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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 February 2009 (19.02.2009)

PCT

(10) International Publication Number
WO 2009/023300 A2

(51) International Patent Classification:
A61K 39/00 (2006.01)

(21) International Application Number:
PCT/US2008/060250

(22) International Filing Date: 14 April 2008 (14.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/923,281 13 April 2007 (13.04.2007) US

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

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **TWETEN, Rodney, K.** [US/US]; 1701 Deer Park Dr., Edmond, OK 73003 (US).

(74) Agent: **CORBETT, Christopher, W.**; Dunlap, Codding & Rogers, 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, BR, BW, BY, BZ, CA, CH, 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, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, 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, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) **Title:** MUTANTS OF CHOLESTEROL-DEPENDENT CYTOLYSINS AND USES THEREOF

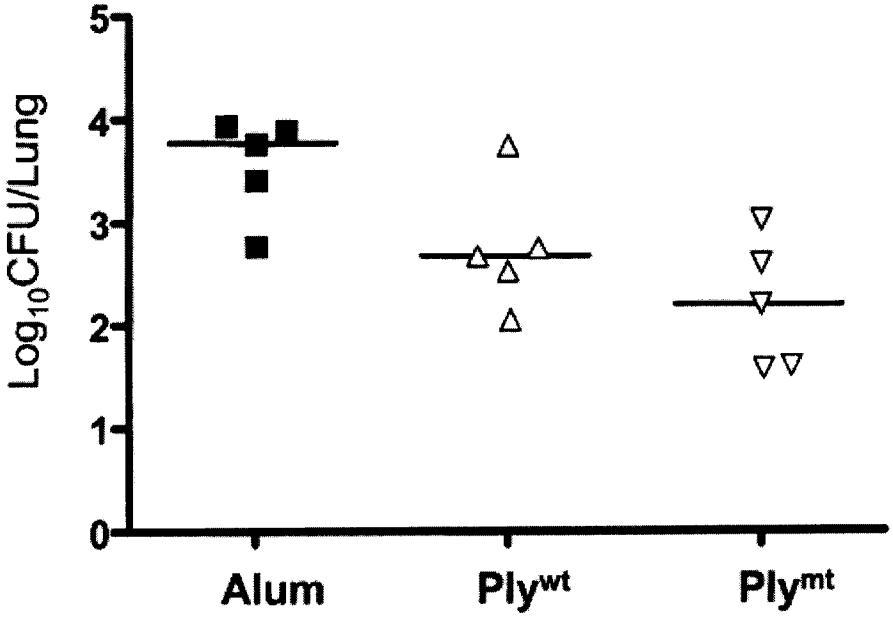


Figure 7

(57) **Abstract:** Mutants of cholesterol-dependent cytolysins comprising at least one amino acid substitution in at least one of Loop 1, Loop 2, or Loop 3 of Domain 4, nucleic acids encoding such polypeptide mutants, and compositions and vaccines comprising such polypeptide mutants.

WO 2009/023300 A2

MUTANTS OF CHOLESTEROL-DEPENDENT CYTOLYSINS AND USES THEREOF

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] Some aspects of this invention were made in the course of Grant 5R01A1037657 awarded by the National Institutes of Health and therefore the Government has certain rights in some aspects of this invention.

BACKGROUND OF THE INVENTION

[0002] 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.

[0003] 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.

[0004] CDC's 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.

[0005] 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.

[0006] During the process of the CDC monomer interaction with the membrane, the undecapeptide and the three other short loops (L1, L2, 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.

[0007] Domain 4 of the CDCs mediates membrane recognition, whether it is via cholesterol or another receptor, as in the case of ILY.

[0008] The CDCs are also capable of the lysis of a wide variety of nucleated cell types in vitro, and this capacity has in turn has 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 population; the rate was higher for persons aged greater than or equal to 65 years (50-83 cases per 100,000 population) and for children aged less than or equal to 2 years (160 cases per 100,000 population). 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 population) 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 population, 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 population; the incidence for children aged 1-2 years in these groups is 2,396 cases per 100,000 population.

[0014] In the United States, the estimated overall annual incidence of pneumococcal meningitis is one to two cases per 100,000 population. 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- (minimum inhibitory concentration {MIC}=0.1-1.0 ug/mL) or high-level (MIC greater than or equal to 2 ug/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, manufactured by both Merck and Company, Inc. (Pneumovax 23™) and Lederle Laboratories (Pnu-Immune 23™), 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 ug 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 is becoming increasingly important since vaccines based on the capsular polysaccharide are losing effectiveness due to genetic variation and are difficult to generate since 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. Hence, the focus for vaccine development has shifted away from the bacterial capsule to

various pneumococcal proteins, pneumolysin being considered as a necessary component for all subunit vaccines. Currently, a single mutant 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 has long been thought to mediate binding to mammalian membranes. Other mutants of pneumolysin are described in U.S. Patent No. 6,716,432, for example.

[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 pneumolysin, which are both non-toxic and which do not bind to cell membranes and which are non-hemolytic yet which stimulate an immune response against corresponding disease organisms would be of great benefit.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1A-E is an amino acid alignment comparison of native amino acid sequences of various cholesterol-dependent cytolysins.

