The variant undecapeptide sequence of the *Arcanobacterium pyogenes* haemolysin, pyolysin, is required for full cytolytic activity

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The cholesterol-dependent cytolysins (CDCs) are characterized by an undecapeptide sequence (ECTGLAWEWWR) that is located near the C terminus and within domain 4 of these proteins. Pyolysin (PLO), the CDC of *Arcanobacterium pyogenes*, has a variant undecapeptide sequence (EATGLAWDPWW). Site-directed mutants were constructed in undecapeptide residues in a recombinant PLO molecule containing a hexahistidine tag (His-PLO). Mutations in each of the three undecapeptide tryptophan residues resulted in low haemolytic activity, confirming the importance of these residues in the protein. Deletion of a proline residue (P₄₉₉), inserted in PLO, or substitution of this residue with either phenylalanine or glycine resulted in mutant proteins with undetectable or low haemolytic activities, indicating that P₄₉₉ is essential for His-PLO haemolytic activity. Substitution of the PLO undecapeptide sequence with a consensus undecapeptide resulted in a His-PLO protein with only 0.1% activity, confirming that the variant PLO undecapeptide is required for the full cytolytic activity of this toxin. The presence of the conserved undecapeptide cysteine residue either alone (His-PLO.C₄₉₂) or in a consensus sequence resulted in His-PLO molecules which were activated in the presence of reducing compounds, confirming the importance of this residue in the thiol-activated nature of many CDC toxins. The ability of His-PLO mutant proteins to bind cholesterol mimicked haemolytic activity, with the exception of His-PLO.C₄₉₂, which, despite having reduced haemolytic activity, showed an increased ability to bind cholesterol compared to His-PLO. Despite reductions in haemolytic activity and cholesterol-binding, all mutant proteins were still able to bind to erythrocyte membranes, suggesting that other regions of PLO may recognize host-cell membranes, through receptors other than cholesterol.

Keywords: cholesterol-dependent cytolysin, virulence factor, amino acid substitution

INTRODUCTION

Arcanobacterium pyogenes is commensal on the mucous membranes of the upper respiratory and urogenital tracts of cattle, sheep, swine and other domestic animals (Carter & Chengappa, 1991). This organism is also commensal on the ruminal wall of cattle, from where it can participate in liver abscessation, secondary to ruminal acidosis (Narayanan *et al.*, 1998). The recent isolation of *A. pyogenes* from the porcine gastric mucosa

(Jost *et al.*, 2002) suggests that it may also be a common inhabitant of the gastrointestinal tract of domestic animals. *A. pyogenes* is an important opportunistic pathogen of livestock and wild ungulates, causing diseases ranging from liver abscessation and mastitis in cattle (Jonsson *et al.*, 1991; Lechtenberg *et al.*, 1988; Nagaraja *et al.*, 1996) to intracranial abscessation in white-tailed deer (Baumann *et al.*, 2001).

A. pyogenes secretes a haemolytic exotoxin, pyolysin (PLO) (Billington *et al.*, 1997; Ding & Lämmler, 1996), which is an important virulence factor, as a knockout mutation in the *plo* gene resulted in reduced virulence of the mutant in a mouse model (Jost *et al.*, 1999). Recombinant PLO is also an effective vaccine in the

Abbreviations: BME, β -mercaptoethanol; CDC, cholesterol-dependent cytolysin; ILY, intermedilysin; LLO, listeriolysin O; PFO, perfringolysin O; PLO, pyolysin; PLY, pneumolysin; SRBC, sheep red blood cell.

(a)		(b)	
SLO	529	ECTGLAWE	- W W R
PLY	427	ECTGLAWE	- W W R
SLY	455	ECTGLAWE	- W W R
LLO	483	ECTGLAWE	- W W R
LSO	484	ECTGLFWE	- W W R
ILO	458	ECTGLAWE	- W W R
PFO	458	ECTGLAWE	- W W R
ALY	460	ECTGLAWE	- W W R
CLY	465	ECTGLAWE	- W W R
PLO	491	EATGLAWD	P W W -
ILY	485	GATGLAWE	P W - R

His-PLO	EATGLAWDPWW	-
His-PLO.C ₄₉₂	E C TGLAWDPWW	-
His-PLO.F ₄₉₇	EATGLA F D PWW	-
His-PLO.F ₅₀₀	EATGLAWDP F W	-
His-PLO.F ₅₀₁	EATGLAWDPW F	-
His-PLO.ΔP ₄₉₉ ΩR ₅₀₂	EATGLAWD - WW R	
His-PLO.ΩR ₅₀₂	EATGLAWDPWW R	
His-PLO.ΔP ₄₉₉	EATGLAWD - WW	-
His-PLO.F ₄₉₉	EATGLAWD F WW	-
His-PLO.G ₄₉₉	EATGLAWD G WW	-
His-PLO _{CON}	E C TGLAWE - WW R	
His-PLO _{CON+A}	EATGLAWE - WW R	

Fig. 1. Alignment of CDC undecapeptide sequences and undecapeptide substitutions in mutant His-PLO molecules. (a) Amino acid sequence alignment of the undecapeptide sequence of CDCs. Amino acids which match the consensus undecapeptide sequence are highlighted. Amino acid numbers are given for the first amino acid in the sequence for each protein. The amino acid sequences of streptolysin molecules from *Streptococcus canis*, *Streptococcus dysgalactiae* subsp. *equisimilis* and *Streptococcus pyogenes* are identical over the region shown. SLO, streptolysin O; SLY: suilylsin; LSO, seeligerilylsin O; ILO, ivanolysin O; ALY, alveolysin; CLY, cereolysin. (b) Amino acid sequence alignment showing amino acid substitutions in the undecapeptide of His-PLO constructed by site-directed mutagenesis. Substituted amino acids are highlighted.

