  L  M  N  Q  Y  W  L  T   R  Y  V  A  A  R  E  R  Y  D   W  H  T  I  Q  H  D  Y  D  Y
CGTGCGCCTGCTGTCCGCGCCCGCCGTCAG GCACGATTACGAAACCAGCTACGAGGCGCC CGATGCGCCGGACCGCAAGTACGGCGCTGG CACGACCCTGGCCGTGAAAATTCTCAGCGC   8843
 V  R  L  L  S  A  P  A  V  R   H  D  Y  E  T  S  Y  E  A  P   D  A  P  D  R  K  Y  G  A  G   T  T  L  A  V  K  I  L  S  A
CATCGACCATGGCAAGGGCGTCGGCACGGT GCGCTTTGTCCGGACACGGCGCGACGCGGA CGGGCAGGGCGCCGCCGAATCCATCGATATG GGTAGCGACCGTGGCATTCGCCTACGATCA   8963
 I  D  H  G  K  G  V  G  T  V   R  F  V  R  R  R  D  A  D      G  Q  G  A  A  E  S  I  M      V  A  T  V  A  F  A  Y  D  Q
GCCGCGCGCGCTGACCCAGGCCCAGCGCTG GCTCAATCCGCTGGGCTTTGCCGTCACCAG CTATCGCGTCGACGCGGAGGCTGGACAGCC ATGATGGCGGCACGGATGATGGCGGCCGGC   9083
 P  R  A  L  T  Q  A  Q  R  W   L  N  P  L  G  F  A  V  T  S   Y  R  V  D  A  E  A  G  Q  P   *
                                                                                                orff
                                                                                                M  M  A  A  R  M  M  A  A  G
CTGGCGGCCACGGCGCTTTCGGCGCATGCG TTCCGGATACCCACGCCAGGAGAACAGGAC GCCCGCATACAGACCGTGCCGTACCACCCG GAGGAGGTGGTGCTGGTGCGCGCCTGGAAC   9203
 L  A  A  T  A  L  S  A  H  A   F  R  I  P  T  P  G  E  Q  D   A  R  I  Q  T  V  P  Y  H  P   E  E  V  V  L  V  R  A  W  N
GGCTACGTCACCCGGATCGTATTCGACGAG CAGGAGAAGATCATCGACGTCGCCGCGGGG TTCGCCGACGGCTGGCAATTCAGCCCGGAA GGCAATGTGCTGTATATCAAGGCCAAATCC   9323
 G  Y  V  T  R  I  V  F  D  E   Q  E  K  I  I  D  V  A  A  G   F  A  D  G  W  Q  F  S  P  E   G  N  V  L  Y  I  K  A  K  S
TTCCCCGCGCAGGGCTCGCCCGCGCAGGCG CCGGAGCCGGGATTGTGGAACACCAATCTG CTCGTCAAAACCGACCGCCGGCTCTACGAC TTCGACCTGGTGCTGGCCAGCGCGGACGCG   9443
 F  P  A  Q  G  S  P  A  Q  A   P  E  P  G  L  W  N  T  N  L   L  V  K  T  D  R  R  L  Y  D   F  D  L  V  L  A  S  A  D  A
GCGACACCGCAGGCGTTGCAGCGTTCGCGC ATGGCCTATCGCCTGCAATTCCGCTACCCG GCCGCGCCGCAGGCGGCATCCCGCGCCAGC CCCGTCGGCCCGGCTGTTCCCGCCGGCGCA   9563
