Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2968398 A1 2016/05/26

(21) **2 968 398**

(12) DEMANDE DE BREVET CANADIEN CANADIAN PATENT APPLICATION

(13) **A1**

(86) **Date de dépôt PCT/PCT Filing Date:** 2015/11/20
(87) **Date publication PCT/PCT Publication Date:** 2016/05/26
(85) **Entrée phase nationale/National Entry:** 2017/05/18
(86) **N° demande PCT/PCT Application No.:** US 2015/061859
(87) **N° publication PCT/PCT Publication No.:** 2016/081839
(30) **Priorité/Priority:** 2014/11/21 (US62/082,848)

(51) **Cl.Int./Int.Cl.** *A61K 38/00* (2006.01)
(71) **Demandeur/Applicant:**
THE BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA, US
(72) **Inventeur/Inventor:**
TWETEN, RODNEY K., US
(74) **Agent:** OSLER, HOSKIN & HARCOURT LLP

(54) **Titre :** MUTANTS DE PNEUMOLYSINE ET PROCEDES D'UTILISATION CORRESPONDANTS
(54) **Title:** PNEUMOLYSIN MUTANTS AND METHODS OF USE THEREOF

```
                                    |                     |                     |
     Cereolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Anthrolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Thuringiolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Perfringolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
     Alveolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
     Caniolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
  Equisimilysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
 Streptolysin O M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Novyiolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Tetanolysin MN-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
     Ivanolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
  Listeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
Seeligeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
       Suilysin MR-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Pneumolysin MA-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
Mitilysin (PLY) ma-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Intermedilysin MK-------- ---------- ---------- ------TKQ- ---------- ---------- ---------- 5
  Viridanolysin MNQEKRLHRF VKKCGLGVCS AVVAAFLLNA QGVALATEQG NRPVETENIA RGKQASQSST AYGGAATRAV 70
       Pyolysin MKR------- ---------- ---------- ---------- ---------- ---------- ---------- 3

                         |                     |                     |                     |
     Cereolysin ---------- ---------- ---------- ---------- --NIKKNTKR RKFLACLLVS LCTINYSSIS 29
    Anthrolysin ---------- ---------- ---------- ---------- FLNIKKNTKR RKFLACLLVS LCTIHYSSIS 32
 Thuringiolysin ---------- ---------- ---------- ---------- FLNIKKNGKR RKFLTCVLVS LCTLNYSSTS 32
 Perfringolysin ---------- ---------- ---------- ---------- ------RFKK TKLIASIAMA LCLFSQPVIS 26
     Alveolysin ---------- ---------- ---------- ---------- -KKKSNHLKG RKVLVSLLVS LQVFAFASIS 30
     Caniolysin ---------- ---------- ---------- ---------- DMSNKKIFKK YSRVAGLLTA ALIVGNLVTA 32
  Equisimilysin ---------- ---------- ---------- ---------- --SNKKIFKK YSRVAGLLTA ALIVGNLVTA 29
 Streptolysin O ---------- ---------- ---------- ---------- --SNKKTFKK YSRVAGLLTA ALIIGNLVTA 29
    Novyiolysin ---------- ---------- ---------- ---------- KS-LKTIIRS ISFLS--ILT LTCSCNFITS 29
    Tetanolysin ---------- ---------- ---------- ---------- KNVLKFVSRS LLIFS--MTG LISNYNSSNV 30
     Ivanolysin ---------- ---------- ---------- ---------- ---------- -KKIMLLLMT LLLVSLPLAQ 20
  Listeriolysin ---------- ---------- ---------- ---------- ---------- -KKIMLVFIT LILVSLPIAQ 20
Seeligeriolysin ---------- ---------- ---------- ---------- ---------- -KIFGLVIMS LLFVSLPITQ 20
       Suilysin ---------- ---------- ---------- ---------- --------KS SHLILSSIVS LALVGVTPLS 24
    Pneumolysin ---------- ---------- ---------- ---------- N--------- ---------- ---------- 3
Mitilysin (PLY) ---------- ---------- ---------- ---------- n--------- ---------- ---------- 3
 Intermedilysin ---------- ---------- ---------- ---------- -----NIARK LSR--VVLLS TLV------- 21
  Viridanolysin DGNVDSDYGH HSVTHTNFED NAWWQVDLGK TENVGKVKLY NRGDGNVANR LSNFDVVLLN EAKQEVARQH 140
       Pyolysin ---------- ---------- ---------- ---------- --------KA FASLVASVVA AATVTMPTAS 25
```

Figure 1A

(57) **Abrégé/Abstract:**

The present disclosure provides, in various embodiments, immunogenic mutants of cholesterol-dependent cytolysins, such as pneumolysin, which have reduced hemolytic activity and reduced pore-forming activity in comparison to their wild type protein. The present disclosure also provides, in various embodiments, nucleic acids which encode such mutants, and methods of the use of such mutants.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 May 2016 (26.05.2016)

(10) International Publication Number
WO 2016/081839 A1

(51) International Patent Classification:
A61K 38/00 (2006.01)

(21) International Application Number:
PCT/US2015/061859

(22) International Filing Date:
20 November 2015 (20.11.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/082,848 21 November 2014 (21.11.2014) US

(71) Applicant: **THE BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA** [US/US]; 660 Parrington Oval, Room 119, Norman, OK 73019 (US).

(72) Inventor: **TWETEN, Rodney, K.**; 1701 Deer Park Dr., Edmond, OK 73003 (US).

(74) Agent: **HESTER, Kathryn, L.**; Dunlap Codding, P.C., P.O. Box 16370, Oklahoma City, OK 73113 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/081839 A1

(54) **Title**: PNEUMOLYSIN MUTANTS AND METHODS OF USE THEREOF

```
                                     |                     |                     |
     Cereolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Anthrolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Thuringiolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Perfringolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
      Alveolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
     Caniolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
  Equisimilysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
 Streptolysin O M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Novyiolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Tetanolysin MN-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
     Ivanolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
   Listeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
Seeligeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
       Suilysin MR-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Pneumolysin MA-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
Mitilysin (PLY) ma-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Intermedilysin MK-------- ---------- ---------- ------TKQ- ---------- ---------- ---------- 5
  Viridanolysin MNQEKRLHRF VKKCGLGVCS AVVAAFLLNA QGVALATEQG NRPVETENIA RGKQASQSST AYGGAATRAV 70
       Pyolysin MKR------- ---------- ---------- ---------- ---------- ---------- ---------- 3

                          |                     |                     |                     |
     Cereolysin ---------- ---------- ---------- ---------- --NIKKNTKR RKFLACLLVS LCTINYSSIS 29
    Anthrolysin ---------- ---------- ---------- ---------- FLNIKKNTKR RKFLACLLVS LCTIHYSSIS 32
 Thuringiolysin ---------- ---------- ---------- ---------- FLNIKKNGKR RKFLTCVLVS LCTLNYSSTS 32
 Perfringolysin ---------- ---------- ---------- ---------- ------RFKK TKLIASIAMA LCLFSQPVIS 26
      Alveolysin ---------- ---------- ---------- ---------- -KKKSNHLKG RKVLVSLLVS LQVFAFASIS 30
     Caniolysin ---------- ---------- ---------- ---------- DMSNKKIFKK YSRVAGLLTA ALIVGNLVTA 32
  Equisimilysin ---------- ---------- ---------- ---------- --SNKKIFKK YSRVAGLLTA ALIVGNLVTA 29
 Streptolysin O ---------- ---------- ---------- ---------- --SNKKTFKK YSRVAGLLTA ALIIGNLVTA 29
    Novyiolysin ---------- ---------- ---------- ---------- KS-LKTIIRS ISFLS--ILT LTCSCNFITS 29
    Tetanolysin ---------- ---------- ---------- ---------- KNVLKFVSRS LLIFS--MTG LISNYNSSNV 30
     Ivanolysin ---------- ---------- ---------- ---------- ---------- -KKIMLLLMT LLLVSLPLAQ 20
   Listeriolysin ---------- ---------- ---------- ---------- ---------- -KKIMLVFIT LILVSLPIAQ 20
Seeligeriolysin ---------- ---------- ---------- ---------- ---------- -KIFGLVIMS LLFVSLPITQ 20
       Suilysin ---------- ---------- ---------- ---------- --------KS SHLILSSIVS LALVGVTPLS 24
    Pneumolysin ---------- ---------- ---------- ---------- N--------- ---------- ---------- 3
Mitilysin (PLY) ---------- ---------- ---------- ---------- n--------- ---------- ---------- 3
 Intermedilysin ---------- ---------- ---------- ---------- -----NIARK LSR--VVLLS TLV------- 21
  Viridanolysin DGNVDSDYGH HSVTHTNFED NAWWQVDLGK TENVGKVKLY NRGDGNVANR LSNFDVVLLN EAKQEVARQH 140
       Pyolysin ---------- ---------- ---------- ---------- --------KA FASLVASVVA AATVTMPTAS 25
```

Figure 1A

(57) **Abstract**: The present disclosure provides, in various embodiments, immunogenic mutants of cholesterol-dependent cytolysins, such as pneumolysin, which have reduced hemolytic activity and reduced pore-forming activity in comparison to their wild type protein. The present disclosure also provides, in various embodiments, nucleic acids which encode such mutants, and methods of the use of such mutants.

PNEUMOLYSIN MUTANTS AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS/ INCORPORATION BY REFERENCE STATEMENT

[0001] This application claims benefit under 35 USC § 119(e) of US Serial No. 62/082,848, filed November 21, 2014. The entire contents of the above-referenced application are expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Contract Number AI037657 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

BACKGROUND

[0003] The cholesterol-dependent cytolysins (CDCs) are a large family of pore-forming toxins that are produced by more than 20 species from the genera *Clostridium, Streptococcus, Listeria, Bacillus,* and *Arcanobacterium.* The pore-forming mechanism of these toxins exhibits two hallmark characteristics: an absolute dependence on the presence of membrane cholesterol and the formation of an extraordinarily large pore. Each CDC is produced as a soluble monomeric protein that, with the exception of one member, is secreted by a type II secretion system. Upon encountering a eukaryotic cell, the CDCs undergo a transformation from a soluble monomeric protein to a membrane-embedded supramolecular pore complex. The conversion of the monomers to an oligomeric, membrane-inserted pore complex requires some extraordinary changes in the structure of the monomer.

[0004] Although the CDCs are well known as beta-hemolytic proteins, it has become increasingly apparent that bacterial pathogens use these proteins in much more sophisticated ways than as simple hemolysins or general cell-lytic agents. The CDC structure also exhibits a plasticity that has allowed the evolution of unique features for some CDCs, without compromising the fundamental pore-forming mechanism. Some of these features are reflected in CDCs that activate complement, that utilize a nonsterol receptor, that exhibit a pH-sensitive, poreforming mechanism, or that can function as a protein translocation channel.

[0005] CDCs are β-sheet-rich, four-domain proteins. A highly conserved tryptophan-rich undecapeptide is present in domain 4, which participates in the binding of some CDCs to cholesterol-rich membranes. In addition, three other short hydrophobic loops (Loops L1, L2 and L3) juxtaposed to the undecapeptide at the tip of domain 4 have been shown to also insert into the membrane surface and anchor the CDC to the membrane in a perpendicular orientation. After membrane binding, the CDC monomers diffuse laterally to initiate formation of the membrane oligomer.

[0006] Once the prepore complex reaches a large size, presumably a complete ring structure, it then makes the transition to the pore complex. The transmembrane pore is formed when two α-helical bundles in domain 3 of each monomer within the prepore complex are converted to two extended amphipathic transmembrane β-hairpins (TMHs). Upon the conversion of the prepore to the pore, the height of the prepore structure undergoes a vertical collapse of about 40 Angstroms. The collapse of the prepore structure brings the domain 3 TMHs within striking distance of the membrane surface, at which point they undergo a concerted insertion into the membrane that results in the formation of the large transmembrane β-barrel pore. The CDC pore is large: it is comprised of 35 to 50 monomers and exhibits a diameter of 250 to 300 Angstroms.

[0007] During the process of the CDC monomer interaction with the membrane, the undecapeptide and the three other short loops (L1, L2, and L3) at the tip of the domain 4 β-sandwich insert into the membrane upon the interaction of the CDC monomers with the membrane surface. These loops do not penetrate deeply into the membrane and apparently do not directly participate in the structure of the transmembrane pore. One function of the loops appears to be to anchor the monomers to the membrane in an upright position. Domain 4 exists in a perpendicular orientation to the membrane and is surrounded by the aqueous milieu, even in the oligomeric state.

[0008] Domain 4 of the CDCs mediates membrane recognition, whether it is via cholesterol or another receptor, as in the case of ILY (Intermedilysin).

[0009] The CDCs are also capable of lysis of a wide variety of nucleated cell types *in vitro*, and this capacity has in turn been used by many investigators to permeabilize various eukaryotic cell types with CDCs. Despite the ability of these toxins to perform as general cell-lytic agents *in vitro*, it has not yet been demonstrated that cell lysis is a primary function of the CDCs during an infection. The contribution of CDCs to infection has been studied for example in *Listeria monocytogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Arcanobacterium pyogenes,* and *Clostridium perfringens.* The results of some of these studies suggest that the bacteria use the CDCs in more sophisticated ways than as general cytolytic agents. It also appears that the CDC structure has undergone some unique evolutionary transformations that facilitate the pathogenic mechanism of these bacterial species.

[0010] *Streptococcus pneumoniae* is an important agent of disease in humans, especially among infants, the elderly, persons with chronic illness, and immunocompromised persons. It is a bacterium frequently isolated from patients with invasive diseases such as bacteremia/septicemia, pneumonia, and meningitis with high morbidity and mortality throughout the world. Even with appropriate antibiotic therapy, pneumococcal infections still result in many deaths. Although the advent of antimicrobial drugs has reduced the overall mortality from pneumococcal disease, the presence of resistant pneumococcal strains has become a major problem in the world today and underscores the need for treating and preventing pneumococcal infection by methods in addition to antimicrobials. Effective pneumococcal vaccines could have a major impact on the morbidity and mortality associated with *S. pneumoniae* disease. Such vaccines would also potentially be useful to prevent otitis media in infants and young children. New immunogenic pneumococcal vaccines that provide long-term immunity are clearly needed, especially for children aged less than 2 years, because incidence of disease is high and antibody responses to the polysaccharide vaccine antigens are poor in this age group.

[0011] Each year in the United States, pneumococcal disease accounts for an estimated 3,000 cases of meningitis, 50,000 cases of bacteremia, 500,000 cases of pneumonia, and 7 million cases of otitis media.

[0012] Severe pneumococcal infections result from dissemination of bacteria to the bloodstream and the central nervous system. In 1997, data from community-based studies indicated that overall annual incidence of pneumococcal bacteremia in the United States was an estimated 15-30 cases per 100,000; the rate was higher for persons aged greater than or equal to 65 years (50-83 cases per 100,000) and for children aged less than or equal to 2 years (160 cases per 100,000). In adults, 60%-87% of pneumococcal bacteremia was associated with pneumonia; in young children, the primary sites of infection were frequently not identified.

[0013] In the United States, the risk for acquiring bacteremia is lower among white persons than among persons in other racial/ethnic groups (i.e., blacks, Alaskan Natives, and American

Indians). Black adults have a threefold to fivefold higher overall incidence of bacteremia (49-58 cases per 100,000) than whites. Rates of invasive pneumococcal disease are exceptionally high among Alaskan Natives and American Indians. The age-adjusted annual incidence of invasive pneumococcal infection among Alaskan Natives and Alaskan Native children aged less than 2 years was determined by a prospective surveillance study to be 74 cases and 624 cases per 100,000, respectively. Rates for meningitis and bacteremic pneumonia are eightfold to tenfold higher for Alaskan Natives of all ages than for other U.S. population groups. The highest incidence rates for any U.S. population have been reported among specific American Indian groups (e.g., Apache). The overall annual incidence for such groups is 156 cases per 100,000; the incidence for children aged 1-2 years in these groups is 2,396 cases per 100,000.

[0014] In the United States, the estimated overall annual incidence of pneumococcal meningitis is one to two cases per 100,000. The incidence of pneumococcal meningitis is highest among children aged 6-24 months and persons aged greater than or equal to 65 years. Rates for blacks are twice as high as those for whites and Hispanics. Because the incidence of *Haemophilus influenzae* type b (Hib) meningitis in children rapidly decreased following the introduction of Hib conjugate vaccines, *S. pneumoniae* has become the most common cause of bacterial meningitis in the United States (26).

[0015] Strains of drug-resistant *S. pneumoniae* (DRSP) have become increasingly common in the United States and in other parts of the world. In some areas, as many as 35% of pneumococcal isolates have been reported to have intermediate-level (minimum inhibitory concentration {MIC} equal to 0.1-1.0 µg/mL) or high-level (MIC greater than or equal to 2 µg/mL) resistance to penicillin. Many penicillin-resistant pneumococci are also resistant to other antimicrobial drugs (e.g., erythromycin, trimethoprim-sulfamethoxazole, and extended-spectrum cephalosporins). High-level penicillin resistance and multidrug resistance often complicate the management of pneumococcal infection and make choosing empiric antimicrobial therapy for suspected cases of meningitis, pneumonia, and otitis media increasingly difficult. Treating patients infected with nonsusceptible organisms may require the use of expensive alternative antimicrobial agents and may result in prolonged hospitalization and increased medical costs. The impact of antimicrobial resistance on mortality is not clearly defined. Emerging antimicrobial resistance further emphasizes the need for preventing pneumococcal infections by vaccination.

[0016] The currently available pneumococcal vaccines, PNEUMOVAX®23 (Merck & Co., Inc., Kenilworth, N.J.) and PNU-IMMUNE®23 (Lederle-Praxis Biologicals, Pearl River, NY), include 23 purified capsular polysaccharide antigens of *S. pneumoniae* (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). These vaccines were licensed in the United States in 1983 and replaced an earlier 14-valent formulation that was licensed in 1977. One dose (0.5 mL) of the 23-valent vaccine contains 25 μg of each capsular polysaccharide antigen dissolved in isotonic saline solution with phenol (0.25%) or thimerosal (0.01%) added as preservative and no adjuvant. As of 1997, the 23 capsular types in the vaccine represented at least 85%-90% of the serotypes that cause invasive pneumococcal infections among children and adults in the United States. The six serotypes (6B, 9V, 14, 19A, 19F, and 23F) that most frequently caused invasive drug-resistant pneumococcal infection in the United States as of 1997 are represented in the 23-valent vaccine. As noted below, the desirability of a vaccine solely comprised of capsular polysaccharides is limited.

[0017] Pneumolysin in particular is a key component in the pathogenesis of streptococcal pneumonia, which kills over a million humans per year worldwide. The use of pneumolysin as a part of a vaccine for *Streptococcus pneumoniae* lung infections and otitis media could provide important benefits, since vaccines based on the capsular polysaccharide are losing effectiveness due to genetic variation and are difficult to generate, as there are more than 90 different capsular serotypes of *Streptococcus pneumoniae*. The immunity to one capsular type does not protect against another capsular type. The currently available pneumococcal vaccine discussed above, which comprises 23 capsular polysaccharides from the strains that most frequently cause disease, has significant shortcomings related primarily to the poor immunogenicity of some capsular polysaccharides, the diversity of the serotypes and the differences in the distribution of serotypes over time, geographic areas, and age groups. Currently, a point mutation variant of pneumolysin has been used for vaccine development. This pneumolysin mutant (referred to as "Pd-B") contains a single mutation at position 433 (wherein the native tryptophan residue has been changed to a phenylalanine). This mutation in pneumolysin is in the conserved undecapeptide of Domain 4, the structure within the cholesterol-dependent cytolysins (CDCs) which had long been thought to mediate binding to mammalian membranes.

[0018] While the pneumolysin Pd-B mutant is conventionally used for vaccine development, this protein is still able to undergo a variety of structural transitions that occur after binding to the membrane of mammalian cells. These changes dramatically alter its structure and may decrease its ability to stimulate an effective neutralizing immune response in a patient, primarily because the structure of pneumolysin that the patient's immune system may "see" will be that of the terminal cell-bound oligomeric complex instead of the initial structure of the soluble monomeric pneumolysin. More importantly, the current genetically toxoided pneumolysin is still hampered by an unacceptable level of toxicity. The basis for this toxicity is not yet clear, but likely results from the fact that this toxoid can still bind to and oligomerize on mammalian cells.

[0019] Therefore, mutants of cholesterol-dependent cytolysins, such as (but not limited to) pneumolysin, which have reduced toxicity and reduced hemolytic activity, yet which stimulate an immune response against corresponding disease organisms, would be of great benefit.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Several embodiments of the present disclosure are hereby illustrated in the appended drawings. It is to be noted, however, that the appended drawings only illustrate several typical embodiments and are therefore not intended to be considered limiting of the scope of the disclosure. Further, in the appended drawings, like or identical reference numerals may be used to identify common or similar elements, and not all such elements may be so numbered. The figures are not necessarily to scale, and certain features and certain views of the figures may be shown exaggerated in scale or in schematic in the interest of clarity and conciseness.

[0021] Figures 1A-E contain an amino acid alignment comparison of native amino acid sequences of various cholesterol-dependent cytolysins. The amino acid sequences of each protein identified herein correspond to the SEQ ID NO's in Table 1 herein; for example, Cereolysin in Fig. 1A-E corresponds in SEQ ID NO:2 in Table 1, and SEQ ID No:18 (PAF) in Table 1 corresponds to Viridanolysin in Fig. 1A-E.

[0022] Figure 2 shows the crystal structure of ILY (Intermedilysin) and a comparison of the D4 crystal structures of ILY and PFO (Perfringolysin). Shown in (a) is a ribbon representation of the crystal structure of ILY[25] denoting the positions of various structures and residues referred to in these studies. Shown in (b) is an overlay of a ribbon representation of the D4 structures of ILY and PFO based on the crystal structures of both proteins[23, 24]. Shown are the relative locations of the undecapeptide for both proteins and the L1-L3 loops residues of ILY and PFO (the latter in parentheses). The structural images were generated using VMD[25].