prevention of experimental infections in mice (Jost *et al.*, 1999), although its ability to protect domestic animals from natural infection is still to be tested. PLO is a novel member of a group of toxins historically known as the thiol-activated cytolysins. As all members of this family are not subject to thiol-activation, including PLO (Billington *et al.*, 1997; Nagamune *et al.*, 1996), a number of alternative designations have been proposed (Billington *et al.*, 2000; Gilbert *et al.*, 1999; Nagamune, 1997; Shatursky *et al.*, 1999), with cholesterol-dependent cytolysins (CDCs) (Shatursky *et al.*, 1999) being the most widely accepted. This group of toxins, which includes listeriolysin O (LLO), perfringolysin O (PFO), pneumolysin (PLY) and streptolysin O, is characterized by an undecapeptide sequence (ECTGLAWEWWR) located near the C terminus of the protein (Fig. 1a). Oxidation of the cysteine residue within this sequence is responsible for the thiol-activated nature of many CDCs. Both PLO and intermedilysin (ILY) of *Streptococcus intermedius* have variant undecapeptide sequences, in which the conserved cysteine has been substituted with an alanine residue, resulting in oxygen-resistant toxins (Billington *et al.*, 1997; Nagamune *et al.*, 1996; Nagamune, 1997) (Fig. 1a). Many advances have been made in recent years as to the function of the four domains within the CDC tertiary structures and their roles in pore-formation (Tweten *et al.*, 2001). However, while it has been evident for some time that the undecapeptide is critical for CDC activity (Boulnois *et al.*, 1991; de los Toyos *et al.*, 1996; Jacobs *et al.*, 1999; Korchev *et al.*, 1998; Michel *et al.*, 1990; Pinkney *et al.*, 1989; Saunders *et al.*, 1989; Sekino-Suzuki *et al.*, 1996), the precise function of this region of the molecule is unclear.

The crystal structure of the prototype CDC, *Clostridium perfringens* PFO, has been described as an elongated molecule composed of four β -sheet-rich domains, only

one of which, the C-terminal domain 4, is contiguous within the primary amino acid sequence (Rossjohn *et al.*, 1997). A proposed structure for *S. pneumoniae* PLY based on a homology model with PFO indicates that PLY takes on a very similar structure (Rossjohn *et al.*, 1998) and it appears likely, given the conservation of residues that map to the hydrophobic core of PFO, that all CDCs, including PLO, share a similar three-dimensional structure (Rossjohn *et al.*, 1997). Studies of pore formation by PFO (Heuck *et al.*, 2000; Shatursky *et al.*, 1999; Shepard *et al.*, 1998, 2000) indicate that two transmembrane amphipathic β -hairpins (TMHs) from domain 3 span the membrane bilayer and serve as the membrane–aqueous interface of the PFO pore. In the crystal structure of the PFO monomer, the tryptophan-rich undecapeptide region forms a hydrophobic loop at one end of domain 4 (Rossjohn *et al.*, 1997), initially proposed to extend out, as a result of cholesterol binding, to form a dagger which facilitates membrane insertion (Rossjohn *et al.*, 1997). However, it has recently been demonstrated that domain 4 does not penetrate deeply into the lipid bilayer, but is responsible for the initial interaction of CDCs with the host membrane (Heuck *et al.*, 2000). In fact, the conformational changes required to form the domain 3 TMHs do not take place until domain 4 has partially inserted into the lipid bilayer (Heuck *et al.*, 2000). Thus, domain 4 may act to sample the membrane for suitability or to tether monomers to the membrane in a pre-pore complex, prior to the insertion of the TMHs (Heuck *et al.*, 2000; Hotze *et al.*, 2002). The undecapeptide sequence has been implicated in the initial binding of CDCs to host-cell membranes, as mAbs directed against the undecapeptide sequence block binding of PLY to erythrocyte membranes (Jacobs *et al.*, 1999), and site-directed mutations in the undecapeptide sequences, particularly the conserved tryptophan residues, of many CDCs significantly affect the ability of these toxins to

form pores (Boulnois *et al.*, 1991; Michel *et al.*, 1990; Sekino-Suzuki *et al.*, 1996).

Both PLO and the human-specific cytolysin ILY have variant undecapeptide sequences (Billington *et al.*, 1997; Nagamune, 1997) (Fig. 1a). It is not known if this sequence divergence represents an attempt to regulate cytolytic activity or whether, in the case of PLO which is the most divergent member of the CDC family, the sequence divergence compensates for structural differences in other regions of the protein between PLO and the other CDCs. In this study, we examine the activity of various site-specific mutants in the undecapeptide of PLO and determine that the sequence variation in the PLO undecapeptide is required for full cytolytic activity.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains DH5 α and DH5 α F'*lacI*^q (Gibco-BRL) were grown either on Luria-Bertani (LB) agar or in LB broth (Difco) at 37 °C. Ampicillin (100 μ g ml⁻¹) was added to the medium when appropriate.