 A  T  P  Q  A  L  Q  R  S  R   M  A  Y  R  L  Q  F  R  Y  P   A  A  P  Q  A  A  S  R  A  S   P  V  G  P  A  V  P  A  G  A
TTGAACCGGCGCTACGCCATGCAGGTGGGC AACGGGTCGGACGGCATCGCACCCATCGCT GCGTACGACGACGGCCGGCATACCTGGCTC ACGTTCAGACCGGGTCAGCCGTTTCCGGCC   9683
 L  N  R  R  Y  A  M  Q  V  G   N  G  S  D  G  I  A  P  I  A   A  Y  D  D  G  R  H  T  W  L   T  F  R  P  G  Q  P  F  P  A
GTATTCGCGGTAGCGCCGGACGGAACGGAA ACCCTGGTCAATCTGCATATCGACAACCAA TCACTGGTCATACACCGGGTAGCGCCGGTC CTGATGCTGCGGTCGGGCGCCAGCGTCATC   9803
 V  F  A  V  A  P  D  G  T  E   T  L  V  N  L  H  I  D  N  Q   S  L  V  I  H  R  V  A  P  V   L  M  L  R  S  G  A  S  V  I
CGGATCGTCAACCAGAACGGCGATGCGTCC GAGTCCCCAGCCTTCGAATGCCATGCTGAA CCGGCCCTCTAGTCCCGATGGCGGCGAAGC GCACGCTTGGCCGCCGGACCCCGAAATCCC   9923
 R  I  V  N  Q  N  G  D  A  S   E  S  P  A  F  E  C  H  A  E   P  A  L  *
TGTTTTCGCCAATGCCGAGCATGCGCATCG GCGTCCGCTGCGCTGGATGTTCGCCCTTGT CGCCGTGGCCCTGTCATGCCTGCTGGCAAC GGGGATATGGCGCAGCCGCGCCGCGCCGCC   10043
                                                orfg
                                                M  F  A  L  V   A  V  A  L  S  C  L  L  A  T   G  I  W  R  S  R  A  A  P  P
```

FIG. 3. *(Figure continues on the opposite page.)*

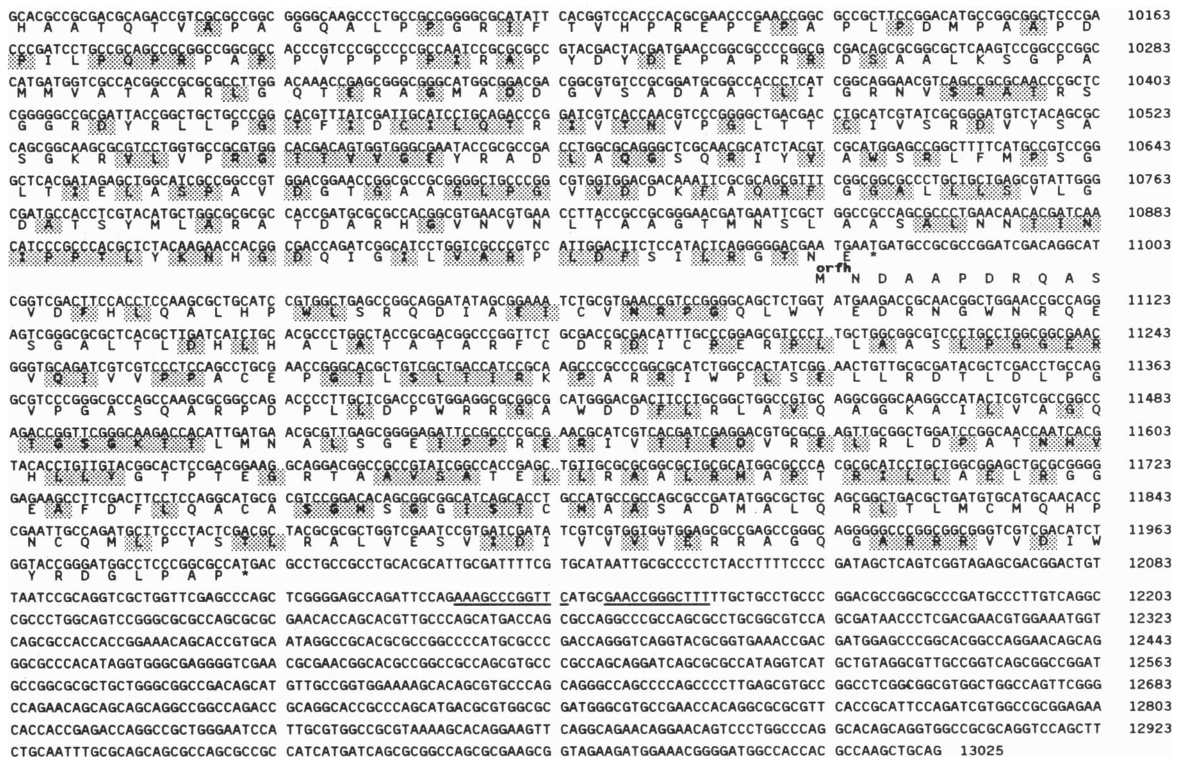

FIG. 3. Nucleotide sequence of the *ptl* operon. The DNA sequence of the *ptl* operon is shown along with the predicted amino acid sequence of each orf. Nucleotides which may represent a transcriptional terminator are underlined. Nucleotides were numbered by using the system of Nicosia *et al.* (4) for the *ptx* operon. The sequence of nucleotides 3684–4692 is taken from that study (4). Amino acid sequences in orfs B, C, D, E, F, G, and H which are identical to sequences in VirB3 (8, 19), VirB4 (8, 19), VirB6 (19), VirB8 (20, 21), VirB9 (20, 21), VirB10 (19), and VirB11 (8, 19), respectively, are shaded.

