Site-directed mutagenesis of *Clostridium perfringens* beta-toxin: expression of wild-type and mutant toxins in *Bacillus subtilis*

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Abstract

Recombinant beta-toxin has been expressed and secreted from *Bacillus subtilis*. Biological activity was tested in vivo and in vitro. The lethal dose in mice was determined. Hemolysis of rabbit and sheep erythrocytes was tested but no effect was observed. Seven mutant proteins were produced. Targets for mutagenesis were mostly selected on the basis of the similarity between beta-toxin and alpha-toxin from *Staphylococcus aureus*, a pore-forming toxin. Mutations of two amino acids affected the lethal dose in mice. Both residues have counterparts in the membrane binding region of alpha-toxin. Alteration of the single cysteine residue did not affect protein function, contrary to previous suggestions.

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1. Introduction

Clostridium perfringens beta-toxin is a major virulence factor of toxigenic type B and C strains. The nature of the toxic effect of beta-toxin has not been elucidated. Native beta-toxin, isolated from a pool of other toxins, has mainly been used in functional analysis of the protein [1–4] and interpretation is complicated by potential contamination from other toxins. Expression of the protein as a fusion protein in *Escherichia coli* facilitated the production of specific polyclonal antiserum but the lack of biological activity in the fusion protein precludes its use in

functional studies [5]. Beta-toxin gene sequencing has revealed similarity (17–29%) with alpha-toxin, gamma-toxin and leukocidin from *Staphylococcus aureus* [6]. Proteins of the gamma-toxin and leukocidin groups act in synergy as two component toxins [7] while alpha-toxin forms a single component heptamer in cell membranes creating cylindrical pores [8–10]. Extensive mutational analysis of the alpha-toxin has yielded considerable information on structure-function relationships in the protein [11–13].

We have undertaken a study of the structure and function of beta-toxin. Expression of beta-toxin in *Bacillus subtilis* is the first report of biologically active cloned beta-toxin, thus facilitating functional analysis. A single cysteine residue has been proposed to be important for the activity of beta-toxin [6,14]

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making it an ideal target for mutagenesis. Single histidine mutations in *S. aureus* alpha-toxin eliminate toxic activity [11,12]. Alpha-toxin and beta-toxin both contain four histidine residues. Although their positions are not strictly conserved between the two proteins this raises questions about possible roles in protein function. In addition, sequence similarity with the pore-forming toxins was used to select four amino acids for mutational analysis.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain DH5α was used for maintaining *E. coli* plasmids. Strain CJ236 was used for preparation of uracil-containing M13 DNA template for mutagenesis and MV1190 was the recipient strain. *B. subtilis* strain 1S53, the *E. coli-B. subtilis* shuttle vector pHB201 and vector pUB110 were obtained from the Bacillus Genetic Stock Center.

2.2. Site-directed mutagenesis

A cysteine 265 to serine mutation was carried out by sequential PCR steps [15]. The beta-toxin gene was amplified from *C. perfringens* DNA in two fragments using two overlapping mutagenic primers (Table 1) and flanking primers spanning the gene. The final product was digested with *Bsp*DI and *Eco*RI and the resulting 770-bp C-terminal fragment cloned

into Bluescript SK$^+$. Other mutations were performed by standard oligonucleotide-directed mutagenesis [15]. A 1.3-kb *Xba*I-*Eco*RI fragment containing the cloned beta-toxin gene from pPB10 (see below) was subcloned into M13mp18 for production of single-stranded template. Mutagenic primers and the resulting mutations are shown in Table 1. All mutations were confirmed by dideoxynucleotide sequencing.

