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Characterization of the Dermonecrotic Toxin in Members of the Genus *Bordetella*

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All members of the genus *Bordetella* and *Pasteurella multocida* (a gram-negative bacillus genetically unrelated to *Bordetella* spp., yet often sharing the same ecological niche) produce a dermonecrotic toxin (DNT). The amount of toxin produced and the time required for appearance of the lesions are identical for *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* but different for *P. multocida* and *B. avium*. DNT has been reported to act by promoting vasoconstriction; however, vasoactive compounds (verapamil, prazosin, hydralazine, tolazoline, or isoxsuprine) are able to reverse the action of the toxin only slightly. Vasoconstrictors (atropine, serotonin, epinephrine, or endothelin) did not produce DNT-like lesions. We have characterized a region of DNA essential for DNT expression. We have determined by Southern analysis that the restriction map of the DNT gene is nearly identical in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, but the sequences are not present in toxigenic *B. avium* and *P. multocida* strains. A gentamicin resistance-origin of transfer cassette cloned into a 1.8-kb *Not*I-*Bam*HI fragment results in constructs which can be mobilized and recombined into the *Bordetella* chromosome, rendering the resultant *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains negative for DNT. A 5-kb *Bam*HI-*Apa*I fragment from the *B. pertussis* chromosome was sequenced and revealed homology to the *Escherichia coli* CNF1 (cytotoxic necrotizing factor 1) toxin.

All *Bordetella* species are associated with upper respiratory disease in their natural hosts (6, 10, 19, 42, 53). They all produce a number of virulence factors, but only two, the dermonecrotic toxin (DNT) and tracheal cytotoxin, a component of the cell wall peptidoglycan, are present in all *Bordetella* species (6, 13, 14, 18, 36, 53). DNT was one of the first virulence factors to be described for *Bordetella pertussis* by Bordet and Gengou in 1909, although they misidentified it as endotoxin (6). DNT is so called because it produces a characteristic skin lesion when injected into test animals such as rabbits, mice, and guinea pigs (6, 30, 38). It is inactivated by heat treatment at 56°C (30) and has been reported to be cytoplasmic rather than secreted by the bacteria (11, 35). The most recent reports suggest that DNT is composed of a single polypeptide chain with a molecular mass of 140,000 Da (22, 58), and a minimal dose of 0.40 pg is required for a skin reaction (58).

The role of DNT in the pathogenesis of whooping cough is uncertain since mutants lacking DNT appear to be as virulent as the wild type in a mouse model of disease (54). There is some suggestion that DNT is an important virulence factor in the swine disease atrophic rhinitis (45), which can be caused by *B. bronchiseptica* (19, 45), an organism which can also cause kennel cough in dogs (19). *B. parapertussis*, both a human pathogen and a sheep pathogen (10), and *B. avium*, a pathogen of domestic fowl (42, 48), also produce DNT, but, as in *B. pertussis* infections, there are no data regarding the role of DNT in infections caused by them.

To compare the DNTs from these species on a genetic level, we have cloned a fragment of DNA which is essential for the expression of the DNT from the *B. pertussis* DNT mutant BPM1809 (56). We report here that while there is tremendous similarity between the DNTs of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, there are genetic and biologic differences between them and the DNT from *B. avium*. Certain strains of *Pasteurella multocida* are implicated in atrophic rhinitis (45), and these strains also produce a toxin, PMT, which causes dermonecrosis in test animals (35, 40, 44). The *B. pertussis* DNT is homologous to the *Escherichia coli* CNF1 (cytotoxic necrotizing factor 1) toxin, which in turn shares homology with the *Pasteurella* DNT, PMT. Interestingly, however, the *Bordetella* DNT is not homologous to PMT. These dermonecrosis-inducing toxins now appear to constitute a large family of related and unrelated members.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. All *Bordetella* and *Pasteurella* spp. were maintained on Bordet-Gengou agar (BBL Microbiology Systems, Cockeysville, Md.), at 35°C, supplemented with 15% sheep blood and appropriate antibiotics at the following concentrations: gentamicin, 10 μg/ml; kanamycin, 25 μg/ml; streptomycin, 300 μg/ml; and nalidixic acid, 25 μg/ml (56). *E. coli* was maintained on Luria agar (31), at 35°C, supplemented with appropriate antibiotics at the following concentrations: gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml. For most experiments, bacteria were harvested from plates and suspended in modified Stainer-Scholte broth (56) without supplements.

Calculation of CFU. Forty-eight-hour cultures of each *Bordetella* species were harvested in Stainer-Scholte broth (56) to an optical density at 600 nm (OD_{600}) of 1.0. These suspensions were serially diluted 10-fold, and 100 μl of each dilution was placed on a Bordet-Gengou agar plate. These plates were incubated for 1 week, and colonies were counted.

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TABLE 1. Bacterial strains used

Strain	Description[a]	Source or reference
B. pertussis		
BP338	Nal^r Tohama I	55
BPM1809	BP338(*dnt1*::Tn*5lac*)	56
BPRA	Ptx$^-$	2
wBP1	BPRA; Fha$^-$	This study
wBP26	wBP1; CyaA$^-$	This study
wBP40	BP338; Dnt$^-$ Gen^r Amp^r	This study
wBP41	BP338; Dnt$^-$ Gen^r	This study
114	Wild type	34
16945	Wild type	ATCC via Mike Brennan
18-323	Wild type	34
B. parapertussis		
253	Wild type	14
77	Wild type	34
wBPa21	77 Nal^r Str^r	This study
wBPa44	wBPa21; Dnt$^-$ Gen^r Amp^r	This study
wBPa45	wBPa21; Dnt$^-$ Gen^r Amp^r	This study
wBPa46	wBPa21; Dnt$^-$ Gen^r	This study
wBPa47	wBPa21; Dnt$^-$ Gen^r Amp^r	This study
17903	Wild type	34
B. bronchiseptica		
11DH	Wild type	34
BB501	11DH; Nal^r	This study
10540	Wild type	ATCC via Mike Brennan
B. avium		
197	Wild type	18
wBA70	197; Nal^r Str^r	This study
008	Wild type	18
045	Wild type	18
ATCC 35086	Wild type	ATCC
W	Wild type	48
P. multocida ATCC 12948	Wild type	ATCC
P. aeruginosa ATCC 19660	Wild type	ATCC
V. vulnificus ATCC 29307	Wild type	ATCC

[a] Abbreviations: Nal, nalidixic acid; Str, streptomycin; Gen, gentamicin; Amp, ampicillin; CyaA, adenylate cyclase toxin; Ptx, pertussis toxin; Fha, filamentous hemagglutinin; Dnt, dermonecrotic toxin.

DNT assay. Infant BALB/cByJ mice bred in our colony were used in the following modified dermonecrotic assay (56). Seven- to 9-day-old mice (weight, approximately 4 g) were patted with Argo cornstarch and injected with 50 μl of crude cell lysates. Crude cell lysates consisted of 20-h cultures of *Bordetella* or *Pasteurella* cells grown on Bordet-Gengou agar, harvested in modified Stainer-Scholte broth (56) to the specified OD_{600} and sonicated on ice for 2 min on a 50% cycle at output 4 of a Branson (Danbury, Conn.) Sonifier. Mice were kept in a covered beaker on a heat block set at approximately 37°C during the assay period and monitored at 3 h and every 30 min thereafter. Frequently, lesions became visible within 3 h but did not reach maximal presentation at that time. Initial DNT assays were terminated within 9 h. Later assays involving *P. multocida* and *B. avium* were terminated within 16 h, and the mice were inspected for the presence of lesions, photographed, and euthanized. Lesions were scored strictly by the following criteria: negative, no lesion; 1+, small red lesion less than 1 cm; 2+, red lesion greater than 1 cm; 3+, black lesion greater than 1 cm; 4+, blue-black lesion 2 cm or greater, often accompanied by death. Each experiment, performed in duplicate, was repeated at least twice to ensure consistent values. No range of values is reported since experiments repeatedly resulted in the same score.