[0023] Figure 3 illustrates that the ILY undecapeptide inserts into cholesterol-depleted membranes. ILY residue Ala-486 was mutated to a cysteine (ILY^{A486C}) and derivatized with NBD (iodoacetamido-*N,N'*-dimethyl-*N*-(7-nitrobenz-2-oxa-1,3-diazolyl)ethylene-diamine). The fluorescence emission of the NBD was determined when $ILY^{A486C\text{-}NBD}$ was incubated alone (solid line), with human red blood cells (hRBCs-dashed line), or with hRBCs depleted of cholesterol (dotted line).

[0024] Figure 4 illustrates that loops L1, L2, and L3 of ILY do not insert into cholesterol-depleted membranes. Each D4 loop residue known to insert into the membrane was substituted for a cysteine and modified with NBD. $ILY^{A428C\text{-}NBD}$ (a), $ILY^{A464C\text{-}NBD}$ (b), or $ILY^{L518C\text{-}NBD}$ (c) was incubated alone (solid line), with hRBCs (dashed line), or with hRBCs depleted of cholesterol (dotted line). Membrane cholesterol was then restored and the insertion of loops L1, L2, and L3 determined. $ILY^{A428C\text{-}NBD}$ (d), $ILY^{A464C\text{-}NBD}$ (e), or $ILY^{L518C\text{-}NBD}$ (f) was incubated alone (solid line) or with cholesterol replete membranes (dashed line).

[0025] Figure 5 shows that the L1-L3 loops mediate PFO binding to cholesterol-rich liposomes, (a) SPR analysis of the binding of native (solid line) and NEM modified PFO (dashed line), (b) SPR analysis of the binding of native PFO (solid line), PFO^{A401D} (long dashed line), PFO^{A437D} (short dashed line) and PFO^{L491D} (dotted line).

[0026] Figure 6 illustrates that chemical modification of the PFO undecapeptide cysteine sulfhydryl blocks the membrane insertion of the undecapeptide tryptophans and conversion of the prepore to pore. The increase in the intrinsic fluorescence emission of the PFO undecapeptide tryptophans has been used to measure their insertion into the membrane[20, 21]. (a) The increase in the intrinsic fluorescence emission of the tryptophans in native PFO is shown as it moves from its soluble form (solid line) to its membrane-bound state (dashed line). (b) The same experiment shown in (a) was repeated with native PFO that had been modified at Cys-459 with NEM.

[0027] Figure 7 shows the immunogenic response in mice immunized with a mutant pneumolysin polypeptide and a wild-type pneumolysin then inoculated with *S. pneumoniae*.

DETAILED DESCRIPTION

[0028] Before explaining at least one embodiment of the inventive concepts in detail by way of exemplary drawings, experimentation, results, and laboratory procedures, it is to be understood that the present disclosure is not limited to the details of composition, components, and methods as set forth in the following description or illustrated in the drawings, experimentation, and/or results. The present disclosure is capable of other embodiments or of being practiced or carried out in various ways. As such, the language used herein is intended to be given the broadest possible scope and meaning, and the embodiments are meant to be exemplary, not exhaustive. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

[0029] Unless otherwise defined herein, scientific and technical terms used in the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Green and Sambrook (Molecular Cloning: A Laboratory Manual (4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012)) and Coligan et al. (Current Protocols in Immunology, Current Protocols, Wiley Interscience (1994)), which are expressly incorporated herein by reference. The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0030] All patents, published patent applications, and non-patent publications mentioned in the present specification are indicative of the level of skill of those skilled in the art to which the present disclosure pertains. All patents, published patent applications, and non-patent publications referenced in any portion of this application are herein expressly incorporated by reference in their entirety to the same extent as if each individual patent or publication was specifically and individually indicated to be incorporated by reference. In particular, the entire contents of the following patents and patent applications are hereby expressly incorporated herein by reference: U.S. Serial No. 13/401,460, filed February 21, 2012; U.S. Serial No. 12/102,696, filed April 14, 2008, now U.S. Patent No. 8,128,939, issued March 6, 2012; U.S. Serial No. 60/923,281, filed April 13, 2007; and U.S. Serial No. 62/082,848, filed Nov. 21, 2014.

[0031] All of the compositions and/or methods described and/or otherwise contemplated herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods disclosed and/or otherwise contemplated herein have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods, and in the steps or in the sequence of steps of the methods, described or otherwise contemplated herein without departing from the concept, spirit, and scope of the present disclosure. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the inventive concepts as defined by the appended claims.

[0032] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0033] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects. For example but not by way of limitation, when the term "about" is utilized, the designated value may vary by plus or minus twelve percent, or eleven percent, or ten percent, or nine percent, or eight percent, or seven percent, or six percent, or five percent, or four percent, or three percent, or two percent, or one percent. The use of the term "at least one" will be understood to include one as well as any quantity more than one, including but not limited to, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 100, etc. The term "at least one" may extend up to 100 or 1000 or more, depending on the term to which it is attached; in addition, the quantities of 100/1000 are not to be considered limiting, as higher limits may also produce satisfactory results. In addition, the use of the term "at least one of X, Y and Z" will be understood to include X alone, Y alone, and Z alone, as well as any combination of X, Y and Z. The use of ordinal number terminology (i.e., "first," "second," "third," "fourth," etc.) is solely for the purpose of differentiating between two or more items and is not meant to imply any sequence or order or importance to one item over another or any order of addition, for example.

[0034] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0035] The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AAB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0036] Throughout the specification and claims, unless the context requires otherwise, the terms "substantially" and "about" will be understood to not be limited to the specific terms qualified by these adjectives/adverbs, but will be understood to indicate a value includes the inherent variation of error for the device, the method being employed to determine the value and/or the variation that exists among study subjects. Thus, said terms allow for minor variations and/or deviations that do not result in a significant impact thereto. For example, in certain instances the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value and/or the variation that exists among study subjects. Similarly, the term “substantially” may also relate to 80% or higher, such as 85% or higher, or 90% or higher, or 95% or higher, or 99% or higher, and the like.

[0037] The terms “purified protein” or “isolated protein” as used herein mean that the protein or fragment is sufficiently free of contaminants or cell components with which the protein normally occurs as to distinguish the protein from the contaminants or cell components. It is not contemplated that "purified" necessitates having a preparation that is technically totally pure (homogeneous), but purified as used herein means the protein or polypeptide fragment is sufficiently separated from contaminants or cell components with which it normally occurs to provide the protein in a state where it can be used in an assay, such as immunoprecipitation or ELISA. For example, the purified protein can be in an electrophoretic gel.

[0038] The term "mutant" when used herein to describe a polypeptide refers to a polypeptide which is less than 100% identical to an amino acid sequence of the corresponding wild type (native) polypeptide, and in particular to a synthetic or recombinant polypeptide wherein one or more amino acid residue positions of the wild type polypeptide have been substituted. The term "variant" may be used interchangeably with the term "mutant."

[0039] The mutant CDCs described herein may be combined with one or more pharmaceutically-acceptable excipients, including carriers, vehicles, and diluents, to form immunogenic compositions. The term pharmaceutically-acceptable excipient as used herein is intended to refer to solvents or other materials in which the mutant CDCs (e.g., mutant pneumolysin polypeptides) disclosed herein can be disposed to improve solubility, deliverability, dispersion, stability, and/or conformational integrity. Examples of such pharmaceutically-acceptable excipients include, but are not limited to, water, saline solutions (such as physiological saline solutions and buffered saline solutions at neutral pH such as phosphate buffered saline (PBS)), ethanol, sugars, dextrose, glycerol, and/or polyalcohols (such as mannitol and sorbitol). Other types of carriers include liposomes or polymers and the like.

[0040] The term "pharmaceutically acceptable" refers to a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected compound without causing any undesirable biological effects or interacting in an undesirable manner with any of the other components of the pharmaceutical composition in which it is contained.

[0041] The mutant CDCs or immunogenic compositions containing said mutant CDCs may further be combined with an adjuvant such as (but not limited to) Freund's incomplete adjuvant, Freund's Complete adjuvant, alum, monophosphoryl lipid A, alum phosphate or hydroxide, QS-21, salts, i.e., $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)_2$, silica, kaolin, and/or carbon polynucleotides (i.e., poly IC and poly AU). Non-limiting examples of adjuvants include QuilA, Alhydrogel, and the like. The term "adjuvant" refers to a substance that is capable of enhancing, accelerating, or prolonging an immune response when give with the immunogen of the composition. Optionally, the mutant CDCs contemplated herein can be combined with immunomodulators and immunostimulants, such as but not limited to, interleukins, interferons, and the like. Many vaccine and other pharmaceutical formulations are known to those of skill in the art.

[0042] By “biologically active” is meant the ability to modify the physiological system of an organism. A molecule can be biologically active through its own functionalities, or may be biologically active based on its ability to activate or inhibit molecules having their own biological activity.

[0043] The term “immunogenic” where used herein is intended to refer to the ability of a substance to elicit an immune response. For example, an “immunogenic composition” is a composition comprising a mutant CDC, such as a mutant pneumolysin polypeptide, which is able to elicit an immune response in a subject animal, such as the production of antibodies, when administered thereto. The term “vaccine” refers to an immunogenic composition for administration to a subject for eliciting an immune response against a particular antigen. For example, a vaccine comprising one or more of the mutant pneumolysin polypeptides disclosed herein is a vaccine for use in the treatment of a disease or condition caused by the bacterium *Streptococcus pneumoniae*.

[0044] The term "patient" or "subject" as used herein includes human and veterinary subjects. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including (but not limited to) humans, domestic animals (such as, but not limited to, dogs and cats), farm animals (such as, but not limited to, cows, horses, pigs, goats, and sheep), laboratory animals (such as, but not limited to, mice, rats, rabbits, guinea pigs, and chinchillas), nonhuman primates, and any other animal that has mammary tissue.

[0045] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include, but are not limited to, individuals already having a particular condition or disorder as well as individuals who are at risk of acquiring a particular condition or disorder (e.g., those needing prophylactic/preventative measures). The term "treating" refers to administering an agent to a patient for therapeutic and/or prophylactic/preventative purposes.

[0046] A "therapeutic composition" or "pharmaceutical composition" refers to an agent that may be administered *in vivo* to bring about a therapeutic and/or prophylactic/preventative effect.

[0047] The phrase "administering a therapeutically effective amount" or "administering a prophylactically effective amount" is intended to provide a therapeutic benefit in the treatment, reduction in occurrence, prevention, or management of a disease. The specific amount that is therapeutically effective can be readily determined by the ordinary medical practitioner, and can vary depending on factors known in the art, such as the type of disease/cancer, the patient's history and age, the stage of disease, and the co-administration of other agents.

[0048] A "disorder" is any condition that would benefit from treatment with the polypeptide. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

[0049] The term "therapeutically effective amount" refers to an amount of a biologically active molecule or conjugate or derivative thereof sufficient to exhibit a desired therapeutic effect without undue adverse side effects (such as toxicity, irritation and allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of the inventive concepts. The therapeutic effect may include, for example but not by way of limitation, inhibiting the growth of undesired tissue or malignant cells. The effective amount for a subject will depend upon the type of subject, the subject's size and health, the nature and severity of the condition to be treated, the method of administration, the duration of treatment, the nature of concurrent therapy (if any), the specific formulations employed, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by one of ordinary skill in the art using routine experimentation based on the information provided herein.

[0050] As used herein, the term "concurrent therapy" is used interchangeably with the terms "combination therapy" and "adjunct therapy," and will be understood to mean that the patient in need of treatment is treated or given another drug for the disease in conjunction with the pharmaceutical compositions of the present disclosure. This concurrent therapy can be sequential therapy, where the patient is treated first with one drug and then the other, or the two drugs are given simultaneously.

[0051] The terms "administration" and "administering," as used herein will be understood to include all routes of administration known in the art, including but not limited to, oral, topical, transdermal, parenteral, subcutaneous, intranasal, mucosal, intramuscular, intraperitoneal, intravitreal and intravenous routes, including both local and systemic applications. In addition, the compositions of the present disclosure (and/or the methods of administration of same) may be designed to provide delayed, controlled or sustained release using formulation techniques which are well known in the art.

[0052] The terms "substitution," "insertion," "addition," and "deletion" are used herein with reference to amino acid or nucleotide sequences. A "substitution" refers to a replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively. An "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring sequence. A "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

[0053] Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

[0054] Substitutions, deletions, insertions, or any combination thereof may be used to arrive at a final mutant polypeptide. Generally, a few amino acids are changed to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances.

[0055] In certain embodiments, amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of an isoleucine with a valine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids.

[0056] In embodiments, substitutions can be made in accordance with known "conservative substitutions." A "conservative substitution" refers to the substitution of an amino acid in one class by an amino acid in the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature.

[0057] In contrast, in certain embodiments, substitutions are non-conservative. A "non-conservative substitution" refers to the substitution of an amino acid in one class with an amino acid from another class.

[0058] The term "polypeptide" as used herein refers to a compound made up of a single chain of amino acid residues linked by peptide bonds. The term "protein" as used herein may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides.

[0059] The term "nucleic acid molecule" includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given mutant CDC protein may be produced. The present disclosure includes every possible variant nucleotide sequence thereof, all of which are possible given the degeneracy of the genetic code.

[0060] A "heterologous" nucleic acid construct or sequence has a portion of thereof which is not native to the cell in which it is expressed. The term "heterologous," with respect to a control sequence, refers to a control sequence (i.e., promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or are not part of the genome in which they are present; rather, the heterologous sequences have been added to the cell, such as by infection, transfection, transformation, microinjection, electroporation, or the like. A "heterologous" nucleic acid construct may contain a control sequence/DNA coding sequence combination that is the same as, or different from, a control sequence/DNA coding sequence combination found in the native cell.

[0061] As used herein, the term “vector” refers to a nucleic acid construct designed for transfer between different host cells. An “expression vector” refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

[0062] Accordingly, an "expression cassette" or "expression vector" is a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter.

[0063] As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

[0064] As used herein, the term "selectable marker-encoding nucleotide sequence" refers to a nucleotide sequence which is capable of expression in cells and where expression of the selectable marker confers to cells containing the expressed gene the ability to grow in the presence of a corresponding selective agent, or under corresponding selective growth conditions.

[0065] As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter, together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences"), is necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

[0066] The terms "chimeric gene" or "heterologous nucleic acid construct," as utilized herein, refer to a non-native gene (i.e., one that has been introduced into a host) that may be composed of parts of different genes, including regulatory elements. A chimeric gene construct for transformation of a host cell is typically composed of a transcriptional regulatory region (promoter) operably linked to a heterologous protein coding sequence, or, in a selectable marker chimeric gene, to a selectable marker gene encoding a protein conferring antibiotic resistance to transformed cells. A typical chimeric gene of the present disclosure, for transformation into a host cell, includes a transcriptional regulatory region that is constitutive or inducible, a protein coding sequence, and a terminator sequence. A chimeric gene construct may also include a second DNA sequence encoding a signal peptide if secretion of the target protein is desired.

[0067] A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA encoding a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors, linkers or primers for PCR are used in accordance with conventional practice.

[0068] As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, that may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

[0069] As used herein, the term "recombinant" includes reference to a cell or vector that has been modified by the introduction of a heterologous nucleic acid sequence; in addition, the term “recombinant” can also refer to a cell that is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed, or not expressed at all as a result of deliberate human intervention.

[0070] As used herein, the terms "transformed," "stably transformed," or "transgenic," with reference to a cell, means the cell has a non-native (heterologous) nucleic acid sequence integrated into its genome or has an episomal plasmid that is maintained through multiple generations.

[0071] As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

[0072] The term "introduced," in the context of inserting a nucleic acid sequence into a cell, refers to any method of insertion of a nucleic acid sequence into a cell, including but not limited to, "transfection," "transformation," and/or "transduction" methods. The term “introduced” also includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

[0073] Turning now to the present disclosure, certain embodiments are directed to compositions comprising one or more non-toxic mutants of cholesterol-dependent cytolysins (CDCs). The compositions may be used, for example, in vaccines directed against corresponding disease pathogens, or may be used in diagnostic or screening methods or other analytical methods such as detection methods.

[0074] The organisms which produce the native forms of the CDCs have various pathological effects, including but not limited to those listed below.

[0075] *Clostridium perfringens* is a causative agent of various human and animal diseases, often characterized by enterotoxemia or soft tissue infections such as gas gangrene. Experimental evidence suggests a role for perfringolysin O in blunting the immune response by affecting neutrophil function.

[0076] *Bacillus cereus* (source of Cereolysin O) is an infrequent cause of serious nongastrointestinal infection, particularly in drug addicts, the immunosuppressed, neonates, and postsurgical patients, especially when prosthetic implants such as ventricular shunts are inserted. Ocular infections are the commonest types of severe infection, including endophthalmitis, anophthalmitis, and keratitis, usually with the characteristic formation of corneal ring abscesses.

[0077] *Bacillus alvei* can cause endophthalmitis and may cause pneumonia and empyema.

[0078] *Streptococcus dysgalactiae* subsp. *equisimilis* has been shown to be involved in many different types of human disease syndromes.

[0079] *Streptococcus canis* typically causes disease in animals, primarily dogs. It can cause disease in humans, most often soft tissue infections, bacteremia, urinary infections, bone infections or pneumonia.

[0080] *Streptococcus* causes a variety of diseases including strep throat, rheumatic fever, soft tissue infections (i.e., the fleshing eating bacteria), and many others. Streptolysin O has been shown to be a major pathogenic factor in many of these diseases.

[0081] Tetanolysin is produced by *Clostridium tetanus* that is the cause of tetanus.

[0082] *Listeria ivanovii* is an infection of animals and primarily causes abortion in sheep.

[0083] *Listeria monocytogenes* causes food borne illness in humans; the most severe food borne illness caused thereby is a meningitis. It is especially problematic for pregnant women where the infection may be subclinical in the mother but fatal for the fetus. Listeriolysin is a critical pathogenic factor for these diseases, without it the bacterium is avirulent.

[0084] *Streptococcus suis* is a cause of septicemia, meningitis, endocarditis, arthritis and, occasionally, other infections in pigs, and is increasingly a problem in humans, more and more outbreaks are being reported with symptoms that include high fever, malaise, nausea and vomiting, followed by nervous symptoms, subcutaneous hemorrhage, septic shock and coma.

[0085] Certain embodiments of the present disclosure provide non-toxic mutants of native (wild type) pneumolysin (“PLY;” SEQ ID NO:1) of *S. pneumoniae* (encoded by mutants of SEQ ID NO:20). These PLY mutants exhibit several potential advantages over the pneumolysin mutant (Pd-B) which has previously been used for vaccine development, particularly in that they substantially lack hemolytic activity in comparison to the wild type PLY protein. For example, the PLY mutants of the present disclosure lack the ability to bind to mammalian membranes, and thus will not undergo any of the structural changes that normally result when the wild type PLY toxin binds to the membrane (as does the Pd-B mutant (Trp433Phe) described above).

[0086] In certain non-limiting embodiments, the present disclosure includes pneumolysin mutants wherein at least one amino acid of positions 293 and 294 (of SEQ ID NO:1), and at least one amino acid at positions 458, 459, and 460, have been substituted with a different amino acid than found in the wild-type PLY sequence (SEQ ID NO:1). More particularly, either or both of the gly residues at positions 293 and 294 can be substituted with an amino acid having a side chain, including but not limited to ala, leu, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, thr, ser, asp, glu, arg, his, and lys. Further, either or both of the thr residues at positions 458 and 459 can be substituted with gly, ala, leu, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, ser, asp, glu, arg, his, and lys. Further, the leu residue at position 460 can be substituted with gly, ala, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, thr, ser, asp, glu, arg, his, and lys. For example, in one non-limiting embodiment, the glycine at position 293 has been replaced with one of ala, leu, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, thr, ser, asp, glu, arg, his, and lys, and the leucine at position 460 has been replaced with one of gly, ala, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, thr, ser, asp, glu, arg, his, and lys. In particular non-limiting embodiments of a mutant pneumolysin, at least one of the glycine residues at positions 293 and 294 has been mutated to a serine or threonine residue, and at least one of the threonine, threonine, and leucine residues at positions 458, 459, and 460, respectively, has been mutated to an aspartate, asparagine, or glutamate residue. For example, when position 293 has been mutated to a serine, and the leucine at position 460 has been mutated to an aspartate, the pneumolysin mutant is designated PLY-G293S/L460D (or PLY-L460D/G293S); the amino acid sequence thereof is provided as SEQ ID NO:40. In alternate non-limiting embodiments, position 460 can be substituted with D, E or N, and position 293 can be substituted with S or T such that the double mutant may comprise a D, E, or N at position 460 and an S or T at position 293.

[0087] In addition to mutants of pneumolysin, the present disclosure provides mutants of other CDCs which have substitutions in analogous positions in Loop 1, Loop 2 and/or Loop 3 of Domain 4, including mutants of Cereolysin (*Bacillus cereus*), Anthrolysin (*Bacillus anthracis*), Thuringiolysin (*Bacillus thuringiensis*), Perfringolysin (*Clostridium perfringens*), Alveolysin (*Bacillus alvei*), Caniolysin (*Streptococcus canis*), Equisimilysin (*Streptococcus equisimilis*), Streptolysin O (*Streptococcus pyogenes*), Tetanolysin (*Clostridium tetani*), Ivanolysin (*Listeria ivanovii*), Listeriolysin (*Listeria monocytogenes*), Seeligeriolysin (*Listeria seeligeri*), Suilysin (*Streptococcus suis*), Mitilysin (*Streptococcus mitis*), Platelet aggregation factor (a.k.a. PAF and Viridanolysin) (*Streptococcus mitis*), Intermedilysin (*Streptococcus intermedius*), Pyolysin (*Arcanobacterium pyogenes*), and Novyiolysin, a.k.a., tetanolysin NT (*Clostridium novyi*).

[0088] Wild-type amino acid sequences of Cereolysin, Anthrolysin, Thuringiolysin, (a.k.a., Thuringolysin or Cereolysin form BT), Perfringolysin, Alveolysin, Caniolysin, Equisimilysin, Streptolysin O, Novyiolysin, Tetanolysin, Ivanolysin, Listeriolysin, Seeligeriolysin, Suilysin, Mitilysin, Intermedilysin, Platelet aggregation factor (a.k.a. Viridanolysin or PAF), and Pyolysin are shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19, respectively.