disrupt the end of the operon in the first copy and the second copy will lack the promoter sequences.

Recombination of the BP338 chromosome with pPTL7, pPTL8, pPTL5, pPTL6, and pPTL9 yields strains BPPTL7, BPPTL8, BPPTL5, BPPTL6, and BPPTL9, respectively, which retain the ability to secrete PT. In contrast, recombination of the *B. pertussis* chromosome with pPTL2, pPTL1, and pPTL4 yields strains BPPTL2, BPPTL1, and BPPTL4, respectively, which are unable to secrete PT. The mutants defining the boundaries of the operons are shown in Fig. 1. Since mutations result only when the cloned region is internal to an operon, the cloned *B. pertussis* fragments present in pPTL2 and pPTL4 are internal to the operon whereas the cloned fragments in pPTL7 and pPTL9 must each extend across a boundary of the operon. The cloned region of pPTL7 contains sequences that encode PT structural subunits and BPPTL7 retains the ability to secrete PT, suggesting that the operon which controls secretion of PT (*ptl* operon) is distinct from the operon which encodes the PT structural subunits (*ptx* operon). The *ptl* operon may therefore span up to 9.5 kb.

In addition, two mutants, BPPTL3-1 and BPPTL3-2, were constructed by allelic exchange (double crossovers). The 437-bp *Bam*HI fragment in orf C was deleted and replaced with a 4.0-kbp *Bam*HI cassette containing the gentamicin resistance gene and the P-incompatibility origin-of-transfer region in both orientations. These mutants did not contain the 437-bp *Bam*HI fragment or plasmid sequences. Both mutants were negative for PT secretion as measured either by PT activity assays or by the colony blot technique.

Information obtained by analyzing BPM3171 allows us to deduce the direction of transcription of the *ptl* operon. Since Tn*5 lac* makes transcriptional fusions, we could determine the direction of transcription by mapping the orientation of the insertion in BPM3171. The *Bam*HI site at the beginning of Tn*5 lac* was determined to be proximal to the PT operon by Southern blot analysis, suggesting that the gene inactivated by the insertion in BPM3171 is transcribed in the same direction as the PT operon. Previous work has demonstrated that the gene into which Tn*5 lac* is inserted is *vir*-regulated (13).

Nucleotide Sequence Analysis of the *ptl* Operon. The region encoding the *ptl* operon was sequenced (Fig. 3). The DNA sequence contains eight orfs. An inverted repeat beginning at nucleotide 12,134 may represent a transcriptional termination site. We propose that the first orf between the end of S3 and orf B is not a trans-acting gene but rather a cis-acting promoter since it contains several transcriptional signals which have loose homology to the ptx −10, −35, and ribosome binding sites, as well as a potential binding site for the positive regulatory element Act (22). A search of the Swiss-Prot data base (Release 23.0) for proteins homologous to the protein predicted by the orfs was conducted. The proteins predicted by orfs B, C, D, E, F, G, and H were found to be homologous to the VirB3, VirB4, VirB6, VirB8, VirB9, VirB10, and VirB11 proteins, respectively, from the plant pathogen *A. tumefaciens* (Fig. 3). The VirB proteins have been implicated in the transfer of a piece of DNA, T-DNA, across the bacterial membranes and ultimately across plant cell membranes (7–9). The T-DNA integrates into the plant cell genome and codes for biosynthesis of plant growth hormones. Overexpression of these hormones leads to a loss of division control and thus to tumors (23).

The *ptl* mutants are deficient in the secretion but not the production of functional PT. Each of the PT subunits is synthesized with a secretion signal sequence (4, 5) which should target it to the periplasm where the subunits may then associate with membrane proteins. The *ptl* pathway appears to be necessary to complete the secretion process. The sequence homology with the protein complex required to transfer the T-DNA of *A. tumefaciens* is provocative. The

T-DNA passes through the two bacterial membranes and through plant membranes. PT presumably only needs assistance through the outer membrane of *B. pertussis*, perhaps explaining why only 7 of the 11 *virB* genes have a *ptl* homologue; however, we cannot exclude the possibility that genes mapping outside of the *ptl* operon may also be required.

Chemotherapy against bacterial infections requires selective toxicity against the microorganism. An antimicrobial agent targeted against only pathogenic bacteria would be an important development. The *ptl* secretion pathway will allow us to screen for antimicrobial agents targeted against a subset of bacteria, Gram-negative bacteria that secrete toxic factors. Since *ptl* mutants are reduced in virulence (24), it seems likely that a therapeutic or vaccine directed against this pathway would also result in less severe infection.

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