Pharmacologic assays. *Bordetella* cells grown on Bordet-Gengou agar for 20 h at 35°C were harvested at an OD_{600} of 0.1 absorbance unit (Spectronic 20; Bausch & Lomb) in modified Stainer-Scholte broth and sonicated on ice. Fifty-microliter aliquots of these suspensions were injected into test animals. Vasoactive agents were prepared at the following concentrations: 50 μg of verapamil per ml, 50 μg of prazosin per ml, 100 μg of hydralazine per ml, 50 μg of prednisolone per ml, 3 mg of tolazoline per ml, and 3 mg of isoxsuprine dissolved in sterile distilled water per ml. All agents were prepared fresh and stored briefly on ice prior to injection. After empiric modification, an optimal time of 60 min between the injection of the DNT preparation and 50-μl aliquots of the pharmacologic agents was used for the reported values. Lesions were allowed 8 h to develop and then scored in comparison with sites of control injections of *Bordetella* lysates and *Bordetella* lysates plus modified Stainer-Scholte broth. To determine whether known vasoconstrictors could produce DNT-like lesions, we injected 50 μl of atropine (500 μg/ml), serotonin (500 μg/ml), epinephrine (100 μg/ml), or endothelin (250 μg/ml).

DNA isolation and manipulation. Small-scale preparations of plasmid DNA were prepared by the alkaline lysis-boiling method (47). Large-scale isolation of plasmid DNA was obtained by cesium chloride density centrifugation (47) after alkaline lysis. The plasmids used in this study are listed in Table 2. Chromosomal DNA was isolated from *Bordetella* and *Pasteurella* spp. by using a modification of the Murray and Thompson procedure (4). Enzyme reactions were performed as described in the specifications of the manufacturers. Chromosomal digests were electrophoresed in agarose gels composed of 0.6 to 0.8% SeaKem GTG agarose in 1× Tris-borate-EDTA (31) or Tris-acetate-EDTA (4) buffer. The DNA in these gels was transferred to charged nylon by using 0.4 N NaOH, and the gels were baked for at least 15 min at 80°C (4). SeaPlaque low-melting-point agarose gels were used for some subcloning experiments. In these cases, 1× Tris-acetate-EDTA buffer was used to electrophorese DNA in 0.75% low-melting-point agarose gels. Appropriate bands were excised from the gel, melted at 70°C, suspended in the appropriate buffer, and subsequently treated with restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase, or T4 kinase. Double-stranded DNA was labelled with ^{32}P by using primer extension, and oligonucleotide probes were labelled with ^{32}P by using T4 polynucleotide kinase. DNA sequencing was performed by Lark Sequencing Technologies (Houston, Tex.).

Hybridizations. High-stringency hybridizations (31) of charged nylon membranes (Biotrace-HP; Gelman Sciences) were performed in a mixture of 50% formamide, 2.5× Denhardt's solution (0.05% Ficoll, 0.05% polyvinylpyrrolidone, 0.05% bovine serum albumin), 2% sodium dodecyl sulfate (SDS), 40 μg of salmon sperm DNA per ml, and 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight at 42°C. These membranes were then washed, first in 2× SSC–0.1% SDS solution at ambient temperature and then in 0.1× SSC–0.1% SDS solution at 60°C. Low-stringency hybridizations as described by Ausubel et al. (4) on charged nylon membranes were performed in a solution of 30% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and 0.3% SDS. These membranes were

TABLE 2. Plasmids used

Plasmid	Vector	Contents[a]	Reference
pGEM5Zf(+)			Promega, Madison, Wis.
pHC79			BRL, Gaithersburg, Md.
pBluescript IIKS(+)			Stratagene, La Jolla, Calif.
pUC-4K			Pharmacia, Piscataway, N.J.
pSS1129		pRTP1 with Gen^r	Scott Stibitz
pEB360	pSS1129	Gen^r/oriT on 3.7-kb *Bam*HI fragment	5
pJR1	pHC79	FhaB gene inactivated with Gen^r/oriT fragment	This study
pEB317	pRTP1	CyaA gene inactivated with Kan^r fragment from pUC-4K	5
pKBlu1	pGEM5Zf(+)	13.5-kb *Not*I	This study
pKEW32	pBluescript II KS(+)	1.8-kb *Not*I-*Bam*HI	This study
pKEW4	pHC79	1.8-kb *Not*I-*Bam*HI, 3.7-kb Gen^r/oriT	This study
pKEW42	pBluescript II KS(+)	1.8-kb *Not*I-*Bam*HI, 3.7-kb Gen^r/oriT	This study
pDNT103	pBluescript II KS(+)	3.1-kb *Not*I-*Apa*I	This study
pDNT115	pBluescript II KS(+)	2.5-kb *Bam*HI-*Bgl*II	This study

[a] Abbreviations: Gen, gentamicin; Kan, kanamycin.

washed, first in 2× SSPE–0.2% SDS and then in 1× SSPE–0.1% SDS, both at room temperature.

Oligonucleotide probe hybridization. In situ hybridizations using both ^{32}P-labelled double-stranded DNA probes and ^{32}P-labelled single-stranded oligonucleotide probes were performed in the following manner (4). Agarose gels were treated for 30 min with 0.4 N NaOH, briefly neutralized with 2× SSC, and rinsed thoroughly with water. The gels were then dried on a gel dryer (Savant, Farmingdale, N.Y.) for 1 h at 45°C and soaked in hybridization solution (6× SSC, 20 mM sodium phosphate buffer, 5× Denhardt's solution, 0.1% SDS, 250 μg of salmon sperm DNA per ml) for 2 h at 42°C. Fresh hybridization solution was added with the radioactively labelled probe and incubated overnight at 42°C. These gels were washed, first with a 6× SSC solution at room temperature and then with 2× SSC–0.1% SDS at 50°C. All samples were subjected to autoradiography at −70°C. Oligonucleotide probes were synthesized by the Medical College of Virginia-Virginia Commonwealth University nucleic acid core laboratory and purified as described previously (56).

Western blots (immunoblots) with monoclonal antibodies against PMT were performed as described previously (56) with an anti-PMT monoclonal antibody kindly supplied by Anthony G. Ostle of Ambico Inc., Dallas, Tex.

Triparental matings. Triparental matings were used to facilitate recombination between DNT sequences cloned in *E. coli* and the chromosomes of *Bordetella* species (49). A 24-h culture of one of the *Bordetella* species was harvested at an OD_{600} of 1.0 in Stainer-Scholte broth at 37°C, and an 800-μl aliquot was used as a recipient. Two *E. coli* strains were grown on L agar and harvested at the same OD. A 100-μl aliquot from each was mixed with the *Bordetella* cells. The mixture of the three strains was incubated at 35°C for 4.5 h on a solid support of Stainer-Scholte agar supplemented with 0.15% bovine serum albumin and 20 mM $MgSO_4$ (56). The mixture was then transferred to Bordet-Gengou agar containing appropriate antibiotics. After 96 h, all transconjugant colonies were examined for retention of the virulent phenotype and tested for DNT. The simplest way to monitor retained virulence in transconjugants is to inspect colonies for the presence of hemolysis.