DNA techniques. Procedures for *E. coli* transformation and plasmid extraction, DNA restriction, ligation, agarose gel electrophoresis and Southern blotting were performed as described by Ausubel *et al.* (1994). Plasmids pJGS59, encoding a 543 aa protein (His-PLO) composed of 507 aa of the mature PLO with an N-terminal extension of 36 aa encoded by pTrcHis B, including a hexahistidine sequence, and pJGS89, a site-directed mutant of pJGS59 that encodes a mutant version of His-PLO with an alanine to cysteine change at position 492 (His-PLO.C₄₉₂), have been described previously (Billington *et al.*, 1997). pJGS59 and pJGS89 were used as templates for the construction of site-directed mutants, using the mutagenic primers indicated in Table 1 and the Transformer Site Directed Mutagenesis Kit (Clontech), according to the manufacturer's instructions. Two selection primers were used. Primer 5'-GACTTGGTTGAGGCCTCACCAGTCAC-3' was designed to change the *ScaI* site of pTrcHis B to *StuI* and was used in conjunction with forward primers listed in Table 1. A second

selection primer, 5'-GAGTGCACCATGGGCGGTGTGA-AAT-3', was designed to change the *NdeI* site of pTrcHis B to *NcoI* and was used in conjunction with the reverse primers listed in Table 1. Introduction of appropriate mutations into either pJGS59 or pJGS89 was confirmed by automated DNA sequencing on a 373A DNA sequencer (Applied Biosystems) at the University of Arizona's DNA sequencing facility.

Production and purification of His-PLO and mutant proteins. Cultures (500 ml) of *E. coli* DH5 α or DH5 α F'*lacI*^q carrying pJGS59, or the appropriate site-directed mutant plasmid, were grown to an OD₆₀₀ value of 0.6 prior to induction with 2.5 mM IPTG for 3 h. Cells were harvested by centrifugation at 5000 g and the cell pellet was resuspended in binding buffer (20 mM Tris/HCl, 100 mM NaCl, pH 8.0). The cells were disrupted by two passages through a French Pressure Cell (Aminco) at 138 MPa and the insoluble material was removed by centrifugation at 12000 g. His-PLO or mutant proteins were purified from the soluble fraction using TALON Metal Affinity Resin (Clontech) according to the manufacturer's instructions, and eluted from the column in binding buffer with 50 mM imidazole. Protein concentration was determined using the Bradford Protein Assay Reagent (Bio-Rad).

Haemolytic assays. Microtitration haemolytic assays were performed essentially as described previously (Billington *et al.*, 1997), except that dilutions of toxins were performed in 1 mg BSA ml⁻¹ to aid in stabilization of the toxin. One haemolytic unit (HU) was defined as the amount of haemolysin (in ng) required to release 50% of the haemoglobin from 200 μ l of 0.25% sheep red blood cells (SRBCs) in 1 h at 37 °C, and specific haemolytic activity was expressed as HU (μ g protein)⁻¹. The specific haemolytic activity of all His-PLO mutant proteins was expressed as a percentage of His-PLO haemolytic activity, which was designated as 100%. The effects of reducing compounds on haemolytic activity was assayed by incubating 10 μ g toxin preparations ml⁻¹ with final concentrations of either 2.5 mM DTT or 2% (v/v) β -mercaptoethanol (BME) for 10 min at room temperature, prior to performance of the haemolytic assay.

Cholesterol-binding assays. The relative cholesterol-binding affinity of His-PLO and mutant proteins was assayed by a modification of an ELISA technique developed by Shimada *et*

Table 1. Construction of site-specific undecapeptide mutants in His-PLO

Toxin	Plasmid	Mutagenic primer*
His-PLO	pJGS59	E A T G L A W D P W W† 5'-GAGGCAACTGGCCTAGCGTGGGATCCGTGGTGG-3'
His-PLO.C ₄₉₂	pJGS89	5'-GAAGCAGGCGAGTGCCTAGC-3'
His-PLO.F ₄₉₇	pJGS135	5'-GCCTAGCGTTCGATCCGTGG-3'
His-PLO.F ₅₀₀	pJGS187	3'-CCCTAGGCAAGACCTGGCAATA-5'
His-PLO.F ₅₀₁	pJGS199	3'-CCCTAGGCACCAAGTGGCAATA-5'
His-PLO. Δ P ₄₉₉ Ω R ₅₀₂	pJGS138	3'-GCACCCTA---ACCACCGCTTGGCAATAG-5'
His-PLO. Ω R ₅₀₂	pJGS215	3'-CTAGGCACCACCGCTTGGCAATAGTTAT-5'
His-PLO. Δ P ₄₉₉	pJGS214	3'-GATCGCACCCTA---ACCACCTGGC-5'
His-PLO.F ₄₉₉	pJGS257	3'-CGCACCCTAAGACACCTGGC-5'
His-PLO.G ₄₉₉	pJGS258	3'-CGCACCCTACCGACACCTGGC-5'
His-PLO _{CON}	pJGS210	5'-GAAGCAGGCGAGTGCCTAGC-3' 3'-GCACCCTC---ACCACCGCTTGGCAATAG-5'
His-PLO _{CON+A}	pJGS209	3'-GCACCCTC---ACCACCGCTTGGCAATAG-5'

* Underlined sequences represent mutated codons.

† The His-PLO undecapeptide sequence and its corresponding DNA sequence are shown.

al. (1999). Microtitre plate wells were coated with 1 μmol of cholesterol, overnight at 37 °C. Following two washes in 0.1 M PBS (pH 7.2) containing 0.3 % (v/v) Tween 20 (PBS-T), the wells were blocked for 1 h with 3 % (w/v) BSA in PBS at 4 °C. The wells were washed twice with PBS-T and incubated for 1 h at 37 °C with dilutions of toxin in 1 mg BSA ml⁻¹ (1 μg to 1 ng toxin or, where necessary, 10 μg to 10 ng toxin). Following nine washes with PBS-T, bound toxin was reacted with a 1/500 dilution of polyclonal goat anti-PLO serum (Billington *et al.*, 1997) at 37 °C for 1 h. Primary antibody was detected using a horseradish-peroxidase-conjugated rabbit-anti-goat IgG (H+L) peroxidase (KPL) and *o*-phenylenediamine as substrate (Sigma).

