gate slow waves. In addition, the critical frequency associated with voids should be well above the region considered here, probably above 40 MHz, the frequency at which the wavelength becomes comparable to the largest voids, the Haversian canals. The theory of Biot (*1*) for wave propagation in a fluid-saturated porous solid, by contrast, predicts effects of the sort observed here.

The full significance of this observation is not yet understood. However, study of these slow waves is important because such waves may be useful for probing the dynamic fluid-solid interaction in bone. Piekarski and Munro (*12*) suggested that fluid pumping through the canaliculi in response to physiological stresses may be the mechanism for providing nutrition and biological information to cells as well as for the removal of waste products. Johnson and co-workers (*13*) considered streaming potentials responsible for the electromechanical effects in saturated bone. However, these are all modeled as occurring in the range of physiological frequencies, from approximately 0.1 to 100 Hz. The information contained in, or the physiological role of, such waves at megahertz frequencies should be investigated.

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References and Notes

1. M. A. Biot, *J. Acoust. Soc. Am.* **28**, 168 (1956); *ibid.*, p. 179.
2. T. J. Plona, *Appl. Phys. Lett.* **26**, 259 (1980).
3. N. R. Paterson, *Geophysics* **21**, 691 (1956).
4. J. G. Berryman, *Appl. Phys. Lett.* **37**, 382 (1980).
5. T. J. Plona and D. L. Johnson, in *1980 Ultrasonics Symposium Proceedings* (IEEE, New York, 1980), p. 868.
6. H. S. Yoon and J. L. Katz, *J. Biomech.* **9**, 407 (1976); *ibid.*, p. 459; in *1976 Ultrasonics Symposium Proceedings* (IEEE, New York, 1976), p. 48.
7. H. Frost, *Henry Ford Hosp. Med. Bull.* **8**, 208 (1960); I. J. Singh and D. L. Gunberg, *Am. J. Phys. Anthropol.* **33**, 373 (1970).
8. R. Lakes, H. S. Yoon, J. L. Katz, in preparation.
9. R. L. Maharidge, thesis, Rensselaer Polytechnic Institute, Troy, N.Y. (1983).
10. J. W. Nunziato and E. K. Walsh, *J. Appl. Mech.* **44**, 559 (1977).
11. J. W. Nunziato and S. C. Cowin, *Arch. Ration. Mech. Anal.* **72**, 177 (1979).
12. K. Piekarski and M. Munro, *Nature (London)* **269**, 80 (1977); M. Munro and K. Piekarski, *J. Appl. Mech.* **APM-32**, 1 (1977).
13. D. A. Chakkalakal, M. W. Johnson, R. A. Harper, J. L. Katz, *IEEE Trans. Biomed. Eng.* **BME-27**, 95 (1980); M. W. Johnson, D. A. Chakkalakal, R. A. Harper, J. L. Katz, *J. Biomech.* **13**, 437 (1980).
14. We thank P. Christel for providing specimens and A. Meunier for many illuminating discussions. This research was supported in part by NIHR grant G008200028 and by NSF grant INT-7920799.
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Cloned Fragment A of Diphtheria Toxin Is Expressed and Secreted into the Periplasmic Space of *Escherichia coli* K12

Abstract. *An 831-base pair segment of the corynebacteriophage* β^{tox-45} *genome encoding fragment A of diphtheria toxin was cloned into plasmid pUC8 in* Escherichia coli *K12. Strains containing recombinant plasmids expressed the adenosine diphosphate ribosyl transferase activity characteristic of fragment A; this activity could be inhibited by polyvalent antiserum to fragment A as well as by the appropriate monoclonal antibodies to diphtheria toxin. The transferase activity was secreted into the periplasmic space of* E. coli. *These findings have implications for the future construction of genetically engineered chimeric toxins.*

Diphtheria toxin production depends on a complex association between a family of closely related toxigenic corynebacteriophages and their sensitive *Corynebacterium diphtheriae* host (*1–5*). Lysogenic conversion with toxigenic phages or with phages that carry mutations within the *tox* gene permits *tox* gene products to be expressed and studied (*6, 7*); however, the inability to ob-

