subclass-specific enzyme-linked immunosorbent assays. *Immunology*, **79**, 89–94.
Harvey, A. N., Costa, N. D., and Savage, J. R. (1994). Electroporation and streptolysin O – a comparison of poration techniques. *Mutat. Res.*, **315**, 17–23.
Iwamoto, M., Ohno Iwashita, Y. and Ando, S. (1987). Role of the essential thiol group in the thiol-activated cytolysin from Clostridium perfringens. *Eur. J. Biochem.*, **167**, 425–30.
Kehoe, M. A., Miller, L., Walker, J. A., and Boulnois, G. J. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. *Infect. Immun.*, **55**, 3228–32.
Momburg, F., Roelse, J., Hammerling, G. J., and Neefjes, J. J. (1994). Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J. Exp. Med.*, **179**, 1613–23.
Palmer, M., Valeva, A., Kehoe, M., and Bhakdi, S. (1995). Kinetics of streptolysin O self-assembly. *Eur. J. Biochem.*, **231**, 388–95.
Pimplikar, S. W., Ikonen, E., and Simons, K. (1994). Basolateral protein transport in streptolysin O-permeabilized MDCK cells. *J. Cell Biol.*, **125**, 1025–35.
Pinkney, M., Kapur, V., Smith, J., Weller, U., Palmer, M., Glanville, M., *et al.* (1995). Different forms of streptolysin O produced by *Streptococcus pyogenes* and by *Escherichia coli* expressing recombinant toxin: cleavage by streptococcal cytein protease. *Infect. Immun.*, **63**, 2776–9.
Rapp, S., Soto, U., and Just, W. W. (1993). Import of firefly luciferase into peroxisomes of permeabilized Chinese hamster ovary cells: a model system to study peroxisomal protein import in vitro. *Exp. Cell Res.*, **205**, 59–65.
Smyth, C. J. and Duncan, J. L. (1978). Thiol-activated (oxygen-labile) cytolysins. In *Bacterial toxins and cell membranes* (ed. J. Jeljaszewicz and T. Wadström), pp. 129–83, Academic Press, London.
Tan, A., Bolscher, J., Feltkamp, C., and Ploegh, H. (1992). Retrograde transport from the Golgi region to the endoplasmic reticulum is sensitive to GTP gamma S. *J. Cell Biol.* **116**, 1357–67.
Walev, I., Palmer, M., Valeva, A., Weller, U., and Bhakdi, S. (1995). Binding, oligomerization, and pore formation by streptolysin O in erythrocytes and fibroblast membranes: detection of nonlytic polymers. *Infect. Immun.*, **63**, 1188–94.
Wendland, M. and Subramani, S. (1993). Cytosol-dependent peroxisomal protein import in a permeabilized cell system. *J. Cell Biol.*, **120**, 675–85.

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This family of 50 to 60 kDa pore-forming bacterial toxins (sulfhydryl-activated cytolysins) comprises 19 structurally and antigenically related, chromosomally encoded single-chain soluble proteins endowed with potent lethal and lytic properties resulting from the disruption of the cytoplasmic membrane of eukaryotic cells and that of certain cell organelles. Membrane cholesterol appears as a specific binding site of these toxins and is thought to contribute to their oligomerization in target membranes.

The 19 toxins identified to date are produced by gram-positive aerobic or anaerobic, sporulating or non-sporulating bacteria from the genera *Streptococcus, Bacillus, Clostridium*, and *Listeria* (Smyth and Duncan 1978, Alouf and Geoffroy 1991) listed below in Table 1.

Except for PLY, which is intracytoplasmic, the other toxins are secreted in culture medium during bacterial growth. Among the toxin-producing bacteria, only *Listeria* are intracellular pathogens which grow and release their toxins in host phagocytes or possibly in other cells.

The lethal (cardiotoxic) and cytolytic properties of the toxins are suppressed by oxidants or by thiol-group blocking agents and restored by reducing agents. The toxins share common epitopes and elicit in humans or in animals neutralizing and precipitating cross-reacting antibodies. The biological properties are irreversibly lost in the presence of very low concentrations of cholesterol and other related 3β-hydroxysterols which interfere with toxin binding on target cells (Alouf and Geoffroy 1991).

