Cloning and Expression in *Escherichia coli* of Three Fragments of Diphtheria Toxin Truncated within Fragment B

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We have constructed three different truncated versions of diphtheria toxin (a 535-amino-acid polypeptide) which correspond to the N-terminal 290, 377, and 485 amino acids of the toxin. These lengths include one, three, and all four of the putative membrane-spanning sequences of the toxin which are thought to play a role in the translocation of fragment A into cells. Each of these three genes has been modified at its 3' end to code for a C-terminal cysteine (to allow for disulfide linkage of a targeting ligand) or a gene fusion with α -melanocyte-stimulating hormone. We have also substituted the native diphtheria *tox* promoter (p_{tox}) with the lambda p_R promoter in an effort to overexpress these proteins. The truncated genes are expressed in *Escherichia coli* from both the *tox* promoter in a constitutive fashion and from the p_R promoter by using the heat-inducible *cI857* repressor. The clones produce proteins which react with anti-diphtheria toxin serum, which migrate at the anticipated M_r on Western blots, and which have ADP-ribosyltransferase activity. Constitutive synthesis from p_{tox} leads to severe proteolytic degradation even in a protease-deficient strain. High-level expression from the p_R promoter in the same *lon htpR* strain allows the full-length polypeptides to accumulate but also stops the growth of the cells. It appears that removal of as few as 50 amino acids from the C-terminus of diphtheria toxin alters its conformation, making it a target for proteases and causing overexpression lethality in the host cells.

Diphtheria toxin is a 535-amino-acid ADP-ribosylating toxin that can be cleaved into an N-terminal, enzymatically active A fragment of 193 amino acids and a C-terminal B fragment of 342 amino acids (26, 38). Because of the high specific toxicity of the toxin, there have been numerous attempts to link fragments of it to different ligands in an effort to target its toxicity to particular cells. Most frequently, diphtheria toxin fragment A, cross-reacting material-45 (CRM45; a 45-kilodalton [kDa] *tox* nonsense mutant [48]), or whole toxin has been used as the toxic subunit of these hybrid molecules. With a few exceptions (17), conjugates to diphtheria toxin fragment A have been nontoxic (8, 9, 42), while conjugates to CRM45 have shown moderate levels of toxicity and high specificity (2, 11). Conjugates made to whole diphtheria toxin often have very high potency (37, 45) and in some cases show specificity (34, 54), but it is unlikely that they can be made selective enough for use in humans.

The gradient of increasing toxicity as larger portions of diphtheria toxin are used in these conjugates is believed to result from the inclusion of different domains of fragment B which mediate the translocation of fragment A into the cytosol. Eisenberg et al. (14) have identified four potential membrane-spanning sequences in fragment B, three of which are present in CRM45. In addition, Lambotte et al. (29) described a region in fragment B, also contained within CRM45, which shows homology to human apolipoprotein A1 and represents a potential membrane surface binding

domain. The diphtheria toxin receptor binding domain, which enables the toxin to bind to a wide variety of eucaryotic cell types, has been localized to the C-terminal sequences beyond amino acid 386. This boundary is defined by the failure of CRM45 to bind to eucaryotic cells (49). Only one of the four putative transmembrane regions of fragment B resides in this C-terminal portion of the toxin. Ideally, the targeting domain of a hybrid toxin should be placed at this still poorly defined boundary between the membrane translocation domains and the receptor binding domain.

This work describes part of a study to determine how much of fragment B should be retained to optimize the delivery of fragment A to target cells while minimizing the general toxicity associated with the native toxin receptor binding domain. To address this question, we have used recombinant DNA techniques to truncate the diphtheria toxin gene at three different sites within the coding sequences for fragment B. The three truncation sites are at amino acid 290 after transmembrane region 1, at amino acid 377 after transmembrane region 3, and at amino acid 485 after transmembrane region 4 and beyond disulfide loop 2 in the toxin (Fig. 1). The truncated proteins at each length end in both a C-terminal cysteine or a translational fusion to the 13 amino acids of α -melanocyte-stimulating hormone (α -MSH). We have also placed these genes under the control of both the native *tox* promoter (p_{tox}) and the p_R promoter of bacteriophage lambda in the presence of the thermoinducible *cI857* repressor and have compared the levels of production from the two promoters. To further aid in this comparison, we describe the levels of production of similar constructs which encode fragment A alone from either p_{tox} or lambda p_R .

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FIG. 1. (A) A partial restriction map of the *tox* gene showing 6-bp restriction sites and *Sau3AI* sites. The *Sau3AI* site at nucleotide 831 occurs within the coding sequences for the disulfide loop between fragments A and B. The numbers indicate the distance (in base pairs) from the G of the 5' *Sau3AI* site to the first base pair of the given restriction recognition site. (B) A diagram of diphtheria toxin showing the 25-amino-acid signal sequence, the position of the two disulfide loops, and the location of the potential transmembrane regions (shaded) identified by Eisenberg et al. (14). The sites at which the modifications were made and the truncation site for CRM45 are shown. The numbers refer to amino acid residues. (C) The recombinant modifications made in plasmids PDT201, PABC313, and PABC402 (a similar sequence is shown for PABM508 in reference 36). Primed numbers indicate amino acids not encoded by the *tox* gene.