The cholesterol-binding activity was calculated as the amount of haemolysin (in ng) required to give an ELISA OD₄₂₀ value 50 % that given by 1 μg His-PLO. The binding activity of all His-PLO mutant proteins was expressed as a percentage of His-PLO binding activity, which was designated as 100 %. An ELISA was also performed on known amounts of each mutant toxin to confirm that His-PLO and each mutant reacted equally with the anti-PLO serum.

Membrane-binding assays. These were performed essentially as described by de los Toyos *et al.* (1996). His-PLO and mutant toxins were diluted in 1 mg BSA ml⁻¹ to a concentration of 2.5 μg ml⁻¹. Toxin (0.5 ml) was added to an equal volume of 10 % SRBC and incubated on ice for 20 min. SRBCs were pelleted by centrifugation at 14000 *g* for 5 min at 4 °C. Cell pellets were lysed by boiling in sample buffer and were then subjected to SDS-PAGE and Western blot analysis with the anti-PLO serum to detect bound toxin.

RESULTS

The tryptophan residues of PLO are important in cytolytic activity

Mutations in any of the hydrophobic tryptophan residues of the undecapeptide have significant effects on the pore-forming ability of LLO, PFO and PLY (Boulnois *et al.*, 1991; Korchev *et al.*, 1998; Michel *et al.*, 1990; Sekino-Suzuki *et al.*, 1996). To determine if this was also true for PLO, each of the three tryptophan residues was changed individually to phenylalanine to create His-PLO.F₄₉₇, His-PLO.F₅₀₀ and His-PLO.F₅₀₁ (Fig. 1b). Haemolytic assays were performed on purified

protein from each of these mutants (Table 2). Like other CDCs, each mutant had significantly reduced haemolytic activity compared to wild-type His-PLO. The most severe reduction was observed with His-PLO.F₄₉₇, with only 0.2 % of His-PLO activity. The haemolytic activity of this mutant protein is consistent with observations from other CDCs, which suggest that the first tryptophan residue of the undecapeptide is essential for cytolytic activity (Boulnois *et al.*, 1991; Sekino-Suzuki *et al.*, 1996). The haemolytic activities of His-PLO.F₅₀₀ and His-PLO.F₅₀₁ at 2.3 and 5.0 % of wild-type His-PLO activity, respectively, also suggest that these residues are important for PLO activity.

The proline residue in the PLO undecapeptide is essential for activity

The major structural and charge differences between the PLO undecapeptide and the consensus undecapeptide for the CDC family appear to be the insertion of a proline (P₄₉₉), which increases the spacing between the three tryptophan residues, and the lack of the terminal arginine (Fig. 1a). A mutant His-PLO protein was created in which the proline was deleted and the arginine inserted (His-PLO. Δ P₄₉₉ Ω R₅₀₂) (Fig. 1b). This mutant protein showed only 2.5 % haemolytic activity compared to wild-type His-PLO (Table 2). To determine which of the two mutations was having the greatest effect on activity, the arginine insertion (His-PLO. Ω R₅₀₂) and proline deletion (His-PLO. Δ P₄₉₉) mutations were introduced independently into His-PLO (Fig. 1b). The His-PLO. Ω R₅₀₂ mutant protein, while reduced in activity compared to His-PLO, still had significant haemolytic activity at 27.5 % of wild-type (Table 2). The His-PLO. Δ P₄₉₉ protein, however, had no detectable haemolytic activity, even at toxin concentrations greater than 2 mg ml⁻¹. Thus, the relative haemolytic activity of the protein was less than 0.003 % of wild-type His-PLO. These data suggest that P₄₉₉ plays an essential role in the cytolytic activity of PLO. To confirm the importance of P₄₉₉, site-specific mutants were created where the proline was substituted with either phenylalanine (His-PLO.F₄₉₉) or glycine (His-PLO.G₄₉₉) (Fig. 1b). His-

Table 2. Mean haemolytic and cholesterol-binding activities of His-PLO mutant proteins

Protein	Haemolytic activity*	Cholesterol-binding activity*
His-PLO	100.00 $\pm$ 12.36	100.00 $\pm$ 8.82
His-PLO.F ₄₉₇	0.22 $\pm$ 0.03	1.60 $\pm$ 0.26
His-PLO.F ₅₀₀	2.34 $\pm$ 0.42	7.15 $\pm$ 0.93
His-PLO.F ₅₀₁	5.00 $\pm$ 0.69	7.50 $\pm$ 3.06
His-PLO. Δ P ₄₉₉ Ω R ₅₀₂	2.46 $\pm$ 0.12	4.17 $\pm$ 0.70
His-PLO. Ω R ₅₀₂	27.50 $\pm$ 1.71	26.23 $\pm$ 0.31
His-PLO. Δ P ₄₉₉	< 0.003	< 0.06
His-PLO.F ₄₉₉	1.30 $\pm$ 0.09	5.93 $\pm$ 1.12
His-PLO.G ₄₉₉	0.36 $\pm$ 0.04	0.30 $\pm$ 0.06
His-PLO _{CON+A}	0.14 $\pm$ 0.01	1.02 $\pm$ 0.25

* Haemolytic and cholesterol-binding activities are expressed as a percentage of His-PLO activity from an average of three experiments $\pm$ 1 SD from the mean.