Table 1. Inhibition of ADPR-transferase activity of CRM45 (2 μg) and the periplasmic fraction of *E. coli* SM529 (pDT201) (50 μl) by monoclonal antibody to diphtheria toxin and polyvalent antiserum to fragment A (*18*). Purified preparations of monoclonal antibody to fragment A (1.7 mg/ml) and of monoclonal antibody to fragment B (1.5 mg/ml) were used. Antibody preparations were incubated with an equal volume of purified CRM45 (40 μg/ml) or periplasmic fraction for 1 hour at 25°C before being assayed for ADPR-transferase activity. The ADPR-transferase assay was performed in duplicate with 50 μl of a standard assay mixture containing 10 m*M* tris-HCl *p*H 8.0, 2 μ*M* [adenine-^{14}C]NAD (~ 2.5 μCi/mmole) and elongation factor 2 as a crude extract of wheat germ. The sample (50 μl) was added and the mixture was incubated at 37°C. After 30 minutes, trichloroacetic acid was added, the precipitate was filtered and washed, and the radioactivity was determined. The background was approximately 200 count/min, and maximal incorporation was approximately 12,000 count/min.

Treatment	Radioactivity (count/min)	
	CRM 45	Periplasmic fraction
None	10,544 ± 265	10,458 ± 294
Polyvalent antiserum to fragment A	899 ± 115	877 ± 3
Monoclonal antibody to fragment A	2,315 ± 277	3,867 ± 242
Monoclonal antibody to fragment B	8,354 ± 552	9,059 ± 126

Table 2. Distribution of glucose-6-phosphate dehydrogenase, alkaline phosphatase, and ADPR-transferase activity in *E. coli* SM529 (*19*) carrying plasmid pDT201. Cells to be fractionated were grown at 37°C in 250 ml of M9 minimal medium plus 1 percent glucose, 1 percent casamino acids, 0.0002 percent thiamine, and 10 μg/ml ampicillin to obtain an absorbance at 590 nm of 0.4 to 0.57 (~ 2×10^8 cells per milliliter). The culture supernatant fluid obtained after centrifugation was concentrated tenfold by ultrafiltration on an Amicon YM-10 membrane before being assayed. Cells were washed in 30 m*M* tris-HCl (*p*H 8.0) and 20 percent sucrose and suspended in 5 ml of the same buffer. The periplasmic fraction was obtained by lysozyme-EDTA treatment (*20*). Spheroplasts were lysed in 10 m*M* tris-HCl (*p*H 8.0) by freeze-thawing three times in the presence of deoxyribonuclease *T* (0.1 mg/ml). The pellet obtained after centrifugation for 15 minutes at 13,000*g* was suspended in 10 m*M* tris-HCl (*p*H 8.0), and 0.1 percent Triton X-100 and designated the membrane fraction. Samples to be assayed for alkaline phosphatase were dialyzed extensively against 10 m*M* tris-HCl (*p*H 8.0). The assays for alkaline phosphatase and glucose-6-phosphate dehydrogenase were performed in triplicate as described (*21*). Total activity in a typical assay for alkaline phosphatase was 600.7 units per 10^8 cells (units expressed as the amount giving a change in absorbance at 420 nm of 1.0 per hour). Typical values for the total activity of glucose-6-dehydrogenase was 10 moles of nicotinamide adenine dinucleotide phosphate reduced per hour per 10^8 cells. The ADPR-transferase assay was performed as described (legend to Table 1), except that the standard assay mixture contained 100 m*M* dithiothreitol. *Escherichia coli* SM529, both untransformed and transformed with pUC8, is devoid of ADPR-transferase activity.

Fraction	Enzymatic activity (%)		
	Glucose-6-phosphate dehydrogenase	Alkaline phosphatase	ADPR-transferase
Supernatant fluid	0	1.9 ± 1.3	0
Periplasmic	0	92.1 ± 5.0	86.6 ± 3.3
Cytoplasmic	96.5 ± 2.7	4.6 ± 4.6	8.9 ± 2.1
Membrane	1.6 ± 1.6	2.9 ± 2.7	3.5 ± 0.6

tain conjugation, transduction, transformation, or transfection with corynebacteria limits the usefulness of these lysogens for genetic analysis. Recombinant DNA technology should allow the *tox* gene sequences to be manipulated more easily. We describe the application of these techniques to the study of diphtheria *tox* gene products.