TABLE 1. Strains of *E. coli* K-12 used in this study

Strain	Genotype	Source or reference
SY327	F ⁻ <i>araD</i> Δ (<i>lac-pro</i>)XIII Rif ^r <i>nalA</i> <i>argE</i> (Am) <i>recA56</i>	25
TB1	λ^- <i>ara</i> Δ (<i>lac-pro</i>) <i>rpsL</i> <i>thi</i> ϕ 80 <i>lacZ</i> Δ M15 r ⁻ m ⁺ (=JM83, r ⁻ m ⁺)	53; T. Baldwin
GM119	F ⁻ <i>dam-3</i> <i>dcm-6</i> <i>metB1</i> <i>galK2</i> <i>galT22</i> <i>lacY1</i> <i>tsx-78</i> <i>supE44</i> [<i>thi-1 tonA31 mtl-1</i>]?]	1
71-18	<i>supE</i> <i>thi</i> Δ (<i>lac-proAB</i>) [F' <i>proAB</i> ⁺ <i>lacI</i> ^{qZ} Δ M15]	33
JM101	<i>supE</i> <i>thi</i> Δ (<i>lac-proAB</i>) [F' <i>traD36</i> <i>proAB</i> ⁺ <i>lacI</i> ^{qZ} Δ M15]	53
LC133	F ⁻ <i>lac</i> (Am) <i>trp</i> (Am) <i>pho</i> (Am) <i>mal</i> (Am) <i>rpsL</i> <i>supC</i> (Ts) <i>tsx</i> : :Tn10	18
LC137	F ⁻ <i>lac</i> (Am) <i>trp</i> (Am) <i>pho</i> (Am) <i>supC</i> (Ts) <i>lon</i> Δ 100	18
SG932	F ⁻ <i>lac</i> (Am) <i>trp</i> (Am) <i>pho</i> (Am) <i>supC</i> (Ts) <i>lon</i> Δ 100	S. Goff and A. Goldberg

MATERIALS AND METHODS

Strains. The strains used are shown in Table 1. Cloning experiments were done with strains SY327 and TB1. pBSC302, a plasmid carrying a *Bcl*I site, was made from the *dam* strain GM119. For single-stranded template preparation, strain JM101 or 71-18 was used. Strains LC133, LC137, and SG932, kindly provided by A. Goldberg, were used for expression studies.

Oligonucleotide synthesis. Oligonucleotides were synthesized by using a solid-phase DNA synthesizer (model 380A; Applied Biosystems, Foster City, Calif.) with phosphoramidite nucleotides and controlled-pore glass columns (Applied Biosystems). The single-stranded oligonucleotides were purified by polyacrylamide gel electrophoresis, as described previously (36), and used as either linkers for cloning or primers for sequencing.

Plasmid constructions. Plasmids pEMBL8 (12) and pEMBLex3 (13) were gifts from R. Rappuoli. Restriction endonucleases, T4 DNA ligase, and *Escherichia coli* DNA polymerase I large fragment were purchased from New England BioLabs, Inc. (Beverly, Mass.), and were used according to the specifications of the manufacturer. Plasmids were isolated by alkaline lysis and purified by CsCl density gradient centrifugation as described previously (32).

DNA sequencing. Plasmids derived from pEMBL8 were transferred to strain JM101 or 71-18. Early-exponential-phase cells were infected with the f1-derived phage IR1, and phage particles were harvested by polyethylene glycol precipitation as described previously (12). Before phenol extraction to remove the phage proteins, the redissolved polyethylene glycol precipitates were mixed with an equal

volume of a solution containing 5% sodium dodecyl sulfate, 50% glycerol, 0.1 M EDTA (pH 8), and 0.1% xylene cyanol and screened by electrophoresis in 1% agarose gels. Only preparations which contained greater amounts of single-stranded plasmid than single-stranded phage DNA were used as templates. Sanger sequencing was carried out on these templates, as described previously (40), with a 22-base-pair (bp) version of the universal primer. Deoxy and dideoxy nucleotides were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Nomenclature. For the plasmids carrying diphtheria toxin sequences truncated within fragment B, we have adopted a method of systematic nomenclature. Plasmids containing *tox* sequences under the control of the *tox* promoter are numbered between 0 and 999, and plasmids with the *tox* sequences under the control of lambda *p_R* are numbered between 1000 and 1999. The presence of fragment A is indicated by an A in the name, fragment B is indicated by a B, a spacer is indicated by an S, the cysteine-stop encoding sequence is indicated by a C, and the α -MSH sequence is indicated by an M. The last three digits in the plasmid number give the amino acid number in the precursor form of toxin at which the modifications begin. Thus, pABC1313 contains fragment A and fragment B sequences fused to the cysteine-stop oligonucleotide beginning at amino acid 313 and is under the control of the lambda *p_R* promoter. Plasmids used are listed in Table 2.

Western blots. Cells were grown in Luria-Bertani medium at 30°C for 2 h to an *A*₅₅₀ of about 0.5. Temperature induction at 42°C or continued growth at 30°C was then carried out for an additional 2 h. After measuring the final *A*₅₅₀, the cells were centrifuged and lysed with 1 ml of 2 $\times$ reducing loading

TABLE 2. Plasmids used in this study

Plasmid	Promoter	<i>tox</i> amino acids	C-terminal amino acids	C-terminus	Molecular mass ^a (daltons)	Reference
pDT201	<i>p_{tox}</i>	194	29	Random amino acids	24,576	30
pDT1201	<i>p_R</i>	194	29	Random amino acids	24,576	This study
pABC313	<i>p_{tox}</i>	290	4	(Ala) ₃ -Cys	32,205	33a
pABC1313	<i>p_R</i>	290	4	(Ala) ₃ -Cys	32,205	33a
pABM313	<i>p_{tox}</i>	290	16	(Ala) ₃ - α -MSH ^b	33,708	35
pABM1313	<i>p_R</i>	290	16	(Ala) ₃ - α -MSH	33,708	This study
pABC402	<i>p_{tox}</i>	377	9	X ^c -(Ala) ₂ -Cys	41,995	This study
pABC1402	<i>p_R</i>	377	9	X-(Ala) ₂ -Cys	41,995	This study
pABM1402	<i>p_R</i>	377	21	X-(Ala) ₂ - α -MSH	43,498	This study
pABC508	<i>p_{tox}</i>	485	4	(Ala) ₃ -Cys	53,058	This study
pABC1508	<i>p_R</i>	485	4	(Ala) ₃ -Cys	53,058	This study
pABM508	<i>p_{tox}</i>	485	16	(Ala) ₃ - α -MSH	54,561	36
pABM1508	<i>p_R</i>	485	16	(Ala) ₃ - α -MSH	54,561	33a

^a Deduced molecular masses of mature proteins without the diphtheria toxin signal sequence.

^b α -MSH, Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val.

^c X, Arg-Ser-Val-Asp-Arg-Pro.