Essential for the transfer of the cloned DNA to the *Bordetella* cell are the characteristics of the two *E. coli* strains. One contains the broad-host-range mobilizing plasmid pRK2013 (29), which facilitates the movement of the plasmid from the second *E. coli* strain. The second strain contains a plasmid with two additional cloned elements: first, the *Bordetella* DNA fragment of interest; and second, a P incompatibility plasmid origin of transfer. The second element was constructed in the following manner. A DNA fragment containing a gentamicin resistance determinant and a P conjugation origin of transfer from pSS1129 (5, 49) was cloned in such a manner as to create a 3.7-kb *Bam*HI fragment in the plasmid pEB360 (5). The presence of this *Bam*HI fragment allows cloned sequences to be introduced into the *Bordetella* chromosome and replaced by allelic exchange and gives a selection for the recombinant (5, 49).

Virulence factor mutant construction. To determine whether other *Bordetella* virulence factors affect DNT lesion production, we constructed a series of strains mutant in one or more virulence factors. The constructions are summarized in Fig. 1. A pertussis toxin-deleted Tohama I derivative strain, *B. pertussis* BPRA (2), was sent to us by Camille Locht. This strain has as much DNT activity as our wild-type *B. pertussis* BP338, also a Tohama I derivative (56). We used a plasmid, pJR1, as a donor to produce a BPRA-derived filamentous hemagglutinin (FHA)-negative strain. pJR1 is a pHC79 derivative with a 3.7-kb *Bam*HI gentamicin resistance-origin of transfer (Gen^r/oriT) cassette (5) inserted in place of an internal 2.376-kb fragment in the FHA structural gene. The presence of this *Bam*HI fragment allows cloned sequences to be introduced into the *Bordetella* chromosome and replaced by allelic exchange and gives a selection for the recombinant (5, 49). The resulting strain, wBP1 (Ptx$^-$ Fha$^-$), was found to have wild-type DNT activity. We created a triple mutant, wBP26 (Ptx$^-$ Fha$^-$ Cya$^-$) by using both pJR1 and pEB317 in BPRA. Plasmid pEB317 is a pSS1129 suicide plasmid that contains the *Sma*I-*Sau*3A fragment internal to the *cyaA*-to-*cyaE* region mutated by deleting an internal 2.1-kb *Pst*I fragment and replacing it with the kanamycin resistance gene from pUC-4K. Allelic exchange selecting for kanamycin-resistant mutants resulted in a *cyaB*-to-*cyaD* chromosomal deletion and a nonhemolytic phenotype due to failure to secrete adenylate cyclase toxin. This strain is also wild type in DNT production.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number is U10527.

RESULTS

DNT activity in *Bordetella*, *Pasteurella*, *Pseudomonas*, and *Vibrio* spp. All *Bordetella* species produce a DNT. We com-

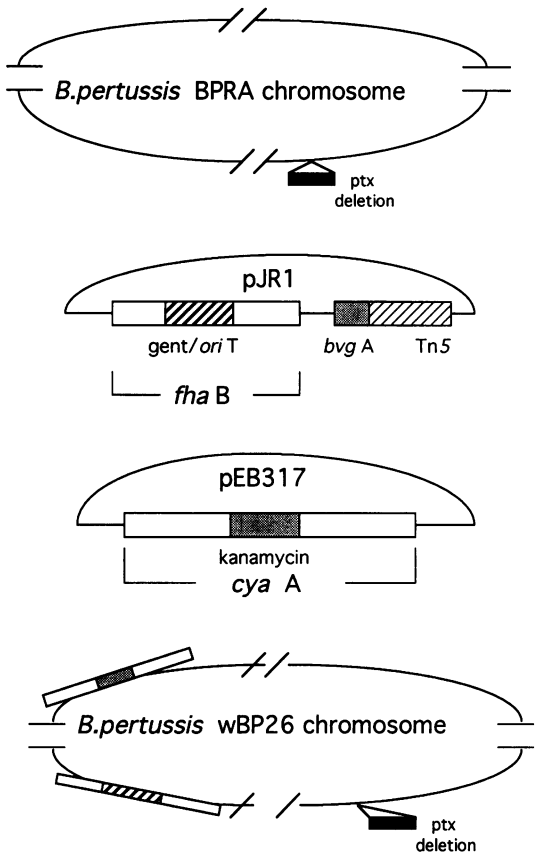

FIG. 1. Construction of mutant *B. pertussis* wBP1 and wBP26. Plasmid pJR1 (containing a mutated copy of the *fhaB* gene) was mated into *B. pertussis* BPRA (deleted for pertussis toxin [ptx]), resulting in wBP1, an FHA- and pertussis toxin-negative strain. wBP1 was mated with an *E. coli* strain containing pEB317, a plasmid with a mutated copy of *cyaA*, resulting in wBP26 an FHA-, pertussis toxin-, and adenylate cyclase toxin-negative strain.

pared the four *Bordetella* species for the ability to cause the characteristic DNT lesion in 7-day-old mice. Equivalent numbers of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* produced similar lesions, but at least two *B. avium* strains, W and 197, appeared to require the lysates from as many as three times the number of cells to produce an equivalent lesion, while other strains were negative in this assay (Table 3). The *B. avium*-induced DNT lesions, although identical in appearance to lesions induced by the other *Bordetella* spp., required at least 13 h to reach maximal presentation. The time required for maximal lesion presentation from *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* was approximately 6 h.

The production of lesions in mice by *P. multocida* ATCC 12948 equivalent to that of *Bordetella* spp. required injection of aliquots with an OD greater than that of the *Bordetella* spp. (Table 3). Upon further investigation, we found that the number of *Pasteurella* CFUs was significantly lower than the number of *Bordetella* CFUs at the same OD, (Table 3), and upon Gram stain, *P. multocida* cells were larger than *Bordetella* cells. PMT appears to be produced in similar amounts on a per cell basis as the DNT of the *Bordetella* species.

Other bacterial genera have been reported to have some type of dermonecrotic activity, and we compared those of two that were obtained from the American Type Culture Collection (ATCC), *Pseudomonas aeruginosa* ATCC 19660 and *Vibrio vulnificus* ATCC 29306, with *Bordetella* DNT activity in a 6-h assay using 50 μl of sonicated cells at an OD of 1. *P. aeruginosa* presented a dissimilar lesion, i.e., more of a red blister than the black necrotic area produced by *Bordetella* species. The *Pseudomonas* lesion was also seen at a distance from the nuchal site of injection. In contrast, *V. vulnificus* had no activity in this assay.

TABLE 3. Comparative levels of DNT activity in *Bordetella* species

Species	Requirements for 4+ lesion[a]		
	OD_{600}	CFU	Time (h)
B. pertussis			
BP338	0.1	2.7×10^8	6
BPRA	0.1	ND[b]	6
wBP1	0.1	ND	6
wBP26	0.1	ND	6
B. bronchiseptica 11DH	0.1	2.2×10^8	6
B. parapertussis			
77	0.1	3.5×10^8	6
253	0.1	ND	6
B. avium			
W	0.5	9.7×10^8	13
197	0.5	7.0×10^8	13
045	0.6	ND	13
008	No activity detected[c]		
ATCC 35086	No activity detected		
P. multocida ATCC 12948	1.2	8.0×10^8	>13

[a] Bacteria were sonicated on ice for 60 s prior to injection of a 50-μl aliquot into infant mice.
[b] ND, not determined.
[c] Cells at an OD of 1.0 had no detectable activity.