Molecular cloning of diphtheria *tox* sequences has been made easier by the availability of detailed restriction maps of the toxigenic corynebacteriophage β_c^{tox+} and the accurate localization of the *tox* operon on these maps (*8, 9*). We have cloned an 831-base pair (bp) segment (Sau 3AI-2) of the 3.9-kilobase (kb) Bam HI-4 fragment of corynebacteriophage β_c^{tox-45}. The Bam HI-4 fragment carries the entire diphtheria *tox* structural gene (*8*). Corynebacteriophage β_c^{tox-45} contains a nonsense mutation in the fragment B portion of the *tox* structural gene, and its *tox* gene product is the nontoxic, serologically related protein CRM45. The Sau 3AI-2 fragment contains the signal sequence for diphtheria toxin and the sequence encoding fragment A of the *tox* structural gene (*10*). In addition, Sau 3AI-2 also contains 177 nucleotides before the start of the *tox* structural gene and thus probably contains the entire regulatory region of the *tox* operon (*11*) (Fig. 1).

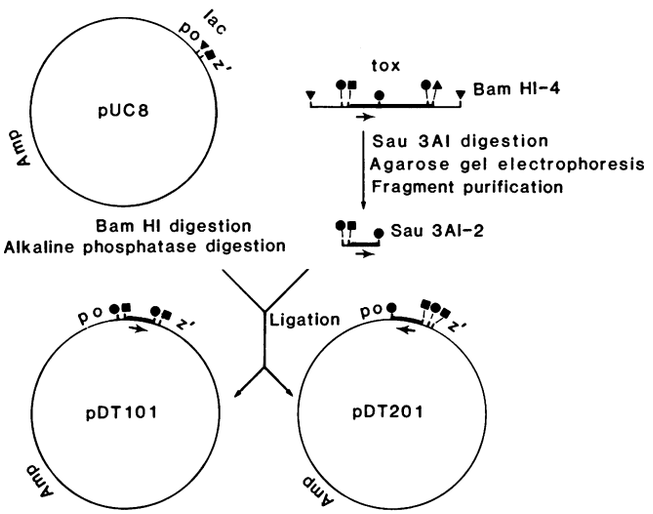

Fig. 1. Schematic representation of the molecular cloning of the DNA encoding fragment A of diphtheria toxin. The 3.9-kb Bam HI-4 fragment of corynebacteriophage β_c^{tox-45} was purified by agarose gel electrophoresis. After electroelution and ethanol precipitation, Bam HI-4 was digested to completion with Sau 3AI. The 831-bp Sau 3AI-2 fragment containing the diphtheria *tox* promoter, signal sequence, and fragment A was purified by agarose gel electrophoresis. After electroelution, Sau 3AI-2 was ligated to Bam HI–digested, alkaline phosphatase–treated plasmid pUC8 with T_4 DNA ligase. Enzymes were obtained from New England Biolabs, Beverly, Massachusetts, and used according to the manufacturer's specifications. Transformants of *E. coli* K12 71-18 were selected on LB plates containing ampicillin (200 μg/ml). Clones carrying the Sau 3AI-2 insert were identified by colony hybridization, with ^{32}P-labeled Bam HI-4 DNA as probe, or by Sau 3AI digestion of small-scale plasmid preparations. As determined by Hind III digestion of plasmid DNA, pDT101 contained the Sau 3AI-2 insert in an orientation such that lac Z′ and diphtheria toxin fragment A were transcribed in the same direction, whereas pDT201 contained the Sau 3AI-2 insert in the opposite orientation. Arrows designate the direction of diphtheria *tox* transcription. Restriction endonuclease sites are (▼) Bam HI, (●) Sau 3AI, (■) Hind III, and (▲) Eco RI.