FIG. 2. Oligonucleotides used in this study. The *ClaI*-*HindIII* linker was used in the construction of the 313 series plasmids, and the *SphI*-*HindIII* linker was used for the 508 series plasmids. ABC plasmids (which end in a C-terminal cysteine codon) were converted to ABM plasmids (which end in the sequence for α -MSH) by cloning the α -MSH linker into the *PstI* sites introduced by the *ClaI*-*HindIII* or *SphI*-*HindIII* linker.

buffer (4% sodium dodecyl sulfate, 5% β -mercaptoethanol, 15% glycerol, 25 μ g of bromophenol blue per ml, 50 mM Tris [pH 6.8] [41]) per 10 ml of culture per A_{550} unit. After boiling, the proteins were separated on 10% polyacrylamide stacking gels (27) in a minigel apparatus (Bio-Rad Laboratories, Richmond, Calif.). Proteins were electrophoretically transferred to nitrocellulose (46) and visualized as described previously (39) by using horse anti-diphtheria toxin serum (2 U/ml; Connaught Laboratories, Swiftwater, Pa.) and peroxidase-conjugated goat anti-horse immunoglobulin G (10 μ g/ml; Cooper Biomedical, Malvern, Pa.).

ADPRT assay. Cultures were grown as described above, and after centrifugation the cell pellets were suspended in 1 ml of ice-cold sonication buffer (30 mM Tris [pH 8], 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) per 10 ml of culture per A_{550} unit. Cells were broken by sonication on ice (four 15-s pulses), and the resulting lysates were assayed directly for ADP-ribosyltransferase (ADPRT) activity as described previously (36). The reference in this assay was ion-exchange-purified CRM45 that was greater than 90% pure by silver-stained sodium dodecyl sulfate-polyacrylamide gel analysis. The CRM45 was quantified by measuring its A_{280} and by the method of Bradford (5).

Containment. When this work was begun, the cloning of *tox* fragments larger than CRM45 (pABC508 and pABC1508) and all fragment B-containing toxin-hormone gene fusions (all ABM plasmids) was restricted to the BL-4 level of containment. In accordance with these directives, much of the work described here was carried out at the BL-4 facility at the National Institute of Allergy and Infectious Diseases, Frederick, Md. Since the level of containment for the BL-4 truncated toxin genes was changed to BL-2 with BL-3 practices (15), recent work with these constructs has been done at the BL-3 level.

RESULTS

Construction of the 313 and 508 series plasmids. Our strategy for constructing the cysteine-stop truncated diphtheria toxin genes was to use synthetic oligonucleotide linkers designed to introduce a cysteine codon followed by an amber stop codon in the correct translational reading

frame. The linkers used are shown in Fig. 2, and their use in constructing some of the truncated toxin genes has been described previously (33a, 36). The plasmids which we modified with these linkers were constructed by Leong et al. (30). pDT201 contains an 831-bp *Sau3AI* restriction fragment that carries the *tox* promoter, signal sequence, and all of the fragment A-encoding sequences. pDT301 contains a 977-bp *Sau3AI* fragment carrying sequences that code for all of fragment B except for the C-terminal 17 amino acids. The vector in both of these plasmids is pUC8, and the orientation of *Sau3AI* fragments is shown in Fig. 3.

From these two plasmids we have derived pAB313 and pAB1313, both of which code for the *tox* signal sequence and *tox* amino acids 1 to 290. pAB313 contains 120 bp of the *tox* 5' upstream sequences which include the *tox* promoter. In pAB1313 the *tox* promoter has been deleted, and the protein-coding sequences have been placed under the control of the lambda *p_R* promoter. This fusion introduces two additional amino acids at positions 2 and 3 in the *tox* signal sequence (Fig. 3). The parent vector for this construct is pEMBLex3, which carries its own *cI857* repressor gene (13).

Because many of these plasmid constructions were restricted to BL-4 containment, we used a two-step strategy to assemble them. First, we made truncated fragment B genes at the BL-1 level, using our oligonucleotides and pDT301; we then recombined these modified C-terminal coding sequences into the unique *ClaI* restriction site of either pAB313 or pAB1313 at the BL-4 level. Thus we used the modified fragment B sequences carried on a *ClaI* fragment from pBM313 (also called pDTM311 [35]) to construct pABM1313. Likewise, an analogous fragment from pBC508 (33a) was used to construct pABC508 and pABC1508.

Construction of the 402 series plasmids. The 402 series plasmids contain the same recombinant stop sequences as the 313 series plasmids, but placed 87 amino acids downstream, after transmembrane region 3 diphtheria toxin. Because there are no unique restriction sites in the *tox* sequence in this region, we developed a novel vector, pBSC302, from which a family of truncated *tox* genes can be isolated with *Bal* 31. Plasmid pBSC302 is a derivative of pDT301 and pEMBL8; it contains a 783-bp spacer sequence from pBR322 interposed between the *tox* sequences and the cysteine-stop sequences. The plasmid also has a unique *BclI* site in place of the former *Sau3AI* site at the beginning of the fragment B sequences (this change was made by altering 1 bp on the vector side of the *Sau3AI* restriction site).

pBSC302 was opened at its unique *SphI* restriction site and digested with *Bal* 31 for various periods of time (Fig. 4). The reaction was stopped with EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], and the downstream remnants of the spacer DNA were removed by digestion with *Bam*HI. Next, the samples were treated with the DNA polymerase I large fragment to give blunt-ended, linearized plasmids with various amounts of fragment B remaining at one end and the cysteine-stop linker at the other. After ligation, the DNA was used to transform *E. coli* JM101.

Ampicillin-resistant colonies were screened first by restriction analysis with *ClaI*-*HindIII* digestion. Clones that contained *ClaI*-*HindIII* fragments between 200 and 400 bp in length were then used to make single-stranded plasmid DNA by infection with IR1 phage. Of the nine clones that were sequenced, one contained the recombinant stop codon at position 387 (that is, it would produce a toxin molecule of exactly the same length as CRM45). However, the frame of this clone, pB402, was off by 1 bp according to the sequence

FIG. 3. Partial restriction maps of some of the plasmids used in this study. A, Fragment A-encoding sequences; B, fragment B-encoding sequences; C, cysteine-stop C terminus; R, lambda *ci*857 repressor gene; S, spacer sequences; Ap, *Apal*; Ba, *Bam*HI; Bc, *Bcl*I; Cl, *Cla*I; Ec, *Eco*RI; Hi, *Hind*III; Sa, *Sau*3AI; Sp, *Sph*I. The parent vector for pDT201 and pDT301 is pUC8; for all other p_{tox} -containing plasmids it is pEMBL8, and for all p_R -containing plasmids it is pEMBLex3.