Table 3. Effects of reducing compounds on the haemolytic and cholesterol-binding activities of cysteine-containing His-PLO mutant proteins

Protein*	Haemolytic activity†			Cholesterol-binding activity†	
	Untreated	+BME‡	+DTT§	Untreated	+BME‡
His-PLO	100.00 ± 12.36	100.78 ± 13.70	104.58 ± 14.94	100.00 ± 8.82	98.03 ± 5.67
His-PLO.C ₄₉₂	4.88 ± 0.98	44.18 ± 2.94	34.67 ± 2.40	ND	166.20 ± 43.56
His-PLO _{CON}	0.14 ± 0.0	1.15 ± 0.05	1.03 ± 0.06	ND	2.90 ± 0.70

ND, Not determined.

* All protein preparations were stored at 4 °C for 2 weeks, prior to performing haemolytic assays.

† The haemolytic and cholesterol-binding activities are expressed as a percentage of His-PLO activity from an average of three experiments ± 1 SD from the mean.

‡ Proteins were pre-incubated with 2% (v/v) BME for 10 min at room temperature, prior to performing haemolytic or cholesterol-binding assays.

§ Proteins were pre-incubated with 2.5 mM DTT for 10 min at room temperature, prior to performing haemolytic assays.

Fig. 2. Membrane binding of His-PLO mutant proteins. His-PLO and mutant toxins (both 1.25 µg) were incubated with 10% SRBCs on ice for 20 min. SRBCs were pelleted, lysed by boiling in sample buffer and subjected to SDS-PAGE and Western blot analysis with anti-PLO serum to detect bound toxin. Lanes: 1, His-PLO; 2, His-PLO.F₄₉₇; 3, His-PLO.F₅₀₀; 4, His-PLO.F₅₀₁; 5, His-PLO.F₄₉₉; 6, His-PLO.G₄₉₉; 7, His-PLO.ΔP₄₉₉; 8, His-PLO.ΔP₄₉₉ΩR₅₀₂; 9, His-PLO.ΩR₅₀₂; 10, His-PLO_{CON+A}; 11, His-PLO_{CON}+BME; 12, His-PLO.C₄₉₂+BME; 13, His-PLO+BME. The positions of molecular mass markers are shown in kDa on the left of the image; the arrow on the right indicates the position of the wild-type and mutant His-PLO proteins.

PLO.F₄₉₉ and His-PLO.G₄₉₉ were also impaired for haemolytic activity, with relative haemolytic activities of 1.3 and 0.4% of His-PLO haemolytic activity, respectively (Table 2).

The variation in the PLO undecapeptide is required for full cytolytic activity

Since there are other changes in the PLO undecapeptide that may compensate for the proline and arginine changes, a consensus undecapeptide was introduced into His-PLO. This protein was constructed in two forms, one containing a purely consensus CDC undecapeptide, His-PLO_{CON}, which includes the conserved cysteine residue and another, His-PLO_{CON+A}, containing all the consensus residues but retaining the alanine of PLO at position 492 (Fig. 1b). Purified His-PLO_{CON} (Table 3) and His-PLO_{CON+A} (Table 2) both had very

low haemolytic activities of 0.1%. This result confirmed that the substituted residues in the PLO undecapeptide were required for full cytolytic activity of this protein. Since His-PLO_{CON} contained the conserved cysteine residue responsible for the thiol-activation of many CDCs, haemolytic assays were performed with purified protein pre-treated with either DTT or BME. In the presence of these reducing agents, the haemolytic activity of His-PLO_{CON} was increased, approximately 10-fold over no treatment, to 1.0–1.1% of His-PLO activity (Table 3). While this activity is still very low, it represents a significant increase in activity over unreduced protein and indicates that His-PLO_{CON} is subject to oxygen inactivation.

Thiol-activation of His-PLO.C₄₉₂

The two members of the CDC family that are not subject to thiol-activation, PLO and ILY, each have divergent undecapeptide sequences, which include substitution of the conserved cysteine residue with an alanine residue (Billington *et al.*, 1997; Nagamune, 1997). We have previously presented evidence that a His-PLO molecule in which the alanine residue has been changed to the conserved cysteine residue (His-PLO.C₄₉₂) is not subject to oxygen inactivation (Billington *et al.*, 1997) (Fig. 1b). However, our results that the activity of His-PLO_{CON} can be activated by reducing agents led us to re-examine the activity of His-PLO.C₄₉₂. Our initial experiments were performed on freshly prepared His-PLO and His-PLO.C₄₉₂ (Billington *et al.*, 1997). However, haemolytic assays performed on purified His-PLO.C₄₉₂ following its storage for a 2-week period at 4 °C indicated that the haemolytic activity of this protein had been reduced to only 4.9% of wild-type His-PLO. This activity could be restored by pre-treatment with either BME or DTT to 44.2 and 34.7% of His-PLO activity, respectively (Table 3), which approximates the activity originally observed with freshly purified His-PLO.C₄₉₂ (Billington *et al.*, 1997). There-

fore, contrary to our previous results (Billington *et al.*, 1997), PLO can be engineered to be thiol-activated by the introduction of the conserved cysteine residue into the undecapeptide but, even when activated, this mutant is not as active as wild-type His-PLO.

Cholesterol-binding activities of His-PLO mutant proteins

Cholesterol is the major host-cell-surface receptor for CDCs and the undecapeptide region of these toxins has been implicated either in direct binding of cholesterol or in membrane insertion as a result of cholesterol binding. Each of the His-PLO mutant proteins was compared with His-PLO for its ability to bind to cholesterol in an ELISA-based assay. The ability of all mutant toxins, with the exception of His-PLO.C₄₉₂, to bind cholesterol was in direct proportion to the haemolytic activity of the toxin (Tables 2 and 3). For example, His-PLO.ΩR₅₀₂ had a haemolytic activity of 27.5% and a cholesterol-binding activity of 26.2% compared to His-PLO, while His-PLO.F₄₉₇ had 0.2 and 1.6% of His-PLO haemolytic and cholesterol-binding activities, respectively. Reduced His-PLO.C₄₉₂, however, appeared to bind cholesterol as well as, if not better than, wild-type His-PLO (mean = 166.2% His-PLO cholesterol-binding activity), despite having a haemolytic activity only 44.2% that of His-PLO (Table 3).

