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Cloning, Expression, and Molecular Characterization of the Dermonecrotic Toxin Gene of *Bordetella* spp.

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A cosmid library of random fragments of *Bordetella bronchiseptica* genomic DNA was prepared and screened with oligonucleotides designed from the sequence of the *B. pertussis* dermonecrotic toxin (DNT) gene. Two cosmid clones which apparently contained the complete *B. bronchiseptica* DNT gene were identified, but they did not express the toxin. A 5-kb fragment containing the DNT gene was subcloned from one of the cosmid clones onto a high-copy-number plasmid, and this resulted in low-level expression of the toxin. The expression level was increased by deletion of a small region upstream of the coding sequence. Assays for biological activity, including the infant mouse dermonecrosis assay, confirmed that the product of the cloned gene was DNT. The complete sequence of the *B. bronchiseptica* DNT gene was determined and was more than 99% homologous to the DNT gene of *B. pertussis*. A putative purine nucleotide-binding motif was shown to be important for toxic activity. Extracts containing the recombinant or the native toxin induced DNA synthesis in Swiss 3T3 cells but inhibited cell division leading to binucleation.

The genus *Bordetella* is composed of several closely related species, which are all respiratory pathogens (38). *Bordetella pertussis* and *B. parapertussis* are human pathogens which cause whooping cough and pertussis-like disease, respectively. *B. avium* causes rhinotracheitis of birds, and *B. bronchiseptica* is a pathogen of several animal species, being particularly associated with atrophic rhinitis in pigs and kennel cough in dogs (16).

Several virulence factors from the genus *Bordetella* have been identified, including adenylate cyclase-hemolysin, filamentous hemagglutinin, fimbriae, pertactin, tracheal cytotoxin, and pertussis toxin (reviewed in reference 52). *B. avium* is a more distant relative to the other three species and does not contain the structural gene for pertussis toxin; *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* all have the gene, but only *B. pertussis* expresses it (3). Expression of these virulence determinants, with the exception of the tracheal cytotoxin, is regulated by the two-component sensory transduction system *bvg* (2, 50). In addition, all *Bordetella* species produce dermonecrotic toxins (DNTs) (5), with similar biological and immunological properties. DNT is a heat-labile toxin which induces dermonecrotic lesions in mice and other laboratory animals when injected intradermally and is lethal for mice at low doses after intravenous inoculation (25). DNT is considered to be a virulence factor for the production of turbinate atrophy in porcine atrophic rhinitis (33, 39). DNT has proved difficult to purify, and reports of its molecular mass have varied widely (89 to 190 kDa) as a result (12, 27, 32). Difficulties in purification are probably due to a low level of expression in *Bordetella* spp. and the association of DNT with other cellular substances. Two groups have, however, reported the purification of DNT to homogeneity (19, 20, 53) and estimated the molecular mass of DNT to be about 140 kDa.

Because of the difficulties with purification and the absence of cloned, recombinant DNT (rDNT), progress in determining the molecular mode of action of the toxin or its role in disease has been relatively slow. It was, however, reported that DNT inhibited elevation of alkaline phosphatase activity and reduced the accumulation of type 1 collagen in an osteoblast-like cell line, suggesting that the toxin might impair the ability of cells to differentiate (21). In addition, DNT stimulated DNA and protein synthesis in these cells, leading to polynucleation, and induced the assembly of actin stress fibers and focal adhesions (22–24). DNT is believed to cause these effects by directly modifying the small GTP-binding protein RhoA (22).

A transposon insertion mutant of *B. pertussis* that was DNT negative was recently analyzed. The region was cloned in three overlapping fragments, and the nucleotide sequence of these was determined (48). An open reading frame was thought to encode the DNT. However, as noted by the authors of that report, since the open reading frame was not cloned on a single insert and the product was not expressed, the identity of the gene product was not proven. The sequence showed limited homology with the cytotoxic necrotizing factors, CNF1 and CNF2, from *Escherichia coli*, which are known to induce multinucleation in target cells (8).

The aim of this work was to clone and express the DNT gene from *B. bronchiseptica* in *E. coli*. Comparison of extracts from the recombinant expressing DNT with those from an isogenic strain of *E. coli* lacking the DNT gene has allowed, for the first time, previously observed biological activities of partially purified toxin preparations to be conclusively ascribed to DNT.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *B. bronchiseptica* B58 is a toxigenic isolate from a pig herd with clinical atrophic rhinitis (33). *E. coli* XL1-Blue (7) was used with the Bluescript II SK− plasmid (Stratagene). *E. coli* LE392 (Gibco-BRL) was used as the host for phage λ during cloning in cosmid pWE15 (Clontech). The expression vector, pKK233-2 (6), was purchased from Clontech. *E. coli* K235(pColK) was obtained from the National Collection of Type Cultures, Colindale, United Kingdom, and the conjugative plasmid, pUB307, was a gift from P. Bennett, University of Bristol. All bacteria were stored as cell suspensions at −70°C in 12% (vol/vol) glycerol. *Bordetella* strains were grown overnight in Hornibrook medium (49) with shaking or on Bordet-Gengou agar (4) supplemented with 15% horse blood for 48 h at 37°C. *E. coli* strains were grown on L agar or in LB broth (44), with appropriate antibiotics added at the following concentrations: ampicillin, 200 μg/ml; tetracycline, 20 μg/ml; kanamycin, 25 μg/ml; and streptomycin, 10 μg/ml.

Enzymes, DNA isolation, and cloning techniques. Small-scale plasmid DNA isolation was performed by the alkaline lysis method as previously described (31).

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Large-scale plasmid isolation was performed by using Qiagen plasmid Midi- or Maxi-kits. Standard cloning techniques were applied using restriction enzymes from Gibco-BRL, New England Biolabs, and Promega. DNA-modifying enzymes such as mung bean nuclease, T4 DNA kinase, and calf intestinal phosphatase were purchased from Promega.

Preparation and screening of cosmid library. All DNT cloning work was carried out under category 3+ regulations as defined by the United Kingdom Advisory Committee for Genetic Manipulation. Chromosomal DNA was isolated from *B. bronchiseptica* B58 as described elsewhere (44) and partially digested with *Sau*IIIA to obtain fragments of approximately 35 to 45 kb. The DNA was fractionated on a sucrose gradient as described elsewhere (44), and fractions containing fragments of the required size range were selected. This DNA was ligated overnight at 15°C to pWE15 cosmid DNA, previously digested with *Bam*HI and treated with calf intestinal phosphatase. The ligation mix was packaged into λ by using an in vitro packaging kit (Gibco-BRL) according to the manufacturer's instructions and plated on *E. coli* LE392 on L agar containing ampicillin (100 μg/ml). About 1,200 Ap^r colonies were obtained. These were stored at −70°C in 12% glycerol in microtiter trays.