After purification of the Sau 3AI-2 fragment and ligation to Bam HI-digested and alkaline phosphatase-treated plasmid pUC8 (*12*), *Escherichia coli* K12 71-18 was transformed. Transformants carrying recombinant plasmids were detected by colony hybridization with ^{32}P-labeled Bam HI-4 as the probe. After detection and colony purification, the orientation of the Sau 3AI-2 fragment in the recombinant plasmids was determined by restriction endonuclease analysis with Hind III. Two plasmids, pDT101 and pDT201, contained the Sau 3AI-2 fragment, in opposite orientations (Fig. 1).

Restriction endonuclease analysis and hybridization studies with pDT201 DNA confirmed that this plasmid contained the desired Sau 3AI-2 fragment of Bam HI-4 (Fig. 2). Comparison of Sau 3AI digestions of pDT101, pDT201, and pUC8 DNA revealed a DNA fragment in pDT101 and pDT201 that had the expected electrophoretic mobility. Furthermore, this fragment contained sequences complementary to those of Bam HI-4.

Since earlier work had shown that diphtheria *tox* gene products could be synthesized in S-30 extracts of *E. coli* programmed with DNA from corynebacteriophage β (*2*), we anticipated the expression in vivo of fragment A from cloned *tox* gene sequences. Evidence that diphtheria toxin fragment A was expressed in *E. coli* carrying pDT101 or pDT201 was obtained by assays for adenosine diphosphate ribosyl (ADPR) transferase activity (*13, 14*). In the presence of nicotinamide adenine dinucleotide (NAD^+) and eukaryotic elongation factor 2 (EF-2), fragment A of diphtheria toxin catalyzes the transfer of the adenosine diphosphate ribose moiety of NAD^+ onto a specific site on EF-2. This reaction is highly specific to diphtheria toxin fragment A and to exotoxin A from *Pseudomonas aeruginosa* (*13–16*). When extracts of *E. coli* containing pDT101 and pDT201 were incubated with crude wheat germ EF-2 and [adenine-^{14}C]-NAD, the transfer of the label into material precipitable with trichloroacetic acid was catalyzed. Additional proof that the ADPR-transferase activity observed in

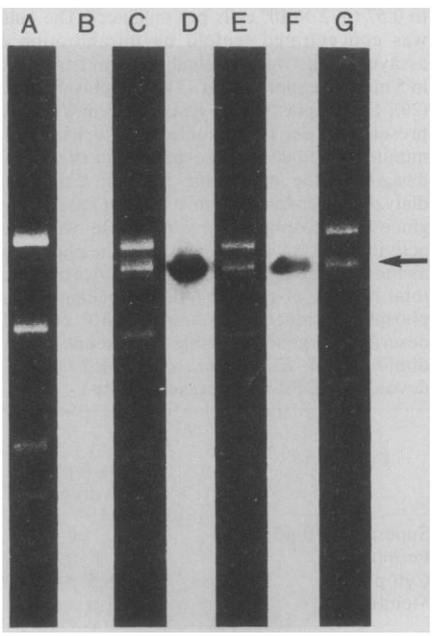

Fig. 2. Restriction endonuclease digestion profiles and Southern blot hybridization pattern of plasmid pUC8, pDT101, and pDT201 with ^{32}P-labeled Bam HI-4 DNA as probe. DNA (~ 1 μg) was digested to completion with Sau 3AI, then heated to 65°C for 5 minutes, quenched on ice, and subjected to electrophoresis on 2 percent agarose gels in TBE buffer [50 m*M* tris-HCl (*p*H 8.3), 89 m*M* boric acid, and 2.5 m*M* Na_2EDTA] for 2 to 3 hours at a constant voltage (170 V). Gels to be photographed were stained with ethidium bromide (2.5 μg/ml) for 30 minutes, illuminated with shortwave ultraviolet light, and photographed through a Hoya R (25 A) filter on Polaroid type 667 film. DNA was transferred from agarose gels to nitrocellulose paper (type BA85, Schleicher & Schuell, Keene, New Hampshire) (*22*). Purified Bam HI-4 was nick-translated with ^{32}P-labeled 5′-nucleotide triphosphates (> 400 Ci/mmole, New England Nuclear) and DNA polymerase I (*23*). (Lane A) pUC8, (lane C) pDT101, (lane E) pDT201, and (lane G) Bam HI-4. Lanes B, D, and F are pUC8, pDT101, and pDT201 probed with ^{32}P-labeled Bam HI-4.