[0089] Another embodiment of the present disclosure is directed to a mutant of Streptolysin O comprising substitutions in at least one of positions 561 and 562 and in at least one of positions 395 and 396 of SEQ ID NO:9 and which is at least 90% identical to the wild type Streptolysin O protein. The substitutions in positions 561 and 562 may be any substitution described herein that can be made in positions 458-460 of PLY (SEQ ID NO:1), and the substitutions in positions 395 and 396 may be any substitution described herein that can be made in positions 293 or 294 of PLY.

[0090] A variant of SEQ ID NO:18 (Platelet Aggregation Factor) which can also be mutated in accordance with the present disclosure is Lectinolysin, which is also obtained from *Streptococcus mitis*. The amino acid sequences of L1, L2 and L3 are the same as PAF. Lectinolysin differs from PAF at 12 positions, including 67, 158, 211, 303, 305-307, 311, 319, 327, 447, and 556 wherein in Lecinolysin the amino acids at these positions are T, D, T, H, E, N, K, N, E, K, T and I, respectively. The present disclosure thus includes mutants of Lectinolysin which are similar to those of the other mutants contemplated herein, and nucleic acids encoding these mutants, and compositions comprising these mutants.

[0091] The pneumolysin mutants contemplated herein also eliminate any toxic activity of the toxin, since they cannot bind to mammalian cells. Although the pneumolysin mutant Pd-B is about 21,000 times less toxic than native pneumolysin, it still exhibits sufficient toxicity to be problematic in the development of any vaccines that include it. It appears that modern vaccine development against *S. pneumoniae* is centered on using pneumolysin with other *S. pneumoniae* derived proteins; thus it appears that regardless of the other proteins used in the vaccine, a pneumolysin will be included in all effective vaccines against *S. pneumoniae* because of its importance to disease establishment and progression.

[0092] As described below, it is shown in perfringolysin, a toxin related to pneumolysin, that the undecapeptide of the protein *does not* mediate binding of these toxins to the mammalian cell, contrary to the conventional wisdom. The structures that do mediate binding are three short hydrophobic loops that are juxtaposed to the undecapeptide. As part of the present disclosure, it is now known that if a negatively charged aspartate or glutamate residue (for example) is placed within any single hydrophobic loop (in a position not already comprising an aspartate or glutamate), binding of the CDC to the membrane is blocked. Hence, this single point mutation eliminates binding of the CDCs, including pneumolysin, to mammalian membranes. For example, a single asparate or glutamate residue substituted for leucine 460 of pneumolysin virtually completely abrogates its hemolytic activity. Since it is known in other systems (described below) that this mutation blocks binding to the membrane of cells, it substantially eliminates any toxic activity (making it at least 200 times less toxic than the Pd-B mutant for example), but also eliminates any possible side effects that might be caused by its binding to the surface of mammalian membranes.

[0093] In certain embodiments, the mutant pneumolysins of the present disclosure lack the hemolytic activity and the pore-forming ability present in a naturally occurring *S. pneumoniae* pneumolysin protein. Generally, the polypeptide component exhibits less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 1%, less than about 0.1%, less than about 0.001%, or less of the hemolytic activity of a naturally occurring *S. pneumoniae* pneumolysin protein.

[0094] In certain embodiments, the mutant pneumolysins of the present disclosure have substitutions in one or more of three residues that flank either side of positions 293, 370, 406 or 460, including positions 290, 291, 292, 294, 295, 296, 367, 368, 369, 371, 372, 373, 403, 404, 405, 407, 408, 409, 457, 458, 459, 461, 462, and 463.

[0095] For example, these residues may be substituted with a negatively-charged amino acid, glutamate, or aspartate (except in position 403, which already comprises aspartate), or a positively charged amino acid lysine, arginine, or histidine (except in positions 367 and 407, which already comprise histidine residues). Alternatively, these residues may be substituted with any other natural amino acid (including gly, ala, leu, ile, val, pro, trp, asn, gln, phe, tyr, met, cys, thr, or ser) which abrogates the binding activity, pore-forming, and/or hemolytic activity of the mutant.

[0096] As noted above, the amino acid sequence for wild type pneumolysin is SEQ ID NO:1, and the reverse complement of the cDNA which encodes the pneumolysin of SEQ ID NO:1 is shown as SEQ ID NO:20. The present disclosure further includes cDNAs of mutant pneumolysins (and reverse complements thereof) and other mutant CDCs described herein which are substituted as necessary to encode the substituted proteins (mutants) described or otherwise enabled herein, and may in turn comprise any conservative base (nucleotide) substitution to make cDNAs which encode such mutants.

[0097] It will be appreciated that the polynucleotide sequences which encode the polypeptides contemplated herein may be altered with degenerate codons yet still encode the mutant polypeptides of the present disclosure. Accordingly, the present disclosure further provides polynucleotides which hybridize to the polynucleotide sequences described herein (or the complementary sequences thereof) having at least 90% identity between sequences, or at least 95% identity, or at least 99% identity.

[0098] Figures 1A through 1E show an alignment of the amino acid sequences of the native versions of the CDCs identified herein. The sequences are aligned along the three hydrophobic loops corresponding to positions 367-373 (second loop, L2), 403-409 (third loop, L3) and 457-

463 (first loop, L1) of pneumolysin, represented in Fig. 1A-E as positions 586-592 (second loop, L2), 622-628 (third loop, L3), and 676-682 (first loop, L1). As noted above, certain particular (but non-limiting) embodiments of the mutants of these CDCs may comprise substitutions at one or more of these positions by the negatively-charged amino acids, glutamic acid, or aspartic acid (except wherein the position already has an aspartic acid), or by the positively-charged amino acids histidine, lysine, or arginine (except by a histidine where the position already has a histidine, by a lysine where the position already has a lysine, or by an arginine where the position already has an arginine) or by any of the other 15 natural amino acids noted above wherein the resulting mutant functions in accordance with the present disclosure.

[0099] The mutants may further comprise more than one of the substitutions described herein such that the mutant has 1, 2, 3, 4, 5, 6, or 7 substituted residues in a single loop (L1, L2, L3), or the mutant may have one or more (1 to 7) substituted residues in two of the loops (e.g., L1 and L2, L1 and L3, L2 and L3), or one or more substituted residues (1 to 7) in each of the three loops (L1, L2, and L3), wherein the substitutions are selected from those listed herein; for example, the mutant may have 1 to 7 substitutions in the first loop (L1), and/or 1 to 7 substitutions in the second loop (L2), and/or 1 to 7 substitutions in the third loop (L3). For example, in certain embodiments, where the native residue is positively-charged, the substituted residue may be negatively-charged, and where the native residue is negatively-charged, the substituted residue may be positively charged. Alternatively, aspartate may be substituted with glutamate, histidine, arginine, or lysine, or glutamate may be substituted with aspartate, lysine, histidine, or asparagine, or arginine may be substituted with a different positively-charged amino acid.

[00100] The amino acid positions of Loop 1, Loop 2, and Loop 3 of each CDC described herein is listed in Table 1.

Table 1: Amino Acid Positions Corresponding to Domain 4 Loops

	SEQ ID NO.	Loop 1	Loop 2	Loop 3
Pneumolysin	1	457-463	367-373	403-409
Cereolysin	2	498-504	408-414	444-450
Anthrolysin	3	501-507	411-417	447-453
Thuringiolysin	4	501-507	411-417	447-453
Perfringolysin	5	488-494	398-404	434-440
Alveolysin	6	490-496	400-406	436-442
Caniolysin	7	562-568	472-478	508-514
Equisimilysin	8	559-565	469-475	505-511
Streptolysin O	9	559-565	469-475	505-511
Novyiolysin	10	502-508	412-418	448-454
Tetanolysin	11	514-520	424-430	460-466

Ivanolysin	12	512-518	422-428	458-464
Listeriolysin O	13	513-519	423-429	459-465
Seeligeriolysin	14	514-520	424-430	460-466
Suilysin	15	484-490	395-401	431-437
Mitilysin	16	457-463	367-373	403-409
Intermedilysin	17	515-521	425-431	461-467
PAF	18	651-657	561-567	597-603
Pyolysin	19	521-527	431-437	467-473

[0101] Thus, provided herein are purified or isolated forms of the protein mutants, and antigenic fragments thereof, immunogenic compositions of these mutants comprising pharmaceutically-acceptable excipients, adjuvants, and/or immunostimulants, and vaccines and sera comprising one or more of the mutants disclosed or otherwise contemplated herein. The mutants or antigenic fragments thereof can be used in analytical methods for detecting the presence of alternative forms of the proteins in biological samples using techniques known in the art, for example ELISA. The present disclosure further provides nucleic acids, host cells, and vectors comprising cDNAs encoding any of the mutants provided herein and methods of their use to produce the mutants contemplated herein. The present disclosure further provides methods of administering the immunogenic compositions for treatment of conditions, diseases, and infections, caused by the CDC-producing organisms described herein.

[0102] As noted above, the present disclosure is also directed to nucleic acid sequences which encode the mutant CDCs contemplated herein. The present disclosure provides nucleic acids which encode allelic variants of the protein mutants disclosed herein, wherein the allelic variants of the protein mutants differ from the protein mutants by less than 15% of their amino acid identity, for example, at least 85% of the amino acids of the allelic variant are identical to the protein mutant, and 100% of the amino acids in the first, second, and third loops (L1, L2, and L3) are identical to those in the protein mutant. For example, the allelic variants may differ from the protein mutants by less than 12% of their amino acid identity, by less than 10% of their amino acid identity, by less than 8% of their amino acid identity, by less than 6% of their amino acid identity, by less than 4% of their identity, by less than 2% of their amino acid identity, or by less than 1% of their amino acid identity from the protein mutants described herein. Further, the present disclosure is further directed to nucleic acids which hybridize under stringent conditions with the nucleic acids which encode the mutant CDCs described herein or with the complements of the nucleic acids encoding the mutant CDCs described herein.

[0103] In one aspect, the CDC mutant polypeptides or proteins of the present disclosure comprise an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least

93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or more percent identity to the sequence presented as SEQ ID NO:1, (as determined by a sequence alignment program), and which have at least one of the mutations described elsewhere herein.

[0104] An alignment of selected sequences in order to determine "% identity" between two or more sequences, may be performed using, for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

[0105] In another embodiment, the term "sequence identity" as used herein means that the sequences are compared as follows. The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default (BLOSUM62) matrix (values –4 to +11) with a gap open penalty of –12 (for the first null of a gap) and a gap extension penalty of –4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

[0106] The immunogenic compositions described or otherwise contemplated herein may include vaccine formulations that can be used in an amount effective to elicit (stimulate) a protective immune response in an animal. For example, the generation of a protective immune response can be measured by the development of antibodies. In certain non-limiting embodiments, the amounts of the mutant CDCs contemplated herein that can form a protective immune response typically are in a unit dosage form of about 0.001 μg to 100 mg per kg of body weight, such as but not limited to, about 0.01 μg to 1 mg/kg of body weight, or about 0.1 μg to about 10 μg/kg body weight, for example, at an interval of about 1 to 6 weeks between immunizations.

[0107] The present disclosure further provides methods of stimulating an immune response against at least one disease organism. In the method, any of the immunogenic compositions disclosed herein can be administered to a patient infected with the disease organism or predisposed to infection with the disease organism. In one non-limiting embodiment, the immunogenic composition comprises a pneumolysin mutant having mutations in positions 293 and 460, such as $PLY_{L460D/G293S}$ (SEQ ID NO:40). In the method, the immunogenic composition is substantially non-toxic (or substantially non-toxic compared to the native PLY protein), does not substantially bind to cell membranes, is substantially non-hemolytic, and/or is as stable as or is substantially more stable than the PLY protein.

[0108] The present disclosure is further directed to at least one method of decreasing the occurrence and/or severity of infection in a patient. In the method, any of the immunogenic compositions disclosed or otherwise contemplated herein is administered to an infected patient or a patient predisposed to infection. In one non-limiting embodiment, the immunogenic composition comprises a pneumolysin mutant having mutations in positions 293 and 460, such as $PLY_{L460D/G293S}$ (SEQ ID NO:40). In the method, the immunogenic composition is substantially non-toxic (or substantially non-toxic compared to the native PLY protein), does not substantially bind to cell membranes, is substantially non-hemolytic, and/or is as stable as or is substantially more stable than the native PLY protein. In certain embodiments, the mutant pneumolysin polypeptides disclosed herein have about 100,000-fold less hemolytic activity than wild type pneumolysin polypeptide. In other embodiments, the mutant pneumolysin polypeptides disclosed herein have about 150,000-fold less hemolytic activity than wild type pneumolysin polypeptide. In other embodiments, the mutant pneumolysin polypeptides disclosed herein have about 200,000-fold less hemolytic activity than wild type pneumolysin polypeptide. In still other embodiments, the mutant pneumolysin polypeptides disclosed herein have about 250,000-fold less hemolytic activity than wild type pneumolysin polypeptide. In at least certain embodiments, the purified mutant pneumolysin polypeptides disclosed herein which have at least two substitutions in amino acid positions 293, 294, 458, 459, and 460, also have an increased yield upon purification over a mutant pneumolysin polypeptide having a substitution in only one of amino acid positions 293, 294, 458, 459, and 460. The increased recombinant yield may be for example, at least about 10X, at least about 15X, at least about 17X, or at least about 20X.

[0109] The immunogenic compositions disclosed herein may be administered to animals which are infected or may become infected by the disease organisms described herein, including but not limited to dogs, cats, rabbits, rodents, horses, livestock (e.g., cattle, sheep, goats, and pigs), zoo animals, ungulates, primates, and humans.

[0110] As noted above, when the mutant is a pneumolysin mutant, the present disclosure includes an immunogenic composition (such as, but not limited to, a vaccine) which can be administered to a subject for stimulating an immunogenic response in the subject. In addition to the one or more pneumolysin mutants, the immunogenic composition/vaccine may comprise other proteins or protein subunits from *S. pneumoniae,* or may comprise capsular polysaccharide material combined with or conjugated to the pneumolysin mutants or other proteins in the immunogenic composition/vaccine. For example, the capsular material may be derived from any one or more of the *S. pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 24F, 27, 33F, or 34, or others known in the art. As noted, the immunogenic composition/vaccine may comprise an adjuvant and/or other pharmaceutically-acceptable excipients. Polysaccharides can be conjugated to the mutant, for example, via a monomeric linkage (only one end of the polysaccharide is attached to the polypeptide), a looped linkage (a single polypeptide is attached to looped polysaccharides), or a cross-linkage (multiple polysaccharides attached to multiple polypeptides).

[0111] The immunogenic compositions or vaccines containing mutant pneumolysin polypeptides of the present disclosure, or fragments thereof, may be used to treat diseases and conditions related to *Streptococcus pneumoniae*, such as, but not limited to, pneumonia, meningitis, bacteremia, and otitis media.

[0112] In certain embodiments, the mutant CDCs disclosed herein are useful for causing stimulation of T-cell proliferation or the generation of antibodies through the stimulation of B cells.

[0113] As noted above, an immunogenic composition of the present disclosure can be formed by combining the mutant CDCs contemplated herein with a pharmaceutically (physiologically) acceptable excipient, such as (but not limited to) physiological saline or buffered saline solutions at neutral pH (such as phosphate buffered saline).

[0114] The present disclosure also includes antigenic fragments of the mutant CDCs described or otherwise contemplated herein. For example, for vaccine compositions, fragments are large enough to stimulate a protective immune response. The polypeptide component must be of a length sufficient to induce such an enhanced immune response. For fragments of a naturally occurring CDC protein, the fragments are at least about 8, at least about 10, at least about 25, at least about 50, at least about 75, at least about 100, at least about 125, at least about 150, at least about 175, at least about 200, at least about 250, at least about 300, at least about 350, at least about 400, at least about 425, at least about 450, at least about 460, at least about 465, or more amino acids in length.

[0115] Fragments may comprise peptide portions from different locations of the mutants that have been joined together. In certain particular (but non-limiting) embodiments, fragments include one or more of the three loops discussed herein.

[0116] The mutant CDCs disclosed or otherwise contemplated herein are also useful to generate neutralizing antibodies which can be used as a passive immune serum to treat or ameliorate symptoms in patients. An immunogenic composition as described above could be administered to an animal (such as a horse or a human) until a neutralizing antibody response is generated. These neutralizing antibodies can then be harvested, purified, and utilized to treat patients exhibiting symptoms.

[0117] Such neutralizing antibodies are administered to patients exhibiting disease symptoms in an amount effective to neutralize the effect of the pathogen. The neutralizing antibodies can be administered intravenously, intramuscularly, intradermal, subcutaneously, and the like. A particular route is intravenously, or for localized infection, topically at the site of tissue damage with debridement. The neutralizing antibody may also be administered in conjunction with antibiotic therapy. The neutralizing antibody can be administered until a decrease in shock or tissue damage is obtained in a single dose or multiple doses. The amount of neutralizing antibodies typically administered is about 1 mg to about 1000 mg antibody per kg of body weight, such as but not limited to, about 50 mg to about 200 mg antibody per kg of body weight.

[0118] The immunogenic compositions of the present disclosure may be prepared as a pharmaceutical composition containing an immunoprotective, non-toxic amount of at least one of the presently disclosed mutant proteins in a non-toxic and sterile pharmaceutically acceptable excipient.

[0119] The immunogenic compositions of the present disclosure can be administered to the appropriate subject in any suitable manner known in the art, including (but not limited to) orally intramuscularly, intravenously, sublingual mucosal, intraarterially, intrathecally, intradermally, intraperitoneally, intranasally, intrapulmonarily, intraocularly, intravaginally, intrarectally, and/or subcutaneously. They can be introduced into the gastrointestinal tract or the respiratory tract, e.g., by inhalation of a solution or powder containing the immunogenic composition. Parenteral administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

[0120] An immunogenic composition (e.g., a vaccine) is administered in an amount sufficient to elicit production of antibodies as part of an immunogenic response. Dosage for any given patient depends upon many factors, including the patient's size, general health, sex, body surface area, age, the particular compound to be administered, time and route of administration, and other drugs being administered concurrently. Determination of optimal dosage is well within the abilities of a pharmacologist of ordinary skill. In certain embodiments, a non-limiting range of effective amounts of a mutant CDC that may be administered to a subject is, for example, about 10 ng of protein to 100 mg per kg of body weight, such as about 0.1 μg of protein to about 1 mg per kg body weight. In at least one non-limiting embodiment, the dosage provided is in a range of from about 0.25 μg to about 25 μg of protein, with or without adjuvants.

[0121] When the immunogenic composition is administered parenterally, via the intramuscular or deep subcutaneous route, the mutant protein may (in certain particular but non-limiting embodiments) be admixed or absorbed with any conventional adjuvant to attract or to enhance the immune response. Such adjuvants include but are not restricted to aluminum hydroxide, aluminum phosphate, muramyl dipeptide, bacterial lipopolysaccharides and derivatives and purified saponins from QuilA. The protein can also be presented to the immune system within microparticles such as liposomes or immunostimulating complexes (ISCOMs). As noted, a formulation containing the mutant protein/peptide fragments of the present disclosure may be designed for oral or intranasal ingestion.

[0122] The therapeutically effective and non-toxic dose of the immunogenic compositions of the present disclosure can be determined by a person of ordinary skill in the art. For example, the specific dose for any subject can depend upon a variety of factors including (but not limited to) age, general health, diet of the patient, time and route of administration, synergistic effects with other drugs being administered, and whether the immunogenic composition is administered repeatedly. If necessary the immunogenic composition will be administered repeatedly with one to three month intervals between each dose and with an optional booster dose later in time. Actual methods of preparing the appropriate dosage forms are known, or will be apparent, to those skilled in this art; see, for example, Remington's Pharmaceutical Sciences latest edition.

[0123] As noted above, the present disclosure includes polynucleotides which encode the herein-described mutant polypeptides and active fragments of the present disclosure. The polynucleotides may be in the form of RNA or in the form of DNA (including, but not limited to, cDNA, genomic DNA, and synthetic DNA). The DNA may be double-stranded or single-stranded, and if single stranded, may be the coding strand or non-coding (anti-sense) strand.

[0124] Shown in Table 2 are DNA sequences (and corresponding amino acid sequences) which directly encode (or encode via the reverse complement) the native sequences of the CDCs contemplated herein and thus which also may be mutated to form the mutant forms described or otherwise contemplated herein.

Table 2: Amino Acid and Nucleic Acid Sequences of Native CDC Forms

	SEQ ID NO: (Amino Acids)	SEQ ID NO: (Nucleic Acid)
Pneumolysin	1	20
Cereolysin	2	21
Anthrolysin	3	22
Thuringiolysin	4	23
Perfringolysin	5	24
Alveolysin	6	25
Caniolysin	7	26
Equisimilysin	8	27
Streptolysin O	9	28
Novyiolysin	10	29
Tetanolysin	11	30
Ivanolysin	12	31
Listeriolysin O	13	32
Seeligeriolysin	14	33
Suilysin	15	34
Mitilysin	16	35
Intermedilysin	17	36
PAF	18	37
Pyolysin	19	38

[0125] Host cells are genetically engineered (transduced, transformed, and/or transfected) with the vectors comprising a polynucleotide encoding a mutant polypeptide of the present disclosure. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media that can be modified as appropriate for activating promoters, selecting transformants, or amplifying the polynucleotides which encode such polypeptides. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. Vectors include chromosomal, nonchromosomal, and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

[0126] The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

[0127] The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli* lac or trp, the phage lambda P_L promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

[0128] In addition, in certain non-limiting embodiments, the expression vectors contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

[0129] The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the proteins.

[0130] As representative (but non-limiting) examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli, Streptomyces, Salmonella typhimurium*; fungal cells, such as yeast; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells, such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host cell is deemed to be within the scope of those skilled in the art from the teachings herein.

[0131] More particularly, the present disclosure also includes recombinant constructs comprising one or more of the sequences as described and enabled herein. The constructs comprise a vector, such as a plasmid or viral vector, into which a polynucleotide sequence has been inserted in a forward or reverse orientation. In one non-limiting embodiment, the construct further comprises regulatory sequences, including, for example, a promoter operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of non-limiting example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen, Inc., Hilden, Germany), pBS, pD10, phagescript, psiX174, pbluescript SK, pBS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene, San Diego, CA); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia, Stockholm, Sweden). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene, San Diego, CA) pSVK3, pBPV, pMSG, pSVL (Pharmacia, Stockholm, Sweden). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

[0132] Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L, and TRP. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

[0133] In a further embodiment, the present disclosure includes host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as (but not limited to) a mammalian cell; a lower eukaryotic cell, such as (but not limited to) a yeast cell; or a prokaryotic cell, such as (but not limited to) a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, electroporation (Davis et al., Basic Methods in Molecular Biology (1986) Elsevier Science Publishing Co., Inc., New York, NY), or any other suitable technique.