The cosmid library was screened by colony hybridization as described elsewhere (17) using two oligonucleotide probes designed from the sequence of the putative *B. pertussis* DNT gene (48). The sequences of the probes were as follows: probe A, 5′-TATGGCGTTTGTGCCGCGACGGCA, which corresponds to nucleotides 888 to 911; probe B, 5′-TCATCCTGACTGGCGGCTCC TTGA, which corresponds to nucleotides 4110 to 4133. The oligonucleotides were end labelled with [γ-^{32}P]ATP (Dupont NEN) by standard techniques and purified by gel filtration chromatography on a Sephadex G-50 column (Pharmacia). Filters were prehybridized for 1 h in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–50 μg of boiled salmon sperm DNA ml^{-1} at 33°C and then hybridized overnight at 33°C in a similar solution, to which the probe was added. Washes were for 1 h each at 60°C in solutions of 3×, 1×, and 0.5× SSC, each containing 0.1% SDS. Hybridizing colonies were detected by autoradiography.

Construction of pKK*nic*$^-$. To comply with the requirements of the United Kingdom Advisory Committee on Genetic Manipulation, the expression vector pKK233-2 was rendered nonmobilizable before we attempted to clone the DNT gene into it. This was achieved by deletion of the *nic-bom* site by digestion with *Nde*I and *Pvu*II, followed by generation of blunt ends with mung bean nuclease and self-ligation. Confirmation that this plasmid (designated pKK*nic*$^-$) was nonmobilizable was obtained by demonstrating that it could not be mobilized from *E. coli* containing pColK and a conjugative plasmid, pUB307, into an Sm^r *E. coli* strain, whereas the unmodified pKK233-2 was efficiently mobilized under the same conditions.

DNA sequence determination. The sequence of the *B. bronchiseptica* DNT gene was determined by using the Sequenase kit (United States Biochemical Corporation) as described by the manufacturer. Template DNA was prepared from the recombinant, XL1-Blue(pBH1), by using a Qiagen plasmid kit and denatured by the addition of a 1/5 volume of 1 M NaOH–1 mM EDTA. Sequencing reactions were carried out directly from the denatured plasmid by using a series of 18-mer oligonucleotide primers designed from the published sequence of the putative *B. pertussis* DNT gene (48). The sequence was analyzed by using the University of Wisconsin computer software.

Site-directed mutagenesis. Site-directed mutagenesis was carried out by using the Chameleon double-stranded site-directed mutagenesis system (Stratagene) according to the manufacturer's instructions. The sequences of the mutagenic oligonucleotides used in this work (mutagenic bases are shown in bold type) were as follows: oligonucleotide A, 5′-GCATTGCGGCAACTTGTC**TA**GATGGCG CTTGTAGGCTAC; oligonucleotide B, 5′-GCCTTCTACCACACTGGC**G**CG TCGACCGAACTCGGGG; oligonucleotide C, 5′-GCCTTCTACCACACTGG CA**G**GTCGACCGAACTCGGGG. The second (or *Kpn*I selection) primer was the reverse complement of that supplied in the kit.

Conjugation. Liquid matings were performed. One-half-milliliter volumes of donor and recipient cultures grown to late exponential phase were mixed and incubated for 2 h at 37°C with gentle agitation. Dilutions of the conjugation mix were spread on selective agar plates and incubated overnight at 37°C.

Preparation of crude protein extracts. Crude extracts were prepared from overnight cultures of *E. coli* or *Bordetella* strains. The cells from 150-ml cultures were pelleted by centrifugation and then resuspended in 50 ml of Tris-Cl (50 mM, pH 7.5). The suspension was sonicated with a Branson sonifier B-12 (Dawe Instruments Ltd.) at 70 W by using the horn attachment. Cell suspensions were cooled on salted ice and subjected to 10 2-min pulses for *Bordetella* spp. or 3 2-min pulses for *E. coli*. The sonicate was centrifuged at 15,000 × *g* for 15 min, and the supernatant was treated with DNase and RNase, each at 10 μg/ml, for 2 h at 37°C. Samples were centrifuged for 5 min, and supernatants were filtered through 0.22-μm-pore-size filters. Extracts were stored at 4°C for up to 1 week.

Protein estimation. Total protein concentrations were estimated by using the Pierce BCA Protein Assay kit according to the manufacturer's standard protocol.

SDS-PAGE and silver staining. Polypeptides were separated by electrophoresis through SDS-polyacrylamide gel electrophoresis (PAGE) gels (5 or 8% polyacrylamide) by standard techniques. Proteins were visualized by silver staining, as described elsewhere (18).

Western blotting (immunoblotting). Proteins were transferred from SDS-PAGE gels to polyvinylidene difluoride membranes (Problott; Applied Biosciences) by a modification of the published method (47) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (10 mM, pH 11) containing 10% methanol. After being blocked overnight at 4°C in TBS (2 mM Tris, 500 mM NaCl [pH 7.5]) containing 1% dry milk, the membrane was washed with TTBS (TBS containing 0.025% Tween 20) three times for 10 min each time and then incubated with anti-DNT monoclonal antibody (MAb) AE9 (1) (20 μl of MAb in 10 ml of TTBS–1% dry milk) for 2 h at room temperature. (MAb AE9 was prepared in this laboratory by injection of mice with DNT which had been purified from *B. bronchiseptica* and detoxifed by formaldehyde treatment. AE9 recognizes both native and rDNT but is not neutralizing.) The membrane was washed as before and incubated with anti-mouse immunoglobulin G-horseradish peroxidase conjugate (10 μl in 10 ml of TTBS–1% dry milk) for 2 h. After washing, bound antibody was detected by enhanced chemiluminescence (Amersham) according to the manufacturer's protocol.