Long-term effects of DNT. It has been reported that intravenous injection of DNT causes death in test animals (38), and we have found that the most severe presentation of a 4+ subcutaneous lesion is accompanied by death. To determine the long-term effects of a sublethal subcutaneous injection of

TABLE 4. Vasoactive agents and DNT lesion formation

Agent (concn)	Lesion description[a]	
	BP338	wBP26
None	4+	4+
Receptor antagonists		
Prazosin, α_1 (50 μg/ml)	Blue-black, <1 cm	Blue-black, <1 cm
Tolazoline, α_1 α_2 (3 mg/ml)	3+	3+
β_2 agonist: isoxsuprine (3 mg/ml)	3+	3+
Vasodilator: hydralazine (3 mg/ml)	Blue-black, <1 cm	Blue-black, <1 cm
Calcium channel blocker: verapamil (50 μg/ml)	3+	3+
Anti-inflammatory agent: prednisolone (50 μg/ml)	4+	4+

[a] Lysates from 2.7×10^8 cells were injected in the nuchal region of mice. After 60 min, a 50-μl aliquot of one vasoactive agent dissolved in distilled water was injected close to the initial site. Control injections consisting of *B. pertussis* cells only or *B. pertussis* cells followed 60 min later by distilled sterile water produced 4+ lesions.

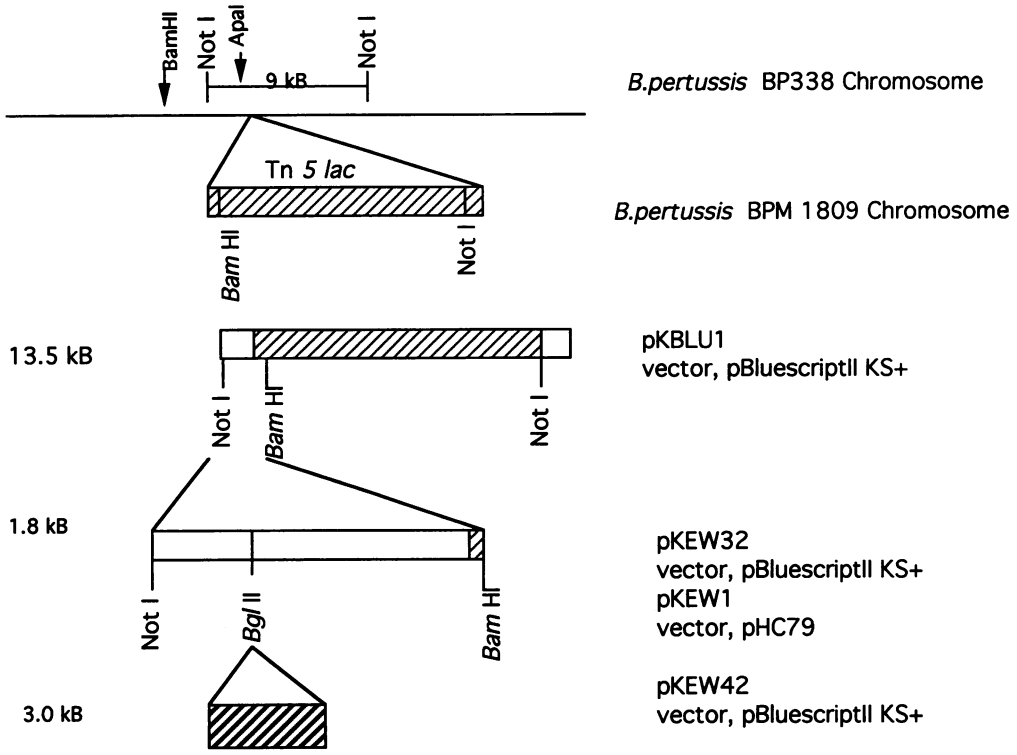

FIG. 2. Cloning of the DNT gene. Chromosomal DNA of BPM1809 was digested with *Not*I and cloned into pGEM5Zf(+). A Tn*5lac*-containing construct, pKBlu1, was digested with *Not*I and *Bam*HI, and a 1.8-kb fragment was cloned into pBluescript II KS(+) digested with *Not*I and *Bam*HI, generating pKEW32. pKEW32 was digested with *Bgl*II and ligated to the 3.7-kb *Bam*HI Gen^r/oriT cassette. This generated pKEW42, which was used to mutagenize *Bordetella* species. The upstream (to *Bam*HI site) and downstream (to *Apa*I site) regions were cloned from BP338 as separate constructs and used to determine the sequence of the DNT gene.

B. pertussis BP338 on infant mice, we returned injected mice (with 3+ lesions) to maternal care after a 6-h assay period. The DNT lesions did not resolve within 3 weeks, indicating that the skin damage caused by the toxin may be long-term. We also observed that the presence of the DNT lesion does not appear to affect the overall growth of the infant mouse, since injected mice gained weight and thrived as well as uninjected littermates. At the end of the 3-week period when the skin covering the lesion appeared ready to exfoliate and we believed that animals were in danger of developing a bacterial infection, we euthanized all of our test animals.

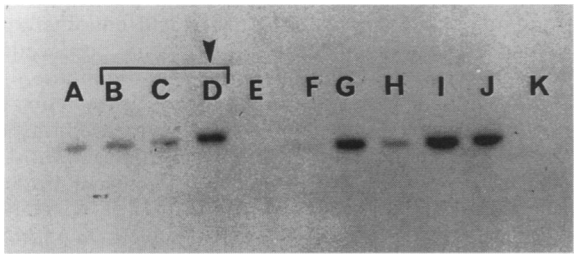

FIG. 3. Chromosomal DNA from all *Bordetella* species digested with *Apa*I. Species included *B. pertussis* 16945 (lane A), 18-323 (lane B), 114 (lane C), and BP338 (lane D); *B. avium* 197 (lane E) and W (lane F); *B. bronchiseptica* 10450 (lane G) and 478 (lane H); *B. parapertussis* wBPa21 (lane I) and 253 (lane J); and *P. multocida* ATCC 12948 (lane K). The brackets surround the *B. pertussis* strains, and the arrowhead denotes the polymorphic strain.

Interaction with other *Bordetella* virulence factors. To determine whether other *Bordetella* virulence factors affect lesion production, we constructed a series of strains mutant in one or more virulence factors (Fig. 1). *B. pertussis* BPRA (Ptx$^-$), wBP1 (Ptx$^-$ Fha$^-$), and wBP26 (Ptx$^-$ Fha$^-$ CyaA$^-$) were all found to have wild-type DNT activity when tested in the 6-h assay (Table 3).

Pharmacologic studies. Since the production of characteristic lesions by DNT has been linked to vasoconstriction by Endoh et al. (14), we wanted to determine which aspect of vasoconstriction was crucial. To that end, we attempted to block production of the lesions by administration of vasoactive compounds to the test animals. We felt that the use of many different classes of vasoactive substances could lead to a more exact understanding of the steps involved in lesion formation.