E. coli carrying pDT101 or pDT201 was due to fragment A of diphtheria toxin was obtained by allowing cell extracts to react with polyvalent antiserum to fragment A and monoclonal antibody to diphtheria toxin before determining enzymatic activity. Both types of antibody specifically inhibited the transferase activity (Table 1).

Two earlier reports had suggested that diphtheria toxin–related molecules would be secreted in *E. coli*. The first demonstrated that toxin is synthesized exclusively on membrane-bound polysomes in *C. diphtheriae* (*10*). This process involves the cotranslational secretion of the polypeptide across the cytoplasmic membrane, cleavage of an NH_2-terminal signal sequence, and release of the toxin into the culture medium. The second report demonstrated that translation of *tox* messenger RNA in vitro in the presence of membrane vesicles resulted in the vectorial transport of the polypeptide into those vesicles (*17*).

Escherichia coli SM529, a PhoR$^-$ derivative of strain MC1000 that is constitutive for alkaline phosphatase, was transformed with pDT201. The localization of ADPR-transferase activity in these strains was compared to that of glucose-6-phosphate dehydrogenase and alkaline phosphatase, which were used as markers for the cytoplasmic and periplasmic fractions, respectively (Table 2). The transferase activity clearly partitioned with alkaline phosphatase in the periplasmic fraction. These experiments strongly support the hypothesis that the signal sequence for diphtheria toxin is recognized by the *E. coli* secretory system and allows fragment A of diphtheria toxin to be translocated across the inner membrane and to be localized as a soluble protein in the periplasmic compartment of *E. coli*.

The study of the structural and functional domains of diphtheria toxin has relied largely on the isolation and characterization of polypeptides produced from mutated *tox* genes (*1*). These studies have been hampered by the inability to isolate a broad spectrum of different types of *tox* mutations. The capability of cloning the NH_2-terminal portion of the diphtheria toxin structural gene in *E. coli* and the resulting expression and secretion of toxin-related polypeptides will allow molecular genetic methods to be applied to this problem. In addition, hybrid toxin genes can be developed in which the enzymatically active fragment A and hydrophobic domain of fragment B are fused to various eukaryotic cell receptor–specific ligands. The expression and secretion of these defined chimeric toxins will advance the study of toxin entry into eukaryotic cells and the development of targeted chimeric toxins for the potential treatment of particular malignancies.

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References and Notes

1. A. M. Pappenheimer, Jr., *Annu. Rev. Biochem* **46**, 69 (1977).
2. J. R. Murphy, A. M. Pappenheimer, Jr., S. Tayart de Borms, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 11 (1974).
3. C. Kanei, T. Uchida, M. Yoneda. *Infect. Immun.* **18**, 203 (1978).
4. J. R. Murphy, J. Skiver, G. McBride. *J. Virol.* **18**, 235 (1976).
5. R. Rappuoli, J. L. Michel, J. R. Murphy. *ibid.* **45**, 524 (1983).
6. J. V. Freeman, *J. Bacteriol.* **61**, 675 (1951).
7. T. Uchida, D. M. Gill, A. M. Pappenheimer, Jr., *Nature (London) New Biol.* **233**, 8 (1971).
8. J. J. Costa, J. L. Michel, R. Rappuoli, J. R. Murphy. *J. Bacteriol.* **148**, 124 (1981).
9. G. A. Buck and N. B. Groman, *ibid.*, p. 153.
10. W. P. Smith, P.-C. Tai, J. R. Murphy, B. D. Davis, *ibid.*, **141**, 184 (1980).
11. J. L. Michel, R. Rappuoli, J. R. Murphy, A. M. Pappenheimer, Jr., *J. Virol.* **42**, 510 (1982).
12. J. Vieira and J. Messing, *Gene* **19**, 259 (1982).
13. R. J. Collier and J. Kandel, *J. Biol. Chem.* **246**, 1496 (1971).
14. D. M. Gill and A. M. Pappenheimer, Jr. *ibid.*, p. 1492.
15. B. H. Iglewski and D. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2284 (1975).
16. D. Chung and R. J. Collier, *Infect. Immun.* **16**, 832 (1977).
17. W. P. Smith, *J. Bacteriol.* **141**, 1142 (1980).
18. Hybridoma cell lines producing monoclonal antibodies to diphtheria toxin were isolated by D. Zucker (manuscript in preparation). A. M. Pappenheimer, Jr., provided polyvalent antibody to fragment A partially purified from rabbit serum.
19. *Escherichia coli* SM529 used in these studies was provided by S. Michaelis and is a derivative of the MC1000 from the collection of J. Beckwith.
20. C. A. Schnaitman, *J. Bacteriol.* **108**, 553 (1971).
21. M. Malamy and B. L. Horecker, *Biochem. Biophys. Res. Commun.* **2**, 104 (1961).
22. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
23. P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg. *ibid.* **113**, 237 (1977).
24. We thank M. Kaczorek and his colleagues for sharing their data on the nucleotide sequence of the diphtheria *tox*-228 allele prior to publication. Supported by a grant from Seragen Inc., Boston, and by a National Science Foundation predoctoral fellowship (to D.L.). All experiments reported here were performed in strict accordance with the NIH guidelines for research involving recombinant DNA molecules as published in volume 47, No. 77, of the *Federal Register*.