Dermonecrosis assay. The dermonecrosis assay was carried out by using the suckling mouse model (9). Strain CD1 (Charles River) 4-day-old suckling mice were used in groups of five. Fifty-microliter samples diluted in Tris-Cl (50 mM, pH 7.5) were injected subcutaneously into the back of the neck of each pup. Pups were returned to the litter and monitored at frequent intervals for the appearance of hemorrhagic lesions for up to 48 h. Lesions were scored from mild through moderate to severe. Mild lesions were defined as small red patches at the site of inoculation. Severe lesions were defined as purple-black lesions of a diameter greater than 0.5 mm. Moderate lesions were defined as those which fell between these two extremes. Pups were killed with halothane (May and Baker Ltd.).

Cell assays. The cytotoxicity of protein samples was determined by using embryonic bovine lung (EBL) cells (43). The assay was carried out in 96-flat-well cell culture plates (Corning), and all samples were assayed in duplicate. Eighty-microliter volumes of Eagle's minimum essential medium (ICN Flow) were added to all the wells. The sample (20 μl) was added to the top row, and fivefold dilutions were made down the plate. Eighty microliters of a suspension of EBL cells in Eagle's minimum essential medium at a concentration of 3×10^5 cells per ml was added to each well. After a 48-h incubation at 37°C in 5% CO_2, the cells were stained in EBL stain (0.2% [wt/vol] crystal violet, 2% [vol/vol] acetic acid, 12% [vol/vol] neutral buffered formalin) for 1 h. Cells were examined for the presence of a cytotoxic effect, visualized by rounding of the cells with spaces in the monolayer, by using an inverted microscope at ×40 magnification (Olympus). The endpoints are presented as the last dilution of sample which produced a cytopathic effect in over 50% of cells in a well.

Protein extracts were assayed for their ability to stimulate DNA synthesis in quiescent Swiss 3T3 fibroblast cells by using a tritiated thymidine incorporation assay (11). Briefly, confluent quiescent cultures of Swiss 3T3 cells were washed and incubated at 37°C in 2 ml of Dulbecco's modified Eagle's medium-Waymouth's medium (1:1, vol/vol) containing 37 kBq of [^{3}H]thymidine per ml and various concentrations of toxin. After 40 h, DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into the acid-soluble material.

Nucleotide sequence accession number. The sequence reported here was deposited in the EMBL/GenBank database under accession number U59687.

RESULTS

Cloning the *B. bronchiseptica* DNT gene. A cosmid library of random genomic *B. bronchiseptica* B58 DNA fragments in pWE15 was constructed and screened with two oligonucleotide probes as described in Materials and Methods. Two clones (B7 and F5) hybridized to both probes. Crude lysates of B7 and F5 were not cytotoxic for EBL cells.

Restriction maps of the cosmid DNA from clones B7 and F5 were determined for *Bam*HI, *Nhe*I, and *Sac*I (Fig. 1). The two contained similar inserts of approximately 38 kb. In F5 but not B7, one of the sites of ligation between the vector and insert had regenerated a *Bam*HI site. A comparison of the restriction maps of these cosmids with the sequence of the *B. pertussis* DNT gene, in particular the closely adjacent *Bam*HI and *Nhe*I sites, suggested the location of the DNT gene (Fig. 1). The 5-kb *Bam*HI fragment from clone F5 (Fig. 1), which was expected to contain the complete *B. bronchiseptica* DNT gene, was subcloned into pBluescript in both orientations to produce pBH1 and pBH2. Crude lysates from *E. coli* XL1-Blue containing either pBH1 or pBH2 grown in the presence or absence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were cytotoxic for EBL cells (Table 1), whereas those from XL1-Blue(pBluescript) had no effect. Lysates from *E. coli*(pBH1), in which the DNT gene was in the same orientation as the *lac* promoter, were about fivefold more cytotoxic than those pre-

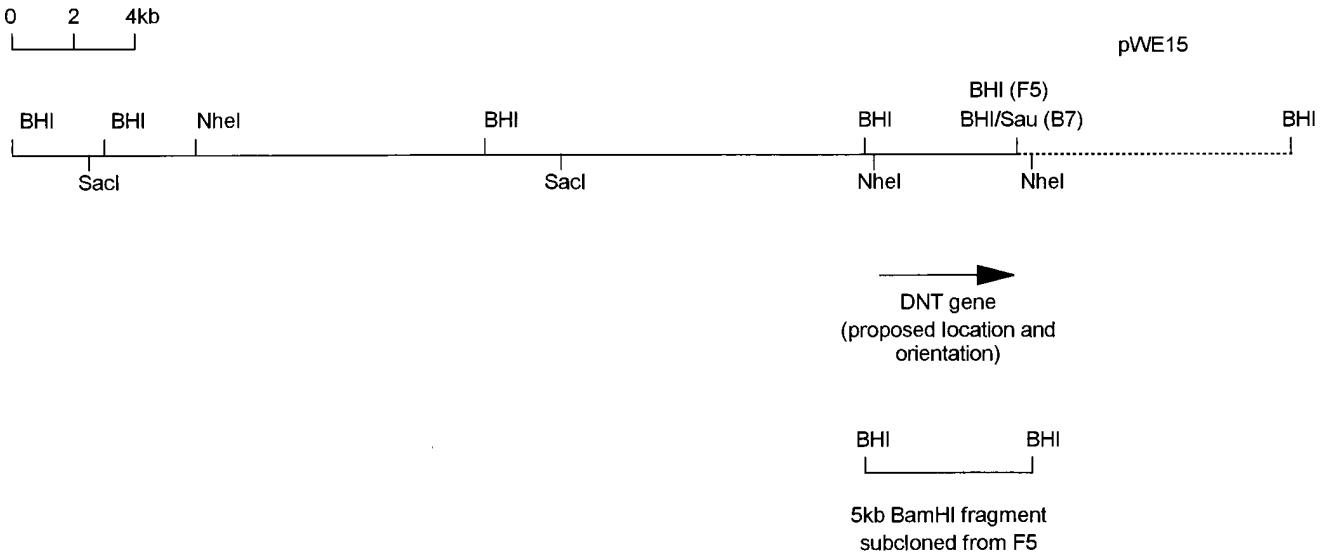


FIG. 1. Restriction map of cosmids F5 and B7. The restriction sites for *Bam*HI (BHI), *Sac*I, and *Nhe*I are shown. The solid line represents the insert DNA, and the dotted line represents the vector. Also indicated are the proposed location and orientation of the DNT gene and the fragment which was subcloned from cosmid F5 to generate plasmids pBH1 and pBH2.

pared from pBH2, and IPTG slightly induced expression from both strains. Comparison of the polypeptides present in the lysates by SDS-PAGE showed no apparent differences among pBH1, pBH2, and pBluescript (Fig. 2).