[0134] The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the present disclosure can be synthetically produced by conventional peptide synthesizers.

[0135] Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present disclosure. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Green and Sambrook (Molecular Cloning: A Laboratory Manual, Fourth Edition, Cold Spring Harbor, N.Y., (2012)), the entire disclosure of which is hereby incorporated by reference.

[0136] Transcription of the DNA encoding the mutant polypeptides of the present disclosure by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0137] Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

[0138] Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host.

[0139] As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Amersham Pharmacia Biotech, Piscataway, N.J., USA) and pGEM1 (Promega, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

[0140] Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction), and cells are cultured for an additional period.

[0141] Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

[0142] Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, a French press, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art. However, it may be desired (but non-limiting) to use host cells which secrete the polypeptides of the present disclosure and permit recovery of the polypeptide from the culture media.

[0143] Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman (Cell (1981) 23:175), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

[0144] The polypeptides can be recovered and/or purified from recombinant cell cultures by well-known protein recovery and purification methods. Such methodology may include ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. In this respect, chaperones may be used in such a refolding procedure. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[0145] The mutant polypeptides that are useful as immunogens in the present disclosure may be products of chemical synthetic procedures, or products of recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect, and mammalian cells in culture), as explained previously. Depending upon the host employed in a recombinant production procedure, the mutant polypeptides of the present disclosure may be glycosylated or may be non-glycosylated.

[0146] The individually expressed polypeptides may be isolated by recombinant expression/isolation methods that are well-known in the art. Typical examples for such isolation methods may utilize an antibody to a conserved area of the protein or to a His tag or cleavable leader or tail that is expressed as part of the protein structure.

[0147] As noted, fragments and variants of the CDC mutant proteins disclosed or otherwise contemplated herein are considered to be a part of the present disclosure. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part, but not all, of the amino acid sequence of the native or mutant polypeptides. The fragments can be "free-standing" or contained within a larger polypeptide of which the fragment forms a part or a region, such as (but not limited to) as a single continuous region. Particular non-limiting fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the present disclosure, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that are antigenic or immunogenic in an animal, particularly a human. In this aspect, the present disclosure includes:

(i) fragments of a mutant CDC, for example (but not by way of limitation) at least about 20-100 amino acids in length, or about 100-200 amino acids in length, and (ii) a pharmaceutical composition comprising the mutant fragment.

[0148] In one embodiment, the nucleic acids which encode the CDC mutants described herein are hybridizable to the corresponding native sequence under high stringency hybridization conditions. An example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5 X SSC, 5 X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured carrier DNA followed by washing two times in 2 X SSC and 0.5% SDS at room temperature and two additional times in 0.1 X SSC and 0.5% SDS at 42°C.

[0149] Nucleic Acid Constructs/Expression Vectors

[0150] As noted, the nucleic acids contemplated herein may be incorporated into heterologous nucleic acid constructs or vectors that are capable of introduction into and replication in a host cell. Any vector may be used as long as it is replicable and viable in the cells into which it is introduced. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The appropriate DNA sequence may be inserted into a plasmid or vector (collectively referred to herein as "vectors") by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by standard procedures. Such procedures and related sub-cloning procedures are deemed to be within the scope of knowledge of those skilled in the art.

[0151] Heterologous nucleic acid constructs of the present disclosure may include the coding sequence for the mutant CDCs contemplated herein or fragments thereof: (i) in isolation; (ii) in combination with additional coding sequences, such as (but not limited to) fusion protein or signal peptide coding sequences, where the mutant CDC coding sequence is the dominant coding sequence; (iii) in combination with non-coding sequences, such as (but not limited to) introns and control elements, such as promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in a suitable host; and/or (iv) in a vector or host environment in which the mutant CDC coding sequence is a heterologous gene.

[0152] Appropriate vectors are typically equipped with a selectable marker-encoding nucleic acid sequence, insertion sites, and suitable control elements, such as promoter and termination sequences. The vector may comprise regulatory sequences, including, for example, non-coding sequences such as introns and control elements, i.e., promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in host cells (and/or in a vector or host cell environment in which a modified soluble protein antigen coding sequence is not normally expressed), operably linked to the coding sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, many of which are commercially available.

[0153] Exemplary promoters include both constitutive promoters and inducible promoters, examples of which include a CMV promoter, an SV40 early promoter, an RSV promoter, an EF-1α promoter, a promoter containing the tet responsive element (TRE) in the tet-on or tet-off system, the beta actin promoter and the metallothionein promoter that can upregulated by addition of certain metal salts. A promoter sequence is a DNA sequence which is recognized by the host cell for expression purposes. It is operably linked to the DNA sequence encoding the mutant polypeptide.

[0154] Unless otherwise indicated, the practice of the compositions and methods of the present disclosure employs conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of one of ordinary skill in the art.

EXAMPLES

[0155] Examples are provided hereinbelow. However, the embodiments of the present disclosure are not limited in application to the specific experimentation, results and laboratory procedures described herein. Rather, the Examples are simply provided as among various embodiments and are meant to be exemplary, not exhaustive, and it will be appreciated that additional and different embodiments of the teachings of the present disclosure will doubtless suggest themselves to those of skill in the art; therefore, such other embodiments are considered to have been inferred from the disclosure herein.

Example 1

[0156] The cholesterol dependent cytolysins (CDCs) are a large family of pore-forming polypeptide toxins produced by more than 20 different species of Gram-positive bacteria[1]. Initially, the bacteria secrete these toxins as stable water-soluble monomers. The monomer binds to membranes and undergoes a specific sequence of structural changes, which promotes oligomerization and pore formation. As the name indicates, the CDC pore forming mechanism is absolutely dependent upon membrane cholesterol for its pore-forming mechanism. The dogma for several decades has been that cholesterol is the receptor for these toxins and that the conserved undecapeptide, located in domain 4 (D4) of the CDCs (Fig. 2), is important to the interaction of the CDCs with cholesterol[2-4]. However, other studies have suggested that the undecapeptide does not mediate the initial binding of these CDCs to cholesterol-rich membranes[5,6]. Hence, the structural components of these CDCs that mediate their binding to cholesterol have been vague prior to the present work.

[0157] The sensitivity of the CDC mechanism to oxidation has been known for over 80 years[7], and this trait was responsible for the title of "thiol-activated cytolysins" that was originally given to these toxins (reviewed in reference 8). The oxidation of this thiol group results in a significant loss of cytolytic activity, often >99%[2]. It was subsequently shown via sequence analysis of a great number of the CDCs that the cysteine having the sensitive thiol group resided in the conserved undecapeptide (ECTGLAWEWWR-SEQ ID NO:39), since this is the only cysteine present in most sequenced CDCs. The loss of cytolytic activity associated oxidation of this thiol group has been suggested to result from alterations in binding to cholesterol-rich membranes[2], thus establishing a putative link between membrane binding and the undecapeptide. The highly conserved nature of the undecapeptide also suggested a highly conserved function, perhaps mediating a direct interaction with membrane cholesterol.

[0158] The dogma that cholesterol is the receptor for the CDCs was complicated by the discovery of intermedilysin (ILY), a CDC that is secreted by *Streptococcus intermedius*. In contrast to other CDCs, ILY is human cell specific[9,10], a feature that is explained by its ability to specifically bind to human CD59, a species-specific inhibitor of the complement membrane attack complex[11,12], rather than cholesterol-rich membranes[13]. Therefore, at least two classes of CDCs now exist, ILY that binds to a specific non-sterol receptor and PFO-like CDCs that bind directly to cholesterol-rich membranes. Yet, the cytolytic mechanisms of both types of CDCs are sensitive to membrane cholesterol and neither is active on membranes that are substantially depleted of cholesterol[14]. These studies, therefore, presented an enigma; does cholesterol contribute to the ILY mechanism in a significantly different way than to the PFO-like CDCs, or is there a unifying molecular basis for the contribution of cholesterol to both classes of CDCs?

[0159] Giddings et al.[14] showed that cholesterol-depletion of hRBC membranes blocked prepore to pore conversion for all CDCs, but also affected binding of PFO-like CDCs, to the membrane. Soltani et al.[15] showed that disrupting the membrane insertion of the L1-L3 D4 loops (Fig. 2) of ILY also blocks prepore to pore conversion. Therefore, two distinct phenomena block prepore to pore conversion in ILY, depletion of membrane cholesterol[14] and disruption of the membrane insertion of the L1-L3 loops[15].

[0160] Based on these observations, a detailed investigation of the interaction of the D4 loops and undecapeptide of ILY and PFO with membranes was performed. The results of these studies indicate that the L1-L3 loops at the base of domain 4 are the primary structures that recognize cholesterol-rich membranes, rather than the undecapeptide. The interaction of these loops with cholesterol-rich membranes mediates the interaction of PFO with cholesterol-rich membranes whereas their insertion into the membrane is also necessary for the prepore to pore conversion of both PFO and ILY. Hence, these results now provide the structural basis for cholesterol sensitivity of the CDCs and provide a unifying explanation for the effect of cholesterol on both ILY and PFO-like CDCs, which use different membrane receptors.

[0161] Materials and Methods of Example 1

[0162] *Bacterial strains, plasmids, and chemicals*

[0163] The genes for ILY and PFO were cloned into pTrcHisA (Invitrogen) as described previously[14,16]. All mutations were made in the native ILY (naturally cysteine-less) or the cysteine-less PFO (PFO^{C459A}) background. Native PFO contains a cysteine at residue 459 that has been changed to alanine to generate the cysteine-less PFO derivative PFO^{C459A}. Both PFO and PFO^{C459A} exhibit similar cytolytic activities[16]. All chemicals and enzymes were obtained from Sigma, VWR, and Research Organics. All fluorescent probes were obtained from Molecular Probes (Invitrogen).

[0164] *Generation and purification of ILY and its derivatives*

[0165] Using PCR QuikChange mutagenesis (Stratagene), various amino acid substitutions were made in native ILY or PFO^{C459A}. DNA sequences of the mutant versions of the ILY gene were analyzed by the Oklahoma Medical Research Foundation Core DNA Sequencing Facility. The expression and purification of recombinant ILY and its derivatives from *Escherichia coli* were carried out as described[15,16]. The eluted protein was dialyzed into buffer (300mM NaCl, 10mM MES, 1mM EDTA, pH 6.5) overnight at 4°C. The protein was then stored in 5mM DTT and 10% (vol/vol) sterile glycerol at -80°C.

[0166] *Chemical modification of ILY and PFO and their derivatives with sulfhydryl specific reagents.*

[0167] The cysteine derivatives of ILY were modified with the environmentally sensitive probe iodoacetamido-*N,N'*-dimethyl-*N*-(7-nitrobenz-2-oxa-1,3-diazolyl)ethylene-diamine (NBD) via the sulfhydryl group. The reaction was carried out as previously described[14]. The modified protein was stored in 10% (vol/vol) sterile glycerol, quick frozen in liquid nitrogen, and stored at -80°C. Proteins were labeled at an efficiency of 75% or greater.

[0168] *Fluorescence measurements*

[0169] All fluorescence intensity measurements were performed using an SLM-8100 photon counting spectrofluorimeter as previously described[16]. For NBD measurements, an excitation wavelength of 460-480 nm and an emission wavelength of 540 nm were used with a bandpass of 4 nm. Emission scans from 500-600 nm for each sample were carried out at a resolution of 1 nm with an integration time of 1 s. Samples containing 10 μg of total toxin were incubated with human red blood cell (hRBC) ghost membranes (equivalent to 303.25 μg of membrane protein) in PBS [10 mM Na_2HPO_4, 2mM KH_2PO_4, 137 mM NaCl, 3 mM KCl (pH 7.5)] at 37°C for 5-10 minutes before making spectral measurements.

[0170] *Liposome preparation*

[0171] Liposomes containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; Avanti Polar Lipids) and cholesterol at a ratio of 45:55 mol % were prepared as described[16].

[0172] *HRBC ghost membrane preparation*

[0173] HRBC ghost membranes were prepared as previously described. Membrane protein content was quantified using the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc.) also previously described[14,16].

[0174] *Cholesterol Depletion and Repletion*

[0175] Cholesterol extraction was performed with methyl-β-cyclodextrin (MβCD) as previously described[14]. Briefly, human huRBC ghost membranes were incubated with a final concentration of 20 mM – 40 mM MβCD (made fresh for each use) at 37°C for 2 hours. The membranes were washed three times by repeated centrifugation (14,000 rpm for 20 min at 4°C) and resuspended in PBS to remove excess MβCD. Ghost membranes were finally suspended in PBS. Cholesterol content was measured using Cholesterol/Cholesteryl Ester Quantitation Kit (Calbiochem, Billerica, MA). Typically the cholesterol content of the membranes was decreased >90% by this method.

[0176] Cholesterol repletion was performed using cholesterol loaded MβCD. This method has been described previously[14]. Briefly, freshly made MβCD was added to buffer A (140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, pH 6.5) to a final concentration of 5 mM. 100mM stock of cholesterol was made in a 1: 2 (vol/vol) of chloroform: methanol. Buffer A + MβCD was heated to 80°C in a glass container. Once heated to 80°C, suspended cholesterol was added to a final concentration of 4 mM. The solution was homogenized by sonication (4 X 20 s). Then the solution was filtered using .22 μm filter. MβCD loaded with cholesterol was added to pelleted cholesterol depleted ghost membranes and incubated for 2 hours at 37°C. The membranes were washed by repeated centrifugation as before and finally, resuspended in PBS.

[0177] *Immobilization of Liposomes on L1 SPR Sensor Chip*

[0178] Surface plasmon resonance (SPR) was measured with a BIAcore 3000 system using a L1 sensor chip (BIAcore, Uppsala, Sweden). The L1 sensor chip contains a dextran matrix to which hydrophobic residues are covalently bound and has routinely been used for immobilization of liposomes. In preparation of the L1 chip for liposomes, 10 µl of 20 mM CHAPS was injected at a flow rate of 10 µl/min. Liposomes (0.5 mM final lipid concentration) were then injected at the same flow rate for 10 min. After injection of liposomes, 50 mM NaOH was injected for 3 min to remove the multiple layers of lipids. This was followed by injection of 0.1 mg/ml BSA to coat the nonspecific binding sites. All injections were performed at 25°C. The L1 chip was regenerated and striped of liposomes by repeated injections of 20 mM CHAPS and 50 mM NaOH until original RU reading was reached. The regeneration procedure did not result in loss of sensor chip binding capacity.

[0179] *SPR Analysis*

[0180] All analysis of interaction between the liposomes and PFO derivatives were performed in HBS at 25°C. Wild type PFO (50 ng/µl) and the PFO aspartate mutants (50 ng/µl) were injected over the liposome coated chip at a flow rate of 30 µl/min for 4 mins.

[0181] Results of Example 1

[0182] *Experimental strategy.* ILY does not depend on membrane cholesterol to bind to native membranes, but its mechanism still remains sensitive to cholesterol. Unlike the PFO-like CDCs that do not bind to membranes that lack cholesterol, receptor binding, and oligomerization of ILY still occurs on cholesterol-depleted membranes[14]. Therefore, ILY was used to first identify structures that were responsible for its cholesterol-dependence. Once the structures of ILY that were sensitive to membrane cholesterol were identified, the effect of disrupting these structures was examined in PFO on its ability to bind to cholesterol-rich liposomal membranes. In this way it could be determined if the same structures in both ILY and PFO were responsible for their cholesterol dependence.

[0183] *Cholesterol is not required for the membrane insertion of the ILY undecapeptide.* Previous studies with ILY have shown the undecapeptide must insert into the membrane in order for the prepore to form[15]. Therefore, it was determined whether or not its insertion was sensitive to membrane cholesterol. A cysteine residue was substituted for Ala-486, which is located within the undecapeptide, and labeled with NBD via its sulfhydryl group. This residue has been shown to insert into the membrane in native ILY[15]. The fluorescence intensity of the NBD in $ILY^{A486C-NBD}$ was measured in the absence and presence of cholesterol-containing membranes or cholesterol-depleted membranes. As shown in Fig. 3, in the presence of hRBC ghost membranes, the undecapeptide inserts into the membrane as shown by the increase in fluorescence emission intensity compared to that observed for ILY in its soluble state. When the membrane is depleted of cholesterol, the same increase in fluorescence emission is observed. These results demonstrate that the membrane insertion of the undecapeptide region near Ala-486 is independent of membrane cholesterol content.

[0184] *Cholesterol is required for the insertion of loops L1, L2, and L3.* The membrane insertion of the three short hydrophobic loops at the tip of D4 (Fig. 2) occurs in concert and is required to anchor and properly orient the CDC monomers on the membrane[15,17]. Their insertion, in concert with the insertion of the undecapeptide, is necessary for the subsequent membrane insertion of the D3 transmembrane β-hairpins (TMHs) that leads to the formation of the transmembrane β-barrel pore[15]. Cholesterol is also required for the insertion of the TMHs and formation of the pore complex[14]. Hence, both membrane cholesterol and the membrane insertion of the L1-L3 loops are prerequisites for prepore to pore conversion[14,15]. Since the membrane insertion of the L1-L3 loops precedes the insertion D3 TMHs, it appeared reasonable that the depletion of membrane cholesterol may block the insertion of the L1-L3 loops that, in turn, would prevent the insertion of the D3 TMHs and block prepore to pore transition. Therefore it was hypothesized that cholesterol is required for membrane insertion of the L1-L3 loops.

[0185] To test this hypothesis, the membrane insertion of the L1-L3 loops into native and cholesterol-depleted huRBC ghost membranes was measured individually. It was recently shown that the ILY residues Leu-518, Ala-424 and Ala-464, located within loops L1, L2, and L3, respectively, insert into the membrane[17]. To measure insertion of each loop, a residue in each loop was mutated to a cysteine (ILY^{A428C}, ILY^{A464C}, ILY^{L518C})[15], and the sulfhydryl group derivatized with NBD. As the NBD located at these sites enters the membrane, its fluorescence emission intensity increases significantly[15,17]. The emission intensity of the NBD was compared between soluble monomeric toxin, toxin bound to huRBC ghost membranes, and toxin bound to cholesterol-depleted ghost membranes.

[0186] In stark contrast to the increase in fluorescence emission intensity seen when each loop inserts into the membrane of native hRBC ghosts, depletion of approximately 90% of the membrane of cholesterol abrogates the membrane insertion of all three loops (Fig. 4, panels a-c). Restoration of cholesterol to the cholesterol-depleted membranes restores the ability of the loops to insert into the membrane (Fig. 4, panels d-f)). Hence, membrane cholesterol is required for the insertion of the L1-L3 loops, and, as shown previously, this insertion is necessary for prepore to pore conversion[14,15].

[0187] *Aspartate substitution of residues in loops L1-L3 of PFO prevents its binding to cholesterol-rich membranes.* The membrane insertion of the L1-L3 loops of ILY was sensitive to cholesterol depletion in native membranes, suggesting that in PFO these same loops might mediate its binding directly to cholesterol-rich membranes. However, this problem could not be approached in PFO in a similar manner to that used with ILY, since cholesterol depletion decreases the binding of PFO to the membrane. Therefore, the effect of mutating these same loops on binding of PFO to cholesterol-rich liposomes was determined. This was accomplished by the introduction of aspartate into loops L1-L3 of PFO, previously shown in ILY to prevent their insertion into the membrane[15]. The insertion of loops L1-L3 is coupled in ILY, and the introduction of an asparate for any single loop residue, Ala-428 (L2), Ala-464 (L3) or Leu518 (L1), blocked their membrane insertion. Therefore, it was predicted that if aspartate was substituted for any one of the analogous residues in PFO, Ala-401, Ala-437 or Leu-491, it would disrupt binding of PFO to cholesterol-rich liposomes.

[0188] Individual substitution of the analogous residues in PFO, Ala-401 (L2), Ala-437 (L3) and Leu-491 (L1), resulted in a loss of greater than 99.7% of the hemolytic activity for each mutant (data not shown). Binding of the PFO mutants to cholesterol-PC liposomes was measured by surface plasmon resonance (SPR). As shown in Fig. 5a, these mutations significantly reduced binding to cholesterol-PC liposomes when examined by SPR. Substitution of aspartate for Ala-401 (L2) or Leu-491 (L1) completely abrogated binding of PFO to the liposomes membranes, and binding by the aspartate substituted Ala-437 (L3) was less than 7% that of wild type (Fig. 5b). This result indicates the D4 L1-L3 loops are critical to the interaction of PFO-like CDCs with cholesterol-rich membranes.

[0189] *Modification of Cys-459 of PFO blocks the membrane insertion of the undecapeptide tryptophan residues, but not membrane binding of PFO.* The conserved undecapeptide of the PFO-like CDCs has been long thought to participate in their binding to cholesterol rich membranes, primarily because chemical modification of the sulfhydryl group of the native cysteine (Cys-459) of the undecapeptide was reported to significantly impact PFO binding to low cell numbers of sheep RBCs, but not to high cell numbers[2]. Others, however, have shown that its modification does not appear to affect binding of other CDCs to cells[5,6]. Therefore, the abilities of native PFO and PFO modified via the sulfhydryl group of Cys-459 of the undecapeptide to bind to cholesterol-PC liposomes via SPR were compared.

[0190] Modification of the PFO undecapeptide Cys-459 thiol with the sulfhydryl specific reagent N-ethylmaleimide (NEM) reduced the hemolytic activity by 99% (data not shown), similar to other reports in which the cysteine sulfhydryl of PFO and SLO were chemically modified[2,18]. The rate and extent of binding, however, of the NEM-modified toxin was increased over that of native toxin, as determined by SPR analysis (Fig. 6A-B). Therefore, chemical modification of Cys-459 did not disrupt binding of PFO to the membrane.