The process of vasoconstriction is not fully defined, but it involves several steps. Two different receptor types, adrenoceptor types α_1 and α_2, are essential for constriction of smooth muscle, including vascular smooth muscle (28). The activation of α_1 results in an increased cytosolic Ca^{2+} concentration and, in some circumstances, the generation of diacyl glycerol and inositol triphosphate from phosphoinositol triphosphate. Adrenoceptor α_2 inhibits adenylate cyclase via the G_1 subunit. In addition, we investigated the possible effects of other vasoactive agents on lesion formation. We tested two agents which fall under the broad general class of vasodilator, i.e., isoxsuprine and hydralazine, which dilate both veins and arteries. We also selected verapamil, a calcium channel blocker. Calcium influx is essential for constriction of arterial smooth muscle as well as other muscle types, so the addition of verapamil dilates

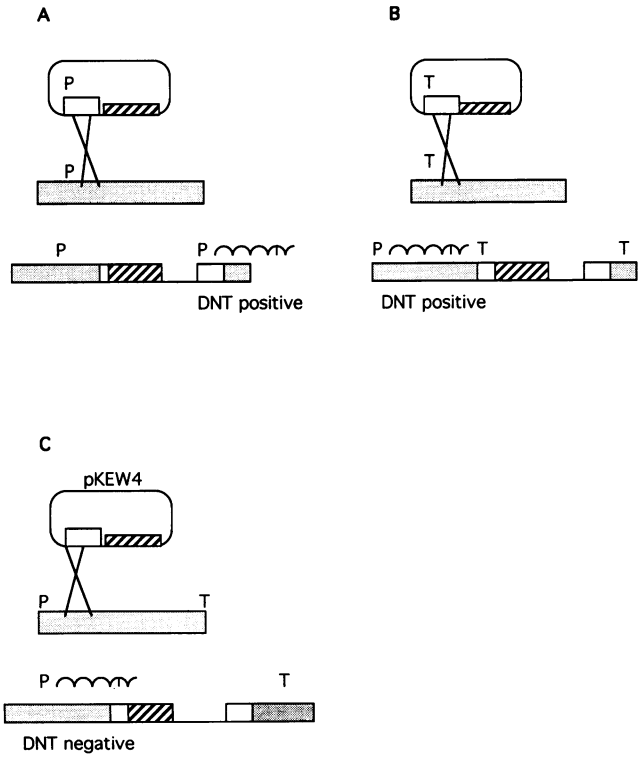

FIG. 4. DNT promoter localization in *B. pertussis*. Cloned DNA is represented by the open boxes, and the stippled boxes represent the chromosomal sequences. The slashed boxes represent the Gen^r/oriT cassette, allowing sequences to be returned to the *Bordetella* chromosome. (A) Diagram depicting the type of recombinational event and resulting DNT phenotype expected if the cloned sequence contains the promoter of the DNT gene; (B) diagram depicting the type of recombinational event and resulting DNT phenotype expected if the cloned sequence contains the terminator of the DNT gene; (C) diagram depicting the type of recombinational event and resulting DNT phenotype expected if the cloned sequence contains neither the promoter nor the terminator of the DNT gene.

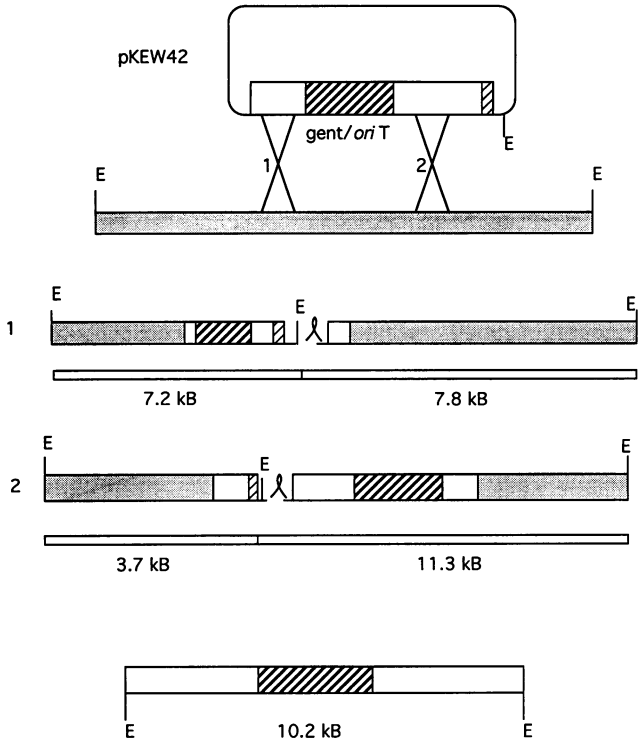

FIG. 5. Expected pKEW42 mutants. Two types of single-crossover recombinants could be produced with pKEW42. Class 1 should generate two fragments when digested with *Eco*RI, one of 7.2 kb (containing the Gen^r/oriT fragment) and one of 7.8 kb. Class 2 should generate two fragments, one of 3.7 kb and one of 11.3 kb (containing the Gen^r/oriT fragment). A 10.2-kb *Eco*RI fragment is generated from a double-crossover mutant. The open boxes represent the cloned DNA, the stippled boxes represent the chromosomal sequences, and the slashed boxes represent the Gen^r/oriT cassette.

peripheral arteries in mammals. Since prednisolone, an anti-inflammatory agent, has already been reported to block the lethal action of DNT (38), it was included for completeness. Prednisolone is a synthetic glucocorticoid, a class of compounds which inhibits phospholipase A_2 activity. This inhibition has important effects on intermediary carbohydrate, protein, and fat metabolism.

We used both wild-type *B. pertussis* BP338 and triple mutant wBP26 in a series of assays to determine which agent was most effective in reducing the severity of the DNT lesion and whether the lack of pertussis toxin, FHA, and adenylate cyclase toxin affected the pharmacology of DNT. We injected lysates of a cell suspension corresponding to the minimum OD_{600} required for a 4+ reaction and asked if the compound could reduce the lesion to 3+ or lower.

As can be seen in Table 4, the addition of vasoactive compounds always affected DNT lesion formation. For some agents, the resulting lesions were of a lower intensity. For others, however, the strength of the DNT reaction was unchanged, but the size of the lesion was considerably smaller, which could result in scoring anomalies. Tolazoline (a reversible α_1 α_2 antagonist), isoxsuprine (a β_2 agonist), and verapamil (a calcium channel blocker) all reduced the severity of a 4+ lesion to a 3+ lesion. However, prazosin (a reversible α_1 antagonist) and hydralazine (a vasodilator) decreased the size of the lesion although the lesion color remained the same. Interestingly, prednisolone (an anti-inflammatant), previously reported to block the lethal action of DNT (38), had no effect on lesion formation, although the method of drug delivery was different in the previous study.

In an effort to determine what similarities exist between *Bordetella* DNT and known vasoconstrictors, we attempted to induce necrotic lesions in mice by injection of vasoconstrictive agents. We injected 50 μl of atropine (500 μg/ml), serotonin (500 μg/ml), epinephrine (100 μg/ml), and endothelin (250 μg/ml) and found that none of these agents produced skin lesions similar to lesions caused by DNT in the mouse.