A considerable progress in our understanding of structure–activity relationship resulted from the cloning, sequencing, and expression of *tox* genes of 8 out of the 19 toxins of the family.

Table 1

Bacterial genus	Species	Toxin name	Established or suggested[b] toxin and gene acronyms	
Streptococcus	*S. pyogenes*	Streptolysin 0[a]	SLO	*sio*
	S. pneumoniae	Pneumolysin[a]	PLY	*ply*
	S. suis	Suilysin[a]	SUI	*sui*
Bacillus	*B. cereus*	Cereolysin 0[a]	CLO	*clo*
	B. alvei	Alveolysin[a]	ALV	*alv*
	B. thuringiensis	Thuringiolysin 0[a]	TLO	*tlo*
	B. laterosporus	Laterosporolysin	LSL	*lsi*
Clostridium	*C.tetani*	Tetanolysin*	TTL	*ttl*
	C. botulinum	Botulinolysin	BTL	*btl*
	C. perfringens	Perfringolysin 0[a]	PFO	*Pfo*
	C. septicum	Septicolysin 0	SPL	*spl*
	C. histolyticum	Histolyticolysin 0	HTL	*htl*
	C. novyi A (oedematiens)	Novyilysin	NVL	*nvl*
	C. chauvoei	Chauveolysin	CVL	*cvl*
	C. bifermentans	Bifermentolysin	BFL	*bfl*
	C. sordellii	Sordellilysin	SDL	*sdl*
Listeria	*L. monocytogenes*	Listeriolysin 0[a]	LLO	*llo/lisA*
	L. ivanovii	Ivanolysin[a]	ILO	*ilo*
	L. seeligeri	Seeligerolysin	LSO	*lso*

Some strains of streptococci of groups C and G also produce streptolysin 0.
[a]Native toxins reported to be purified to apparent homogeneity [b]Author's proposal.

The deduced number of amino acid residues and mol. wt. of mature toxins are the following: SLO (538 AA, 60151 Da; databank accession number M18638; see preceding entry p. 5), PLY (471 AA, 52800 Da, databank accession number: M17717; Walker *et al.* 1987), PFO (472 AA, 52469 Da; databank accession number: M36704; Tweten 1988*b*), ALV (469 AA, 51766 Da; databank accession number M62709; Geoffroy *et al.* 1990), LLO (504 AA, 55842 Da; databank accession number M24199; Mengaud *et al.* 1988; Domann and Chakraborty 1989), ILO (505 AA, 55961 Da; databank accession number X60461; Haas *et al.* 1992), and LSO (505 AA, 56371 Da; databank accession number X60462; Haas *et al.* 1992) Cereolysin gene *(clo)* has been cloned and expressed in *E. coli* and *B. subtilis* (Kreft *et al.* 1983) but to our knowledge no sequence has been so far reported. A considerable AA sequence homology (stronger at the C-terminal part) was found. It was more pronounced when structurally related AA were taken into account. At the nucleotide sequence level, the homology was lower although detectable, indicating that *tox* genes have undergone extensive divergence from a common ancestor (Boulnois *et al.* 1991).

An 11 AA sequence (—ECTGLAWEWWR—) was the longest common motif conserved in each protein (except for single amino acid change in seeligerolysin). It contained the unique Cys residue of the toxins (except for ILO which possesses a second Cys residue beyond the consensus sequence closer to the C-terminus). The thiol group of the Cys residue of the conserved undecapeptide was logically considered as an 'essential' group required for lytic activity as supported by its abrogation by thiol blocking agents. This contention was not supported by site-directed mutagenesis undertaken on PLY, SLO, and LLO (see Michel *et al.* 1990; Boulnois *et al.* 1991; Sheehan *et al.* 1994). Changing the Cys residue to either alanine, serine, or glycine did not affect or only reduced lytic activity suggesting that there is no absolute requirement for the thiol group in the *in vitro* activity of the toxins. In contrast, the overall structure of the motif appears important (at least in part) for interaction with cholesterol and pore-formation. On the other hand, the use of genetically truncated recombinant PLY showed that the deletion of the six C-terminal AA reduced binding by 96 per cent as also found for PFO (Owen *et al.* 1994).