2.3. Construction of plasmids

A wild-type beta-toxin clone containing the open reading frame and upstream sequences was constructed by subcloning the C-terminal *Bsp*DI-*Eco*RI fragment of pB12 into pUC18 containing a previously cloned N-terminal *Xba*I-*Bsp*DI fragment [5]. The resulting wt clone, pPB10, consisted of nt 58–1343 of the published beta-toxin sequence [6]. This fragment was further subcloned as a *Sph*I-*Eco*RI fragment into pHB201 to make shuttle plasmid pPB14. A full-length clone containing the C265S mutation was constructed by replacing the *Bsp*DI-*Eco*RI fragment in pPB10 with the corresponding fragment containing the mutation. The resulting clone was subcloned as an *Xba*I-*Eco*RI fragment into Bluescript KS$^-$ (pPB21) and further as a *Sac*I-*Eco*RI fragment into pHB201 creating shuttle plasmid pPB22. Other mutant clones were subcloned from M13mp18 RF as *Sph*I-*Eco*RI fragments into pHB201. Plasmid pXB10 and pXB30, containing wild-type beta-toxin and R212E respectively in

Table 1
Oligonucleotide sequences used for mutagenesis

Mutation	Oligonucleotides[a]	Codon change
Cys-265 to Ser[b]	5′-GAAACT**CG**TATTATCTT-3′	TGT → TCG
	5′-TAATA**CG**AGTTTCTTTC-3′	
His-85 to Leu	5′-ACAATTTGC**T**TGATGTTACTA-3′	CAT → CTT
Tyr-134 to Asn	5′-ACAATGGGA**A**ACAAAATAGGA-3′	TAC → AAC
Asp-167 to Asn	5′-TGTCCAACCT**A**ATTTTTCTAC-3′	GAT → AAT
His-175 to Leu	5′-CAGACAGATCTTTCAACCTCT-3′	CAT → CTT
Tyr-203 to Cys	5′-AACCCTGTAT (**G/T/C**) TGGAAATGAAATGT-3′	TAT → TGT
Tyr-203 to Phe		TAT → TTT
Arg-212 to Glu	5′-GTTTATGTACGGA (**G/C**) **A**ATATACTAATGT-3′	AGA → GAA
Arg-212 to Gln		AGA → CAA
His-293 to Leu	5′-GTAGATAGTC**T**TATATTTACA-3′	CAT → CTT
His-304 to Leu	5′-GGCTTACTC**T**TAAAGTAACAG-3′	CAT → CTT

[a]Base changes are in boldface.

[b]This mutation was constructed by overlap PCR, which requires two complementary mutagenic oligonucleotides.

pUB110, were constructed by subcloning the beta-toxin gene as a *Sal*I-*Eco*RI fragment from the pHB201 vector into Bluescript KS⁻ (pPB15 and pPB30) and further as an *Xba*I fragment into pUB110. *B. subtilis* strain 1S53 was transformed with plasmid DNA using the method of Gryczan et al. [16].

2.4. Protein expression and analysis

Freshly grown colonies of 1S53 transformants expressing beta-toxin were inoculated in $2\times$ concentrated Luria broth without NaCl, supplemented with chloramphenicol ($5~\mu g~ml^{-1}$) and 0.2% glucose. The culture was incubated at 35°C in a shaking incubator at 100 rpm and allowed to grow for 24 h. After pelleting cells, the supernatant was stored at −20°C. Protein analysis was performed by SDS-PAGE analysis, followed by Western blotting and detection with polyclonal anti-beta-toxin antiserum [5].

2.5. Estimation of beta-toxin concentration

Total protein concentration in beta-toxin-containing culture supernatants was determined by the method of Bradford [17] with bovine serum albumin (BSA) as a standard. Following SDS-PAGE, protein was detected by silver staining and the ratio of the beta-toxin band to total protein determined by densitometry scanning. Beta-toxin comprised 47% of the total protein in one preparation. This preparation was then used as a standard in an ELISA test for determination of beta-toxin concentration in other preparations. The ELISA test was performed as described by Hansen et al. [18] using monoclonal and polyclonal anti-beta-toxin as first and second antibody respectively. A good correlation was obtained when beta-toxin concentration in different preparations, as determined by the method above, was compared to results from the ELISA test, indicating that the immunological assays yield correct estimates of the mutant protein concentrations.