FIG. 4. The use of pBSC302 to generate *Bal* 31 deletions in the gene for fragment B. Abbreviations are as for Fig. 3.

data. This was verified by the fact that this clone gave blue colonies on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates because the *tox* gene is fused in frame with the *lacZ'* sequence but out of frame with the oligonucleotide-encoded cysteine codon and TAG. The frame was corrected by filling in the *Sal*I site which lies immediately downstream of the *Bam*HI site in pBSC302 with DNA polymerase I large fragment. The resulting plasmid, pBC402, gave white colonies on X-Gal plates and contains a new *Pvu*I site resulting from the filled-in *Sal*I site. The *Cla*I fragment

FIG. 5. Restriction analysis of the truncated *tox* genes on a 1% agarose gel. Lanes A to E contain *Eco*RI-*Hind*III double digests of *p*_{tox}-containing plasmids and lanes G to L contain *Hind*III digests of *p*_R-containing plasmids. The samples were loaded as follows (the expected length [in base pairs] of the fragment carrying the *tox* gene is given in parentheses). Lanes: A, pABC313 (1,089); B, pABM313 (1,139); C, pABC402 (1,365); D, pABC508 (1,674); E, pABM508 (1,724); F, *Hind*III digest of lambda DNA; G, pABC1313 (1,426); H, pABM1313 (1,476); I, pABC1402 (1,702); J, pABM1402 (1,752); K, pABC1508 (2,011); L, pABM1508 (2,061). The size (in base pairs) of some of the lambda fragments is shown.

from pBC402 was ligated into the *Cla*I site of pAB313 and pAB1313 to give pABC402 and pABC1402, respectively.

pABM1402 was constructed by inserting the α -MSH oligonucleotide into the unique *Pst*I site of pBC402 to give pBM402 and then isolating the modified *Cla*I fragment from pBM402. This fragment was cloned into pAB1313 at the BL-4 level of containment to yield pABM1402.

The truncated *tox* genes driven by *p*_{tox} are bracketed by *Eco*RI and *Hind*III restriction sites, and the genes driven by lambda *p*_R are bracketed by *Hind*III sites (Fig. 3). Figure 5 shows a restriction analysis of the 313, 402, and 508 series plasmids in which the different sized *tox* genes have been excised by either *Eco*RI-*Hind*III digestion or *Hind*III digestion alone.

Construction of pDT1201. pDT1201 contains the coding sequences for fragment A of the diphtheria toxin up to amino acid 194 under the control of the *tox* promoter. Unlike the 313, 402, and 508 plasmids, which contain deliberately placed stop codons, this plasmid directs the synthesis of a fusion protein that is composed of fragment A plus 29 foreign amino acids encoded by pUC8 sequences. pDT1201 directs the synthesis of the same fusion protein, but under the control of the lambda *p*_R promoter. This plasmid was made by purifying a 1,001-bp *Cla*I-*Acc*I fragment that contains the *cI857* gene and *p*_R promoter fused to the N-terminal sequences of *tox* from plasmid pABC1313-3C (pABC1313-3C is pABC1313 with a third *Cla*I site at the 3' end of the *cI857* repressor gene) and cloning it into *Acc*I-digested pDT201. Because the *Acc*I sites in pDT201 have different recognition sequences, the vector in this ligation reaction could not close without an insert. Furthermore, only the upstream *Acc*I site in pDT201 is compatible with *Cla*I ends; thus, most of the recombinant colonies isolated from this ligation reaction contained the correct insert in the correct orientation.

Expression. The proteins encoded by these genes have been detected both by their ADPRT activity in whole cell lysates and by Western blot analysis. Clones synthesizing constructs from the *tox* promoter at 30°C produced about 4 mg of CRM45 equivalents per liter, whereas the lambda *p*_R promoter directed the production of about 1 mg of CRM45 equivalents per liter after induction at 42°C (Table 3). Con-

trol experiments showed that the empty vectors in the same *E. coli* host produce negligible or undetectable amounts of ADPRT activity.

Western blotting revealed that with the exception of pDT201 and pDT1201 which code for fragment A only, the larger truncated toxin proteins containing portions of fragment B undergo proteolytic degradation in *E. coli*. This proteolysis is most pronounced in wild-type strains, in strains grown at high temperatures, and in strains making low levels of protein constitutively off the *tox* promoter (data not shown). However, in the *lon htpR* mutant (LC137) the degradation is attenuated, and a major product on the Western blots corresponds to the full-length proteins (Fig. 6). A *lon* mutant with a functional *htpR* allele (SG932) also provides partial rescue of ABM1508 from proteolysis (data not shown), indicating that the La protease is involved in degrading these proteins.

In proteins ABC1313 and ABM1313 as well as in ABC1508 and ABM1508, a difference in migration between the ABC and ABM proteins corresponding to the additional 12 amino acids in the ABM proteins is visible. Protein ABM1402 (Fig. 6, lane G) appears to migrate at the same molecular mass as that of ABC1402 (42 kDa; Fig. 6, lane F); however, on close inspection of the ABM1402 lane, there is a faint band running above the 42-kDa band which corresponds to full-length ABM1402. Because the 402 series proteins (unlike the 313 and 508 series) carry six foreign amino acids in the linker arm between diphtheria toxin and the C-terminal α -MSH or cysteine, we believe they are more susceptible to proteolytic degradation. Not only does the junction between the *tox* gene and this six-amino-acid linker produce an Arg₃₇₇-Arg₃₇₈ sequence, but there is also another arginine at position 382. Thus, the 42-kDa band in both the ABC1402 and ABM1402 lanes probably corresponds to cleavage at one of these three arginines. We have been unable to observe specific staining

FIG. 6. Western blot of the p_R -produced truncated toxins. *E. coli* LC137 carrying the plasmids indicated were grown for 2 h at 30°C and then induced for 2 h at 42°C. Equivalent numbers of cells from each culture were lysed in sodium dodecyl sulfate and subjected to electrophoresis on a 10% polyacrylamide gel under reducing conditions. Each lane corresponds to the total protein from 0.2 ml of a culture at an A_{550} of 1.0. Lanes: A and J, partial trypsin digest of diphtheria toxin giving bands for whole toxin (58 kDa), fragment B (37 kDa), and fragment A (21 kDa); B, pEMBLex3 (empty vector); C, pDT1201; D, pABC1313; E, pABM1313; F, pABC1402; G, pABM1402; H, pABC1508; I, pABM1508. The molecular masses given (in kilodaltons) are deduced.

of any of the ABM series proteins with a variety of anti- α -MSH antibodies tested.