The genetic regulation of toxin expression has been particularly investigated for LLO and PFO. The former was shown to be positively regulated by a 27.1 kDa protein encoded by *prfa* gene the deletion of which besides LLO affects at least four other virulence factors (Schwan *et al.* 1994; Sheehan *et al.* 1994). PFO expression was also under positive control of a regulatory gene *(pfor)* which also affected the expression of clostridal collagenase and hemagglutinin (Shimizu *et al.* 1994).

Purification and sources

The native toxins purified so far are isolated to apparent homogeneity by standard protein chemistry techniques from culture supernatants (except for PLY obtained from disrupted pneumococci) of appropriate toxin-producing strains, grown under culture conditions specific for each bacterial species described in the hereafter cited publications. Most procedures comprise a combination of crude material concentration (ultrafiltration and or salting-out by ammonium sulfate), then ion-exchange column chromatography, followed by gel molecular

sieving and (or) hydrophobic (low- or high-pressure) column chromatography. Covalent thiopropyl gel chromatography has been widely used taking advantage of toxin cysteinyl residue. Appropriate purification techniques have been described for PLY (Kanclerski and Möllby 1987; Rubins *et al.* 1994), PFO (Tweten 1988*a*), ALV (Geoffroy and Alouf 1983), LLO (Geoffroy *et al.* 1987; Kreft *et al.* 1989), ILO (Kreft *et al.* 1989; Vazquez-Boland *et al.* 1989), BTL (Haque *et al.* 1992), and suilysin (Jacobs *et al.* 1994). The production and purification of fully bioactive recombinant PFO expressed in *E.coli* (Tweten 1988*a*) or of PLY expressed in this micoorganism (Mitchell *et al.* 1989; Rubins *et al.* 1994) and *B.subtilis* (Taira *et al.* 1989) has been reported. The toxins are not commercially available to our knowledge.

The pH optimum for the lytic activity of the various toxins so far studied falls between 6.5 and 7.4 except for LLO with a pH optimum about 5 (no activity at pH 7.0) which may reflect the acid nature of the phagolysosome where it acts (Geoffroy *et al.* 1987; Sheehan *et al.* 1994).

Toxicity

Microgram quantities of the toxins are lethal for mice, rabbits, and other laboratory animals. The LD_{50} (i.v. route) ranges from 0.2 to 0.8 μg (Smyth and Duncan 1978; Geoffroy *et al.* 1987).

Hemolytic activity on sheep or rabbit erythrocytes ranges from 0.5 to 2×10^6 hemolytic units/mg of protein (Alouf and Geoffroy 1991). The use of the toxins is safe for operators following normal safety recommendations for bacterial manipulations.

Use in cell biology

The lytic properties of the toxins and their specific binding of cholesterol on to eukaryotic cells and organelle membranes have been used for the permeabilization of these structures for the analysis of various cell functions and metabolism, the introduction of exogeneous molecular effectors and the generation of limited lesions in various cells for the isolation of membrane receptors intracellular enzymes or organelles which could not be easily obtained by other methods (see Alouf and Geoffroy 1991; Berthou *et al.* 1992; Launay *et al.* 1992; Ahnert-Hilger *et al.* 1993).

References

Ahnert-Hilger, G., Stecher, B., Beyer, C., and Gratzl, M. (1993). Exocytotic membrane fusion as studied in toxin-permealized cells. *Methods Enzymol.*, **221**, 139–49.

Alouf, J. E. and Geoffroy, C. (1991). The family of antigenically-related, cholesterol-binding (sulfhydryl-activated) cytolytic toxins. In *Sourcebook of bacterial protein toxins* (ed. J. E. Alouf and J. H. Freer), pp. 147–86, Academic Press, London.