[0191] If modification of Cys-459 did not affect binding, it raised the question of what this modification does to PFO that effectively blocked its activity. Since the discovery of the CDCs nearly 90 years ago, it has been known that their cytolytic mechanism was sensitive to oxidation. The oxidation sensitive residue was ultimately linked to the highly conserved undecapeptide cysteine residue[1]. The structural effects of the cysteine modification on PFO were further examined to determine if its modification prevented a structural change in PFO that could impact its activity. The membrane insertion of the undecapeptide tryptophans 464, 466 and 467 is conformationally coupled to the insertion of the D3 TMHs. Previous studies have shown that mutations in the D3 TMH1 residues that increase their rate of insertion also increase the rate of membrane insertion of the undecapeptide tryptophan residues[19]. Since Cys-459 is juxtaposed to the tryptophan residues, it was determined if chemical modification of the cysteine thiol group blocked the membrane insertion of the tryptophan residues.

[0192] The membrane insertion of the undecapeptide tryptophan residues can be monitored by the increase in their intrinsic fluorescence intensity as they move into the nonpolar environment of the membrane[20,21]. The insertion of these tryptophans was measured in the NEM-modified and native PFO (Fig. 6a and 6b). The modification of Cys-459 blocked the insertion of the undecapeptide tryptophans, but did not prevent it from forming an SDS-resistant oligomer, similar to native PFO (data not shown). Hence, these data show that the conformational change in the PFO structure that is reflected by the loss of the insertion of the undecapeptide tryptophan residues affects the subsequent conversion of the prepore oligomer to the pore complex.

[0193] Immunization with Pneumolysin Mutant Leu 460Asp

[0194] CBA/CAHN-XID mice were immunized subcutaneously with 5 μg of pneumolysin or pneumolysin mutant, using alum (aluminum hydroxide) as the adjuvant on days 0 and 14. On day 21, the mice were immunized with the proteins in diluent alone (no adjuvant). All injections were given in 0.2 ml volume. On day 35, mice were challenged with capsular type 19F strain

EF3030. Seven days later, the mice were euthanized with carbon dioxide gas. The lungs were homogenized, and the numbers of colony forming units (CFU) in the lungs of each mouse was determined by plating the homogenized tissue on blood agar plates. The mice were also bled. No pneumococci were observed in the blood, demonstrating that this is a model of pneumonia and not pneumonia and sepsis. The results show that both wild-type and the mutant pneumolysin were able to protect against pneumonia in a focal pneumonia model in mice (Fig. 7).

[0195] Two long-standing hallmarks of the CDCs are the dependence of their pore-forming mechanism on the presence of membrane cholesterol and the reversible inactivation of most CDCs by oxidation of the undecapeptide cysteine. The studies herein resolve the molecular basis for both phenomena. Without wishing to be bound by theory, the membrane insertion of the L1-L3 loops, located at the base of domain 4, appears to be the primary event that is sensitive to the presence of membrane cholesterol for ILY. Upon cholesterol depletion, these loops do not insert into the membrane, and, as shown previously, cholesterol extraction from hRBC membranes[15] prevents the prepore to pore conversion of ILY. These results indicate that both effects also result from the inability of these loops to insert into cholesterol-depleted membranes. These data further indicate that the oxidation of the conserved cysteine in PFO, and presumably other PFO-like CDCs, blocks the membrane insertion of the tryptophan residues that trap PFO in a prepore state, but does not affect binding to cholesterol-rich liposomes.

[0196] The discovery of ILY, a human cell specific toxin, presented a conundrum of how ILY could discriminate between human and animal cells if cholesterol was its receptor. The human cell specificity of ILY was explained by the discovery that human CD59, a late stage, species-specific complement inhibitor, was its receptor[13]. Even though cholesterol was not the ILY receptor, its pore-forming mechanism remained sensitive to membrane cholesterol[14], and showed that cholesterol was required for a much later stage of the pore-forming mechanism in ILY; substantial depletion of membrane cholesterol blocked prepore to pore conversion. Interestingly, this was also observed for SLO and PFO[14], two CDCs that can bind directly to cholesterol-rich membranes. Although depletion of membrane cholesterol from hRBCs blocked prepore to pore conversion of PFO, it also decreased PFO binding. Therefore, cholesterol is necessary for prepore to pore conversion for all three CDCs and in addition it also contributes to membrane binding by the PFO-like CDCs.

[0197] Recently Soltani et al.[15] showed that the membrane insertion of the L1-L3 D4 loops of ILY is necessary for prepore to pore conversion. Hence, both cholesterol and membrane insertion of the L1-L3 loops were necessary for prepore to pore conversion of ILY. Without wishing to be bound by theory, the data presented herein indicates that a unifying explanation for these observations is that the membrane insertion of these loops only occurs in cholesterol-rich membranes, and this insertion is necessary for the prepore to pore conversion of both ILY and PFO-like CDCs. In addition, the ability of these loops to insert into cholesterol-rich membranes also mediates the initial binding of PFO, and presumably the PFO-like CDCs, to cholesterol-rich membrane surfaces. Therefore, these data indicate that in both ILY and PFO-like CDCs, the L1-L3 loops must insert into the membrane in order for the successful formation of the pore complex. In the case of ILY, binding is mediated first by huCD59 followed by the insertion of the L1-L3 loops into cholesterol-rich membranes, whereas these two events, binding and insertion, are one and the same in PFO and are mediated primarily by the L1-L3 loops.

[0198] It has been traditionally accepted that the undecapeptide of the PFO-like CDCs contributed or directly mediated the recognition of cholesterol-rich membranes[2,3,21]. The studies herein indicate that the L1-L3 loops are the primary structures that mediate the interaction between the CDCs and cholesterol-rich membranes. Although chemical modification of the PFO undecapeptide cysteine with NEM decreases its hemolytic activity by more than 99%, its binding to cholesterol-PC liposomes is largely unimpaired. Hence, in contrast to existing dogma, the interaction of PFO, and other PFO-like CDCs, is primarily mediated by loops L1-L3 and not the undecapeptide. Mutations within the undecapeptide could influence the interaction of L1-L3 with cholesterol rich membranes. It has been shown that mutation of undecapeptide Trp-491 of ILY blocks the insertion of L1-L3[15], and the altered structure of the native ILY undecapeptide apparently prevents the direct interaction of L1-L3 with cholesterol-rich membranes, thus allowing it to first bind to huCD59. This latter idea is reinforced by the fact that when the consensus undecapeptide structure was introduced into ILY, it enabled it to bind to nonhuman cells[22].

[0199] It is curious why the L1-L3 loops of ILY do not mediate binding to cholesterol rich membranes similar to PFO. As suggested above, it appears that the major difference in domain 4 between is the primary structure of the highly conserved undecapeptide. It is clear that ILY has lost the ability to bind directly to cholesterol-rich membranes; otherwise, it would not exhibit the human cell specificity mediated via huCD59. The crystal structures of D4 of ILY and PFO may provide an explanation for this difference in the L1-L3 loops to mediate direct binding of these two CDCs to cholesterol-rich membranes. The location and orientation of L1-L3 residues (Leu-518, Ala-428, and Ala-464) of ILY are nearly identical to the analogous residues in PFO (Leu-

491, Ala-401, and Ala-437) (Fig. 4b). In fact, the majority of the D4 structure of the two CDCs is nearly identical (rms deviation of less than 0.6 Å, reference 23), with the exception of the undecapeptide loop and a β-tongue structure at the top of domain 4. The undecapeptide loop of ILY extends down from the base of D4 4-5Å further than the PFO undecapeptide. Hence, the ILY undecapeptide may sterically hinder the interaction of the L1-L3 loops of ILY with the cholesterol-rich surface. Perhaps only after binding to receptor is the ILY undecapeptide structure altered in such a way as to permit the insertion of the L1-L3 loops.

[0200] The present disclosure reveals a structural basis for the severe effect on activity that oxidation of the undecapeptide cysteine exhibits on the cytolytic mechanism of PFO, and presumably other PFO-like CDCs. Originally the CDCs were termed the thiol-activated cytolysins due to this feature, but the molecular basis for this effect was unknown. Early studies suggested that binding to RBCs was affected, but at the same time binding to cholesterol was unaffected, and non-lytic oligomers were still observed on the surface of the cells[2]. As shown herein, this modification prevents the insertion of the undecapeptide tryptophans and results in a prepore-trapped oligomeric structure. Although the precise structural basis for this effect is not known, previous studies have shown that the membrane insertion of the domain 3 TMHs, that form the transmembrane â-barrel pore, is conformationally coupled to the membrane insertion of the domain 4 undecapeptide tryptophan residues[19]. Hence, preventing the membrane insertion of these tryptophans may prevent the insertion of the domain 3 TMHs, thus trapping PFO in the prepore state.

Example 2

[0201] His-tagged $PLY_{wildtype}$ and PLY mutants (PLY_{L460D} and $PLY_{L460D/G293S}$) were purified using an affinity column. The average protein yield was $PLY_{wildtype}$: 1 mg/ml, PLY_{L460D}: 1 mg/ml, and $PLY_{L460D/G293S}$: 4 mg/ml. The purified proteins were tested for hemolytic activity by incubation of serially titrated toxins with human red blood cells (RBCs). The EC_{50} (effective concentration required to lyse 50% RBCs) was calculated for each protein from a non-linear sigmoidal dose-response curve. The fold change from wildtype PLY was determined for each mutant (fold change = EC_{50} wildtype/EC_{50} mutant). Results for the two PLY mutants are reported as being more than a certain fold less active than wildtype PLY as the 100% RBCs lysis required for an accurate dose-response curve was unattainable at the highest protein concentrations of these derivatives. The decrease in hemolytic activity of the mutant in comparison to the wild type protein (fold-less active than $PLY_{wildtype}$) for PLY_{L460D} was >10,000 fold and for $PLY_{L460D/G293S}$ >260,000 fold.

[0202] The relative stability of a protein is inferred from calculating the melting temperature (T_M Celsius), the temperature at which 50% of the protein has unfolded. Protein melt curves were generated using a Protein Thermal Shift Assay Dye Kit (Applied Biosystems). As the temperature is increased the protein unfolds and the dye is able to bind exposed hydrophobic regions and fluoresce. The T_m for $PLY_{wildtype}$, PLY_{L460D}, and $PLY_{L460D/G293S}$ were 47.50 ± 0.2, 47.69 ± 0.2, 47.97 ± 0.2, respectively. The insignificant differences in T_M reported for the three PLY proteins indicate that introducing the indicated mutations into $PLY_{wildtype}$ had no effect on the stability of the proteins.

[0203] As indicated above, in one non-limiting embodiment, the pneumolysin mutant of the present disclosure is a double mutant designated as PLY-L460D/G293S ($PLY_{L460D/G293S}$) wherein the L of position 460 is substituted with D and the G of position 293 is substituted with S. By itself the G293S substitution only decreases hemolytic activity of the PLY mutant by about 50-fold. The L460D substitution, by itself, decreases activity of the PLY mutant by about 5000-10,000 fold. But in a PLY mutant with both substitutions, the decrease in activity exceeds 260,000-fold less than the native PLY toxin. This is a geometric decrease, not merely an "additive" decrease in activity.

[0204] Without wishing to be bound by theory, this precipitous decrease in activity is due to the blockage of two essential functions of PLY. First, the L460D substitution blocks binding to cholesterol and second, the G293S substitution traps PLY in a prepore state that cannot insert the β-barrel pore (the prepore is defined as membrane bound monomers that have oligomerized in the ring like structure, but cannot insert the β-barrel pore). Therefore, the inhibitory effect is geometric: 50 X 5000-10,000 >250,000 fold less toxic (less hemolytic), which is in accordance with measurements that indicate it is >260,000-fold less toxic than native PLY. The G293S substitution also stabilizes the monomer structure of the L460D mutant protein and increases the yield upon purification. For example, upon purification from a 1.0 liter E. coli culture, the yield of the PLY-L460D/G293S mutant was approximately 52 mg vs. 3 mg of the PLY–L460D mutant (about 17X greater).

[0205] Accordingly, in certain embodiments, the present disclosure is directed to a purified or isolated mutant pneumolysin polypeptide comprising an amino acid sequence that is at least about 90% identical to SEQ ID NO:1 and having an amino acid substitution in at least one of amino acid positions 458, 459, and 460, and in at least one of amino acid positions 293 and 294.

The mutant pneumolysin polypeptide may have reduced hemolytic activity and reduced pore forming activity as compared to a wild type pneumolysin polypeptide. The amino acid sequence may be at least about 91% identical, at least about 92% identical, at least about 93% identical, at least about 94% identical, at least about 95% identical, at least about 96% identical, at least about 97% identical, at least about 98% identical, or at least about 99% identical to SEQ ID NO:1. The mutant pneumolysin polypeptide may include amino acid substitutions in amino acid positions 293 and 458; 293 and 459; 293 and 460; 294 and 458; 294 and 459; or 294 and 460. The mutant pneumolysin polypeptide may include amino acid substitutions in amino acid positions 293, 458, and 459; 293, 459, and 460; or 293, 458, and 460. The mutant pneumolysin polypeptide may include amino acid substitutions in amino acid positions 294, 458, and 459; 294, 459, and 460; or 294, 458, and 460. The mutant pneumolysin polypeptide may include amino acid substitutions in amino acid positions 293, 294, 458, and 459; 293, 294, 459, and 460; or 293, 294, 458, and 460. The mutant pneumolysin polypeptide may include amino acid substitutions in amino acid positions 293, 294, 458, 459, and 460. The mutant pneumolysin polypeptide may comprise a serine or threonine in amino acid position 293, and an aspartic acid, glutamic acid, or asparagine in amino acid position 460. The amino acid sequence of the purified mutant pneumolysin polypeptide may be SEQ ID NO:40. The mutant pneumolysin polypeptide may have an increased yield over a wild type pneumolysin or over a mutant pneumolysin polypeptide having a substitution in only one of amino acid positions 293, 294, 458, 459, and 460. The mutant pneumolysin polypeptide may have about 250,000 fold less hemolytic activity than a wild type pneumolysin polypeptide.

[0206] In another embodiment, one or more of the mutant pneumolysin polypeptides described herein above or otherwise contemplated herein may be disposed in a pharmaceutically-acceptable excipient to form an immunogenic composition. Another embodiment includes a vaccine that includes the immunogenic composition, and which may optionally contain an adjuvant. Yet another embodiment is a nucleic acid sequence which encodes any of the mutant pneumolysin polypeptides described or otherwise contemplated herein; a further embodiment is directed to a host cell that includes said nucleic acid sequence. A yet further embodiment is directed to a method of treating, prophylactically preventing, or reducing the occurrence of a condition, disease, or infection caused by *Streptococcus pneumoniae*; in the method, a therapeutically-effective amount of any of the immunogenic compositions described or otherwise contemplated herein is administered to a subject.

[0207] In other embodiments, the present disclosure is directed to a purified or isolated mutant streptolysin O polypeptide comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 9 and having an amino acid substitution in at least one of amino acid positions 561 and 562, and in at least one of amino acid positions 395 and 396. The mutant streptolysin O polypeptide may have reduced hemolytic activity and reduced pore forming activity as compared to a wild type streptolysin O polypeptide. The amino acid sequence may be at least about 91% identical, at least about 92% identical, at least about 93% identical, at least about 94% identical, at least about 95% identical, at least about 96% identical, at least about 97% identical, at least about 98% identical, or at least about 99% identical to SEQ ID NO: 9. The mutant streptolysin O polypeptide may include amino acid substitutions in amino acid positions 395 and 561; 395 and 562; 396 and 561; and/or 396 and 562. The mutant streptolysin O polypeptide may include amino acid substitutions in amino acid positions 395, 561, and 562; 396, 561, and 562; 395, 396, and 561; 395, 396, and 562; and 395, 396, 561 and 562. The mutant streptolysin O polypeptide may have an increased yield over a wild type streptolysin O or over a mutant streptolysin O polypeptide having a substitution in only one of amino acid positions 395, 396, 561, and 562. The mutant streptolysin O polypeptide may have about 250,000 fold less hemolytic activity than a wild type streptolysin O polypeptide.

[0208] In another embodiment, one or more of the mutant streptolysin O polypeptides described herein above or otherwise contemplated herein may be disposed in a pharmaceutically-acceptable excipient to form an immunogenic composition. Another embodiment includes a vaccine that includes the immunogenic composition, and which may optionally contain an adjuvant. Yet another embodiment is a nucleic acid sequence which encodes any of the mutant streptolysin O polypeptides described or otherwise contemplated herein; a further embodiment is directed to a host cell that includes said nucleic acid sequence. A yet further embodiment is directed to a method of treating, prophylactically preventing, or reducing the occurrence of a condition, disease, or infection caused by *Streptococcus pyogenes*; in the method, a therapeutically-effective amount of any of the immunogenic compositions described or otherwise contemplated herein is administered to a subject.

[0209] Although the present disclosure has been described in detail, it should be understood that various changes, substitutions and alterations can be made in the embodiments described herein without departing from the spirit and scope of the present disclosure. Moreover, the scope of the present disclosure is not intended to be limited to the particular embodiments of the process, compositions of matter, means, methods and steps described in the specification particularly in regard to the specific amino acid or nucleic acid sequences described or enabled herein. As one of ordinary skill in the art will readily appreciate from the present disclosure, processes, compositions of matter, means, methods, sequences, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein. Accordingly, the appended claims are intended to include within their scope all such processes, compositions of matter, means, methods, method steps, amino acid sequences, and nucleic acid sequences.

REFERENCES

The following references are specifically incorporated herein by reference, particularly in regard to the exemplary procedural or other details supplementary to those set forth herein.

1. Alouf, J.E., Billington, S.J. & Jost, B.H. Repertoire and general features of the cholesterol-dependent cytolysins in *Bacterial Toxins: A Comprehensive Sourcebook* (eds. Alouf, J.E. & Popoff, M.R.) 643-658 (Academic Press, London, 2005).
2. Iwamoto, M., Ohno-Iwashita, Y. & Ando, S. Role of the essential thiol group in the thiol-activated cytolysin from Clostridium perfringens. *Eur J Biochem* **167**, 425-430 (1987).
3. Sekino-Suzuki, N., Nakamura, M., Mitsui, K.I. & Ohno-Iwashita, Y. Contribution of individual tryptophan residues to the structure and activity of theta-toxin (perfringolysin o), a cholesterol-binding cytolysin. *Eur J Biochem* **241**, 941-947 (1996).
4. Jacobs, T. et al. The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Lett.* **459**, 463-466 (1999).
5. Vazquez-Boland, J.A., Dominguez, L., Rodriguez-Ferri, E.F., Fernandez-Garayzabal, J.F. & Suarez, G. Preliminary evidence that different domains are involved in cytolytic activity and receptor (cholesterol) binding in listeriolysin O, the Listeria monocytogenes thiol-activated toxin. *FEMS Microbiol Lett* **53**, 95-9 (1989).
6. Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W. & Boulnois, G.J. Pneumolysin, the thiol-activated toxin of Streptococcus pneumoniae, does not require a thiol group for *in vitro* activity. *Infect. Immun.* **57**, 2547-2552 (1989).
7. Neill, J.M. & Fleming, W.L. Studies on the oxidation and reduction of immunological substances: II The hematoxin of the *Welch bacillus. J. Exp. Med.* **44**, 215-226 (1926).
8. Alouf, J.E. & Geoffrey, C. Structure activity relationships in the sulfhydryl-activated toxins. in *Bacterial Protein Toxins* (eds. Alouf, J.E., Fehrenbach, F.J., Freer, J.H. & Jeljaszewicz, J.) 165-171 (Academic Press, London, 1984).
9. Nagamune, H. et al. Intermedilysin. A cytolytic toxin specific for human cells of a *Streptococcus intermedius* isolated from human liver abscess. *Adv Exp Med Biol* **418**, 773-775 (1997).
10. Nagamune, H. et al. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect Immun* **64**, 3093-3100 (1996).
11. Rollins, S.A., Zhao, J., Ninomiya, H. & Sims, P.J. Inhibition of homologous complement by CD59 is mediated by a species-selective recognition conferred through binding to C8 within C5b-8 or C9 within C5b-9. *J Immunol* **146**, 2345-51 (1991).
12. Rollins, S.A. & Sims, P.J. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J Immunol* **144**, 3478-83 (1990).
13. Giddings, K.S., Zhao, J., Sims, P.J. & Tweten, R.K. Human CD59 is a receptor for the cholesterol–dependent cytolysin intermedilysin. *Nat Struct Mol Biol* **12**, 1173-1178 (2004).

14. Giddings, K.S., Johnson, A.E. & Tweten, R.K. Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *Proc Natl Acad Sci U S A* **100**, 11315-11320 (2003).

15. Soltani, C.E., Hotze, E.M., Johnson, A.E. & Tweten, R.K. Specific protein-membrane contacts are required for prepore and pore assembly by a cholesterol-dependent cytolysin. *J. Biol. Chem* . Paper is press 282 (21), 15709-15716, April 5, 2007.

16. Shepard, L.A. et al. Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: an á-helical to â-sheet transition identified by fluorescence spectroscopy. *Biochemistry* **37**, 14563-14574 (1998).

17. Ramachandran, R., Heuck, A.P., Tweten, R.K. & Johnson, A.E. Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. *Nat Struct Biol* **9**, 823-7 (2002).

18. Harris, J.R., Adrian, M., Bhakdi, S. & Palmer, M. Cholesterol-Streptolysin O Interaction: An EM Study of Wild-Type and Mutant Streptolysin O. *J Struct Biol* **121**, 343-55 (1998).

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

20. Heuck, A.P., Tweten, R.K. & Johnson, A.E. Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J Biol Chem* **278**, 31218–31225 (2003).

21. Nakamura, M., Sekino, N., Iwamoto, M. & Ohno-Iwashita, Y. Interaction of theta-toxin (perfringolysin O), a cholesterol-binding cytolysin, with liposomal membranes: change in the aromatic side chains upon binding and insertion. *Biochemistry* **34**, 6513-6520 (1995).

22. Nagamune, H. et al. The human-specific action of intermedilysin, a homolog of streptolysin o, is dictated by domain 4 of the protein. *Microbiol Immunol* **48**, 677-92 (2004).

23. Polekhina, G., Giddings, K.S., Tweten, R.K. & Parker, M.W. Insights into the action of the superfamily of cholesterol-dependent cytolysins from studies of intermedilysin. *Proc Natl Acad Sci* **102**, 600-605 (2005).

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

25. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J Mol Graph* **14**, 33-8, 27-8 (1996).

26. Anonymous: Prevention of Pneumococcol Disease: Recommendations of the Advisory Committee on Immunization Practices (ACIP). Morbidity and Mortality Weekly Report-Recommendations and Reports: **46**:1-24 (April 4, 1997).