Sequence analysis of *B. bronchiseptica* DNT gene. The complete nucleotide sequence of the DNT gene was determined (Fig. 3). The base composition of the gene was 63% G+C, which is within the range determined for members of the genus *Bordetella* (61.6 to 69.0% G+C [26]). The nucleotide sequence was more than 99% identical (23 differences) to that of the equivalent *B. pertussis* sequence (48). However, because of conservative changes, the deduced 1,451-amino-acid sequence of the *B. bronchiseptica* DNT had only 11 amino acid differences from the *B. pertussis* DNT. The most notable difference was the cysteine-to-arginine substitution at amino acid 850. Analysis of the region upstream of the first methionine codon of the open reading frame showed no apparent *E. coli*-like promoter or ribosome binding sites. There is a putative binding site for the regulatory protein, BvgA, which is known to activate expression of DNT (40, 41). Thus, the sequence TTTCC TGTTTCCGG at nucleotides 197 to 210 in the *B. bronchiseptica* sequence is similar to a direct repeat of the consensus BvgA-binding heptamer, TTTCCTA. There was a G+C-rich inverted repeat downstream of the open reading frame, which is a possible transcriptional terminator. The *B. bronchiseptica* DNT gene contains the same putative ATP-or GTP-binding motif (45) as the *B. pertussis* gene, and the region of homology between the amino acid sequence of DNT and the cytotoxic necrotizing toxins, CNF1 and CNF2, was conserved between the two *Bordetella* species (35% identity over 100 amino acids). No other significant homologies were found.

Increased expression of rDNT. Two strategies were employed to try to maximize expression of rDNT.

The first strategy was to insert the DNT gene into an inducible expression vector, pKK233-3, which had been rendered nonmobilizable (pKK*nic*$^-$) as described in Materials and Methods. In order to isolate a fragment containing the DNT

TABLE 1. Relative cytotoxicities of total proteins from various bacteria on EBL cells[a]

Sonicate	IPTG induction	Cytotoxicity (endpoint dilution factor)
B. bronchiseptica B58	−	625
XL1-Blue(pBluescript)	−	—[b]
	+	—
XL1-Blue(pBH1)	−	125
	+	625
XL1-Blue(pBH2)	−	25
	+	125
XL1-Blue(pDNT1)	−	3,125
	+	15,625
XL1-Blue(pDNT2)	−	125
	+	625

[a] Sonicates were first adjusted to contain similar total protein concentrations (approximately 500 μg/ml), and then fivefold dilutions were assayed as described in Materials and Methods. IPTG was added to the medium immediately before seeding (to a final concentration of 1 mM) where indicated. Sonicates from these strains were assayed in triplicate, and the results were identical each time.

[b] —, lysate was inactive.

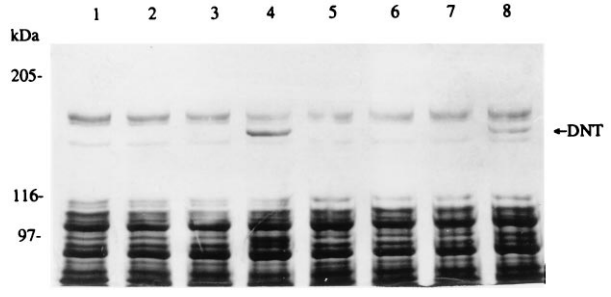
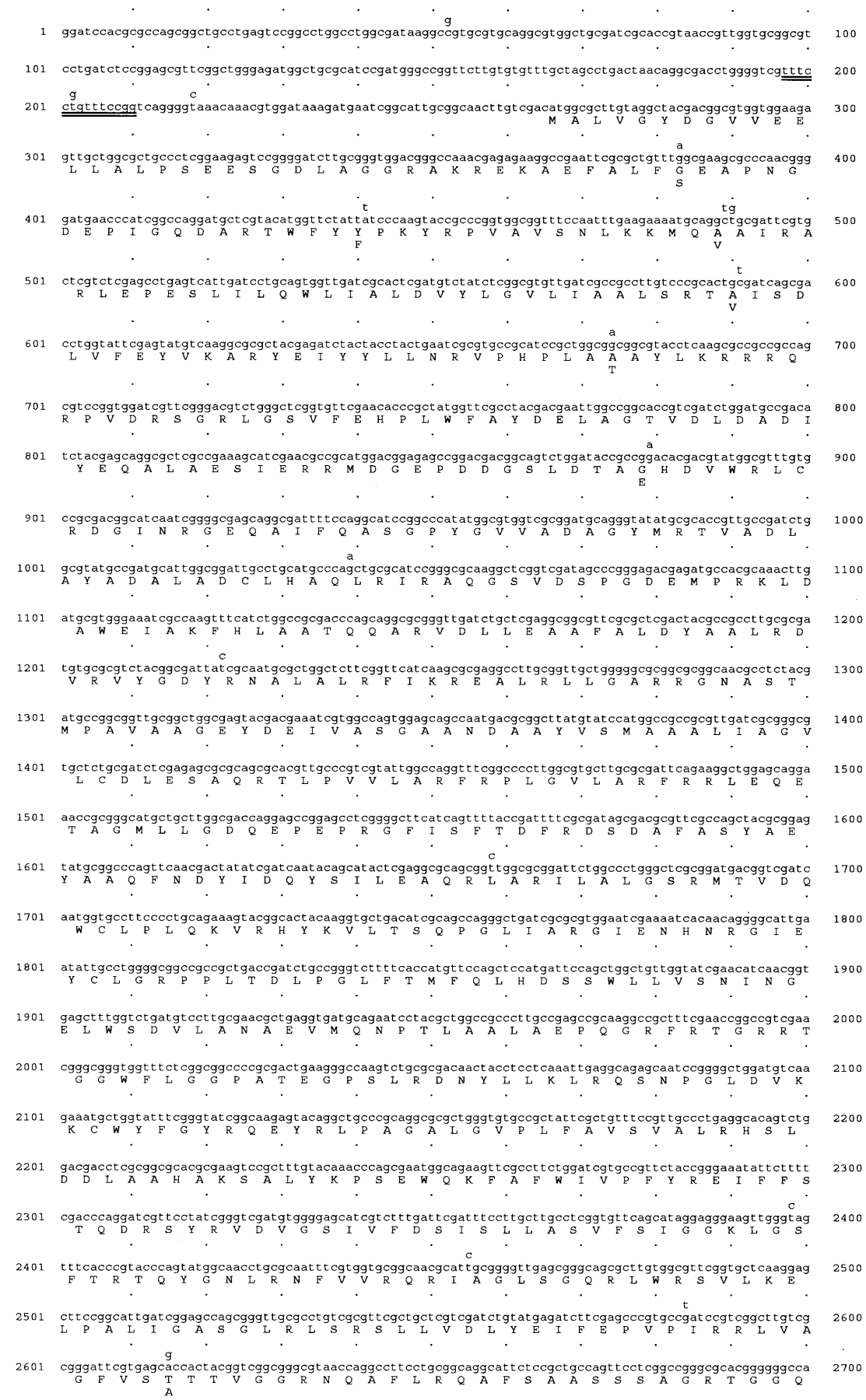