Berthou, L., Corvaia, N., Geoffroy, C., Mutel, V., Launay, J. M., and Alouf, J. E. (1992). The phosphoinositide pathway of lymphoid cells: labeling after permeabilization by alveolysin, a bacterial sulfhydryl-activated cytolysin. *Eur. J. Cell. Biol.*, **58**, 377–82.

Boulnois, G. J., Paton, J. C., Mitchell, T. J., and Andrew, P. W. (1991). Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae. Mol. Microbiol.*, **59**, 2611–16.

Domann, E. and Chakraborty, T. (1989). Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1/2a strain. *Nucleic Acids Res.*, **17**, 6406.

Geoffroy, C. and Alouf, J. E. (1983). Selective purification by thiol–disulfide interchange chromatography of alveolysin, a sulfhydryl-activated toxin of *B. alvei. J. Biol. Chem.*, **255**, 9968–72.

Geoffroy, C., Gaillard, J. L., Alouf, J. E., and Berche, P. (1987). Purification, characterization and toxicity of the sulfhydrl-activated hemolysin listeriolysin O from *Listeria monocytogenes. Infect. Immun.*, **55**, 1641–46.

Geoffroy, C., Mengaud, J., Alouf, J. E., and Cossart, P. (1990). Alveolysin, the thiol-activated toxin *of Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O and contains a single cysteine. *J. Bacteriol.*, **172**, 7301–5.

Haas, A., Dumbsky, M., and Kreft, J. (1992). Listeriolysin genes : complete sequence of ilo from *Listeria ivanovii* and of lso from *Listeria seeligeri. Biochim. Biophys. Acta.*, **1130**, 81–4.

Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S., *et al.* (1992). Production, purification, and characterization of botulinolysin, a thiol-activated hemolysin *of Clostridium botulinum. Infect. Immun.*, **60**, 71–8.

Jacobs, A. A. C., Loeffen, P. L. W., Van Den Berg, A. J. G., and Storm, P. K. (1994). Identification, purification and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis. Infect. Immun.*, **62**, 1742–8.

Kanclerski, K. and Möllby, R. (1987). Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). *J. Clin. Microbiol.*, **25**, 222–5.

Kreft, J., Berger, H., Härtlein, M., Müller, B., Weidinger, G., and Goebel, W. (1983). Cloning and expression in *Escherichia coli* and *Bacillus subtilis* of the hemolysin (cereolysin) determinant from *Bacillus cereus. J. Bacteriol.*, **155**, 681–9.

Kreft, J., Funke, D., Haas, A., Lottspeich, F., and Goebel, W. (1989). Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b. *FEMS Microbiol. Lett.*, **57**, 197–202.

Launay, J. M., Geoffroy, C., Mutel, V., Buckle, M., Cesum, A., Alouf, J. E., *et al.* (1992). One-step purification of the serotonin transporter located at the human platelet plasma membrane. *J. Biol. Chem.*, **267**, 11344–51.

Mengaud, J., Vincente, M. F., Chenevert, J., Moniz Pereira, J., Geoffroy, C., Giquel-Sanszey, B., *et al.* (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. *Immun.*, **56**, 766–72.

Michel, E., Reich, K. A., Favier, R., Berche, P., and Cossart, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. *Mol. Microbiol.*, **4**, 2167–78.

Mitchell, T. J., Walker, J. A., Saunders, F. K., Andrew, P. W., and Boulnois, G. J. (1989). Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties. *Biochim. Biophys. Acta.*, **1007**, 67–72.

Owen, R. H. G., Boulnois, G. J., Andrew, P. W., and Mitchell, T. J. (1994). A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae. FEMS Microbiol. Lett.*, **121**, 217–22.

Rubins, J. B., Mitchell, T. J., Andrew, P. W., and Niewoehner, D. E. (1994). Pneumolysin activates phospholipase A in pulmonary artery endothelial cells. *Infect. Immun.*, **62**, 3829–36.