Membrane binding of His-PLO mutant proteins

Cholesterol is the proposed major target cell receptor for CDCs, although it is not known if initial interactions occur through other host molecules. To test the ability of the mutant toxins to bind to host-cell membranes, SRBCs incubated with each toxin were analysed by Western blotting using anti-PLO serum (Fig. 2). Despite the variations in haemolytic activity (undetectable to 44.2% of His-PLO) and cholesterol binding (undetectable to 166.2% of His-PLO), all mutant toxins bound to SRBC membranes, and no obvious defects in binding compared to His-PLO could be determined from this assay.

DISCUSSION

The undecapeptide of CDCs has for some time been implicated in the cytolytic activity of these toxins. This region is the largest stretch of identity among the toxins that originally formed the thiol-activated cytolysin family. However, with the discovery of the variant undecapeptide sequences of PLO (Billington *et al.*, 1997) and ILY (Nagamune, 1997), the requirement for identity within this undecapeptide region to produce a functional cytolysin has been questioned (Billington *et al.*, 2000). The haemolytic activity of PLO against SRBCs is approximately 10% that of PFO (S. J. Billington, unpublished observation), so it is possible that the variant undecapeptide of PLO is responsible for this reduced activity. However, PLO is the most divergent member of the CDC family (Billington *et al.*, 1997), and it is also possible that the variations in the PLO

undecapeptide sequence are required to compensate for structural differences imposed on the undecapeptide by other regions of the molecule. To assess the requirement for its variant undecapeptide, we have introduced various mutations into the undecapeptide of His-PLO. All mutations introduced had a deleterious effect on the cytolytic activity of His-PLO. In particular, the introduction of a consensus undecapeptide resulted in very low haemolytic activity, indicating that the variant undecapeptide of PLO is required for its full cytolytic activity.

Of the changes in the undecapeptide sequence of PLO compared to the CDC consensus, the proline insertion appears to have the greatest effect, since removal of this amino acid resulted in a protein that was non-haemolytic, even at very high toxin concentrations. The importance of P₄₉₉ may be to provide the correct spacing between important residues, such as the three undecapeptide tryptophan residues. However, the substitution of P₄₉₉ with either the similarly bulky amino acid phenylalanine or the flexible amino acid glycine did not result in functional proteins, suggesting that spacing alone does not explain the importance of P₄₉₉. Proline residues are important in the structural integrity of many proteins, as they can force peptide bends. In fact, the general conservation of proline residues within CDC primary sequences suggests that they may be involved in the maintenance of higher-order structures (Billington *et al.*, 1997). The undecapeptide proline residue may be required to align the PLO undecapeptide to compensate for structural differences imposed on the PLO undecapeptide by other regions of the PLO molecule, which vary considerably in primary amino acid sequence from the structural backbone of other CDCs. Interestingly, ILY, which is not nearly as divergent in its primary sequence, also contains a proline at the same position in the undecapeptide as PLO (Fig. 1a; Nagamune, 1997). However, in ILY the terminal arginine is retained and the third tryptophan residue is missing, which may have implications for its binding to host cells. Indeed, ILY appears to show more specificity in host-cell type than other members of the CDC family (Nagamune, 1997; Nagamune *et al.*, 1996).

Despite the differences in the PLO undecapeptide, it shares the three hydrophobic tryptophan residues which have been demonstrated to play a pivotal role in the cytolytic activity of many CDCs (Boulnois *et al.*, 1991; Korchev *et al.*, 1998; Michel *et al.*, 1990; Nakamura *et al.*, 1998; Sekino-Suzuki *et al.*, 1996). However, the spacing between the first and second tryptophan is altered by insertion of P₄₉₉. Mutations in each of the tryptophan residues resulted in vastly reduced cytolytic activity in LLO, PFO and PLY (Boulnois *et al.*, 1991; Korchev *et al.*, 1998; Michel *et al.*, 1990; Sekino-Suzuki *et al.*, 1996). Consistent with results from other CDCs, substitution of any of the tryptophan residues of PLO with phenylalanine resulted in a substantial reduction in haemolytic activity compared to the wild-type, suggesting that the undecapeptide plays a similar role in PLO as it does in other members

of the family. The role of the undecapeptide sequence in the initial binding, tethering or membrane sampling of CDCs has yet to be proven. However, the position of the first undecapeptide tryptophan residue at the very tip of the undecapeptide loop (Rossjohn *et al.*, 1997) implies a prominent role in any initial interaction with the target cell membrane precipitated by domain 4. The marked effect of mutations at this position in PLO (Table 2) and other CDCs (Boulnois *et al.*, 1991; Sekino-Suzuki *et al.*, 1996) supports the importance of this residue in CDC cytolytic activity.