Cloning of the sequences essential for DNT production from *B. pertussis*. Previously, mutagenesis with the transposon Tn*5lac* produced a DNT-negative *B. pertussis* strain, BPM1809 (56). This strain has been characterized and shown to be deficient only in the production of the DNT (56). A 13.5-kb *Not*I fragment from BPM1809 containing upstream *B. pertussis* and internal Tn*5lac* sequences was cloned into pGEM5Zf(+) by selecting for kanamycin-resistant transformants (Fig. 2, pKBlu1). In addition, transformants containing this fragment had a Lac$^+$ phenotype due to the presence of Tn*5lac*. pKBlu1 was digested with *Not*I and *Bam*HI to liberate a 1.8-kb fragment containing approximately 1.77 kb of *B. pertussis* DNA and 60 bp of Tn*5lac* DNA. The 1.8-kb *Not*I-*Bam*HI fragment

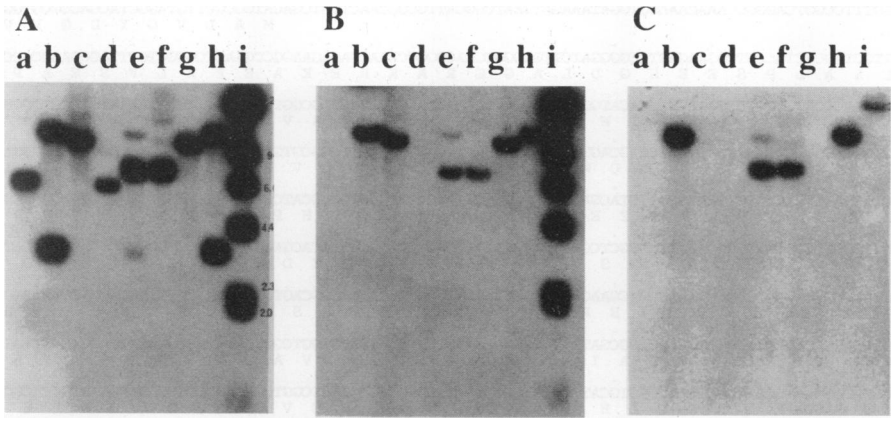

FIG. 6. Autoradiographs of pKEW42 mutants. Autoradiographs of Southern blots of pKEW42-generated mutant chromosomal DNA digested with *Eco*RI probed with ^{32}P-labelled *Not*I-*Bam*HI fragment (A), Gen^r/oriT cassette (B), or pBluescript II KS(+) (C). Lanes: a to c, *B. pertussis* BP338 (lane a), wBP40 (lane b), and wBP41 (lane c); d to h, *B. parapertussis* wBPa21 (lane d), wBPa45 (lane e), wBPa44 (lane f), wBPa46 (lane g), and wBPa47 (lane h); i, molecular size markers, lambda *Hin*dIII, of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb from top to bottom.

was cloned into pBluescript II KS(+) digested with *Not*I and *Bam*HI to generate pKEW32. The same 1.8-kb *Not*I-*Bam*HI fragment was also cloned into pHC79 digested with *Bam*HI and *Eag*I, resulting in the construct pKEW1.

The DNT gene is similar in *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* but not *B. avium* or *P. multocida*. High-stringency Southern analysis and in situ hybridization of *Cla*I, *Eco*RI, *Sal*I, and *Bam*HI digests using the 1.8-kb *Bam*HI-*Not*I *B. pertussis* fragment as a probe reveal identically sized fragments in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (data not shown). Digestion with *Apa*I, however, revealed some polymorphisms (Fig. 3). Except for *B. avium*, the genome of each species contains an *Apa*I fragment which hybridizes to the 1.8-kb *Not*I-*Bam*HI DNT fragment. A polymorphism between *B. pertussis* BP338 (Fig. 3, lane D) and other *B. pertussis* (Fig. 3, lanes A to C) and *Bordetella* (Fig. 3, lanes G to J) strains was detected. Repeated low-stringency and in situ hybridization failed to detect bands in *B. avium* 197 (Fig. 3, lane E) or in *P. multocida* ATCC 12948 (Fig. 3, lane K). A faint band of similar size was detected in *B. avium* W (Fig. 3, lane F). Autoradiographies of low-stringency Southern hybridization analysis and in situ hybridization revealed bands only in *B. avium* W (data not shown) and not in strain 197 or 045, which are also DNT positive, or in strain 008 or ATCC 35086, which are DNT negative. These data suggest that if the probe is detecting DNT in strain W, similar sequences are not present in the other DNT-positive or -negative isolates.

Chromosomal DNA from *P. multocida*, regardless of the hybridization conditions used, contained no fragment which hybridized to the *Bordetella* 1.8-kb *Not*I-*Bam*HI probe (Fig. 3, lane K). The *P. multocida* DNT (termed PMT) gene has been cloned and sequenced (40). An oligonucleotide probe (GGGG GCTCTCCTTATAGTCCTTTCCG) with homology to the *P. multocida* DNT gene revealed no bands by in situ hybridization of *Bordetella* chromosomal digests, including *B. avium*, but did hybridize to *P. multocida* ATCC 12948 (data not shown). In addition, a monoclonal antibody directed against PMT failed to react with *Bordetella* spp. in Western blots (data not shown).

Genetic characterization of the *Not*I-*Bam*HI fragment in *B. pertussis*. To confirm that the cloned fragment was essential for DNT expression, pKEW4, a plasmid which can be mobilized into but cannot replicate in *Bordetella* spp., was constructed by adding a 3.7-kb *Bam*HI Gen^r/oriT fragment to the *Bam*HI site of pKEW1 (Fig. 2). pKEW4 was returned to the *B. pertussis* chromosome by using a triparental mating system. Figure 4 depicts the three hypothetical classes of mutants generated by triparental matings. If the sequence returned to the chromosome contains the whole gene, the recombinant will contain two copies of the gene and will have a wild-type phenotype. If it contains either the promoter (Fig. 4A) or the terminator (Fig. 4B) of a given gene, the resulting strain will have a wild-type phenotype since its genome contains one wild-type and one mutant copy of the gene. Only an internal fragment, which contains neither the promoter nor the terminator (Fig. 4C), produces a mutant phenotype by disrupting the sequence. Our construct, pKEW4, when mated into wild-type *B. pertussis* BP338, produced a strain which was DNT negative, suggesting it contains neither the beginning nor the end of the DNT gene, and this was confirmed by the sequence analysis to be described.

Construction of *Bordetella* DNT mutants. To construct a mutator plasmid, we digested pKEW32 with *Bgl*II. A 3.7-kb *Bam*HI cassette containing a Gen^r/oriT sequence from pEB360 was ligated to the *Bgl*II site, creating pKEW42 (Fig. 2). The Gen^r/oriT cassette both creates a mutation in the DNT gene and allows return of the sequences to the *Bordetella* chromosome. pKEW42 was mated into *B. pertussis* BP338, *B. parapertussis* wBPa21, and *B. bronchiseptica* BB501. We were able to produce both single- and double-crossover recombinants, and both single- and double-crossover classes of mutants were DNT negative.

Two classes of single-crossover mutants could be obtained by using pKEW42 as a mutagen (Fig. 5). The first class corresponds to a mutant in which the recombinational event occurred 5′ to the Gen^r/oriT fragment, and when probed with the 1.8-kb *Not*I-*Bam*HI fragment, 7.8- and 7.2-kb *Eco*RI fragments will be detected. The second class of single crossovers corresponds to a mutant in which the recombinational event occurred 3′ to the Gen^r/oriT insert, and when probed with the 1.8-kb *Not*I-*Bam*HI fragment, one band of 3.7 kb and one band of 11.3 kb will be detected. Both classes were easily obtained in *B. parapertussis*, and the second class was found in *B. pertussis* (Fig. 6A, lanes b, e, f, and h). Both of these classes indicate both the presence of the entire pKEW42 plasmid and the duplication of the *Not*I-*Bam*HI fragment in the chromosome. We also obtained double-crossover recombinants (Fig.