[0021] 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].

[0022] Figure 3. The ILY undecapeptide inserts into cholesterol-depleted membranes. ILY residue Ala-486 was mutated to a cysteine (ILY^{A486C}) and derivatized with NBD. 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).

[0023] Figure 4. 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 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).

[0024] Figure 5. 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).

[0025] Figure 6. 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.

[0026] Figure 7 shows the immunogenic response in mice immunized with a mutant and a wild-type pneumolysin then inoculated with *S. pneumoniae*.

DETAILED DESCRIPTION OF THE INVENTION

[0027] The present invention is directed in one embodiment to compositions comprising one or more non-toxic mutants of cholesterol-dependent cytolysins (CDCs). The compositions may be used in vaccines

directed against corresponding disease pathogens, or may be used in diagnostic or screening methods or other analytical methods such as detection methods.

[0028] The organisms which produce the native forms of the CDCs have various pathological effects.

[0029] *Clostridium perfringens* is a causitive 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.

[0030] *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.

[0031] *Bacillus alvei* can cause endophthalmitis and may cause pneumonia and empyema.

[0032] *Streptococcus dysgalactiae* subsp. *Equisimilis* has been shown to be involved in many different types of human disease syndromes.

[0033] *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.

[0034] *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.

[0035] Tetanolysin is produced by *Clostridium tetanus* that is the cause of tetanus.

[0036] *Listeria ivanovii* is an infection of animals and primarily causes abortion in sheep.

[0037] *Listeria monocytogenes* causes food borne illness in humans, the most severe 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.

[0038] *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.

[0039] In one embodiment, the invention comprises genetically toxoided mutants of native pneumolysin (SEQ ID NO:1) of *S. pneumoniae* (encoded by mutants of SEQ ID NO:20) that have been developed based on our extensive studies into the molecular mechanism of the cholesterol dependent cytolysins, which includes pneumolysin. These mutants exhibit several potential advantages over the current pneumolysin mutant being used for vaccine development. First, in one version, the leucine at position 460 has been mutated, for example, to an aspartate residue, and lacks the ability to bind to mammalian membranes. This mutant, therefore will not undergo any of the structural changes that normally result when these toxins bind to the membrane (as does the Pd-B mutant (Trp433Phe) described above).

[0040] In addition to mutants of pneumolysin, the present invention further comprises 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*).

[0041] 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.

[0042] A variant of SEQ ID NO:18 (Platelet Aggregation Factor) which can also be mutated in accordance with the present invention 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 invention thus contemplates 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.

[0043] The pneumolysin mutants contemplated herein also eliminate any toxic activity of the toxin since they cannot bind to mammalian cells. Although the currently used 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.

[0044] As described below, we have 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. We now know 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 completely abrogates its hemolytic activity. Since we know 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.

[0045] The polypeptide component preferably lacks the hemolytic activity and the pore-forming ability present in a naturally occurring *S. pneumoniae* pneumolysin protein. Generally, the polypeptide component exhibits less than 30%, 20%, 10%, 5%, or 1%, or less of the hemolytic activity of a naturally occurring *S. pneumoniae* pneumolysin protein.

[0046] The present invention also contemplates pneumolysin mutants having substitutions in several positions other than position 460 which have the same effect and include for example positions 370 and 406 of pneumolysin, as well as substitutions in one or more of three residues that flank either side of positions 370, 406 or 460, including positions 367, 368, 369, 371, 372, 373, 403, 404, 405, 407, 408, 409, 457, 458, 459, 461, 462, and 463.

[0047] 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 hemolytic activity of the mutant.

[0048] The amino acid sequence for 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 invention further comprises cDNAs of pneumolysin (and reverse complements thereof) and other mutant CDCs contemplated herein which are substituted as necessary to encode the substituted proteins (mutants) contemplated herein, and may in turn comprise any conservative base (nucleotide) substitution to make cDNAs which encode such mutants.

[0049] It will be appreciated that the polynucleotide sequences which encode the polypeptides contemplated herein may be altered with degenerate

codons yet still encode the polypeptides of the invention. Accordingly the present invention further provides polynucleotides which hybridize to the polynucleotide sequences described herein (or the complement sequences thereof) having 90% identity between sequences, more preferably 95% identity, and more preferably 99% identity.

[0050] Figures 1A and 1E show aligned 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, mutants of these CDCs preferably 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 invention.

[0051] The mutants may further comprise more than one of the substitutions contemplated 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 substututed 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). Where the native residue is positively-charged, the substituted residue is preferably negatively-charged and where the native residue is negatively-charged, the substitute is preferably positively charged. Alternatively, aspartate may be substituted with glutamate, or 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.

[0052] The amino acid positions of Loop 1, Loop 2 and Loop 3 of each CDC described herein is listed in Table 1.

[0053] 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	494-490	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

[0054] The present invention thus comprises purified or isolated forms of the protein mutants as described herein, and antigenic fragments thereof, compositions of these mutants comprising pharmaceutically-acceptable carriers, and vaccines and sera comprising one or more of the mutants contemplated herein as well as adjuvants and immunostimulants. The mutants, or 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 invention further comprises host cells and vectors comprising cDNAs encoding any of the mutants contemplated herein and methods of their use to produce the mutants contemplated herein.