The expression of pDT1201, which codes for the first 194 amino acids of toxin (fragment A; 21 kDa) fused to 29 foreign amino acids produces a doublet with the major band running at 24 kDa and the minor band at 21 kDa (Fig. 6, lane C). Because the three proteolytically sensitive arginines present in the Cys₁₈₆-Cys₂₀₁ disulfide loop of the toxin were also present in this fragment, the doublet probably arises from partial cleavage at one of these sites to yield fragment A alone. This cleavage reaction has been well demonstrated with the pDT201 protein *in vitro* (31).

Overexpression lethality. All of the high-expression plasmids carrying the lambda p_R promoter fused to *tox* sequences inhibited the continued growth of their host cells upon induction at 42°C (Table 3). This effect was not seen with the same constructs under the control of the *tox* promoter at either 30°C or 42°C nor was it observed with the parent vector, pEMBLex3, which makes the *lacZ'* polypeptide in a heat-inducible fashion.

DISCUSSION

In targeted toxins constructed from diphtheria toxin fragments, it has become clear that toxicity is enhanced when the ligand is attached by a disulfide bond (16). Moreover, Bacha et al. (2) have shown that CRM26 (a 26-kDa *tox* nonsense fragment) linked to thyrotropin-releasing hormone is virtually nontoxic to pituitary cells, whereas CRM45-thyrotropin-releasing hormone is toxic. This indicates that the fragment B lipid-associating regions of diphtheria toxin, which are present in CRM45 but absent in CRM26, are important for toxicity, presumably because they facilitate conjugate toxin entry. Using a recombinant CRM45-length truncated toxin ending in cysteine as well as fragment A and whole toxin as the toxic portions of their immunotoxins, Colombatti et al. (11) have demonstrated that the N-terminal 21 kDa of fragment B is necessary for specific toxicity and that this toxicity is enhanced 100-fold when whole toxin is used as the toxic moiety. This suggests that domains both

TABLE 3. Growth ratios and ADPRT activity

Culture	Plasmid	Growth ratio ^a	ADPRT activity ^b	ADPRT sp act ^c
1	pDT201	1.83	14	1.5
2	pABC313	1.96	55	7.7
3	pABM313	1.82	60	6.7
4	pABC402	2.03	37	4.8
5	pABC508	1.98	50	7.5
6	pABM508	1.74	42	4.5
7	pEMBL8	1.91	0.5	0.08
8	pDT1201	1.13	6	1.2
9	pABC1313	1.13	13	2.1
10	pABM1313	1.22	11	1.6
11	pABC1402	1.14	17	3.2
12	pABM1402	1.02	18	3.0
13	pABC1508	0.96	12	2.0
14	pABM1508	0.83	9	1.0
15	pEMBLex3	1.73	<0.4	<0.05

^a *E. coli* LC137 cultures carrying the plasmids indicated were grown for 2 h at 30°C and then for an additional 2 h at either 30°C (cultures 1 to 7) or 42°C (cultures 8 to 15). Growth ratio is A_{550} at 4 h/ A_{550} at 2 h.

^b ADPRT activity is given in micrograms of CRM45 equivalents per milliliter. ADPRT activity was measured in lysates of equivalent numbers of cells and standardized to the activity of a purified preparation of CRM45. A 1-ml volume of lysate corresponds to 10 ml of a culture at A_{550} = 1.0. The lower limit of detection of the ADPRT assay is 0.4 μ g of CRM45 per ml.

^c ADPRT specific activity is given in micrograms of CRM45 equivalents per milliliter per A_{280} unit. To standardize for protein concentration, each lysate was centrifuged, and the ADPRT activity was normalized by the A_{280} of the supernatant.

proximal and distal to the CRM45 truncation site are important for the delivery of fragment A. Recently, we have shown that partially purified ABM1508 (toxin amino acids 1 to 485 fused to α -MSH) is toxic for α -MSH receptor-positive melanoma cells but is nontoxic to cells that do not contain α -MSH receptors at concentrations as high as 10^{-7} M (36). This latter result indicates that the C-terminal 50 amino acids of diphtheria toxin contain sequences essential for binding to the native toxin receptor and that nontargeted toxin fragments up to amino acid 485 are likely to be active in the translocation of fragment A but unable to bind to cells via the toxin receptor. Nevertheless, it remains unclear what the relative efficiencies of fragment A translocation are with fragments shorter than 485 amino acids.

To test the role of fragment B sequences more fully, a series of diphtheria toxin N-terminal fragments truncated within fragment B would be useful. There are a number of *tox* nonsense mutants which produce toxin molecules truncated within fragment B. These include CRM26 (CRM2), CRM30, CRM34 (CRM3), CRM45, and CRM47 (CRM11) (23, 28, 48, 49). The major drawback for using these molecules to construct conjugate toxins is that they do not have unique cysteines where one can attach the targeting ligand.

To overcome this problem, we have constructed three truncated genes for diphtheria toxin and have expressed them in *E. coli*. Each of the three truncated genes has been modified to end in either a C-terminal cysteine codon to allow disulfide linkage to a ligand or in a fusion to sequences encoding α -MSH. The resulting six genes are expressed constitutively in *E. coli* from the native *tox* promoter. We have also fused the lambda p_R promoter to the 5' end of these same truncated genes. This fusion adds two amino acids to the *tox* signal sequence and expresses the *tox* proteins in a heat-inducible fashion owing to the presence of a *c1857* repressor gene in the plasmid vector.