FIG. 2. Levels of DNT expression from various recombinants in the presence and absence of IPTG induction. Sonicates were adjusted to contain the same amount of total protein, separated by SDS-PAGE through 5% polyacrylamide gels, and silver stained. The samples were *E. coli* XL1-Blue containing plasmids pBluescript (lanes 1 and 5), pBH1 (lanes 2 and 6), pBH2 (lanes 3 and 7), and pDNT1 (lanes 4 and 8). The samples in lanes 1 to 4 were grown in the presence of 1 mM IPTG.



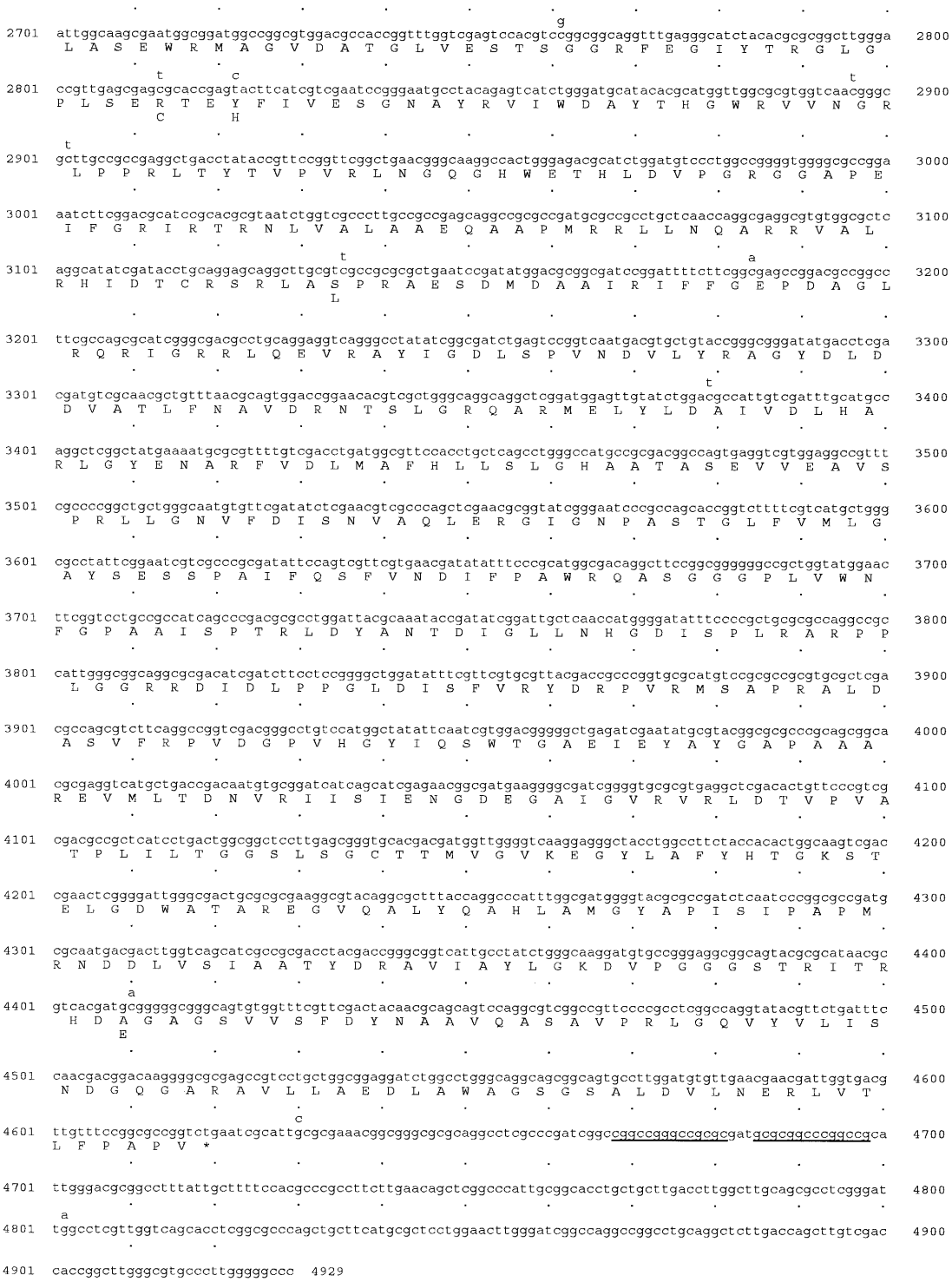


FIG. 3. Nucleotide sequence of the 4,929-bp fragment from *B. bronchiseptica* containing the complete DNT gene. The derived amino acid sequence of the DNT gene is also shown. Above the *B. bronchiseptica* nucleotide sequence are the nucleotide differences present in the corresponding *B. pertussis* sequence (48), and below are the amino acid differences. Doubly underlined nucleotides indicate the possible BvgA-binding site, and the potential transcriptional terminator is underlined.

gene, a unique *Xba*I restriction site was introduced into pBH1 just upstream of the start codon by site-directed mutagenesis using oligonucleotide A. The *Xba*I-*Bam*HI fragment from this mutant containing the DNT gene was ligated into pKK*nic*$^-$ at the *Nco*I site after treatment of both vector and insert with mung bean nuclease. However, all the resultant recombinants which contained inserts had the DNT gene in the wrong orientation for expression from the strong P_{trc} promoter. A repeat experiment gave the same result.