[0055] "Purified protein" or "isolated protein" as used herein means 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.

[0056] As noted above, the invention contemplated herein is also directed to nucleic acid sequences which encode the mutant CDCs contemplated herein. The invention further contemplates nucleic acids which encode allelic variants of the protein mutants contemplated 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. More preferably the allelic variants differ from the protein mutants by less than 12% of their amino acid identity. More preferably the allelic variants differ in less than 10% of their amino acid identity. In another embodiment the allelic variants differ in less than 8% of their amino acid identity. More preferably the allelic variants differ in less than 6% of their amino acid identity, or more preferably in less than 4% of their identity, and even more preferably in less than 2% of their identity, and most preferably the allelic variants differ in less than 1% of their amino acid identity from the protein mutants described herein. Further, the present invention is directed to nucleic acids which hybridize under stringent conditions with the nucleic acids which encode the mutant CDCs described herein.

[0057] In one aspect, the CDC mutant polypeptides or proteins of the present invention comprise a sequence having at least 90%, or 95%, or 96%, or 97%, or 98%, or 99% or more % 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 in Loop 1, Loop 2, or Loop 3 contemplated herein.

[0058] A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is 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.

[0059] 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.

[0060] The mutant CDCs contemplated herein may be combined with pharmaceutically-acceptable carriers or diluents. Pharmaceutically-acceptable carriers or diluents include physiological saline solutions, and buffered saline solutions at neutral pH such as phosphate buffered saline (PBS). Other types of carriers include liposomes or polymers and the like.

[0061] The pharmaceutically acceptable carrier or adjuvant in the vaccine of the present invention can be selected by standard criteria. By "pharmaceutically acceptable" is meant 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 a undesirable manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier or adjuvant may depend on the method of administration and the particular patient.

[0062] Optionally, the mutant CDCs contemplated herein can be combined with an adjuvant such as 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, carbon polynucleotides, i.e., poly IC and poly AU. Preferred adjuvants include QuilA and Alhydrogel and the like. Optionally, the mutant CDCs contemplated herein can be combined with immunomodulators and immunostimulants such as interleukins, interferons and the like. Many vaccine formulations are known to those of skill in the art.

[0063] The mutant CDCs contemplated herein are added to a vaccine formulation in an amount effective to stimulate a protective immune response in an animal. Generation of a protective immune response can be measured by the development of antibodies. 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, more preferably .01 μg

to 1 mg/kg of body weight, and more preferably about 0.1 μg to about 10 μg/kg body weight, for example, at an interval of about 1 to 6 weeks intervals between immunizations.

[0064] The vaccine compositions are administered to animals which 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. The preferred animal is a human.

[0065] As noted above, when the mutant is a pneumolysin mutant, the invention contemplates a vaccine which can be administered for stimulating an immunogenic response in a human subject. In addition to the one or more pneumolysin mutants, the vaccine may comprise other proteins or protein subunits from *S. peumoniae*, or may comprise capsular polysaccharide material combined with or conjugated to the pneumolysin mutants or other proteins in the 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 vaccine may comprise an adjuvant and/or other pharmaceutically-acceptable carriers. 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 cross-linked (multiple polysaccharides attached to multiple polypeptides).

[0066] The mutant CDCs contemplated herein are also useful to form other pharmaceutical compositions, such as for causing stimulation of T-cell proliferation.

[0067] A pharmaceutical composition is formed by combining the mutant CDCs contemplated herein with a pharmaceutically (physiologically) acceptable carrier such as physiological saline, buffered saline solutions as neutral pH such as phosphate buffered saline. The preferred range of effective amounts is 100 ng to 100 mg per kg of body weight, more preferably 1 μg to 1 mg/kg body weight.

[0068] The invention also includes antigenic fragments of the mutant CDCs contemplated herein. 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 8, 10, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 425, 450, 460, 465, or more amino acids in length. Fragments are peptides that are about 4 to 250 amino acids, more preferably about 10-100 amino acids.

[0069] Fragments may comprise peptide portions from different locations of the mutants joined together. Preferably, fragments include one or more of the three loops discussed herein.

[0070] The mutant CDCs contemplated herein are also useful to generate neutralizing antibodies which can be used as a passive immune serum to treat or ameliorate the symptoms in patients. A vaccine 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.

[0071] The 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, intradermally, subcutaneously, and the like. The preferred route is intravenously or for localized infection, topically at the site of tissue damage with debridement. It is also preferred that the neutralizing antibody 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 or multiple dose. The preferred amount of neutralizing antibodies typically administered is about 1 mg of antibody to 1000 mg/kg, more preferably about 50-200 mg/kg of body weight.

[0072] The vaccine of the invention is preferably prepared as a pharmaceutical composition containing an immunoprotective, non-toxic amount of the protein of the invention in a non toxic and sterile pharmaceutically acceptable carrier.