2.6. Hemolytic activity determination

Hemolysis of rabbit and sheep erythrocytes was determined essentially as described by Walker and Bayley [13], using serial twofold dilutions of beta-toxin ($16~\mu g~ml^{-1}$). Culture supernatant without beta-toxin served as a negative control and *C. perfringens* type C culture supernatant as a positive control in both experiments. The cells were incubated with toxin preparations for 3 h at 22°C or 37°C. Cell lysis was determined visually.

2.7. Biological activity

Male and female NMRI mice weighing 17–22 g were injected intravenously (i.v.) with 0.4 ml of filter-sterilized *B. subtilis* culture supernatant diluted in PBS. Death by 24 h was recorded. The 50% lethal dose (LD_{50}), was determined by injecting 2–6 mice with each dilution tested. Tests were repeated on different days and results combined for analysis. The total number of mice tested was 82 for the wild-type toxin and 30–46 for the mutant toxins (see legend to Fig. 3). The LD_{50} for each toxin tested and 95% confidence limits were determined using the VacMan computer program as described by Spouge [19].

3. Results

3.1. Expression of wild-type beta-toxin in B. subtilis

In order to express beta-toxin in *B. subtilis* the beta-toxin gene and its upstream sequences were cloned into an *E. coli-B. subtilis* shuttle vector as described in Section 2. The resulting clone, pPB14, was transformed into the sporulation-negative *B.*
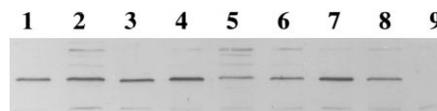

Fig. 1. Western analysis of protein induced by *B. subtilis* strains harboring *C. perfringens* beta-toxin genes. Proteins were separated by SDS-PAGE on gels containing 12% acrylamide. Each lane contains 2.5 µl culture supernatant of the relevant strain. Beta-toxin (MW 34 kDa) was detected using polyclonal anti-beta-toxin antibody. Lanes: 1, wild-type beta-toxin expressed from pPB14; 2, H85L; 3, H175L; 4, Y203F; 5, R212E; 6, R212Q; 7, C265S; 8, H293L; 9, pHB201 (negative control). Labels of mutant strains indicate wt amino acid residue, its position in mature protein and mutant residue.

subtilis strain 1S53 and grown in liquid culture. Beta-toxin was detected in the culture supernatant by immunoblotting (Fig. 1 lane 1), but only in minimal amounts in cell extracts (not shown), indicating efficient secretion of the protein by *B. subtilis*.

3.2. Site-directed mutagenesis

Amino acid sequence alignment of beta-toxin and *S. aureus* alpha-toxin, leukocidin and hemolysin components revealed several conserved residues between these proteins [6]. Selection of amino acids for mutagenesis was partially based on this homology. Sequence alignment of mature alpha-toxin and beta-toxin is shown in Fig. 2 and amino acids selected for mutagenesis are indicated. Two conserved tyrosine residues, Y134 and Y203, one arginine residue,

R212, and aspartic acid residue D167, which has a counterpart in alpha-toxin only, were altered. Some of the initial mutagenesis of alpha-toxin showed the importance of histidine residues for the toxic effect and therefore we chose to alter these amino acids in beta-toxin. The only cysteine residue in the protein, C265, was also mutated. Mutations were primarily chosen on the basis of altering functional groups while conserving volume.

3.3. Expression of mutant beta-toxin

Clones of 11 single mutations (Table 1) were isolated and subcloned into the shuttle vector pHB201. Mutant proteins were expressed in *B. subtilis* and detected in culture supernatants by immunoblotting. Seven mutant proteins were produced in detectable

Fig. 2. Amino acid sequence comparison between *C. perfringens* beta-toxin (GenBank accession number X83275) and *S. aureus* alpha-toxin (GenBank accession number X01645). The sequence alignment was done with the Gene Inspector program (Textco) using a BLOSUM62 scoring table with a gap insertion penalty of 9.00 and a gap extension penalty of 0.80. Asterisks indicate amino acids selected for mutagenesis in beta-toxin. Boxed amino acids denote two sides of the triangle region in alpha-toxin defined by Song et al. [10]. The membrane spanning stem in alpha-toxin is underlined, double underlining shows the base of the stem.