The second approach was to modify plasmid pBH1 by removing much of the intervening DNA between the *lac* promoter of pBluescript and the DNT start codon. For this, the

*Sac*I-*Nhe*I fragment of approximately 200 bp just upstream of the DNT gene was deleted from pBH1 by digestion with both restriction endonucleases, followed by treatment with mung bean nuclease to generate blunt ends and self-ligation. This deletion removed most of the pBluescript multiple cloning region as well as nucleotides 1 to 170 of the insert. This plasmid was designated pDNT1. *E. coli* XL1-Blue(pDNT1) lysates were about 25-fold more cytotoxic on EBL cells than those of XL1-Blue(pBH1) grown under similar conditions (Table 1). This construct also expressed more DNT than pBH1 as assessed by electrophoresis on SDS–5% PAGE gels, in which a band of approximately 145 kDa was visible (Fig. 2). The expression of DNT from pDNT1 was induced slightly by the addition of IPTG.

The recombinant, XL1-Blue(pDNT1) grown in the presence of 1 mM IPTG, was therefore used as the source of rDNT in subsequent biological experiments.

Biological activities of rDNT. The biological activities of native DNT and rDNT were compared in several different assays. For these experiments, crude lysates from *B. bronchiseptica* B58 and from XL1-Blue(pDNT1) were adjusted so that they contained similar total protein concentrations. XL1-Blue(pBluescript) was used as a negative control.

Both DNT and rDNT were cytotoxic, resulting in a rounding of EBL cells (Fig. 4). A similar effect was observed with partially purified native DNT (15). The XL1-Blue(pBluescript) extract was noncytotoxic. Typical endpoints (expressed as the reciprocal of the endpoint dilution factor) were 625 and 15,625 for B58 and pDNT1, respectively, showing that the recombinant was approximately 25-fold more cytotoxic than *B. bronchiseptica*. Heat treatment of DNT- and of rDNT-containing samples for 30 min at 56°C completely abolished the cytotoxic effect of the toxin on EBL cells.

Bacterial lysates containing native DNT or rDNT induced [^{3}H]thymidine incorporation into quiescent Swiss 3T3 cells, showing that DNA synthesis was stimulated by the toxin (Fig. 5). However, the toxin was not mitogenic but inhibited cell division leading to binucleation in this cell system (Fig. 6). The negative control had no effect on incorporation of [^{3}H]thymidine. DNT treatment led to a similar binucleation in primary derived chicken osteoblasts (35).

Crude lysates were also assayed in the infant mouse dermonecrosis assay. The results (Table 2) demonstrate that the recombinant toxin is dermonecrotic, producing lesions similar to those produced by native DNT. The difference between the dermonecrotic effect of DNT1 and B58 sonicates was not as great as expected from the difference in cytotoxicity on EBL cells, suggesting that these two assays do not correspond quantitatively. The negative control, XL1-Blue(pBluescript), had no effect in this assay. The dermonecrotic effect of both native DNT and rDNT was abolished by heat treatment.

Thus, the recombinant toxin had activities similar to those of the native *B. bronchiseptica* toxin in all assays used.

Functional significance of nucleotide-binding motif. The role of the possible nucleotide-binding motif of DNT {residues 1304 to 1311 **AFYHTGKS** [the consensus sequence is (A/G) XXXXGK(S/T)]} was investigated by making base substitutions in the conserved lysine residue. Lysine 1310 of pDNT1 was mutated to alanine (by incorporation of oligonucleotide B) (K1310A) and to arginine (by incorporation of oligonucleotide C). The mutations were confirmed by sequencing this region of the plasmids from the mutants. One isolate with the K1310R substitution and two independent isolates with K1310A were identified. In addition, a further isolate from the attempted lysine-to-alanine mutagenesis had a 3-nucleotide deletion, resulting in the loss of the glycine immediately preceding the lysine (this mutant was designated GK→A).

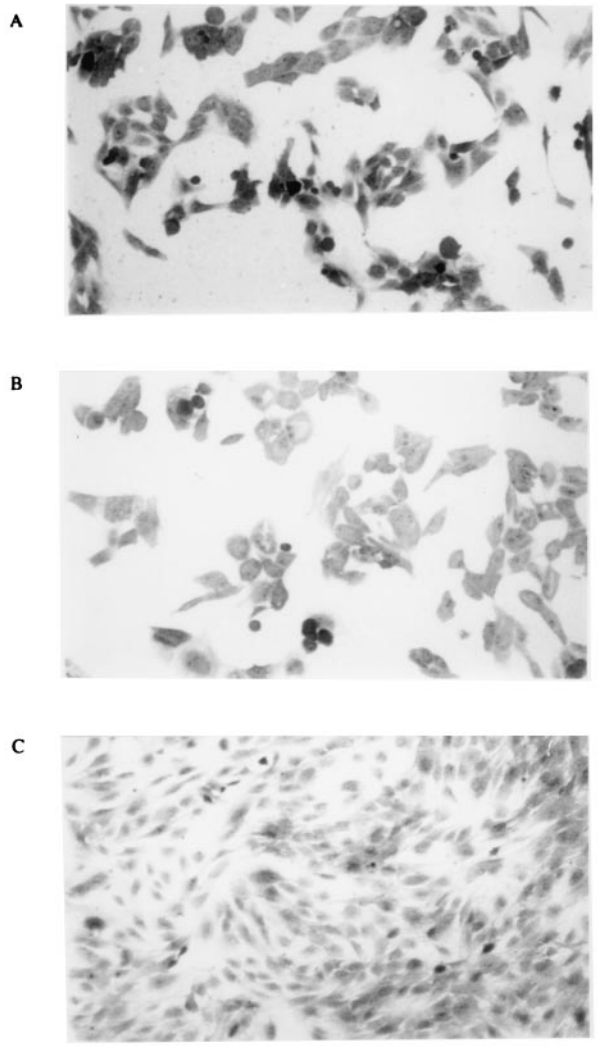


FIG. 4. Cytopathic effect of native DNT and rDNT on EBL cells. Sonicates of bacteria adjusted to contain similar amounts of total protein were assayed on EBL cells, and the cells were stained after 48 h as described in Methods and Materials. (A) *B. bronchiseptica* B58; (B) *E. coli* XL1-Blue(pDNT1); (C) *E. coli* XL1-Blue(pBluescript).