[0073] The vaccines of the present invention can be administered to the appropriate subject in any manner known in the art, e.g., orally intramuscularly, intravenously, sublingual mucosal, intraarterially, intrathecally, intradermally, intraperitoneally, intranasally, intrapulmonarily, intraocularly, intravaginally, intrarectally 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 conjugates. In some embodiments, the compositions can be administered via absorption via a skin patch. 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. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained.

[0074] A pharmaceutical 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.

[0075] When the vaccine is administered parenterally, via the intramuscular or deep subcutaneous route, the protein is preferably 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 Quil A. The protein can also be presented to the immune system within microparticles such as liposomes or ISCOMs. A vaccine formulation containing the protein of the invention may be designed for oral or intranasal ingestion.

[0076] The therapeutically effective and non-toxic dose of the vaccine can be determined by a person of ordinary skill in the art. However the specific dose for any person will depend upon a variety of factors including age, general health, diet of the patient, time and route of administration, synergistic effects with other drugs being administered and whether the vaccine is administered repeatedly. If necessary the vaccine 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; for example, see Remington's Pharmaceutical Sciences latest edition.

[0077] As noted above the present invention provides polynucleotides which encode the hereinabove described polypeptides and active fragments of the invention. The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes 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.

[0078] 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 contemplated herein.

[0079] 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

[0080] Host cells are genetically engineered (transduced or transformed or transfected) with the vectors comprising a polynucleotide encoding a polypeptide of the invention. 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 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.

[0081] 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.

[0082] 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.

[0083] 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.

[0084] In addition, the expression vectors preferably 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*.

[0085] 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.

[0086] As representative 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 is deemed to be within the scope of those skilled in the art from the teachings herein.

[0087] More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this 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 example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen, Inc.), pBS, pD10, phagescript, psiX174, pbluescript SK, pBS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

[0088] 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.

[0089] In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

[0090] 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 invention can be synthetically produced by conventional peptide synthesizers.

[0091] 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 invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

[0092] Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is 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.

[0093] 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.

[0094] 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.

[0095] As a representative but nonlimiting 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.

[0096] 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.

[0097] Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

[0098] 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 know to those skilled in the art. However, preferred are host cells which secrete the polypeptide of the invention and permit recovery of the polypeptide from the culture media.

[0099] 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, 23:175 (1981), 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.

[0100] 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.

[0101] The polypeptides that are useful as immunogens in the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated.

[0102] Procedures for the isolation of the individually expressed polypeptides may be isolated by recombinant expression/isolation methods that are well-known in the art. Typical examples for such isolation 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.

[0103] Typically, a mutant CDC, (or allelic variant) as described herein has a derivative sequence containing at least one amino acid substitution, addition, deletion or insertion, preferably at least one substitution.

[0104] It is well-known in the art that certain amino acid substitutions may be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function.

[0105] Fragments and variants of the CDC mutant proteins contemplated herein are considered to be a part of the invention. 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 previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region. Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, 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 his aspect, the invention includes (i) fragments of a mutant CDC, preferably at least about 20-100 amino acids in length, more preferably about 100-200 amino acids in length, and (ii) a pharmaceutical composition comprising the mutant fragment.

[0106] The term "substitution" as used herein means a replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

[0107] 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.

[0108] 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.

[0109] 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.

[0110] Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances.

[0111] 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.

[0112] Substitutions are generally 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.

[0113] A "non-conservative substitution" refers to the substitution of an amino acid in one class with an amino acid from another class.

[0114] 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.

[0115] 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 invention contemplates every possible variant nucleotide sequence, thereof, all of which are possible given the degeneracy of the genetic code.

[0116] A "heterologous" nucleic acid construct or sequence has a portion of the sequence which is not native to the cell in which it is expressed. 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 part of the genome in which they are present, and have been added to the cell, 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.

[0117] 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.

[0118] 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.

[0119] 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.

[0120] 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.

[0121] 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") are 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.

[0122] "Chimeric gene" or "heterologous nucleic acid construct", as defined herein refers 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 invention, 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.

[0123] 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.

[0124] 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).

[0125] In one embodiment, the nucleic acids contemplated herein 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.

[0126] As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell 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.

[0127] 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 as an episomal plasmid that is maintained through multiple generations.

[0128] 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.

[0129] The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and 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).

[0130] Nucleic Acid Constructs/Expression Vectors

[0131] The nucleic acids contemplated herein may be incorporated into heterologous nucleic acid constructs or vectors, 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.

[0132] Heterologous nucleic acid constructs of the present invention may include the coding sequence for the mutant CDC contemplated herein or a fragment thereof: (i) in isolation; (ii) in combination with additional coding sequences; such as 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 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.

[0133] 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.

[0134] 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 metallothionine 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 the DNA sequence encoding the mutant polypeptide.

[0135] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art.

[0136] Specific embodiments of the invention will now be further described in more detail in the following non-limiting examples and it will be appreciated that additional and different embodiments of the teachings of the present invention will doubtless suggest themselves to those of skill in the art and such other embodiments are considered to have been inferred from the disclosure herein.

[0137] 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 (reviewed in [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.

[0138] 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 [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.

[0139] 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?

[0140] 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].

[0141] 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.