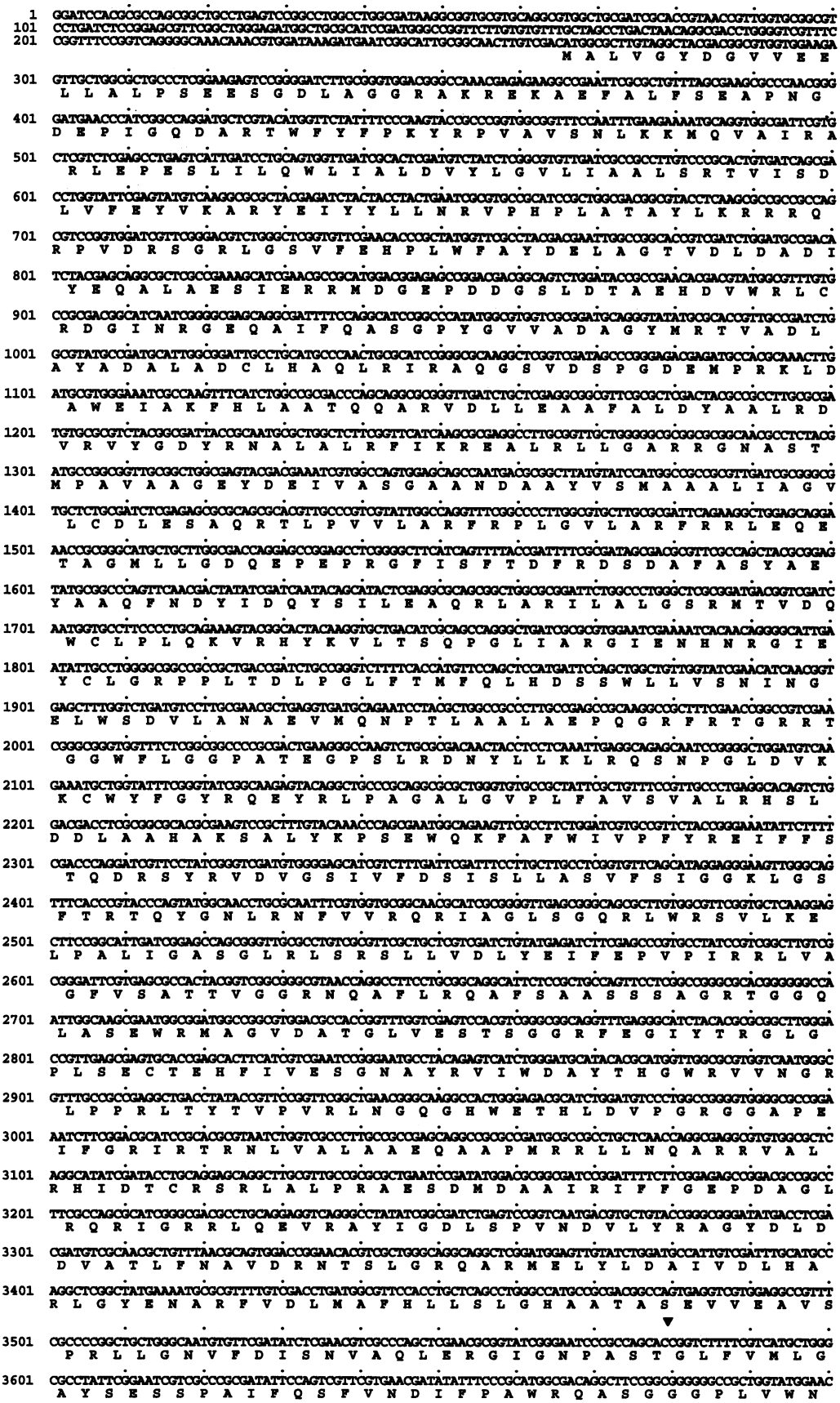

FIG. 7. Sequence of the 4,929-bp DNT region of the *B. pertussis* chromosome. The predicted 1,451-amino-acid sequence for the DNT gene is shown below. The triangle denotes the site of the Tn*5lac* insertion in BPM1809.

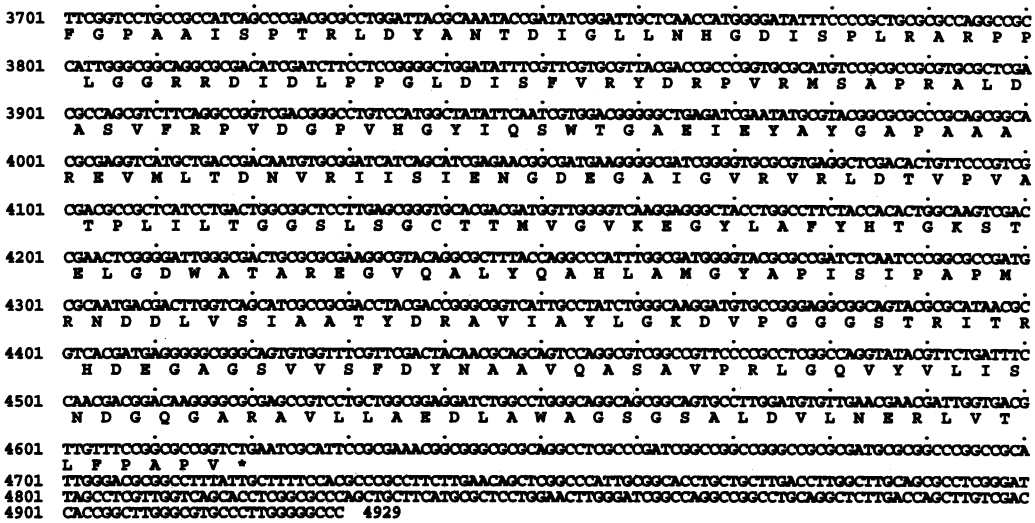

FIG. 7—*Continued.*

6A, lanes c and g). These 10.2-kb fragments correspond exactly to the size of the wild-type *Bordetella* DNT fragment (6.5 kb) increased by the size of the Gen^r/oriT fragment (3.7 kb). Additional blots probed with ^{32}P-labelled sequences from the Gen^r/oriT fragment and pBluescript II KS(+) sequences verify the class of mutants (Fig. 6B, lanes b, c, e, f, g, and h, and Fig. 6C, lanes b, e, f, and h). No transconjugants were obtained by using *B. avium* W and 197 as recipients.

Sequencing the DNT gene. The DNT gene was sequenced in three parts. First the 1.8-kb *Not*I-to-*Bam*HI region was sequenced, resulting in a single open reading frame in the *B. pertussis* sequence. The site of the Tn*5lac* insertion was determined since 59 bp of this clone, including the *Bam*HI site, were derived from Tn*5lac*. Cloning upstream and downstream regions was accomplished by using homologous recombination with the previously cloned DNT sequences to introduce Bluescript vectors into the chromosome, resulting in constructs similar to those depicted in Fig. 4 and 5. Chromosomal DNA was prepared and digested with restriction enzymes which recognize only a single site in the plasmid, and the adjacent *Bordetella* sequences were ligated to the vector and isolated as ampicillin-resistant *E. coli* transformants. Specifically, the downstream region was obtained by introducing pDNT102 (pKEW32 containing the *Bam*HI Gen^r/oriT fragment) into wild-type *B. pertussis* BP338. Chromosomal DNA was isolated, digested with *Apa*I, and ligated to itself. One ampicillin-resistant transformant, pDNT103, which contained a 3.2-kb *Not*I-to-*Apa*I downstream DNT region of the *B. pertussis* chromosome (Fig. 2), was characterized and sequenced. Similarly, the upstream region of the chromosome was isolated. The 753-bp *Not*I-to-*Bgl*II region from pKEW32 was cloned into Bluescript SK+, and the Gen^r/oriT region was also cloned into this construct as a *Sal*I fragment into the *Sal*I site of Bluescript SK+. The chromosomal DNA from this recombinant was isolated and digested with *Bam*HI, and the 2-kb *Bam*HI-to-*Not*I region (Fig. 2) was sequenced. Note that each clone overlapped, and redundant sequences from each clone were obtained to ensure there were no closely spaced multiple restriction sites.