Sonicates of *E. coli* XL1-Blue containing the four mutated plasmids were assayed for cytotoxicity. Both isolates of K1310A and also GK→A were noncytotoxic, whereas the isolate with the K1310R substitution, which was a conservative mutation, retained most of its cytotoxic activity (Table 3). Electrophoretic analysis of these sonicates showed that all four mutants expressed similar amounts of a protein which comigrated with the DNT band from pDNT1 (Fig. 7). The identity of this band was confirmed by Western blot analysis. The anti-DNT MAb recognized this band from pDNT1 and all four mutants but did not react with any proteins in the vector control (Fig. 7). These results show that the nonconservative mutation of lysine 1310 to alanine completely inhibits the cytotoxic activity of the DNT.

DISCUSSION

We have described the cloning and expression of a *Bordetella* DNT gene for the first time. The recombinant toxin exhibited

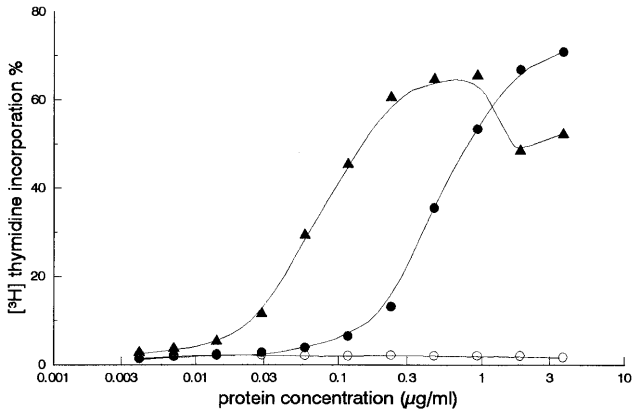


FIG. 5. Effect of DNT on DNA synthesis in quiescent Swiss 3T3 cells, measured by incorporation of tritiated thymidine. Cells were treated with the indicated concentrations of B58 (●), XL1-Blue(pDNT1) (▲), and XL1-Blue(pBluescript) (○) sonicates. Results are expressed as a percentage of incorporation induced by 10% fetal calf serum. The results of a representative experiment are shown. Replicate experiments gave similar curves.

cellular effects indistinguishable from those of purified preparations of native DNT. This confirms that the sequence described by Walker and Weiss (48) does represent the *B. pertussis* DNT gene.

A moderate level of rDNT expression was obtained from pDNT1, in which the DNT gene was cloned in a high-copy-number vector with an inducible *lac* promoter. Although expression was slightly inducible with IPTG, the basal level of expression under noninducing conditions was quite high. There was no apparent *E. coli*-like promoter or Shine-Dalgarno sequence upstream of the first methionine codon of the open reading frame, but consensus *Bordetella* promoters and ribosome binding sites have not been defined and might be quite different from those in *E. coli*. Alternatively, it is possible that a different methionine codon represents the translational start, and the apparent molecular mass of the toxin (140 kDa) is consistent with this possibility. We have so far been unable to obtain N-terminal amino acid sequence from rDNT or native, purified DNT. Presumably, translation of rDNT from pDNT1 starts at the correct methionine, since rDNT comigrates with native, purified DNT on SDS-PAGE (1).

Attempts to improve expression levels beyond those obtained from pDNT1 were unsuccessful. The observation that the DNT gene could be inserted into the expression vector pKK*nic*$^-$ only in the wrong orientation for expression suggested that the basal level of DNT expression from this vector might be too high to be tolerated by *E. coli*. DNT might be toxic to *E. coli*, either directly or indirectly, and a more strictly controlled system might be required to achieve a higher level of expression.

The expression of many *Bordetella* virulence factors is regulated by the two-component signal transduction system, BvgA-BvgS, in response to environmental signals (2). In addition, nonvirulent isolates or phase variants sometimes occur because of mutations in the *bvg* locus (29, 34, 46). There was a possible BvgA-binding site upstream of the DNT gene, and we have found that nonvirulent phase variants of *B. bronchiseptica* express some DNT but in much reduced amounts compared with those of wild-type bacteria, suggesting that DNT production is controlled by this system (15, 52). Interestingly, the *B. pertussis* BvgA-binding site is slightly less similar to the consensus than is the *B. bronchiseptica* sequence, with an additional mismatch in the first repeat (TTTCCGGTTTCCGG).

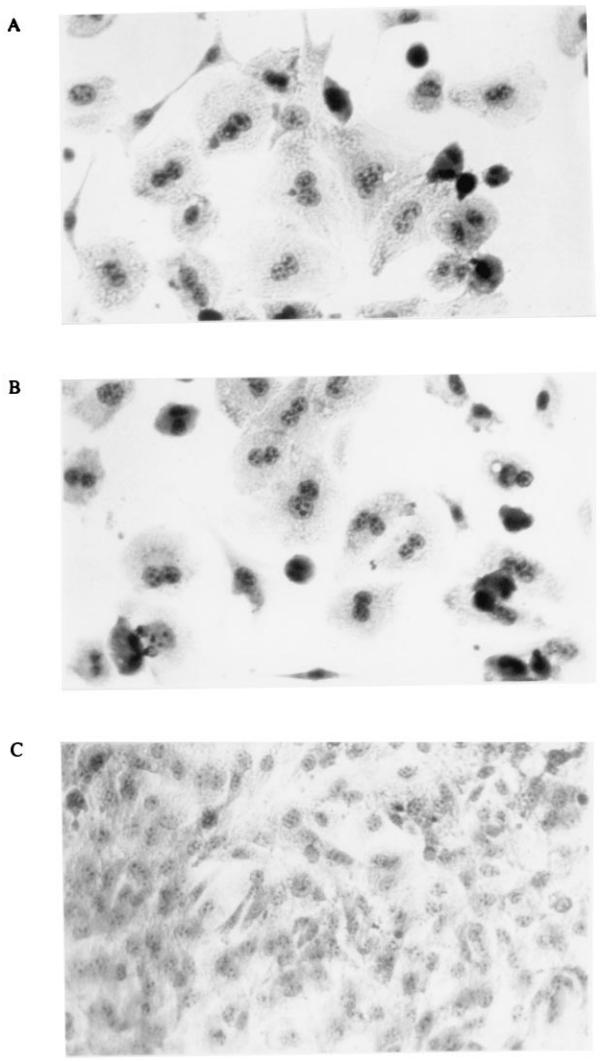


FIG. 6. Binucleation effect of DNT. Sonicates of bacteria were adjusted to contain similar amounts of protein and added to Swiss 3T3 cells. After 3 days, the cells were stained with crystal violet, as for staining of EBL cells. (A) *B. bronchiseptica* B58; (B) *E. coli* XL1-Blue(pDNT1); (C) *E. coli* XL1-Blue(pBluescript).