[0142] **Materials and Methods**

[0143] *Bacterial strains, plasmids, and chemicals*

[0144] 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).

[0145] *Generation and purification of ILY and its derivatives*

[0146] 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.

[0147] *Chemical modification of ILY and PFO and their derivatives with sulfhydryl specific reagents.*

[0148] 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.

[0149] *Fluorescence measurements*

[0150] 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.

[0151] *Liposome preparation*

[0152] 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].

[0153] *HRBC ghost membrane preparation*

[0154] 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].

[0155] *Cholesterol Depletion and Repletion*

[0156] 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). Typically the cholesterol content of the membranes was decreased >90% by this method.

[0157] 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.

[0158] *Immobilization of Liposomes on L1 SPR Sensor Chip*

[0159] Surface plamon 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.

[0160] *SPR Analysis*

[0161] 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.

[0162] **Results**

[0163] *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, we used ILY to first identify structures that were responsible for its cholesterol-dependence. Once we identified the structures of ILY that were sensitive to membrane cholesterol we examined the effect of disrupting these structures in PFO on its ability to bind to cholesterol-rich liposomal membranes. In this way we could determine if the same structures in both ILY and PFO were responsible for their cholesterol dependence.

[0164] *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, was its insertion 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 $ILY^{A486C\text{-}NBD}$ 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.

[0165] *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 we hypothesized that cholesterol is required for membrane insertion of the L1-L3 loops.

[0166] To test this hypothesis we individually measured the membrane insertion of the L1-L3 loops into native and cholesterol-depleted huRBC ghost membranes. We recently showed 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.

[0167] 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].

[0168] *Aspartate substitution of residues in loops L1-L3 of PFO prevent 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. We could not approach this problem in PFO in a similar manner to that use with ILY since cholesterol depletion decreases the binding of PFO to the membrane. Therefore, we determined the effect of mutating these same loops on binding of PFO to cholesterol-rich liposomes. 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 lops L1-L3 is coupled in ILY and the introduction of an asparate for any single loop residues, Ala-428 (L2), Ala-464 (L3) or Leu518 (L1), blocked their membrane insertion. We, therefore, 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.

[0169] 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.

[0170] *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, we first compared ability native PFO, and PFO modified via the sulfhydryl group of Cys-459 of the undecapeptide, to bind to cholesterol-PC liposomes via SPR.

[0171] Modification of the PFO undecapeptide Cys-459 thiol with the sulfhydryl specific reagent N-ethylmaleimide (NEM) reduced the hemolytic activity 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.

[0172] If modification of Cys-459 did not affect binding, what then did this modification do 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. He oxidation sensitive residue was ultimately linked to the highly conserved undecapeptide cysteine residue (reviewed in [1]). We further examined the structural effects of the cysteine modification on PFO 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 we determined if chemical modification of the cysteine thiol group blocked the membrane insertion of the tryptophan residues.

[0173] 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.

[0174] Immunization with Pneumolysin Mutant Leu 460Asp

[0175] 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 are able to protect against pneumonia in a focal pneumonia model in mice (Fig. 7).

[0176] 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] prevent the prepore to pore conversion of ILY. Our results indicate that both effects also result from the inability of these loops to insert into cholesterol-depleted membranes. Our 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 traps PFO in a prepore state, but does not affect binding to cholesterol-rich liposomes.

[0177] The discovery of ILY, a human cell specific toxin, presented a conundrum; how could ILY 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] 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.

[0178] 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.

[0179] 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. We have 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].

[0180] Why don't the L1-L3 loops of ILY 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, [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 to permit the insertion of the L1-L3 loops.

[0181] We have provided 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 nonlytic oligomers were still observed on the surface 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.

[0182] In summary, these studies have provided a structural basis for the two main hallmarks of the CDCs, the sensitivity to the presence of membrane cholesterol and to oxidation of the undecapeptide cysteine thiol.

[0183] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application 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 contemplated herein. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, 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 may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, compositions of matter, means, methods, amino acid or nucleic acid sequences, or steps.

[0184] All patents, patent applications, articles and publications mentioned herein, are hereby expressly incorporated herein by reference in their entireties.

References:

[0185] 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).

[0186] 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).

[0187] 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).

[0188] 4. Jacobs, T. et al. The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Lett.* **459**, 463-466 (1999).

[0189] 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).

[0190] 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).

[0191] 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).

[0192] 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).

[0193] 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).

[0194] 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).

[0195] 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).

[0196] 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).

[0197] 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).

[0198] 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).

[0199] 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.

[0200] 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).

[0201] 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).

[0202] 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).

[0203] 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).

[0204] 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).

[0205] 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).

[0206] 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).

[0207] 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).

[0208] 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).

[0209] 25. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J Mol Graph* **14**, 33-8, 27-8 (1996).

[0210] 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 cholesterol-dependent cytolysin, comprising:

a polypeptide having at least one amino acid substitution in at least one of a first hydrophobic loop, a second hydrophobic loop, or a third hydrophobic loop of an amino acid sequence comprising SEQ ID NO:1, 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, or SEQ ID NO:19, wherein when the amino acid sequence is SEQ ID NO:1, the at least one amino acid substitution is in at least one of the first loop or third loop thereof.