We also have described the construction of pBSC302, a novel *E. coli* plasmid designed to produce *Bal* 31 deletions in the gene for fragment B and to fuse specific C-terminal sequences to the 3' end of the deletion without the need for further subcloning steps. This pEMBL8-derived vector also allows for rapid Sanger sequencing of the fusion junction by infection with phage IR1 to produce single-stranded templates directly from the plasmid-carrying bacteria. We have used pBSC302 to produce CRM45-length truncated toxin genes ending with either a cysteine codon or the sequence for α -MSH. One unfortunate limitation of these particular fusion genes is that the junction between the *tox* sequences and the 3' stop sequences in the 402 series plasmids gives rise to an Arg-Arg linkage that has proven highly susceptible to proteolytic cleavage upon expression in *E. coli*.

The expression of genes for fragment A in *E. coli* gives a stable, active protein (31). In marked contrast, our genes coding for proteins composed of fragment A plus 97, 184, or 292 amino acids of fragment B give many intermediate-length polypeptides, in addition to the full-length species, upon expression in *E. coli*. We believe the intermediate-length fragments result from proteolytic degradation, not incomplete synthesis, because more full-length product appears when the genes are expressed in *lon* or *lon htpR* strains of *E. coli*. Such strains have been shown to be proteolytically deficient (3, 10, 18).

Typically, the proteolytic fragments detected by our polyclonal anti-diphtheria toxin serum migrate between fragment A and the full-length protein, and a major band is seen at the level of or slightly above fragment A. This suggests that cleavage is occurring primarily from the C terminus and that

fragment A is a limit digestion product. We have been unable to verify this by using α -MSH-specific sera which would detect only the C-terminal fragments of the ABM proteins. α -MSH isolated from mammalian sources contains a C-terminal amide (22) which appears to be an important immunological epitope since a wide variety of antibodies raised against amidated α -MSH fail to recognize the free acid form of the peptide (K. Parker, personal communication). The fact that our ABM fusion proteins end in the free-acid form of α -MSH explains our inability to detect them with the antisera to α -MSH now available. Without this immunological verification, our evidence that these recombinant proteins actually end with the predicted C terminus currently rests on our DNA sequencing data and upon the observation that the ABM proteins migrate about 1 kDa higher than do their corresponding ABC proteins.

All of the constructs in this study contain the *tox* signal sequence and consequently should be exported to the periplasmic space of *E. coli* as seen with previously described cloned *tox* fragments (30, 47). The proteolytic activity of the periplasmic space has been studied by Swamy and Goldberg (43), who identified eight soluble proteolytic activities in *E. coli*, only two of which were completely periplasmic and five of which were completely cytoplasmic. Moreover, Talmadge and Gilbert (44) have shown that recombinant proinsulin made in *E. coli* is 10 times more long lived when directed to the periplasmic space by fusion to the *E. coli* penicillinase signal sequence than when confined to the cytoplasm.

Despite this apparent safety of the periplasmic space for certain proteins, our results show that little or no full-length fragment B-containing truncated toxins can be detected immunologically when produced constitutively from the *tox* promoter. Similar degradation was seen by Tweten and Collier (47) with a 26- and a 43-kDa cloned segment of diphtheria toxin expressed in *E. coli* from p_{tox} . We do, however, observe the full-length proteins when they are overexpressed from the p_R promoter. Thus, even in a proteolytically deficient strain grown at 30°C, low-level constitutive synthesis of these products from p_{tox} leads to rampant proteolysis, while the high-level expression system seems to allow full-length molecules to accumulate even though the 42°C heat induction is known to further increase proteolytic activity.

In view of these observations based on immunological detection, we were surprised to find that the low-expression p_{tox} constructs produced about four times more enzymatic activity than did the high-expression p_R constructs. One explanation for this is that the inclusion of fragment B sequences leads to a conformation of fragment A that is inactive upon expression in *E. coli*. In this scheme, the p_{tox} -produced lysates would give higher activities because they are more completely degraded to active fragment A. Although whole diphtheria toxin requires proteolytic nicking at the junction between fragments A and B for enzymatic activity (38), CRM45 does not. Thus, this postulated inactive conformation of the truncated toxins would have to be a unique feature of their expression in *E. coli*.

A second possible explanation for the low specific activity of the p_R -produced lysates is that the insertion of Asp-Pro at positions 2 and 3 of the *tox* signal sequence in our p_R promoter fusions produces a signal sequence that cannot be processed normally. The hydrophilic portion of the *tox* signal sequence (fMet₁-Ser₂-Arg₃-Lys₄) has a +2 charge which is changed to +1 in the p_R fusions. Incorrect processing or failure to cleave the signal peptide might reduce the enzymatic activity of our p_R -produced proteins. However,

N-terminal protein sequencing of ABM1508 purified from the periplasmic space of *E. coli* shows that the new signal peptide is correctly removed in spite of the alteration in sequence and charge (M. Borowski, unpublished results). Hall et al. (21) have shown that changing Arg₆ to Ser in the hydrophilic portion of the *lamB* signal sequence reduces protein expression by about 80%. Furthermore, when the charge of the corresponding hydrophilic region of the lipoprotein signal peptide is changed from +2 to +1 or -1 by changing Lys₂-Ala₃ to Lys-Asp or Glu-Asp, levels of production drop by 25 and 90%, respectively (24). Vlasuk et al. (51) have gone on to show that mutations which reduce or abolish the positive charge of the hydrophilic portion of the signal peptide alter the coupling between translation and secretion in *E. coli*. Thus while our altered signal sequence probably does not affect the specific activity of fragment A, it may reduce the level of expression of the *p_R*-driven genes.

The third possibility, which we favor, is that high-temperature induction and overexpression leads to insoluble, inactive aggregates of our recombinant proteins. In preliminary experiments with 42°C fermenter growths of *E. coli* harboring pABM1508 (*p_R* driven), we have observed that the majority of the immunodetectable product remains associated with the spheroplast fraction after lysozyme-EDTA treatment or with the membrane fraction after French press lysis (M. Borowski and W. R. Bishai, unpublished results). Whole diphtheria toxin itself has been demonstrated to form dimers and higher aggregates after prolonged storage (7, 20), and we believe our truncated toxin molecules have even greater propensities to aggregate because their transmembrane domains are likely to be more exposed than are those in native diphtheria toxin. The aggregation of recombinant proteins into insoluble forms after expression in *E. coli* has been observed with other genes (6, 50).