DNT is a member of a newly recognized family of bacterial toxins, which includes CNF1 and CNF2 of *E. coli* (13, 36) and the *Pasteurella multocida* toxin (PMT) (42). These toxins are all large polypeptides which affect regulation of cell growth or division. There is limited homology among the members of the group at the amino acid sequence level. The PMT and the CNF sequences have a region of significant homology near their N termini (13, 30, 36), and there is a region of significant homology between the CNFs and DNT near their C termini (48). However, there is no significant homology between PMT and DNT. All these toxins stimulate quiescent cells to undergo DNA synthesis. PMT is a potent mitogen causing marked cell proliferation (42), whereas DNT and the CNFs induce nuclear division without subsequent cell division, resulting in bi- or multinucleation (23, 36). Another similar cellular effect of all three toxins is the stimulation of actin stress fiber formation (14, 22, 28). Recently, it has been suggested that both DNT and CNF target and modify Rho proteins (22, 36), a family of small GTP-binding proteins which are involved in the regulation of

TABLE 2. Dermonecrotic effects of DNT and rDNT in the infant mouse assay[a]

Inoculum	Dilution	Time (h) to severe lesions
B58	None (undiluted)	4
	10^{-1}	4
	10^{-2}	12
	10^{-3}	—[b]
XL1-Blue(pDNT1)	10^{-1}	4
	10^{-2}	4
	10^{-3}	48[c]
	10^{-4}	—
B58, heat treated[d]	None (undiluted)	—
XL1-Blue(pDNT1), heat treated	None (undiluted)	—
XL1-Blue(pBluescript)	None (undiluted)	—
Tris buffer[e]		—

[a] Groups of five mice were inoculated with sonicates, adjusted to contain similar total protein concentrations, from the indicated strains.
[b] —, no lesions.
[c] Lesions were mild.
[d] Heat treatment was at 56°C for 30 min.
[e] 50 mM Tris, pH 7.5.

various cell functions, including assembly of actin stress fibers and focal adhesions, membrane ruffling, cell motility, and cytokinesis. Despite the similarities, these toxins are unlikely to be of recent common origin since CNF, like PMT, is encoded by A+T-rich DNA (13, 30, 36) whereas the gene for DNT is 63% G+C.

The DNT genes of *B. bronchiseptica* and of *B. pertussis* (48) contain a putative purine nucleotide-binding motif, the P-loop (45). We demonstrated by site-directed mutagenesis that changing the conserved lysine residue (which is thought to interact directly with one of the phosphate groups of the nucleotide) to alanine completely inhibited the cytotoxic activity of the toxin. This strongly suggested that DNT binds either ATP or GTP, and that this nucleotide binding is essential for activity. Detailed comparison of the sequences of nucleotide-binding proteins has revealed another conserved motif (NKXD) that confers GTP specificity (10). This motif is absent from DNT, suggesting that this toxin may be an ATP-binding protein. We are currently attempting to demonstrate nucleotide binding to the purified toxin directly. The role of nucleotide binding remains unknown, but it seems likely that ATP hydrolysis could be used as a source of energy for some element of toxin action.

The retention of such a large gene by different species of the genus *Bordetella* strongly suggests that it has an important role. With *B. bronchiseptica*, there is much evidence that DNT plays a role in bone breakdown in atrophic rhinitis in pigs (33, 39). However, the role of DNT in human disease caused by *B. pertussis* is less clear. A DNT-negative transposon insertion mutant of *B. pertussis* was fully virulent in both the infant mouse and coughing rat models of whooping cough (37, 51). However, these models might not be directly relevant to human disease. The toxin is located in the cytoplasm (9) and is probably released only after lysis of the bacteria or possibly as a result of direct contact between the bacterium and ciliated mucosal cells. Any pathological effect of the toxin is therefore likely to be late in infection.

The cloning and expression of the DNT gene offer many possibilities for further analysis of the toxin, including detailed analysis of its enzymatic mode of action, its target, and the downstream signalling events it induces.

TABLE 3. Cytotoxicity of site-directed mutants of pDNT1 on EBL cells[a]

Crude lysate	Cytotoxicity (endpoint dilution)
DNT1	15,625
XL1-Blue(pBluescript)	—[b]
K1310A mutant 1	—
K1310A mutant 2	—
K1310R	3,125
GK→A	—

[a] Sonicates of *E. coli* XL1-Blue containing the plasmids indicated were adjusted to contain similar amounts of protein and assayed for cytotoxicity as described in Materials and Methods. Identical results were obtained from three replicate assays.
[b] —, lysate was inactive.

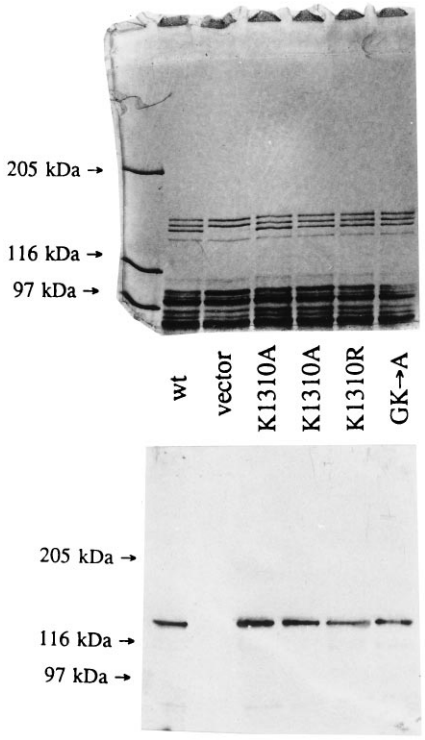


FIG. 7. Sonicates of *E. coli* XL1-Blue containing the plasmids indicated between the two panels were separated by SDS-PAGE on duplicate 5% polyacrylamide gels. One of the gels was silver stained (top), and the second was Western blotted and reacted with anti-DNT MAb (bottom). wt, pDNT1; vector, pBluescript. The positions of molecular mass markers are indicated.

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