2. The purified mutant cholesterol-dependent cytolysin of claim 1 wherein the peptide is substantially non-hemolytic, non-pore forming, and non-binding to cell membranes.

3. A composition comprising one or more of the mutants of claim 1 disposed in a pharmaceutically-acceptable carrier.

4. A vaccine comprising the purified mutant cholesterol-dependent cytolysin of claim 1.

5. A purified mutant cholesterol-dependent cytolysin, comprising:

a polypeptide having at least one amino acid substitution in at least one of a first loop, a second loop, or a third loop of an amino acid sequence comprising SEQ ID NO:1, SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:17 or SEQ ID NO:18, wherein in SEQ ID NO:1, the first loop, second loop, and third loop comprise amino acid positions 457-463, 367-373, and 403-409, respectively, and wherein in SEQ ID NO:9, the first loop, second loop, and third loop comprise amino acid positions 559-565, 469-475, and 505-511, respectively, and wherein in SEQ ID NO:13, the first loop, second loop and third loop comprise amino acid positions 513-519, 423-429, and 459-465, respectively, and wherein in SEQ ID NO:17, the first loop, second

loop, and third loop comprise amino acid positions 515-521, 425-431, and 461-467, respectively, and wherein in SEQ ID NO:18, the first loop, second loop, and third loop comprise amino acid positions 651-657, 561-567, and 597-603, respectively, and wherein when the amino acid sequence is SEQ ID NO:1, the at least one amino acid substitution is in at least one of the first loop or third loop thereof.

6. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the amino acid sequence is SEQ ID NO:1 and comprises 1 to 7 amino acid substitutions in at least one of the first loop and the third loop.

7. The purified mutant cholesterol-dependent cytolysin of claim 6 further comprising 1 to 7 amino acid substitutions in the second loop.

8. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the amino acid sequence is SEQ ID NO:9 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

9. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the amino acid sequence is SEQ ID NO:13 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

10. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the amino acid sequence is SEQ ID NO:17 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

11. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the amino acid sequence is SEQ ID NO:18 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

12. The purified mutant cholesterol-dependent cytolysin of claim 5 wherein the polypeptide is substantially non-hemolytic, non-pore forming, and non-binding to cell membranes.

13. A composition comprising the purified mutant cholesterol-dependent cytolysin of claim 5, and a pharmaceutically-acceptable carrier or vehicle.

14. A vaccine comprising the purified mutant cholesterol-dependent cytolysin of claim 5.

15. The vaccine of claim 14 further comprising capsular polysaccharride serotypes, or immunogenic proteins or protein subunits or fragments.

16. A method vaccinating a subject, comprising:
providing the vaccine of claim 14 and administering the vaccine to the subject wherein an immunogenic response is induced in the subject.

17. A purified mutant cholesterol-dependent cytolysin, comprising:
a polypeptide having at least one amino acid substitution in at least one of a first loop, a second loop, or a third loop of an amino acid sequence comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6, wherein in SEQ ID NO:2, the first loop, second loop, and third loop comprise amino acid positions 498-504, 408-414, and 444-450, respectively, and wherein in SEQ ID NO:3, the first loop, second loop, and third loop comprise amino acid positions 501-507, 411-417, and 447-453, respectively, and wherein in SEQ ID NO:4, the first loop, second loop and third loop comprise amino acid positions 501-507, 411-417, and 447-453, respectively, and wherein in SEQ ID NO:5, the first loop, second loop, and third loop comprise amino acid positions 488-494, 398-404, and 434-440, respectively, and wherein in SEQ ID NO:6, the first loop, second loop, and third loop comprise amino acid positions 490-496, 400-406, and 436-442, respectively.

18. The purified mutant cholesterol-dependent cytolysin of claim 17 wherein the amino acid sequence is SEQ ID NO:2 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

19. The purified mutant cholesterol-dependent cytolysin of claim 17 wherein

the amino acid sequence is SEQ ID NO:3 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

20. The purified mutant cholesterol-dependent cytolysin of claim 17 wherein the amino acid sequence is SEQ ID NO:4 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

21. The purified mutant cholesterol-dependent cytolysin of claim 17 wherein the amino acid sequence is SEQ ID NO:5 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

22. The purified mutant cholesterol-dependent cytolysin of claim 17 wherein the amino acid sequence is SEQ ID NO:6 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

23. A purified mutant cholesterol-dependent cytolysin, comprising:

a polypeptide having at least one amino acid substitution in at least one of a first loop, a second loop, or a third loop of an amino acid sequence comprising SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:12, wherein in SEQ ID NO:7, the first loop, second loop, and third loop comprise amino acid positions 562-568, 472-478, and 508-514, respectively, and wherein in SEQ ID NO:8, the first loop, second loop, and third loop comprise amino acid positions 559-565, 469-475, and 505-511, respectively, and wherein in SEQ ID NO:10, the first loop, second loop and third loop comprise amino acid positions 502-508, 412-418, and 448-454, respectively, and wherein in SEQ ID NO:11, the first loop, second loop, and third loop comprise amino acid positions 514-520, 424-430, and 460-466, respectively, and wherein in SEQ ID NO:12, the first loop, second loop, and third loop comprise amino acid positions 512-518, 422-428, and 458-464, respectively.