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Platelet Thromboxane Synthetase Inhibitors with Low Doses of Aspirin: Possible Resolution of the "Aspirin Dilemma"

Abstract. *Selective pharmacological inhibition of thromboxane A_2 synthesis did not prevent arachidonate-induced aggregation of human platelets in vitro. Prevention was instead achieved by a combination of thromboxane A_2 inhibitors with low concentrations of aspirin. The latter partially reduced the proaggregatory cyclooxygenase products that accumulated when thromboxane A_2 synthesis was blocked. The aspirin concentrations did not affect per se either platelet aggregation or prostacyclin synthesis in cultured human endothelial cells. The combination of thromboxane synthetase inhibitors with low doses of aspirin may offer greater antithrombotic potential than either drug alone.*

Aspirin, by inhibiting cyclooxygenase (*1*), prevents the formation of two arachidonic acid metabolites that have opposite biological effects, namely, the aggregating thromboxane (Tx) A_2 in platelets (*2*) and the antiaggregatory prostaglandin (PG) I_2 or prostacyclin in vascular cells (*3*). Simultaneous inhibition of TxA_2 and PGI_2 synthesis might be the reason for the disappointing results of clinical trials of the antithrombotic effect of aspirin (*4*). Studies with cultured endothelial cells

Table 1. Effect of aspirin (2.5 μM), dazoxiben (40 μM), and a combination of the two drugs on PGI_2 generation by human endothelial cells stimulated by sodium arachidonate. Human endothelial cells derived from umbilical veins were cultured in 2-mm^2 wells (NUNC-Libco) as described (*21*) and used when confluent. At the beginning of the experiment the cells were washed once with phosphate saline buffer (free of Ca^{2+} and Mg^{2+}) and incubated with the drugs or the vehicle in 0.3 ml of buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 4 percent bovine albumin (Sigma) for 10 minutes at 37°C. Sodium arachidonate (200 μM) was then added for 5 minutes at 37°C, and the buffer was removed and stored at −20°C until assayed for 6-keto-$PGF_{1\alpha}$ (the stable derivative of PGI_2) by radioimmunoassay (*19*). At the end of the experiment the cells were detached by 0.05 percent trypsin and 0.2 percent EDTA and counted in a hemacytometer. Each well contained approximately 10^5 cells. The results are expressed as means (± standard error of the mean) of four different replicates. No statistical differences were detected by Duncan's new multiple range test.

Group	6-keto-$PGF_{1\alpha}$ (pmole/10^5 cells)
Control	11.53 ± 1.27
Aspirin	7.72 ± 1.62
Dazoxiben	10.81 ± 1.10
Aspirin plus dazoxiben	7.91 ± 1.12