amounts (Fig. 1). Protein yields were similar to wild-type, as determined by ELISA, with the exception of the R212 mutants and H293L, which routinely produced 40% of the wild-type level. The lack of beta-toxin in the supernatant from the four remaining mutant clones was not due to poor cell growth or to inefficient protein secretion since no protein was detected in cell extracts. It is possible that the mutant proteins were unstable but no attempt was made to distinguish between alternative reasons for the absence of these mutant proteins. Increased expression of R212E was obtained by transferring the cloned fragment containing the mutation to the *B. subtilis* expression vector pUB110, yielding sufficient protein concentrations for LD_{50} determination.

3.4. Lethality in mice

B. subtilis culture supernatant was used for determination of LD_{50} for each beta-toxin-expressing strain (Fig. 3). LD_{50} for the wild-type protein expressed in *B. subtilis* was 1.1 µg kg^{-1}. The LD_{50} for i.v. injected native beta-toxin has been reported to be 0.3 µg kg^{-1} [3]. In our hands the lethal dose of *C. perfringens* culture supernatant containing native beta-toxin was similar to that obtained with the recombinant protein (not shown). The difference in lethal dose observed in the two studies may be at least partially due to different methods used for determination of beta-toxin concentration.

Alteration of the three histidine residues and the single cysteine did not affect the lethal dose of the protein as compared to wild-type. Changing tyrosine 203 to phenylalanine (Y203F) caused a 2.5-fold increase in the lethal dose. The effect of the arginine 212 to glutamic acid mutation (R212E) was more pronounced, the lethal effect of this mutant protein was approximately 12-fold lower than the wild-type. Substitution of arginine 212 with glutamine (R212Q) resulted in a 5.5-fold increase in the lethal dose.

3.5. Hemolysis assay

Hemolysis was observed on sheep and rabbit erythrocytes when incubated with *C. perfringens* exotoxins while no lysis was detected when incubated with *B. subtilis* culture supernatant containing beta-
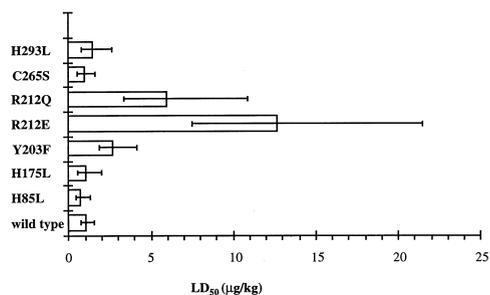

Fig. 3. Lethal dose of wild-type and mutant beta-toxins in mice. LD_{50} was determined by i.v. injection of culture supernatant from *B. subtilis* strains producing beta-toxin. 95% confidence limits are shown. Wild type, $n = 82$; H85L, $n = 43$; H175L, $n = 30$; Y203F, $n = 46$; R212E, $n = 36$; R212Q, $n = 34$; C265S, $n = 37$; H293L, $n = 30$. Labels of mutant strains indicate wt amino acid residue, its position in mature protein and mutant residue.

toxin. Similarly, culture supernatant without beta-toxin had no effect. These results indicate that beta-toxin is not hemolytic on sheep or rabbit erythrocytes.

4. Discussion

B. subtilis cells can produce secretory proteins of other Gram-positive bacteria in the extracellular medium by using the promoters and secretion signals present in their encoding genes [20]. We have found that active beta-toxin is expressed and secreted efficiently using its native promoter and its own protein processing signals in this system, thus making it ideal for studies on beta-toxin structure and function. Although this study is limited to a few amino acid substitutions at selected sites, valuable information was gained.

Site-directed mutagenesis revealed the importance of arginine 212 for the biological activity of the protein. Replacement of this residue with glutamic acid resulted in 11.5-fold reduction in toxicity while replacement with glutamine reduced the activity 5.5-fold compared to wild-type. The corresponding residue in *S. aureus* alpha-toxin (R200) is important for binding and oligomerization of the protein as well as for hemolysis [13].