24. The purified mutant cholesterol-dependent cytolysin of claim 23 wherein the amino acid sequence is SEQ ID NO:7 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

25. The purified mutant cholesterol- dependent cytolysin of claim 23 wherein the amino acid sequence is SEQ ID NO:8 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

26. The purified mutant cholesterol-dependent cytolysin of claim 23 wherein the amino acid sequence is SEQ ID NO:10 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

27. The purified mutant cholesterol-dependent cytolysin of claim 23 wherein the amino acid sequence is SEQ ID NO:11 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

28. The purified mutant cholesterol-dependent cytolysin of claim 23 wherein the amino acid sequence is SEQ ID NO:12 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

29. A purified mutant cholesterol-dependent cytolysin, comprising:

a polypeptide having at least one amino acid substitution in at least one of a first loop, a second loop, or a third loop of an amino acid sequence comprising SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:19, wherein in SEQ ID NO:14, the first loop, second loop, and third loop comprise amino acid positions 514-520, 424-430, and 460-466, respectively, and wherein in SEQ ID NO:15, the first loop, second loop, and third loop comprise amino acid positions 494-490, 395-401, and 431-437, respectively, and wherein in SEQ ID NO:16, the first loop, second loop and third loop comprise amino acid positions 457-463, 367-373, and 403-409, respectively, and wherein in SEQ ID NO:19, the first loop, second loop, and third loop comprise amino acid positions 521-527, 431-437, and 467-473, respectively.

30. The purified mutant cholesterol-dependent cytolysin of claim 29 wherein the amino acid sequence is SEQ ID NO:14 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

31. The purified mutant cholesterol- dependent cytolysin of claim 29 wherein the amino acid sequence is SEQ ID NO:15 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

32. The purified mutant cholesterol-dependent cytolysin of claim 29 wherein the amino acid sequence is SEQ ID NO:16 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

33. The purified mutant cholesterol-dependent cytolysin of claim 29 wherein the amino acid sequence is SEQ ID NO:19 and comprises 1 to 7 amino acid substitutions in at least one of the first loop, the second loop, and the third loop.