Contrary to results with PFO (Sekino-Suzuki *et al.*, 1996; Shimada *et al.*, 1999), mutations in undecapeptide amino acids of His-PLO that resulted in decreased cytolytic activity, with the exception of the alanine to cysteine change in His-PLO.C₄₉₂, also resulted in a concomitant reduction in cholesterol binding. There are several possible explanations for this observation. The undecapeptide of PLO may be intimately involved in cholesterol binding or mutations in undecapeptide residues may directly affect the cholesterol-binding site of PLO. Alternatively, mutations in the PLO undecapeptide may have a considerable effect on the structure of the PLO molecule, in particular domain 4, which likely contains the cholesterol-binding domain, such that it can no longer bind cholesterol. The variant nature of the PLO undecapeptide may be required not only to properly align the undecapeptide sequence but also to maintain the structure of domain 4 in general. Support for the latter comes from minor substitutions at the C terminus of CDCs (Owen *et al.*, 1994; Shimada *et al.*, 1999), which disrupt the ability of these toxins to bind to cholesterol and host-cell membranes. Aligned with this final suggestion is the possibility that mutations introduced in this study result in the misfolding of domain 4, or indeed the entire PLO protein structure, leading to the reduction in haemolytic and cholesterol-binding activities. This is not the case with the majority of mutations in this region of PFO (Sekino-Suzuki *et al.*, 1996), where reduction in haemolytic activity was observed in the absence of major structural changes. Despite the reduced ability to bind to cholesterol, each of the His-PLO mutant toxins was still able to bind to erythrocyte membranes. These results suggest that CDCs may also bind through additional host-cell molecules (Dubail *et al.*, 2001; Owen *et al.*, 1994). This suggestion is supported by experiments with a truncated LLO molecule composed of domains 1, 2 and 3 but lacking the cholesterol-binding domain 4, which indicated that this truncated molecule could still bind to host-cell membranes (Dubail *et al.*, 2001).

The thiol-activated nature of the original members of the CDC family is the result of oxidation of the cysteine residue at position 2 of the undecapeptide motif (Michel *et al.*, 1990; Pinkney *et al.*, 1989; Saunders *et al.*, 1989). Generally, mutagenesis of this cysteine residue to alanine resulted in a toxin with a similar cytolytic activity, but which was not inactivated by oxygen (Michel *et al.*, 1990; Pinkney *et al.*, 1989; Saunders *et al.*, 1989). The identification of PLO and ILY confirmed speculation by

Pinkney *et al.* (1989) as to the presence of members of the CDC family that lack the cysteine residue and thus would be able to stably express their cytolytic activity. The significance of the thiol-activation of CDCs is unknown. It may be a fortuitous event in evolution, but the simplicity of the substitution to an oxygen-resistant form suggests some selection for the oxygen-sensitive form. It is possible that oxygen sensitivity may regulate toxin function following secretion by pathogenic bacteria in the host, although expression of an oxygen-stable form of LLO had no effect on the pathogenesis of *Listeria monocytogenes* in mice (Michel *et al.*, 1990). The His-PLO.C₄₉₂ toxin, while reduced for haemolytic activity, appeared to bind cholesterol better than wild-type His-PLO. Perhaps in a molecule more amenable to its presence, the cysteine aids in the interaction of domain 4 with the primary host-cell receptor. Anecdotally, the His-PLO.C₄₉₂ protein loses activity much more slowly than His-PLO_{CON}, although both lose approximately 90% of activity to oxygen inactivation. This observation may support the initial assertion that the variant sequence of the PLO undecapeptide affects the stability of PLO to oxygen, even in the presence of the cysteine substitution (Billington *et al.*, 1997).

We conclude that the variant undecapeptide of PLO is required for full cytolytic activity of this toxin. It seems likely that the altered residues in the PLO undecapeptide may compensate for structural changes in other parts of PLO, with respect to other CDCs, as PLO has the most divergent amino acid sequence (Billington *et al.*, 1997). It is also possible that the undecapeptide controls host-cell-membrane sampling. PLO is known to more-readily lyse erythrocytes from some animals species (e.g. rabbit or sheep) than other species (e.g. bovine) (Funk *et al.*, 1996). This suggestion has implications for the variant undecapeptide of ILY and its limited host range (Nagamune, 1997; Nagamune *et al.*, 1996).

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994). *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates and Wiley.
- Baumann, C. D., Davidson, W. R., Roscoe, D. E. & Beheler-Amass, K. (2001). Intracranial abscessation in white-tailed deer of North America. *J Wildl Dis* 37, 661–670.
- Billington, S. J., Jost, B. H., Cuevas, W. A., Bright, K. R. & Songer, J. G. (1997). The *Arcanobacterium* (*Actinomyces*) *pyogenes* hemolysin, pyolysin, is a novel member of the thiol-activated cytolytic family. *J Bacteriol* 179, 6100–6106.
- Billington, S. J., Jost, B. H. & Songer, J. G. (2000). Thiol-activated cytolytic toxins: structure, function and role in pathogenesis. *FEMS Microbiol Lett* 182, 197–205.
- Boulnois, G. J., Paton, J. C., Mitchell, T. J. & Andrew, P. W. (1991).

Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. *Mol Microbiol* 5, 2611–2616.

Carter, G. R. & Chengappa, M. M. (1991). *Essentials of Veterinary Bacteriology and Mycology*, 4th edn. Philadelphia, PA: Lea & Febiger.

de los Toyos, J. R., Mendez, F. J., Aparicio, J. F. & 7 other authors (1996). Functional analysis of pneumolysin by use of monoclonal antibodies. *Infect Immun* 64, 480–484.

Ding, H. & Lämmler, C. (1996). Purification and further characterization of a haemolysin of *Actinomyces pyogenes*. *Zentralbl Veterinarmed (B)* 43, 179–188.

Dubail, I., Autret, N., Beretti, J.-L., Kayal, S., Berche, P. & Charbit, A. (2001). Functional assembly of two membrane-binding domains in listeriolysin O, the cytolysin of *Listeria monocytogenes*. *Microbiology* 147, 2679–2688.

Funk, P. G., Staats, J. J., Howe, M., Nagaraja, T. G. & Chengappa, M. M. (1996). Identification and partial characterization of an *Actinomyces pyogenes* hemolysin. *Vet Microbiol* 50, 129–142.