What is claimed is:

1. A purified mutant pneumolysin polypeptide comprising:
   an amino acid sequence that is at least 90% identical to SEQ ID NO:1 and having:
      an amino acid substitution in at least one of amino acid positions 458, 459, and 460 of SEQ ID NO:1; and
      an amino acid substitution in at least one of amino acid positions 293 and 294 of SEQ ID NO:1.

2. The purified mutant pneumolysin polypeptide of claim 1, wherein said mutant pneumolysin polypeptide has reduced hemolytic activity and reduced pore forming activity as compared to a wild type pneumolysin polypeptide.

3. The purified mutant pneumolysin polypeptide of claim 1, wherein the amino acid sequence is at least 95% identical to SEQ ID NO:1.

4. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 293 and 458.

5. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 293 and 459.

6. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 293 and 460.

7. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 294 and 458.

8. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 294 and 459.

9. The purified mutant pneumolysin polypeptide of claim 1, comprising amino acid substitutions in amino acid positions 294 and 460.

10. The purified mutant pneumolysin polypeptide of claim 1, having increased yield over a mutant pneumolysin polypeptide having a substitution in only one of amino acid positions 293, 294, 458, 459, and 460.

11. The purified mutant pneumolysin polypeptide of claim 1, having about 250,000-fold less hemolytic activity than wild type pneumolysin polypeptide.

12. The purified mutant pneumolysin polypeptide of claim 1, comprising a serine or threonine in amino acid position 293 and an aspartic acid, glutamic acid, or asparagine in amino acid position 460.

13. The purified mutant pneumolysin polypeptide of claim 1, wherein the amino acid sequence is at least 95% identical to SEQ ID NO:1.

14. The purified mutant pneumolysin polypeptide of claim 1, wherein the amino acid sequence thereof comprises SEQ ID NO:40.

15. An immunogenic composition comprising one or more of the mutant pneumolysin polypeptides of claim 1 disposed in a pharmaceutically-acceptable excipient.

16. A vaccine comprising the immunogenic composition of claim 15.

17. The vaccine of claim 16, further comprising an adjuvant.

18. A nucleic acid sequence encoding the mutant pneumolysin polypeptide of claim 1.

19. A host cell, comprising the nucleic acid sequence of claim 18.

20. A method of treating, prophylactically preventing, or reducing the occurrence of a condition, disease, or infection caused by *Streptococcus pneumoniae*, in a subject in need of such treatment, comprising the step of:

administering to the subject a therapeutically-effective amount of the immunogenic composition of claim 15.

21. The purified mutant pneumolysin polypeptide of claim 1 or 2, wherein the amino acid sequence is at least 95% identical to SEQ ID NO:1.

22. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 293 and 458.

23. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 293 and 459.

24. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 293 and 460.

25. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 294 and 458.

26. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 294 and 459.

27. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, comprising amino acid substitutions in amino acid positions 294 and 460.

28. The purified mutant pneumolysin polypeptide of any one of claims 1-2 and 21, having increased yield over a mutant pneumolysin polypeptide having a substitution in only one of amino acid positions 293, 294, 458, 459, and 460.

29. The purified mutant pneumolysin polypeptide of any one of claims 1-10, 12-14, and 21-28, having about 250,000-fold less hemolytic activity than wild type pneumolysin polypeptide.

30. The purified mutant pneumolysin polypeptide of claim 1-11, 13-14, and 21-29, comprising a serine or threonine in amino acid position 293 and an aspartic acid, glutamic acid, or asparagine in amino acid position 460.

31. The purified mutant pneumolysin polypeptide of any one of claims 1-13 and 21-30, wherein the amino acid sequence is at least 95% identical to SEQ ID NO:1.

32. An immunogenic composition comprising one or more of the mutant pneumolysin polypeptides of any one of claims 2-14 and 21-31 disposed in a pharmaceutically-acceptable excipient.

33. A vaccine comprising the immunogenic composition of claim 32.

34. The vaccine of claim 33, further comprising an adjuvant.

35. A nucleic acid sequence encoding the mutant pneumolysin polypeptide of any one of claims 2-14 and 21-31.

36. A host cell, comprising the nucleic acid sequence of claim 35.

37. A method of treating, prophylactically preventing, or reducing the occurrence of a condition, disease, or infection caused by *Streptococcus pneumoniae*, in a subject in need of such treatment, comprising the step of:

administering to the subject a therapeutically-effective amount of the immunogenic composition of claim 32.

```
                                    |                     |                     |
     Cereolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Anthrolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Thuringiolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
 Perfringolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
     Alveolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
     Caniolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
  Equisimilysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
Streptolysin O M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
    Novyiolysin MK-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Tetanolysin MN-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
      Ivanolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
  Listeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
Seeligeriolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
       Suilysin MR-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
    Pneumolysin MA-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
Mitilysin (PLY) ma-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
  Intermedilysin MK-------- ---------- ---------- ------TKQ- ---------- ---------- ---------- 5
  Viridanolysin MNQEKRLHRF VKKCGLGVCS AVVAAFLLNA QGVALATEQG NRPVETENIA RGKQASQSST AYGGAATRAV 70
       Pyolysin MKR------- ---------- ---------- ---------- ---------- ---------- ---------- 3

                         |                     |                     |                     |
     Cereolysin ---------- ---------- ---------- ---------- --NIKKNTKR RKFLACLLVS LCTINYSSIS 29
    Anthrolysin ---------- ---------- ---------- ---------- FLNIKKNTKR RKFLACLLVS LCTIHYSSIS 32
 Thuringiolysin ---------- ---------- ---------- ---------- FLNIKKNGKR RKFLTCVLVS LCTLNYSSTS 32
 Perfringolysin ---------- ---------- ---------- ---------- ------RFKK TKLIASIAMA LCLFSQPVIS 26
     Alveolysin ---------- ---------- ---------- ---------- -KKKSNHLKG RKVLVSLLVS LQVFAFASIS 30
     Caniolysin ---------- ---------- ---------- ---------- DMSNKKIFKK YSRVAGLLTA ALIVGNLVTA 32
  Equisimilysin ---------- ---------- ---------- ---------- --SNKKIFKK YSRVAGLLTA ALIVGNLVTA 29
Streptolysin O ---------- ---------- ---------- ---------- --SNKKTFKK YSRVAGLLTA ALIIGNLVTA 29
    Novyiolysin ---------- ---------- ---------- ---------- KS-LKTIIRS ISFLS--ILT LTCSCNFITS 29
    Tetanolysin ---------- ---------- ---------- ---------- KNVLKFVSRS LLIFS--MTG LISNYNSSNV 30
      Ivanolysin ---------- ---------- ---------- ---------- ---------- -KKIMLLLMT LLLVSLPLAQ 20
  Listeriolysin ---------- ---------- ---------- ---------- ---------- -KKIMLVFIT LILVSLPIAQ 20
Seeligeriolysin ---------- ---------- ---------- ---------- ---------- -KIFGLVIMS LLFVSLPITQ 20
       Suilysin ---------- ---------- ---------- ---------- --------KS SHLILSSIVS LALVGVTPLS 24
    Pneumolysin ---------- ---------- ---------- ---------- N--------- ---------- ---------- 3
Mitilysin (PLY) ---------- ---------- ---------- ---------- n--------- ---------- ---------- 3
  Intermedilysin ---------- ---------- ---------- ---------- -----NIARK LSR--VVLLS TLV------- 21
  Viridanolysin DGNVDSDYGH HSVTHTNFED NAWWQVDLGK TENVGKVKLY NRGDGNVANR LSNFDVVLLN EAKQEVARQH 140
       Pyolysin ---------- ---------- ---------- ---------- --------KA FASLVASVVA AATVTMPTAS 25
```

Figure 1A

```
                                         |                     |                     |
     Cereolysin FAET------ ---------- ---------- -QASNATDVT KNASG----- ---------- ---------- 47
    Anthrolysin FAET------ ---------- ---------- -QAGNATGAI KNASD----- ---------- ---------- 50
 Thuringiolysin FAET------ ---------- ---------- -QAGHATDIT KNASS----- ---------- ---------- 50
 Perfringolysin FSK------- ---------- ---------- -------DIT DKNQS----- ---------- ---------- 37
     Alveolysin SA-------- ---------- ---------- --------AP TEPND----- ---------- ---------- 39
     Caniolysin NADSNKQNTA NTETTTTNEQ PKPESSELTT EKAGQKMDDM LNSNDMIKLA PKEMPLESAE KEEKKSEDNK 102
   Equisimilysin NADSNKQNTA NTETTTTNEQ PKPESSELTT EKAGQKMDDM LNSNDMIKLA PKEMPLESAE KEEKKSEDNK 99
 Streptolysin O NAESNKQNTA STETTTTSEQ PKPESSELTI EKAGQKMDDM LNSNDMIKLA PKEMPLESAE KEEKKSEDKK 99
    Novyiolysin TQK-NV---- ---------- ---------- --------SL LSGPNKVI-- ---------- -----KPKK- 48
    Tetanolysin LAKGNVEE-- ---------- ---------- -------HSL INNGQVVT-- ---------- -----SNTKC 54
     Ivanolysin EAQA-DASVY SYQGI-ISHM APPASPP--- ---------- ---------- ---------- -----AKPKT 50
   Listeriolysin QTEAKDASAF NKENL-ISSM APPASPP--- ---------- ---------- ---------- -----ASPKT 51
 Seeligeriolysin QPEARDVPAY DRSEVTISPA ETPESPP--- ---------- ---------- ---------- -----ATPKT 52
       Suilysin VLA------- ---------- ---------- ---------- ---------- ---------- ---------- 27
    Pneumolysin ---------- ---------- ---------- ---------- ---------- ---------- ---------- 3
 Mitilysin (PLY) ---------- ---------- ---------- ---------- ---------- ---------- ---------- 3
  Intermedilysin ---LSSAAPI SAAFAETPTK PKAAQTEKKP EK-------- -------KP- --------EN SNSEAAK--- 61
   Viridanolysin FDSLNGKAEL EVFF--TAKD ARYVKVELKT KN-------- -------TPL SLAEVEVFRS ATTQVGQDRT 193
       Pyolysin FAAGLGNSSG LTDGLSAPRA SISPTDKVDL KSA------- ---------- ---------- ---------- 58

                         |                     |                     |                     |
     Cereolysin ---------I DTGIANLKYN NQEVLAVNGD KVESFVPKES INSNGKFVVV EREKKSLTTS PVDISIIDSV 108
    Anthrolysin ---------I NTGIANLKYD SRDILAVNGD KVESFIPKES INSNGKFVVV EREKKSLTTS PVDILIIDSV 111
 Thuringiolysin ---------I DTGIGNLTYN NQEVLAVNGD KVESFVPKES INSNGKFVVV EREKKSLTTS PVDISIIDSV 111
 Perfringolysin ---------I DSGISSLSYN RNEVLASNGD KIESFVPKEG KKAGNKFIVV ERQKRSLTTS PVDISIIDSV 98
     Alveolysin ---------I DMGIAGLNYN RNEVLAIQGD QISSFVPKEG IQSNGKFIVV ERDKKSLTTS PVDISIVDSI 100
     Caniolysin KSEEDHTEEI NDKIYSLNYN ELEVLAKNGE TIENFVPKEG VKKADKFIVI ERKKKNINTT PVDISIIDSV 172
   Equisimilysin KSEEDHTEEI NDKIYSLNYN ELEVLAKNGE TIENFVPKEG VKKADKFIVI ERKKKNINTT PVDISIIDSV 169
 Streptolysin O KSEEDHTEEI NDKIYSLNYN ELEVLAKNGE TIENFVPKEG VKKADKFIVI ERKKKNINTT PVDISIIDSV 169
    Novyiolysin ------TKSL DDRIYGLKYD PNKILSFNGE KVENFVPNEG FSTPDKYIVI KREKKSISDS TAD¦AVIDSM 112
    Tetanolysin NLAKDNSSDI DKNIYGLSYD PRKILSYNGE QVENFVPAEG FENPDKFIVV KREKKSISDS TAD¦SIIDSI 124
     Ivanolysin PVEKKNAAQI DQYIQGLDYD KNNILVYDGE AVKNVPPKAG YKEGNQYIVV EKKKKSINQN NAD¦QVINSL 120
   Listeriolysin PIEKKHADEI DKYIQGLDYN KNNVLVYHGD AVTNVPPRKG YKDGNEYIVV EKKKKSINQN NAD¦QVVNAI 121
 Seeligeriolysin PVEKKHAEEI NKYIWGLNYD KNSILVYQGE AVTNVPPKKG YKDGSEYIVV EKKKKGINQN NAD¦SVINAI 122
       Suilysin ----DSKQDI NQYFQSLTYE PQEILTNEGE YIDNPPATTG MLENGRFVVL RREKKNITNN SAD¦AVIDAK 93
    Pneumolysin -------KAV NDFILAMNYD KKKLLTHQGE SIENRFIKEG NQLPDEFVVI ERKKRSLSTN TSD¦SVTATN 66
 Mitilysin (PLY) -------kav ndfilamdyd kkkllthqge sienrfikeg nqlpdefvvi erkkrslstn tsd¦svtatn 66
  Intermedilysin -------KAL NDYIWGLQYD KLNILTHQGE KLKNHSSREA FHRPGEYVVI EKKKQSISNA TSKLSVSSAN 124
   Viridanolysin APVVDQTSAL KDYLFGLTYN PLDILTRKGE TLENRYNTSA KEQNGEFVVV EKIKKTLSTG TADVSING-- 261
       Pyolysin --QETDETGV DKYIRGLKYD PSGVLAVKGE SIENVPVTKD QLKDGTYTVF KHERKSFNNL RSDISAFDAN 126
```

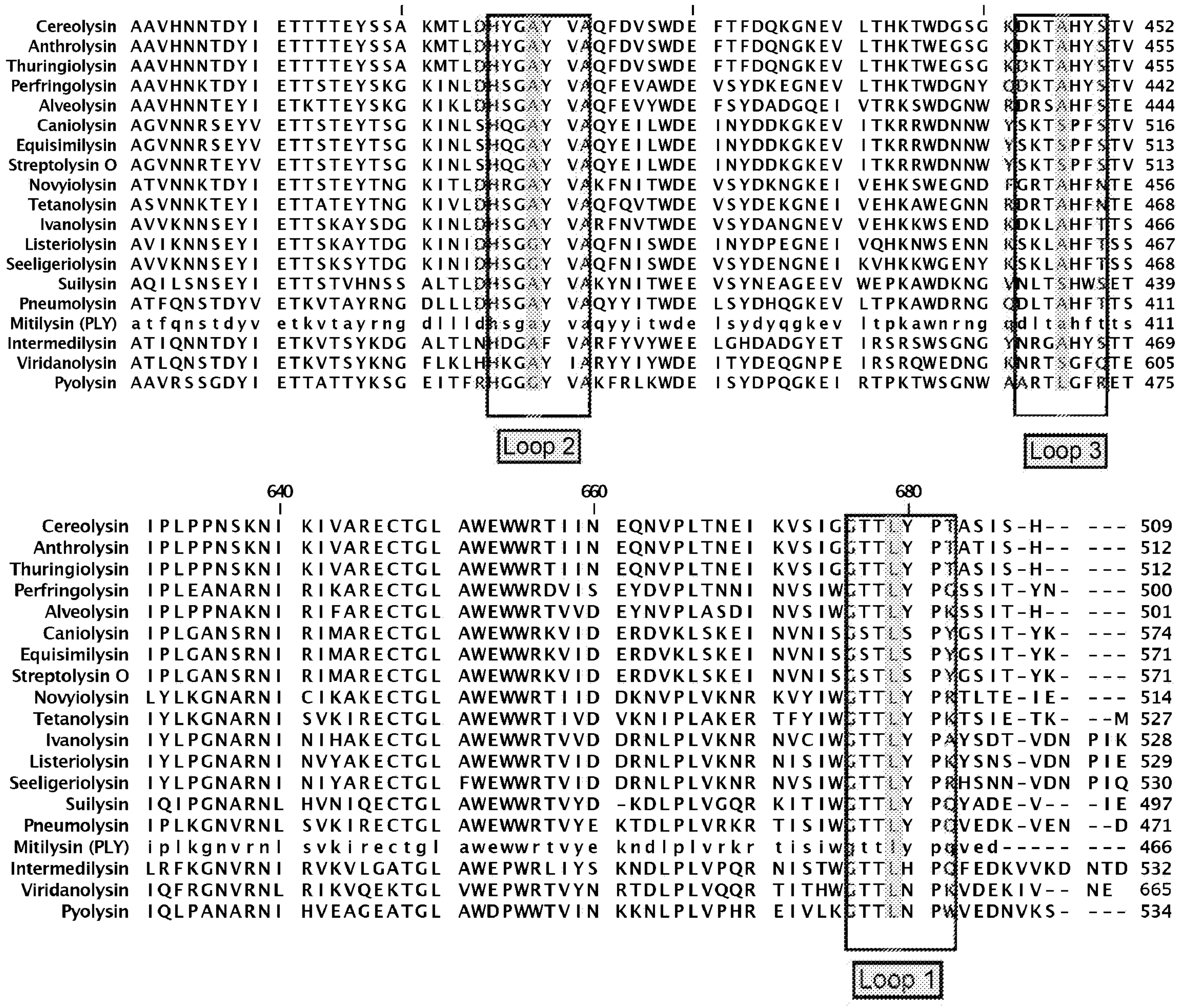
Figure 1B

```
                                    |                     |                     |
     Cereolysin VNRTYPGAVQ LANKAFADNQ PSLLVAKRKP LNISIDLPGM -RKENTITVQ NPTYGNVAGA VDDLVSTWNE 177
    Anthrolysin VNRTYPGAVQ LANKAFADNQ PSLLVAKRKP LNISIDLPGM -RKENTITVQ NPTYGNVAGA VDDLVSTWNE 180
 Thuringiolysin ANRTYPGAVQ LANKAFADNQ PSLLVAKRKP LNISIDLPGM -RKENTITVQ NPTYGNVAGA VDDLVSTWNE 180
 Perfringolysin NDRTYPGALQ LADKAFVENR PTILMVKRKP ININIDLPGL -KGENSIKVD DPTYGKVSGA IDELVSKWNE 167
     Alveolysin TNRTYPGAIQ LANKDFADNQ PSLVMAARKP LDISIDLPGL -KNENTISVQ NPNYGTVSSA IDQLVSTWGE 169
     Caniolysin TDRTYPAALQ LANKGFTENK PDAVVTKRNP QKIHIDLPGM -GDKATVEVN DPTYANVSTA IDNLVNQWHD 241
  Equisimilysin TDRTYPAALQ LANKGFTENK PDAVVTKRNP QKIHIDLPGM -GDKATVEVN DPTYANVSTA IDNLVNQWHD 238
 Streptolysin O TDRTYPAALQ LANKGFTENK PDAVVTKRNP QKIHIDLPGM -GDKATVEVN DPTYANVSTA IDNLVNQWHD 238
    Novyiolysin NDKTYPGAIQ LANRNLIENK PNIVSCERKP ITISIDLPGM -GEEGKTTIT SPTYSSVKAG IDSLLNKWNS 181
    Tetanolysin NDRTYPGAIQ LANRNLMENK PDIISCERKP ITISVDLPGM -AEDGKKVVN SPTYSSVNSA INSILDTWNS 193
     Ivanolysin ASLTYPGALV KANSELVENQ PDVLPVKRDS VTLSIDLPGM VNHDNEIVVQ NATKSNINDG VNTLVDRWNN 190
  Listeriolysin SSLTYPGALV KANSELVENQ PDVLPVKRDS LTLSIDLPGM TNQDNKIVVK NATKSNVNNA VNTLVERWNE 191
Seeligeriolysin SSLTYPGALV KANRELVENQ PNVLPVKRDS LTLSVDLPGM TKKDNKIFVK NPTKSNVNNA VNTLVERWND 192
       Suilysin AANIYPGALL RADQNLLDNN PTLISIARGD LTLSLNLPGL ANGDSHTVVN SPTRSTVRTG VNNLLSKWNN 163
    Pneumolysin DSRLYPGALL VVDETLLENN PTLLAVDRAP MTYSIDLPGL ASSDSFLQVE DPSNSSVRGA VNDLLAKWHQ 136
Mitilysin (PLY) dsrlypgall vvdetllenn ptllavdrap mtysidlpgl assdsflqve dpsnssvrga vndllakwhq 136
 Intermedilysin DDRIFPGALL KADQSLLENL PTLIPVNRGK TTISVNLPGL KNGESNLTVE NPSNSTVRTA VNNLVEKWIQ 194
  Viridanolysin NQNVFLGGLY KANQNLLENQ PELISLARAK GTVSVDLPGM IHSENKIEA- NPTTSGMQEA MNTLVEKWTK 330
       Pyolysin NAHVYPGALV LANKDLAKGS PTSIGIARAP QTVSVDLPGL VDGKNKVVIN NPTKSSVTQG LNGLLDGWIQ 196

                         |                     |                     |                     |
     Cereolysin KYSTTH-TLP ARMQYTESMV YSKSQIASAL NVNAKYLDNS LNIDFNAVAN GEKKVMVAAY KQIFYTVSAE 246
    Anthrolysin KYSTTH-TLP ARMQYTESMV YSKSQIASAL NVNAKYLDNS LNIDFNAVAN GEKKVMVAAY KQIFYTVSAE 249
 Thuringiolysin KYSETH-TLP ARMQYTESMV YSKSQIASAL NVNAKYLDNS LNIDFNAVAN GEKKVMVAAY KQIFYTVSAE 249
 Perfringolysin KYSSTH-TLP ARTQYSESMV YSKSQISSAL NVNAKVLENS LGVDFNAVAN NEKKVMILAY KQIFYTVSAD 236
     Alveolysin KYSSTH-TLP ARLQYAESMV YSQNQISSAL NVNAKVLNGT LGIDFNAVAN GEKKVMVAAY KQIFYTVSAG 238
     Caniolysin NYSGGN-TLP ARTQYTESMV YSKSQIEAAL NVNSKILDGT LGIDFKSISK GEKKVMIAAY KQIFYTVSAN 310
  Equisimilysin NYSGGN-TLP ARTQYTESMV YSKSQIEAAL NVNSKILDGT LGIDFKSISK GEKKVMIAAY KQIFYTVSAN 307
 Streptolysin O NYSGGN-TLP ARTQYTESMV YSKSQIEAAL NVNSKILDGT LGIDFKSISK GEKKVMIAAY KQIFYTVSAN 307
    Novyiolysin HYSSIY-SIP TRFSYSDSMV YSKSQLSAKL GCNFKALNKA LDIDFDSIYK GQKKVMLLAY KQIFYTVNVD 250
    Tetanolysin KYSSKY-TIP TRMSYSDTMV YSQSQLSAAV GCNFKALNKA LNIDFDSIFK GEKKVMLLAY KQIFYTVSVD 262
     Ivanolysin KYSEEYPNIS AKIDYDQEMA YSESQLVAKF GAAFKAVNNS LNVNFGAISE GKVQEEVINF KQIYYTVNVN 260
  Listeriolysin KYAQAYPNVS AKIDYDDEMA YSESQLIAKF GTAFKAVNNS LNVNFGAISE GKMQEEVISF KQIYYNVNVN 261
Seeligeriolysin KYSKAYPNIN AKIDYSDEMA YSESQLIAKF GTAFKAVNNS LNVNFEAISD GKVQEEVISF KQIYYNINVN 262
       Suilysin TYAGEYGNTQ AELQYDETMA YSMSQLKTKF GTSFEKIAVP LDINFDAVNS GEKQVQIVNF KQIYYTVSVD 233
    Pneumolysin DYGQVN-NVP ARMQYEKITA HSMEQLKVKF GSDFEKTGNS LDIDFNSVHS GEKQIQIVNF KQIYYTVSVD 205
Mitilysin (PLY) dygqvn-nvp armqyekita hsmeqlkvkf gsdfektgns ldidfnsvhs gekqiqivnf kqiyytvsvd 205
 Intermedilysin KYSKTH-AVP ARMQYESISA QSMSQLQAKF GADFSKVGAP LNVDFSSVHK GEKQVFIANF RQVYYTASVD 263
  Viridanolysin NYSSSH-SVP ARVQYESTTA YSMNQLKAKF GADFEKAGAP LKIDFEAVQK GEKQIEVVNF KQIYYTATFD 399
       Pyolysin RNSK-YPDHA AKISYDETMV TSKRQLEAKL GLGFEKVSAK LNVDFDAIHK RERQVAIASF KQIYYTASVD 265
```