It appears that the 313, 402, and 508 series plasmids produce proteins which are conformationally unstable. Two lines of evidence support this conclusion. The first is our observation that a *lon* strain partially protects the truncated toxins from proteolytic degradation. The La protease has been shown to play a role in the degradation of abnormal proteins in *E. coli* (19, 52). Its ability to degrade the larger truncated toxins but not fragment A during expression in *E. coli* suggests that as few as the C-terminal 50 amino acids of fragment B are required for correct folding of the whole toxin. Moreover, the fact that this cytoplasmic protease has access to the truncated toxins in *E. coli* indicates that, at least in the *p_R* expression system, translation and secretion are not tightly coupled. This is probably due to the alterations we have made in the hydrophilic region of the *tox* signal sequence.

Secondly, we observe overproduction lethality when the truncated toxins are made in high levels from the *p_R* promoter. This phenomenon has been observed with a number of fusion proteins that have a portion of a secreted protein at the N terminus and most of β -galactosidase at the C terminus (4). These fusion proteins are correctly secreted when expressed at low levels, but jam the export machinery—presumably because of the presence of the abnormal C terminus—when overexpressed. A similar phenomenon is probably occurring with truncated toxin molecules; without the complete diphtheria toxin C terminus their conformation appears to be incompatible with high-level export to the periplasm.

Although whole diphtheria toxin is a stable, secreted protein when expressed from its gram-positive host, *Corynebacterium diphtheriae*, we find that the mere deletion of

its C-terminal 50 amino acids (as in the 508 series constructs) produces molecules which are degraded after expression in *E. coli*. Growth in a proteolytically deficient strain and high-level expression from the lambda *p_R* promoter partially overcomes this degradation, but the overexpression of these abnormal constructs is a lethal event for the cells.

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LITERATURE CITED

1. Arraj, J. A., and M. G. Marinus. 1983. Phenotypic reversal in *dam* mutants of *Escherichia coli* K-12 by a recombinant plasmid containing the *dam*⁺ gene. *J. Bacteriol.* **153**:562–565.
2. Bacha, P., J. R. Murphy, and S. Reichlin. 1983. Thyrotropin-releasing hormone-diphtheria toxin-related polypeptide conjugates: potential role of the hydrophobic domain in toxin entry. *J. Biol. Chem.* **258**:1565–1570.
3. Baker, T. A., A. D. Grossman, and C. A. Gross. 1984. A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis. *Proc. Natl. Acad. Sci. USA* **81**:6779–6783.
4. Benson, S. A., M. N. Hall, and T. J. Silhavy. 1985. Genetic analysis of protein export in *Escherichia coli* K12. *Annu. Rev. Biochem.* **54**:101–134.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
6. Buell, G., and N. Panayotatos. 1986. Mechanism and practice, p. 345–363. In W. Reznikoff and L. Gold (ed.), *Maximizing gene expression*. Butterworth Publishers Inc., Boston.
7. Carroll, S. F., J. T. Barbieri, and R. J. Collier. 1986. Dimeric form of diphtheria toxin: purification and characterization. *Biochemistry* **25**:2425–2430.
8. Cawley, D. B., H. R. Herschman, D. G. Gilliland, and R. J. Collier. 1980. Epidermal growth factor-toxin A chain conjugates: EGF-ricin A is a potent toxin while EGF-diphtheria fragment A is nontoxic. *Cell* **22**:563–570.
9. Chang, T., A. Dazord, and D. M. Neville. 1977. Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate. II. Biochemical and biologic properties of diphtheria toxin fragment A-S-S-human placental lactogen. *J. Biol. Chem.* **252**:1515–1522.
10. Chung, C. H., and A. L. Goldberg. 1981. The product of the *lon* (*capR*) gene in *Escherichia coli* is the ATP-dependent protease, protease La. *Proc. Natl. Acad. Sci. USA* **78**:4931–4935.
11. Colombatti, M., L. Greenfield, and R. J. Youle. 1986. Cloned fragment of diphtheria toxin linked to T cell-specific antibody identifies regions of B chain active in cell entry. *J. Biol. Chem.* **261**:3030–3035.
12. Dente, L., G. Cesareni, and R. Cortese. 1983. pEMBL: a new family of single stranded plasmids. *Nucleic Acids Res.* **11**:1645–1655.
13. Dente, L., M. Sollazzo, C. Baldari, G. Cesareni, and R. Cortese. 1985. The pEMBL family of single-stranded vectors, p. 101–107. In D. M. Glover (ed.), *DNA cloning*, vol. 1. A practical approach. IRL Press Limited, Oxford.
14. Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179**:125–142.
15. Federal Register. 1986. Guidelines for research involving recombinant DNA molecules, appendix F. *Fed. Regist.* **51**:16971–16972.