Gilbert, R. J. C., Jimenez, J. L., Chen, S., Tickle, I. J., Rossjohn, J., Parker, M., Andrew, P. W. & Saibil, H. R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*. *Cell* 97, 647–655.

Heuck, A. P., Hotze, E. M., Tweten, R. K. & Johnson, A. E. (2000). Mechanism of membrane insertion of a multimeric β -barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. *Mol Cell* 6, 1233–1242.

Hotze, E. M., Heuck, A. P., Czajkowsky, D. M., Shao, Z., Johnson, A. E. & Tweten, R. K. (2002). Monomer–monomer interactions drive the prepore to pore conversion of a β -barrel-forming cholesterol-dependent cytolysin. *J Biol Chem* 277, 11597–11605.

Jacobs, T., Cima-Cabal, M. D., Darji, A. & 7 other authors (1999). The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Lett* 459, 463–466.

Jonsson, P., Olsson, S.-O., Olofson, A.-S., Fälth, C., Holmberg, O. & Funke, H. (1991). Bacteriological investigations of clinical mastitis in heifers in Sweden. *J Dairy Res* 58, 179–185.

Jost, B. H., Songer, J. G. & Billington, S. J. (1999). An *Arcanobacterium* (*Actinomyces*) *pyogenes* mutant deficient in production of the pore-forming cytolysin pyolysin has reduced virulence. *Infect Immun* 67, 1723–1728.

Jost, B. H., Post, K. W., Songer, J. G. & Billington, S. J. (2002). Isolation of *Arcanobacterium pyogenes* from the porcine gastric mucosa. *Vet Res Commun* 26, 419–425.

Korchev, Y. E., Bashford, C. L., Pederzoli, C., Pasternak, C. A., Morgan, P. J., Andrew, P. W. & Mitchell, T. J. (1998). A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers. *Biochem J* 329, 571–577.

Lechtenberg, K. F., Nagaraja, T. G., Leipold, H. W. & Chengappa, M. M. (1988). Bacteriologic and histologic studies of hepatic abscesses in cattle. *Am J Vet Res* 49, 58–62.

Michel, E., Reich, K. A., Favier, R., Berche, P. & Cossart, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitution in listeriolysin O. *Mol Microbiol* 4, 2167–2178.

Nagamune, H. (1997). Streptococcal cytolysins. *Seikagaku* 69, 343–348.

Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R. A., Tsuji, A. & Matsuda, Y. (1996). Intermedilysin, a novel

cytotoxin specific for human cells, secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect Immun* 64, 3093–3100.

Nagaraja, T. G., Laudert, S. B. & Parrott, J. C. (1996). Liver abscesses in feedlot cattle. Part I. Causes, pathogenesis, pathology, and diagnosis. *Comp Cont Edu Pract Vet* 18, S230–S241, S256.

Nakamura, M., Sekino-Suzuki, N., Mitsui, K.-I. & Ohno-Iwashita, Y. (1998). Contribution of tryptophan residues to the structural changes in perfringolysin O during interaction with liposomal membranes. *J Biochem* 123, 1145–1155.

Narayanan, S., Nagaraja, T. G., Wallace, N., Staats, J., Chengappa, M. M. & Oberst, R. D. (1998). Biochemical and ribotypic comparison of *Actinomyces pyogenes* and *A. pyogenes*-like organisms from liver abscesses, ruminal wall, and ruminal contents of cattle. *Am J Vet Res* 59, 271–276.

Owen, R. H. G., Boulnois, G. J., Andrew, P. W. & Mitchell, T. J. (1994). A role in the cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 121, 217–221.

Pinkney, M., Beachey, E. & Kehoe, M. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect Immun* 57, 2553–2558.

Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K. & Parker, M. W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* 89, 685–692.

Rossjohn, J., Gilbert, R. J. C., Crane, D. & 7 other authors (1998). The molecular mechanism of pneumolysin, a virulence factor from *Streptococcus pneumoniae*. *J Mol Biol* 284, 449–461.

Saunders, F. K., Mitchell, T. J., Walker, J. A., Andrew, P. W. & Boulnois, G. J. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect Immun* 57, 2547–2552.

Sekino-Suzuki, N., Nakamura, M., Mitsui, K.-I. & Ohno-Iwashita, Y. (1996). Contribution of individual tryptophan residues to the structure and activity of θ -toxin (perfringolysin O), a cholesterol-binding cytolysin. *Eur J Biochem* 241, 941–947.

Shatursky, O., Heuck, A. P., Shepard, L. A., Rossjohn, J., Parker, M. W., Johnson, A. E. & Tweten, R. K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* 99, 293–299.

Shepard, L. A., Heuck, A. P., Hamman, B. D., Rossjohn, J., Parker, M. W., Ryan, K. R., Johnson, A. E. & Tweten, R. K. (1998). Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: an α -helical to β -sheet transition identified by fluorescence spectroscopy. *Biochemistry* 37, 14563–14574.

Shepard, L. A., Shatursky, O., Johnson, A. E. & Tweten, R. K. (2000). The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane β -hairpins. *Biochemistry* 39, 10284–10293.

Shimada, Y., Nakamura, M., Naito, Y., Nomura, K. & Ohno-Iwashita, Y. (1999). C-terminal amino acid residues are required for the folding and cholesterol binding property of perfringolysin O, a pore-forming cytolysin. *J Biol Chem* 274, 18536–18542.

Tweten, R. K., Parker, M. W. & Johnson, A. E. (2001). The cholesterol-dependent cytolysins. *Curr Top Microbiol Immunol* 257, 15–33.

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