Figure 1C

```
                                           |                                |                                 |
      Cereolysin  LPNNPSDLFD  NSVTFDELTR  KGVSNSAPPV  MVSNVAYGRT  IYVKLETTSK  SKDVQAAFKA  LLK----NNS  312
     Anthrolysin  LPNNPSDLFD  NSVTFDELTR  KGVSNSAPPV  MVSNVAYGRT  VYVKLETTSK  SKDVQAAFKA  LLK----NNS  315
   Thuringiolysin LPNNPSDLFD  NSVTFDELTR  KGVSNSAPPV  MVSNVAYGRT  VYVKLETTSK  SKDVQAAFKA  LLK----NNS  315
   Perfringolysin LPKNPSDLFD  DSVTFNDLKQ  KGVSNEAPPL  MVSNVAYGRT  IYVKLETTSS  SKDVQAAFKA  LIK----NTD  302
       Alveolysin LPNNPSDLFD  DSVTFAELAR  KGVSNEAPPL  MVSNVAYGRT  IYVKLETTSK  SNDVQTAFKL  LLN----NPS  304
       Caniolysin LPNNPADVFD  KSVTFKELQA  KGVSNEAPPL  FVSNVAYGRT  VFVKLETSSK  SNDVEAAFSA  ALK----GTD  376
   Equisimilysin  LPNNPADVFD  KSVTFKELQR  KGVSNEAPPL  FVSNVAYGRT  VFVKLETSSK  SNDVEAAFSA  ALK----GTD  373
  Streptolysin O  LPNNPADVFD  KSVTFKDLQR  KGVSNEAPPL  FVSNVAYGRT  VFVKLETSSK  SNDVEAAFSA  ALK----GTD  373
      Novyiolysin APNHPSDFFG  DKVTFNDLAK  KGVNSKNPPV  YVSSVSYGRT  IYVKLETTSK  SANVKAAFKA  LIE----NQN  316
      Tetanolysin PPNRPSDLFG  DSVTFDELAL  KGINNNNPPA  YVSNVAYGRT  IYVKLETTSK  SSHVKAAFKA  LIN----NQD  328
       Ivanolysin EPTSPSRFFG  KSVTKENLQA  LGVNAENPPA  YISSVAYGRD  IFVKLSTSSH  STRVKAAFDT  AFK----GKS  326
    Listeriolysin EPTRPSRFFG  KAVTKEQLQA  LGVNAENPPA  YISSVAYGRQ  VYLKLSTNSH  STKVKAAFDA  AVS----GKS  327
  Seeligeriolysin EPTSPSKFFG  GSVTKEQLDA  LGVNAENPPA  YISSVAYGRQ  VYVKLSSSSH  SNKVKTAFEA  AMS----GKS  328
         Suilysin EPESPSKLFA  EGTTVEDLKR  NGITDEVPPV  YVSSVSYGRS  MFIKLETSSR  STQVQAAFKA  AIK----GVD  299
      Pneumolysin AVKNPGDVFQ  DTVTVEDLKQ  RGISAERPLV  YISSVAYGRQ  VYLKLETTSK  SDEVEAAFEA  LIK----GVK  271
  Mitilysin (PLY) avknpgdvfq  dtvtvedlrq  rgisadrplv  yissvaygrq  vylklettsk  sdeveaafea  lik----gvk  271
    Intermedilysin SPNSPSALFG  SGITPTDLIN  RGVNSKTPPV  YVSNVSYGRA  MYVKFETTSK  STKVQAAIDA  VVK----GAK  329
     Viridanolysin APTNPAAVFD  KSVTPEDLKQ  RGVDSQTPPV  YVSNVSYGRQ  IYVKFESTSK  STELKAAINA  VIK----GAT  465
         Pyolysin TPTSPHSVFG  PNVTAQDLKD  RGVNNKNPLG  YISSVSYGRQ  IFVKLETTST  SNDVQAAFSG  LFKAKFGNLS  335

                           |                                |                                 |                                 |
      Cereolysin  VETSGQYKDI  FEESTFTAVV  LGGDAKEHNK  VVTKDFNEIR  NIIKDNAELS  LKNPAYPISY  TSTFLKDNST  382
     Anthrolysin  VETSGQYKDI  FEESTFTAVV  LGGDAKEHNK  VVTKDFNEIR  NIIKDNAELS  FKNPAYPISY  TSTFLKDNAT  385
   Thuringiolysin VETSGQYKDI  FEESTFTAVV  LGGDAKEHNK  VVTKDFNEIR  NIIKDNAELS  FKNPAYPISY  TSTFLKDNAT  385
   Perfringolysin IKNSQQYKDI  YENSSFTAVV  LGGDAQEHNK  VVTKDFDEIR  KVIKDNATFS  TKNPAYPISY  TSVFLKDNSV  372
       Alveolysin IQASGQYKDI  YENSSFTAVV  LGGDAQTHNQ  VVTKDFNVIQ  SVIKDNAQFS  SKNPAYPISY  TSVFLKDNSI  374
       Caniolysin VKTNGKYSDI  LENSSFTAVV  LGADAAEHNK  VVTKDFDVIR  NVIKANATFS  RKNPAYPISY  TSVFLKNNKI  446
   Equisimilysin  VKTNGKYSDI  LENSSFTAVV  LGGDAAEHNK  VVTKDFDVIR  NVIKDNATFS  RKNPAYPISY  TSVFLKNNKI  443
  Streptolysin O  VKTNGKYSDI  LENSSFTAVV  LGGDAAEHNK  VVTKDFDVIR  NVIKDNATFS  RKNPAYPISY  TSVFLKNNKI  443
      Novyiolysin ISSNSEYKNI  LNQSSFTATV  LGGGAKEHNK  VITKNFDEIR  NIITNNSEYS  PRNPGYPIAY  TTSFLKDNSV  386
      Tetanolysin ISSNAEYKDI  LNQSSFTATV  LGGGAQEHNK  IITKDFDEIR  NIIKNNSVYS  PQNPGYPISY  TTTFLKDNSI  398
       Ivanolysin VKGDTELENI  IQNASFKAVI  YGGSAKDEVE  IIDGDLSKLR  DILKQGANFD  KKNPGVPIAY  TTNFLKDNQL  396
    Listeriolysin VSGDVELTNI  IKNSSFKAVI  YGGSAKDEVQ  IIDGNLGDLR  DILKKGATFN  RETPGVPIAY  TTNFLKDNEL  397
  Seeligeriolysin VKGDVELTNI  IKNSSFKAVI  YGGSAKEEVE  IIDGNLGELR  DILKKGSTYD  RENPGVPISY  TTNFLKDNDL  398
         Suilysin ISGNAEYQDI  LKNTSFSAYI  FGGDAGSAAT  VVSGNIETLK  KIIEEGARYG  KLNPGVPISY  STNFVKDNRP  369
      Pneumolysin VAPQTEWKQI  LDNTEVKAVI  LGGDPSSGAR  VVTGKVDMVE  DLIQEGSRFT  ADHPGLPISY  TTSFLRDNVV  341
  Mitilysin (PLY) vapqtewkqi  ldntevkavi  lggdpssgar  vvtgkvdmve  dliqegsrft  adhpglpisy  ttsflrdnvv  341
    Intermedilysin LKAGTEYENI  LKNTKITAVV  LGGNPGEASK  VITGNIDTLK  DLIQKGSNFS  AQSPAVPISY  TTSFVKDNSI  399
     Viridanolysin IAPNSEWSRL  LKNTSVTAVI  VGGNASGAAK  VVTGTVENLK  ELIREGANFS  AQSPAVPISY  KTAFLKDNAQ  535
         Pyolysin TEFKAKYADI  LNKTRATVYA  VGGSARGGVE  VATGNIDALK  KIIKEESTYS  TKVPAVPVSY  AVNFLKDNQL  405
```

Figure 1D



Cereolysin	AAVHNNTDYI	ETTTTEYSSA	KMTLDHYGAY	VAQFDVSWDE	FTFDQKGNEV	LTHKTWDGSG	KDKTAHYSTV	452
Anthrolysin	AAVHNNTDYI	ETTTTEYSSA	KMTLDHYGAY	VAQFDVSWDE	FTFDQNGKEV	LTHKTWEGSG	KDKTAHYSTV	455
Thuringiolysin	AAVHNNTDYI	ETTTTEYSSA	KMTLDHYGAY	VAQFDVSWDE	FTFDQNGKEV	LTHKTWEGSG	KDKTAHYSTV	455
Perfringolysin	AAVHNKTDYI	ETTSTEYSKG	KINLDHSGAY	VAQFEVAWDE	VSYDKEGNEV	LTHKTWDGNY	QDKTAHYSTV	442
Alveolysin	AAVHNNTEYI	ETKTTEYSKG	KIKLDHSGAY	VAQFEVYWDE	FSYDADGQEI	VTRKSWDGNW	RDRSAHFSTE	444
Caniolysin	AGVNNRSEYV	ETTSTEYTSG	KINLSHQGAY	VAQYEILWDE	INYDDKGKEV	ITKRRWDNNW	YSKTSPFSTV	516
Equisimilysin	AGVNNRSEYV	ETTSTEYTSG	KINLSHQGAY	VAQYEILWDE	INYDDKGKEV	ITKRRWDNNW	YSKTSPFSTV	513
Streptolysin O	AGVNNRTEYV	ETTSTEYTSG	KINLSHQGAY	VAQYEILWDE	INYDDKGKEV	ITKRRWDNNW	YSKTSPFSTV	513
Novyiolysin	ATVNNKTDYI	ETTSTEYTNG	KITLDHRGAY	VAKFNITWDE	VSYDKNGKEI	VEHKSWEGND	FGRTAHFNTE	456
Tetanolysin	ASVNNKTEYI	ETTATEYTNG	KIVLDHSGAY	VAQFQVTWDE	VSYDEKGNEI	VEHKAWEGNN	RDRTAHFNTE	468
Ivanolysin	AVVKNNSEYI	ETTSKAYSDG	KINLDHSGAY	VARFNVTWDE	VSYDANGNEV	VEHKKWSEND	KDKLAHFTTS	466
Listeriolysin	AVIKNNSEYI	ETTSKAYTDG	KINIDHSGGY	VAQFNISWDE	INYDPEGNEI	VQHKNWSENN	KSKLAHFTSS	467
Seeligeriolysin	AVVKNNSEYI	ETTSKSYTDG	KINIDHSGGY	VAQFNISWDE	VSYDENGNEI	KVHKKWGENY	KSKLAHFTSS	468
Suilysin	AQILSNSEYI	ETTSTVHNSS	ALTLDHSGAY	VAKYNITWEE	VSYNEAGEEV	WEPKAWDKNG	VNLTSHWSET	439
Pneumolysin	ATFQNSTDYV	ETKVTAYRNG	DLLLDHSGAY	VAQYYITWDE	LSYDHQGKEV	LTPKAWDRNG	QDLTAHFTTS	411
Mitilysin (PLY)	atfqnstdyv	etkvtayrng	dllldhsgay	vaqyyitwde	lsydyqgkev	ltpkawnrng	qdltahftts	411
Intermedilysin	ATIQNNTDYI	ETKVTSYKDG	ALTLNHDGAF	VARFYVYWEE	LGHDADGYET	IRSRSWSGNG	YNRGAHYSTT	469
Viridanolysin	ATLQNSTDYI	ETKVTSYKNG	FLKLHHKGAY	IARYYIYWDE	ITYDEQGNPE	IRSRQWEDNG	KNRTSGFQTE	605
Pyolysin	AAVRSSGDYI	ETTATTYKSG	EITFRHGGGY	VAKFRLKWDE	ISYDPQGKEI	RTPKTWSGNW	AARTLGFRET	475

Cereolysin	IPLPPNSKNI	KIVARECTGL	AWEWWRTIIN	EQNVPLTNEI	KVSIGGTTLY	PTASIS-H--	---	509
Anthrolysin	IPLPPNSKNI	KIVARECTGL	AWEWWRTIIN	EQNVPLTNEI	KVSIGGTTLY	PTATIS-H--	---	512
Thuringiolysin	IPLPPNSKNI	KIVARECTGL	AWEWWRTIIN	EQNVPLTNEI	KVSIGGTTLY	PTASIS-H--	---	512
Perfringolysin	IPLEANARNI	RIKARECTGL	AWEWWRDVIS	EYDVPLTNNI	NVSIWGTTLY	PQSSIT-YN-	---	500
Alveolysin	IPLPPNAKNI	RIFARECTGL	AWEWWRTVVD	EYNVPLASDI	NVSIWGTTLY	PKSSIT-H--	---	501
Caniolysin	IPLGANSRNI	RIMARECTGL	AWEWWRKVID	ERDVKLSKEI	NVNISGSTLS	PYGSIT-YK-	---	574
Equisimilysin	IPLGANSRNI	RIMARECTGL	AWEWWRKVID	ERDVKLSKEI	NVNISGSTLS	PYGSIT-YK-	---	571
Streptolysin O	IPLGANSRNI	RIMARECTGL	AWEWWRKVID	ERDVKLSKEI	NVNISGSTLS	PYGSIT-YK-	---	571
Novyiolysin	LYLKGNARNI	CIKAKECTGL	AWEWWRTIID	DKNVPLVKNR	KVYIWGTTLY	PRTLTE-IE-	---	514
Tetanolysin	IYLKGNARNI	SVKIRECTGL	AWEWWRTIVD	VKNIPLAKER	TFYIWGTTLY	PKTSIE-TK-	--M	527
Ivanolysin	IYLPGNARNI	NIHAKECTGL	AWEWWRTVVD	DRNLPLVKNR	NVCIWGTTLY	PAYSDT-VDN	PIK	528
Listeriolysin	IYLPGNARNI	NVYAKECTGL	AWEWWRTVID	DRNLPLVKNR	NISIWGTTLY	PKYSNS-VDN	PIE	529
Seeligeriolysin	IYLPGNARNI	NIYARECTGL	FWEWWRTVID	DRNLPLVKNR	NVSIWGTTLY	PRHSNN-VDN	PIQ	530
Suilysin	IQIPGNARNL	HVNIQECTGL	AWEWWRTVYD	-KDLPLVGQR	KITIWGTTLY	PQYADE-V--	-IE	497
Pneumolysin	IPLKGNVRNL	SVKIRECTGL	AWEWWRTVYE	KTDLPLVRKR	TISIWGTTLY	PQVEDK-VEN	--D	471
Mitilysin (PLY)	iplkgnvrnl	svkirectgl	awewwrtvye	kndlplvrkr	tisiwgttly	pqved-----	---	466
Intermedilysin	LRFKGNVRNI	RVKVLGATGL	AWEPWRLIYS	KNDLPLVPQR	NISTWGTTLH	PQFEDKVVKD	NTD	532
Viridanolysin	IQFRGNVRNL	RIKVQEKTGL	VWEPWRTVYN	RTDLPLVQQR	TITHWGTTLN	PKVDEKIV--	NE	665
Pyolysin	IQLPANARNI	HVEAGEATGL	AWDPWWTVIN	KKNLPLVPHR	EIVLKGTTLN	PWVEDNVKS-	---	534

Figure 1E

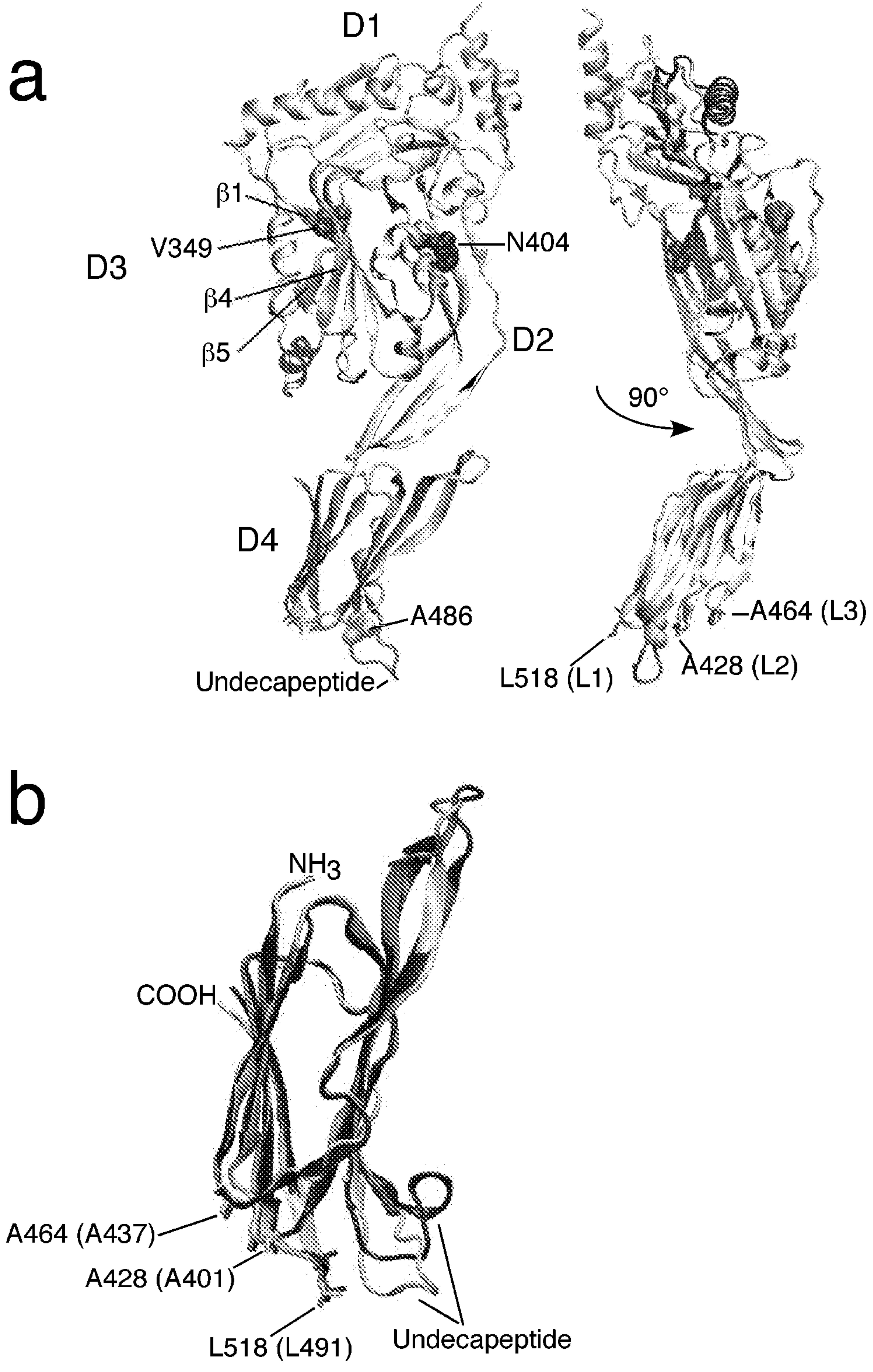

a
D1
β1
V349
D3
β4
β5
N404
D2
90°
D4
A486
Undecapeptide
L518 (L1)
A428 (L2)
A464 (L3)
b
NH3
COOH
A464 (A437)
A428 (A401)
L518 (L491)
Undecapeptide