When tyrosine 203 was changed to phenylalanine the result was a 2.5-fold increase in the LD_{50} dose,

indicating a role for this residue in the activity of the protein. It has been shown that the corresponding residue in S. aureus alpha-toxin (Y191) is located in the putative membrane binding surface of the protein [10]. The effect of the Y203F mutation on beta-toxin function would be consistent with a similar location of Y203 in beta-toxin.

Replacing each individual histidine residue with a leucine did not affect the biological activity of the protein. Alteration of the histidine residues in the S. aureus alpha-toxin all affect protein function to a varying degree, with the N-terminal histidine mutation abolishing toxicity [11,12]. Both proteins contain four histidine residues but their locations are not conserved between the two proteins and thus their roles may differ. It has recently been shown that the two N-terminal histidine residues in alpha-toxin, H35 and H48, are important for interaction of promoters in the heptameric protein [10].

The importance of a cysteine residue in the activity of beta-toxin was first suggested by Sakurai et al. [14]. They showed that the toxin was inactivated by oxidizing agents and could be reactivated by reduction. Our data show that the presence of the single cysteine residue is not required for the function of the protein. It thus appears that the inactivation of the oxidized protein is due to modification of the cysteine residue and that the cysteine itself does not play a critical role in protein function.

A common feature of the proteins that share homology with beta-toxin is hemolytic activity. Beta-toxin is not hemolytic on rabbit or sheep erythrocytes at the concentration tested. This corresponds to 50-fold the concentration of staphylococcal alpha-toxin that causes 100% hemolysis on rabbit erythrocytes [21]. However, a much higher concentration is needed for hemolysis of human erythrocytes by alpha-toxin. It has been suggested that alpha-toxin binds non-specifically to membranes at a high concentration whereas a cellular receptor is involved in binding at a lower concentration [22]. The absence of hemolysis by beta-toxin indicates that if the toxin elicits its effect by generating pores in the cell membrane it most likely requires specific receptors for membrane binding.

The lethal effect of alpha-toxin in experimental animals has been ascribed to circulatory collapse, possibly due to simultaneous cardiac failure and development of acute pulmonary edema [23]. Very little experimental data is available on the in vivo effect of beta-toxin. Intravenous injection of recombinant beta-toxin causes death due to toxic shock or generalized toxemia in mice and cardiac failure in rats (unpublished data). Previous work with purified beta-toxin indicates that it causes a rise in blood pressure in rats [4]. Further work is needed to determine more specifically where beta-toxin elicits its effect.

Hunter et al. [6] reported 28% similarity of beta-toxin to the alpha-toxin of S. aureus. The homology is not limited to well defined conserved regions but dispersed over most parts of the protein. The recent publication of the structure of the heptameric alpha-toxin pore [10] paves the way for a more meaningful interpretation of this comparison. The alteration of two amino acids in the beta-toxin affects the toxicity of the protein indicating that these amino acids may have a functional role, although the possibility of structural changes cannot be ruled out in the absence of physical analysis of the mutant proteins. Both amino acids have counterparts in a putative membrane binding region in alpha-toxin.

It is noteworthy that there is strong homology between the two proteins in the two arms of the triangle adjacent to the membrane spanning region of the alpha-toxin (Fig. 2). According to Song et al. [10] this region participates in crucial protomer-protomer interaction as well as being important in conformational rearrangements involved in multimer formation in the membrane bound form. The only mutation we have made that maps to the triangle region is D167N. No protein was obtained from this mutant clone, perhaps reflecting strict structural requirements in this region. A corresponding alpha-toxin mutant protein (D152C) has been expressed in vitro [13] and was non-lytic. Although the amino acid sequence of the putative membrane spanning loop of beta-toxin has low homology to that of alpha-toxin, it can be fitted to a model where polar amino acid side chains are mainly internal to the pore and hydrophobic side chains in contact with the membrane, as in the crystal structure of alpha-toxin. In summary, our results combined with available data are consistent with the idea that beta-toxin is structurally and functionally related to the alpha-toxin of S. aureus.

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