The sequence of this 4,929-bp region contained a large open reading frame, transcribed in the orientation required to generate a promoter fusion with the β-galactosidase gene of Tn*5lac* (Fig. 7). The site of the transposon insertion is shown by the triangle. This open reading frame was highly predicted by the GCG codon preference program, using either *E. coli* or *B. pertussis* codon frequency data. The 1,451-amino-acid sequence predicted in the large open reading frame has a molecular mass of 159,240 Da and an isoelectric point of 6.63, in good agreement with the 6.3 to 6.7 experimental value (21, 22). The region 5′ to the open reading frame is devoid of conventional *E. coli* transcriptional and translational sequence information, which is not uncommon for *Bordetella* genes. It also appears to lack the BvgA-binding heptamer (43) as well as the two 20-bp repeats required for transcription of pertussis toxin and adenylate cyclase toxin (26). However, the upstream regions of other *Bordetella* genes, such as *aroA* (32) and those for pertactin (9) and fimbriae (57), do not resemble each other or other promoters; therefore, what constitutes a *Bordetella* promoter has not been established.

The larger open reading frames were analyzed by the program BLAST (1) to identify possible homologs. An excellent match was seen with the *E. coli* CNF1 toxin (Fig. 8). CNF2 (37), which was not in the BLAST database, is 85% identical to CNF1 and thus also homologous to DNT. An ATP- or GTP-binding site (P loop) (46) at amino acids 1,304 to 1,310 is predicted by the MOTIFS program. This region overlaps the homology region of CNF1, but interestingly, this would not form a nucleotide binding site in CNF1 since only the glycine

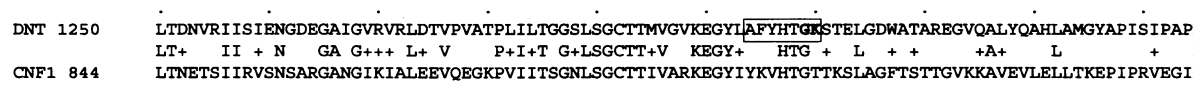

FIG. 8. Regions of homology between DNT and CNF1. Conservative amino acid changes are indicated by a plus. All of the identical amino acids are also present in CNF2 (not shown). The boxed region corresponds to the ATP- or GTP-binding site predicted by the MOTIFS program. The boldface amino acids, G and K, are essential for nucleotide binding.

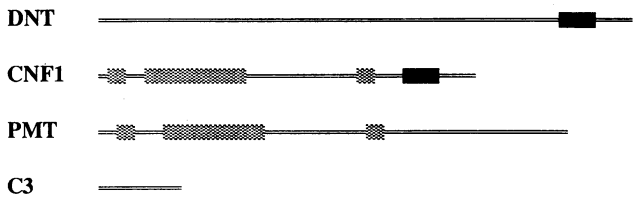

FIG. 9. The dermonecrosis-inducing toxin family. The homologous regions of DNT and CNF1 are depicted by the black boxes. The homologous regions between CNF1 and PMT are depicted by shaded boxes. Unique regions without homologs (including all of C3) are depicted as double lines. All proteins are drawn to the same scale.

residue of the essential glycine and lysine residues (Fig. 8, boldface letters) is present in the CNF1 sequence.

The DNT sequence does not appear to possess an N-terminal secretion signal sequence. CNF1 (15), CNF2 (37), and PMT (40) are also devoid of obvious N-terminal secretion signal sequences. Our evidence and that of others suggest that DNT, like CNF1, CNF2, and PMT, is not secreted (15, 16, 35).

A partial open reading frame (C terminus only) encoded by bases 4929 to 4717 following the large open reading frame associated with DNT activity was also highly predicted to be transcribed and also has a good match (as determined by BLAST) with a hypothetical 35.9-kDa protein in the intergenic region of the *tcbC-tcbE* operon from *Pseudomonas* sp. strain P51 that is able to metabolize chlorinated catechols (52). This chromosomal region is probably unrelated to DNT activity. The open reading frames upstream from DNT neither were highly predicted to be transcribed nor had significant matches as determined by BLAST.

DISCUSSION

A large family of dermonecrosis-inducing toxins which have been shown to stimulate DNA synthesis has been described. In addition to DNT, this toxin family includes CNF1, CNF2, PMT, EDIN (for staphylococcal epidermal cell differentiation inhibitor) (27, 50), and botulinum C3 toxin. CNF1, CNF2, DNT, and C3 toxin stimulate DNA synthesis, but cell division does not occur, resulting in multinuclear cells (3, 7, 8, 12, 17, 23, 24, 51). PMT appears to stimulate DNA synthesis but does not adversely affect cell division (33, 44). The molecular basis of toxicity for C3 (25), EDIN (27, 50), and, recently, CNF2 (37) has been shown to be due to different modifications of the mammalian Rho protein. Rho is a member of the Ras family of small GTPase enzymes (20). Rho has been shown to control cytoskeletal rearrangements (39, 41). C3 and EDIN have been shown to ADP-ribosylate Rho (25, 50), while CNF2 (and presumably CNF1) treatment increases the apparent molecular migration of Rho on protein gels, but the modification does not appear to involve ADP-ribosylation (37). It is interesting that different toxins should target the same protein with different modifications. Whether DNT and PMT carry out the same reaction remains to be determined.

The open reading frame we have sequenced is consistent with the predictions from the purified DNT protein; however, definitive proof that it is a structural gene for the *B. pertussis* DNT will require independent verification such as identity with the amino acid sequence of purified protein or expression of toxin activity from the cloned DNA in *E. coli*. It is interesting that CNF1 and PMT share extensive homology (Fig. 9), and *Bordetella* DNT is homologous to CNF1 but not in a region where CNF1 is homologous to PMT. C3 and EDIN share extensive homology with each other (27) but no homology with any of the other toxins of this group. We have shown that the DNTs of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* appear to be extremely similar. In contrast, Southern analysis of restriction digests of *B. avium* chromosomal DNA failed to reveal homologous sequences in some DNT-positive *B. avium* strains (Fig. 3), suggesting that this might be yet another distant member of the dermonecrosis-inducing toxin family.

How the molecular activity of these toxins can translate into dermonecrotic lesions is unclear. None of the toxins appears to kill cells in culture. DNT has been reported to cause vasoconstriction both in cell lines and organ strips (14). All of the agents we tested which cause vasodilation (regardless of the mechanism of action) affected DNT lesion production to some degree; however, this could be due in part to vasodilation decreasing the local concentration of the toxin. The pharmacological studies suggest that if DNT induces vasoconstriction, it acts at a very early step in the pathway. In light of the sequence predictions, it is tempting to suggest that vasoconstriction is due to disruption of a developmental pathway. DNT has been shown to disrupt the normal developmental pathway in an osteoblast cell line (23, 24) and could have similar effects on endothelial tissue as well.

Our previous results showing that *B. pertussis* mutants unable to synthesize DNT are as virulent as the DNT-positive parental strain in an infant mouse model of infection (54) are still paradoxical and demonstrate that our understanding of the pathogenesis of *B. pertussis* is far from complete.

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