Figure 2

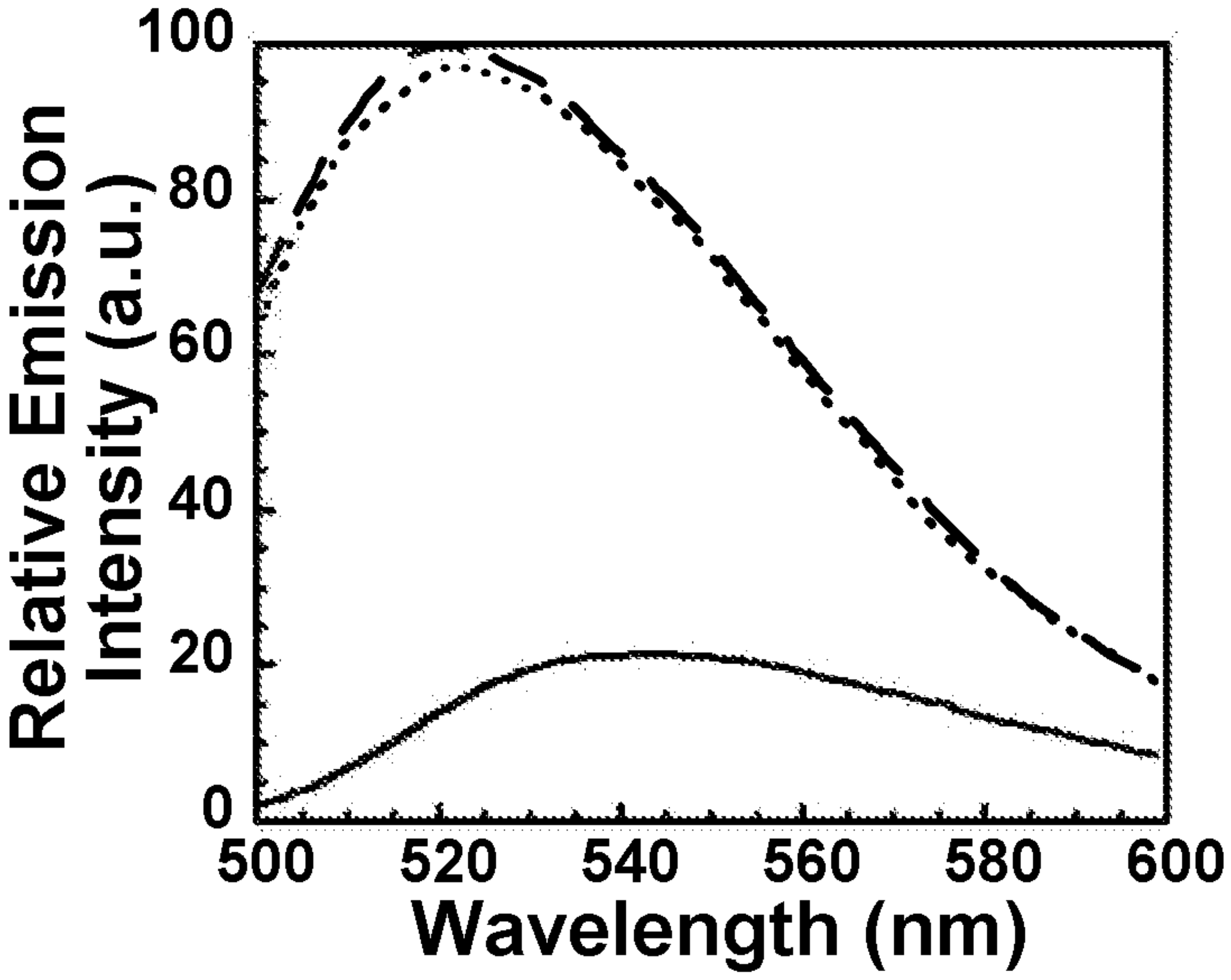


Figure 3

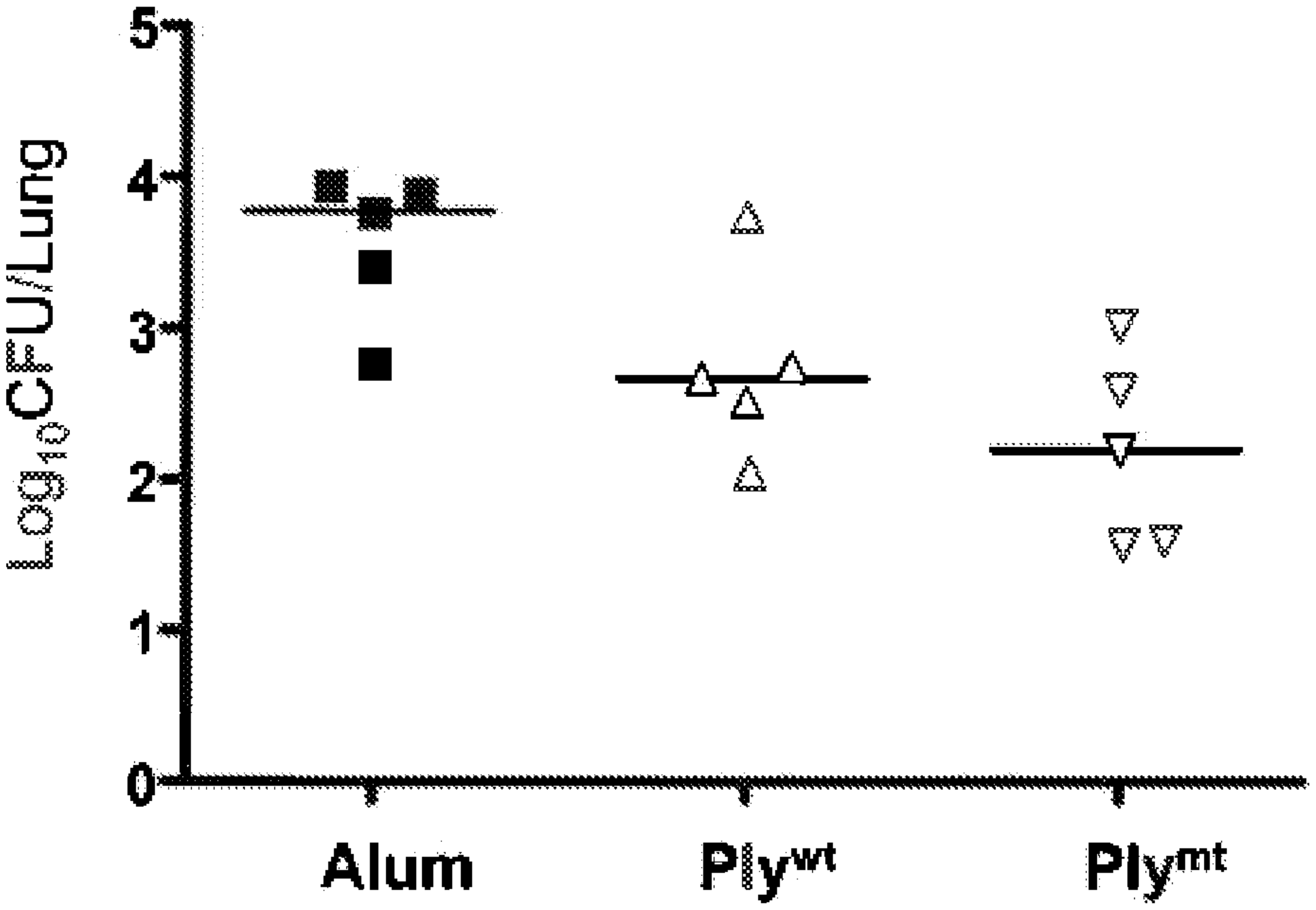


Figure 7

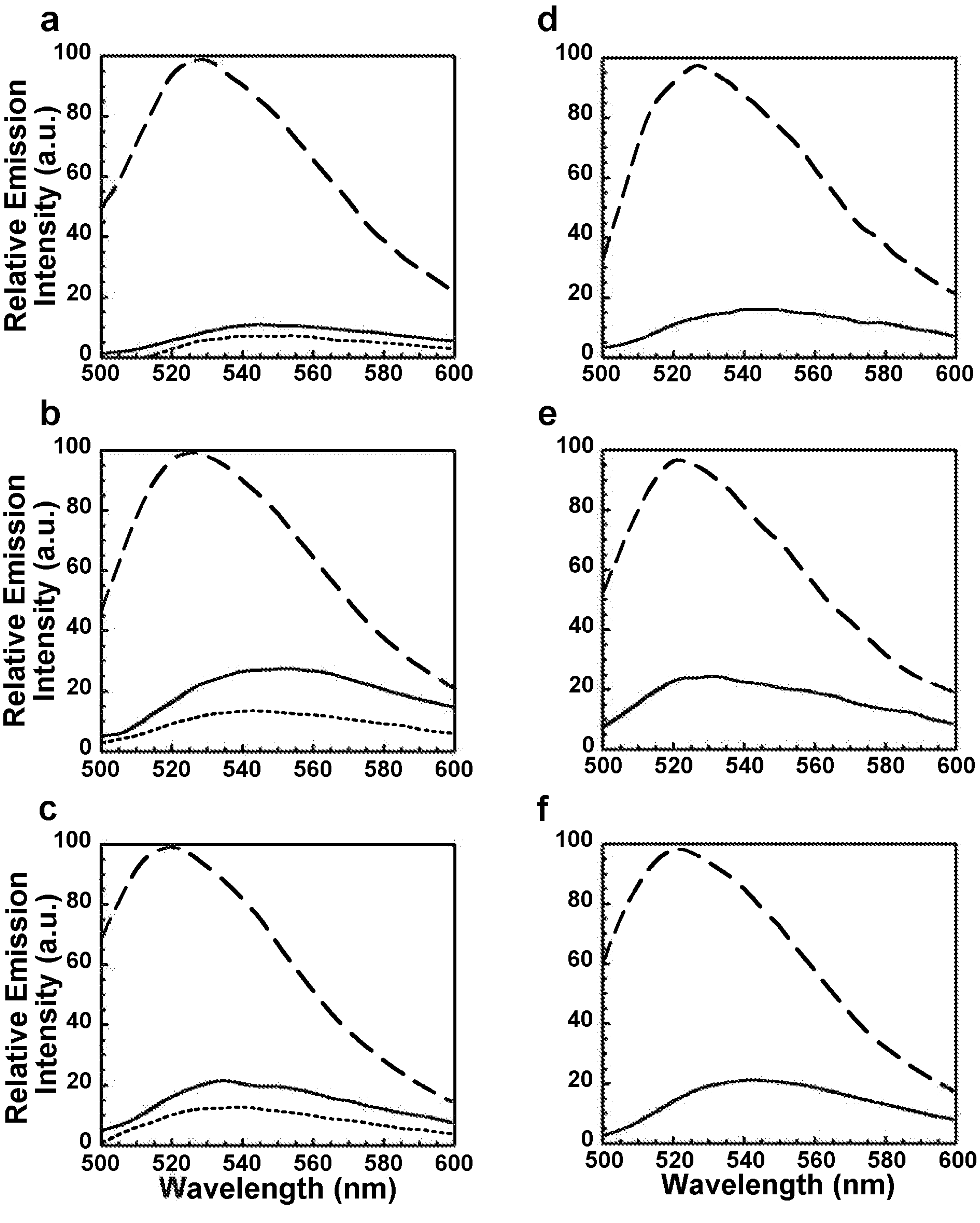

a
b
c
d
e
f
Relative Emission Intensity (a.u.)
0
20
40
60
80
100
500
520
540
560
580
600
Wavelength (nm)

Figure 4

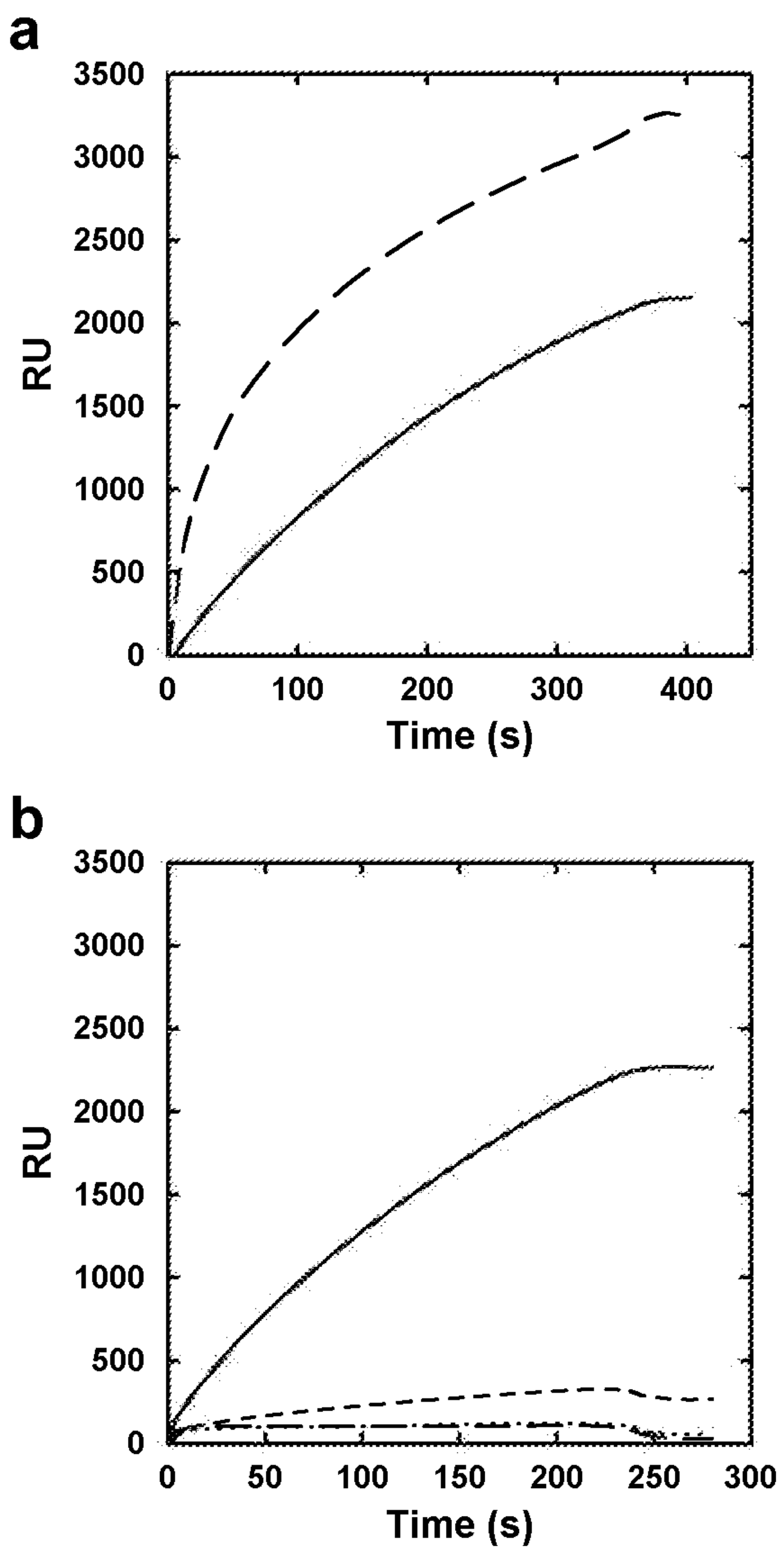


Figure 5

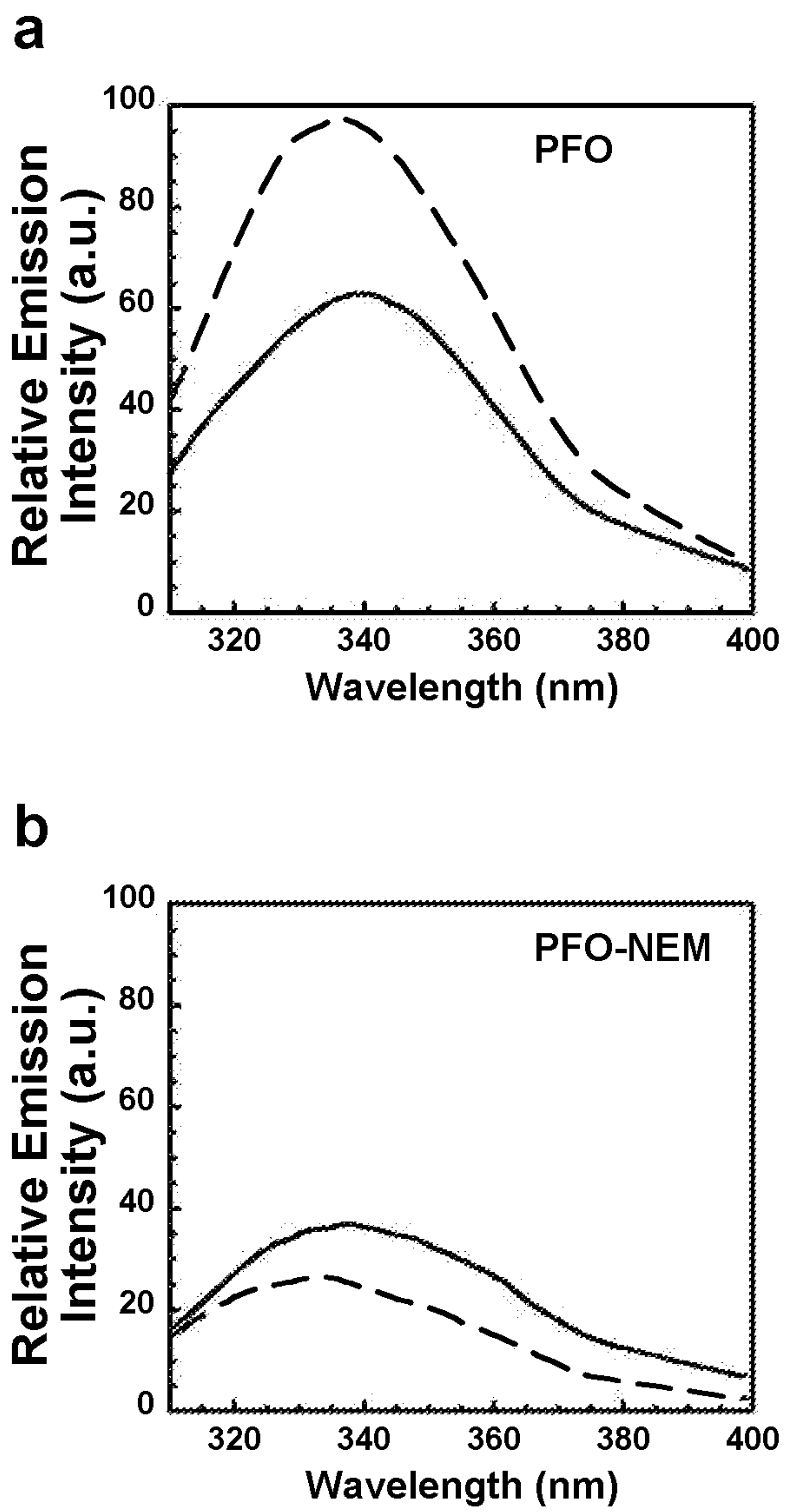


Figure 6

Cereolysin	M---------	----------	----------	----------	----------	----------	----------	1
Anthrolysin	MI--------	----------	----------	----------	----------	----------	----------	2
Thuringiolysin	MI--------	----------	----------	----------	----------	----------	----------	2
Perfringolysin	MI--------	----------	----------	----------	----------	----------	----------	2
Alveolysin	M---------	----------	----------	----------	----------	----------	----------	1
Caniolysin	MK--------	----------	----------	----------	----------	----------	----------	2
Equisimilysin	M---------	----------	----------	----------	----------	----------	----------	1
Streptolysin O	M---------	----------	----------	----------	----------	----------	----------	1
Novyiolysin	MK--------	----------	----------	----------	----------	----------	----------	2
Tetanolysin	MN--------	----------	----------	----------	----------	----------	----------	2
Ivanolysin	M---------	----------	----------	----------	----------	----------	----------	1
Listeriolysin	M---------	----------	----------	----------	----------	----------	----------	1
Seeligeriolysin	M---------	----------	----------	----------	----------	----------	----------	1
Suilysin	MR--------	----------	----------	----------	----------	----------	----------	2
Pneumolysin	MA--------	----------	----------	----------	----------	----------	----------	2
Mitilysin (PLY)	ma--------	----------	----------	----------	----------	----------	----------	2
Intermedilysin	MK--------	----------	----------	------TKQ-	----------	----------	----------	5
Viridanolysin	MNQEKRLHRF	VKKCGLGVCS	AVVAAFLLNA	QGVALATEQG	NRPVETENIA	RGKQASQSST	AYGGAATRAV	70
Pyolysin	MKR-------	----------	----------	----------	----------	----------	----------	3

Cereolysin	----------	----------	----------	----------	--NIKKNTKR	RKFLACLLVS	LCTINYSSIS	29
Anthrolysin	----------	----------	----------	----------	FLNIKKNTKR	RKFLACLLVS	LCTIHYSSIS	32
Thuringiolysin	----------	----------	----------	----------	FLNIKKNGKR	RKFLTCVLVS	LCTLNYSSTS	32
Perfringolysin	----------	----------	----------	----------	------RFKK	TKLIASIAMA	LCLFSQPVIS	26
Alveolysin	----------	----------	----------	----------	-KKKSNHLKG	RKVLVSLLVS	LQVFAFASIS	30
Caniolysin	----------	----------	----------	----------	DMSNKKIFKK	YSRVAGLLTA	ALIVGNLVTA	32
Equisimilysin	----------	----------	----------	----------	--SNKKIFKK	YSRVAGLLTA	ALIVGNLVTA	29
Streptolysin O	----------	----------	----------	----------	--SNKKTFKK	YSRVAGLLTA	ALIIGNLVTA	29
Novyiolysin	----------	----------	----------	----------	KS-LKTIIRS	ISFLS--ILT	LTCSCNFITS	29
Tetanolysin	----------	----------	----------	----------	KNVLKFVSRS	LLIFS--MTG	LISNYNSSNV	30
Ivanolysin	----------	----------	----------	----------	----------	-KKIMLLLMT	LLLVSLPLAQ	20
Listeriolysin	----------	----------	----------	----------	----------	-KKIMLVFIT	LILVSLPIAQ	20
Seeligeriolysin	----------	----------	----------	----------	----------	-KIFGLVIMS	LLFVSLPITQ	20
Suilysin	----------	----------	----------	----------	--------KS	SHLILSSIVS	LALVGVTPLS	24
Pneumolysin	----------	----------	----------	----------	N---------	----------	----------	3
Mitilysin (PLY)	----------	----------	----------	----------	n---------	----------	----------	3
Intermedilysin	----------	----------	----------	----------	-----NIARK	LSR--VVLLS	TLV-------	21
Viridanolysin	DGNVDSDYGH	HSVTHTNFED	NAWWQVDLGK	TENVGKVKLY	NRGDGNVANR	LSNFDVVLLN	EAKQEVARQH	140
Pyolysin	----------	----------	----------	----------	--------KA	FASLVASVVA	AATVTMPTAS	25

Figure 1A