16. Gilliland, D. G., R. J. Collier, J. M. Moehring, and T. J. Moehring. 1978. Chimeric toxins: toxic, disulfide-linked conjugate of concanavalin A with fragment A from diphtheria toxin. *Proc. Natl. Acad. Sci. USA* 75:5319-5323.
17. Gilliland, D. G., Z. Steplewski, R. J. Collier, K. F. Mitchell, T. H. Chang, and H. Koprowski. 1980. Antibody-directed cytotoxic agents: use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells. *Proc. Natl. Acad. Sci. USA* 77:4539-4543.
18. Goff, S. A., L. P. Casson, and A. L. Goldberg. 1984. Heat shock regulatory gene *hspR* influences rates of protein degradation and expression of the *lon* gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 81:6647-6651.
19. Goff, S. A., and A. L. Goldberg. 1985. Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. *Cell* 41:587-595.
20. Goor, R. S. 1968. New form of diphtheria toxin. *Nature (London)* 217:1051-1053.
21. Hall, M. N., J. Gabay, and M. Schwartz. 1983. Evidence for a coupling of synthesis and export of an outer membrane protein in *Escherichia coli*. *EMBO J.* 2:15-19.
22. Harris, J. I., and A. B. Lerner. 1957. Amino-acid sequence of the α -melanocyte-stimulating hormone. *Nature (London)* 179:1346-1347.
23. Holmes, R. K. 1976. Characterization and genetic mapping of nontoxinogenic (*tox*) mutants of corynebacteriophage beta. *J. Virol.* 19:195-207.
24. Inouye, S., X. Soberon, T. Franceschini, K. Nakamura, K. Itakura, and M. Inouye. 1982. Role of positive charge on the amino-terminal region of the signal peptide in protein secretion across the membrane. *Proc. Natl. Acad. Sci. USA* 79:3438-3441.
25. Isberg, R. R., A. L. Lazaar, and M. Syvanen. 1982. Regulation of Tn5 by the right-repeat proteins: control at the level of the transposition reaction? *Cell* 30:883-892.
26. Kaczorek, M., F. Delpyroux, N. Chenciner, R. E. Streeck, J. R. Murphy, P. Boquet, and P. Tiollais. 1983. Nucleotide sequence and expression of the diphtheria *tox228* gene in *Escherichia coli*. *Science* 221:855-858.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
28. Laird, W., and N. Groman. 1976. Isolation and characterization of *tox* mutants of corynebacteriophage beta. *J. Virol.* 19:220-227.
29. Lambotte, P., P. Falmagne, C. Capiou, J. Zanen, J.-M. Ruyschaert, and J. Dirckx. 1980. Primary structure of diphtheria toxin fragment B: structural similarities with lipid-binding domains. *J. Cell. Biol.* 87:837-840.
30. Leong, D., K. D. Coleman, and J. R. Murphy. 1983. Cloned fragment A of diphtheria toxin is expressed and secreted into the periplasmic space of *Escherichia coli* K12. *Science* 220:515-517.
31. Leong, D., K. D. Coleman, and J. R. Murphy. 1983. Cloned diphtheria toxin fragment A is expressed from the *tox* promoter and exported to the periplasm by the *secA* apparatus of *Escherichia coli* K12. *J. Biol. Chem.* 258:15016-15020.
32. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. Messing, J., B. Gronenborn, B. Müller-Hill, and P. H. Hofschneider. 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *HindIII* fragment of the *lac* regulatory region in M13 replicative form *in vitro*. *Proc. Natl. Acad. Sci. USA* 74:3642-3646.
- 33a. Miyahara, A., W. Bishai, J. Boyd, S. Nagle, and J. Murphy. 1986. Genetic construction and expression of a diphtheria toxin-related α -melanocyte stimulating hormone fusion gene, p. 365-374. In P. Falmagne, J. E. Alouf, F. J. Fehrenbach, J. J. Eljaszewicz, and M. Thelestam (ed.), *Bacterial protein toxins*. Gustav Fischer Verlag, Stuttgart.
34. Moolten, F. L., and S. R. Cooperband. 1970. Selective destruction of target cells by diphtheria toxin conjugated to antibody directed against antigens on the cells. *Science* 169:68-70.
35. Murphy, J. R. 1985. The diphtheria toxin structural gene. *Curr. Top. Microbiol. Immunol.* 118:235-251.
36. Murphy, J. R., W. Bishai, M. Borowski, A. Miyahara, J. Boyd, and S. Nagle. 1986. Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria toxin-related α -melanocyte-stimulating hormone fusion protein. *Proc. Natl. Acad. Sci. USA* 83:8258-8262.
37. O'Keefe, D. O., and R. K. Draper. 1985. Characterization of a transferrin-diphtheria toxin conjugate. *J. Biol. Chem.* 260:932-937.
38. Pappenheimer, A. M. 1977. Diphtheria toxin. *Annu. Rev. Biochem.* 46:69-94.
39. Robb, M., J. C. Nichols, S. K. Whoriskey, and J. R. Murphy. 1982. Isolation of hybridoma cell lines and characterization of monoclonal antibodies against cholera enterotoxin and its subunits. *Infect. Immun.* 38:267-272.
40. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
41. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. Simpson, D. L., D. B. Cawley, and H. R. Herschman. 1982. Killing of cultured hepatocytes by conjugates of asialofetuin and EGF linked to the A chains of ricin or diphtheria toxin. *Cell* 29:469-473.
43. Swamy, K. H. S., and A. L. Goldberg. 1982. Subcellular distribution of various proteases in *Escherichia coli*. *J. Bacteriol.* 149:1027-1033.
44. Talmadge, K., and W. Gilbert. 1982. Cellular location affects protein stability in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 79:1830-1833.
45. Thorpe, P. E., W. C. J. Ross, A. J. Cumber, C. A. Hinson, D. C. Edwards, and A. J. S. Davies. 1978. Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin. *Nature (London)* 271:752-755.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
47. Tweten, R. K., and R. J. Collier. 1983. Molecular cloning and expression of gene fragments from corynebacteriophage β encoding enzymatically active peptides of diphtheria toxin. *J. Bacteriol.* 156:680-685.
48. Uchida, T., D. M. Gill, and A. M. Pappenheimer. 1971. Mutation in the structural gene for diphtheria toxin carried by temperate phage β . *Nature (London) New Biol.* 233:8-11.
49. Uchida, T., A. M. Pappenheimer, and R. Greany. 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 248:3838-3844.
50. Varadarajan, R., A. Szabo, and S. G. Boxer. 1985. Cloning, expression in *Escherichia coli*, and reconstitution of human myoglobin. *Proc. Natl. Acad. Sci. USA* 82:5681-5684.
51. Vlasuk, G. P., S. Inouye, H. Ito, K. Itakura, and M. Inouye. 1983. Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli*. *J. Biol. Chem.* 258:7141-7148.
52. Waxman, L., and A. L. Goldberg. 1986. Selectivity of intracellular proteolysis: protein substrates activate the ATP-dependent protease (La). *Science* 232:500-503.
53. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.
54. Youle, R. J., F. M. Uckun, D. A. Vallera, and M. Colombatti. 1986. Immunotoxins show rapid entry of diphtheria toxin but not ricin via the T3 antigen. *J. Immunol.* 136:93-98.