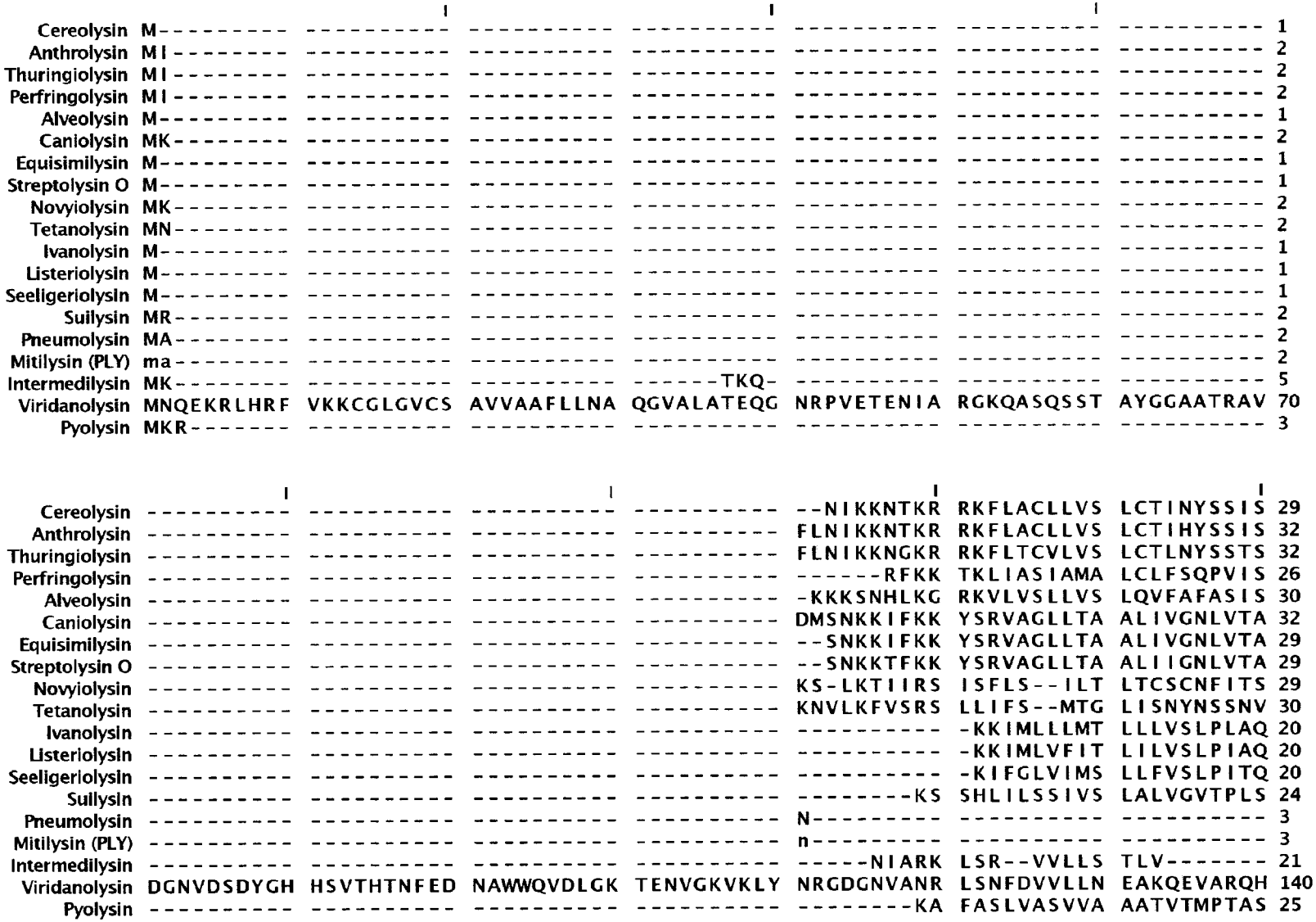

Cereolysin M--------- ---------- ---------- ---------- ---------- ---------- ---------- 1
Anthrolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
Thuringiolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 2
Perfringolysin MI-------- ---------- ---------- ---------- ---------- ---------- ---------- 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

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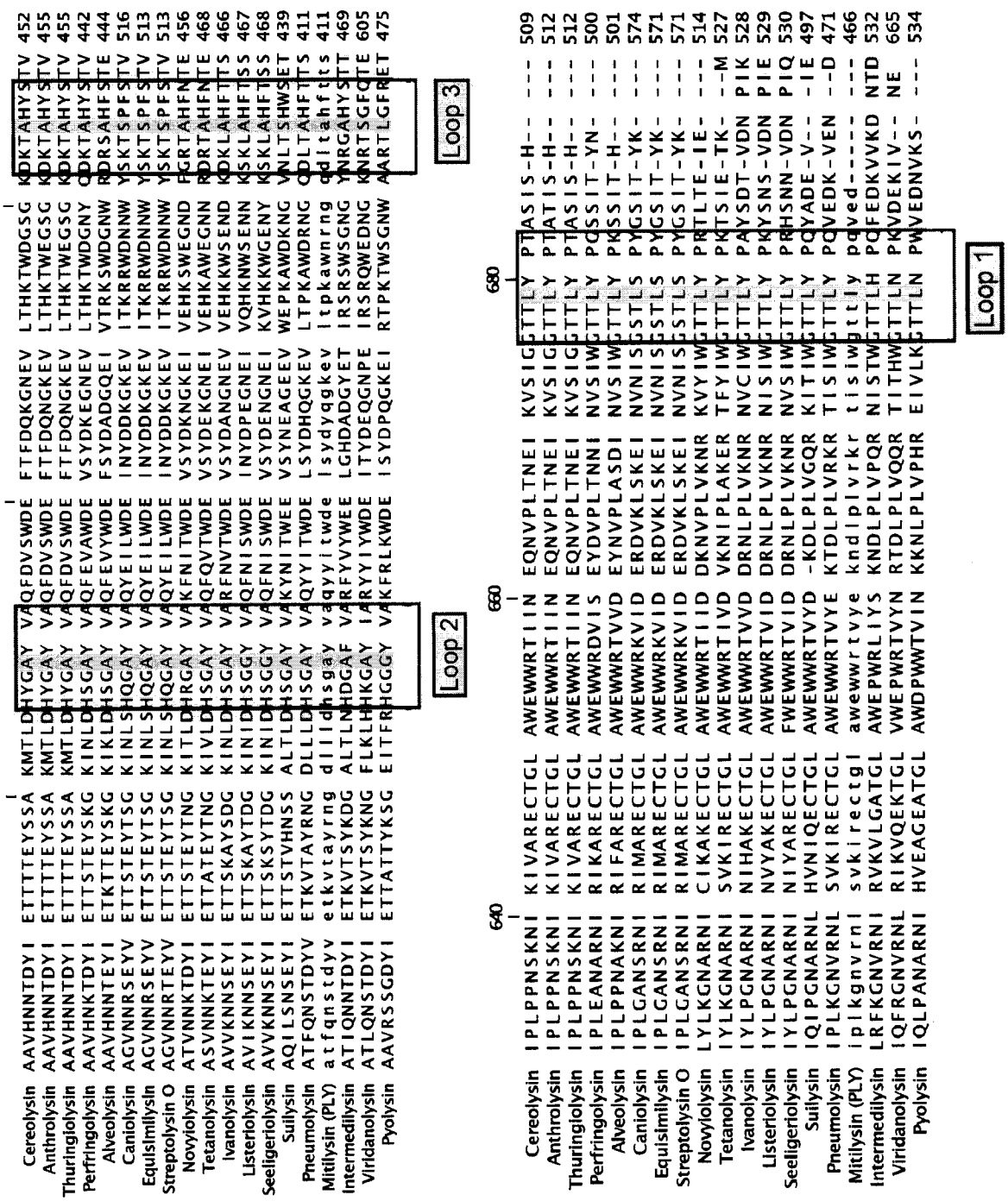


Figure 1E

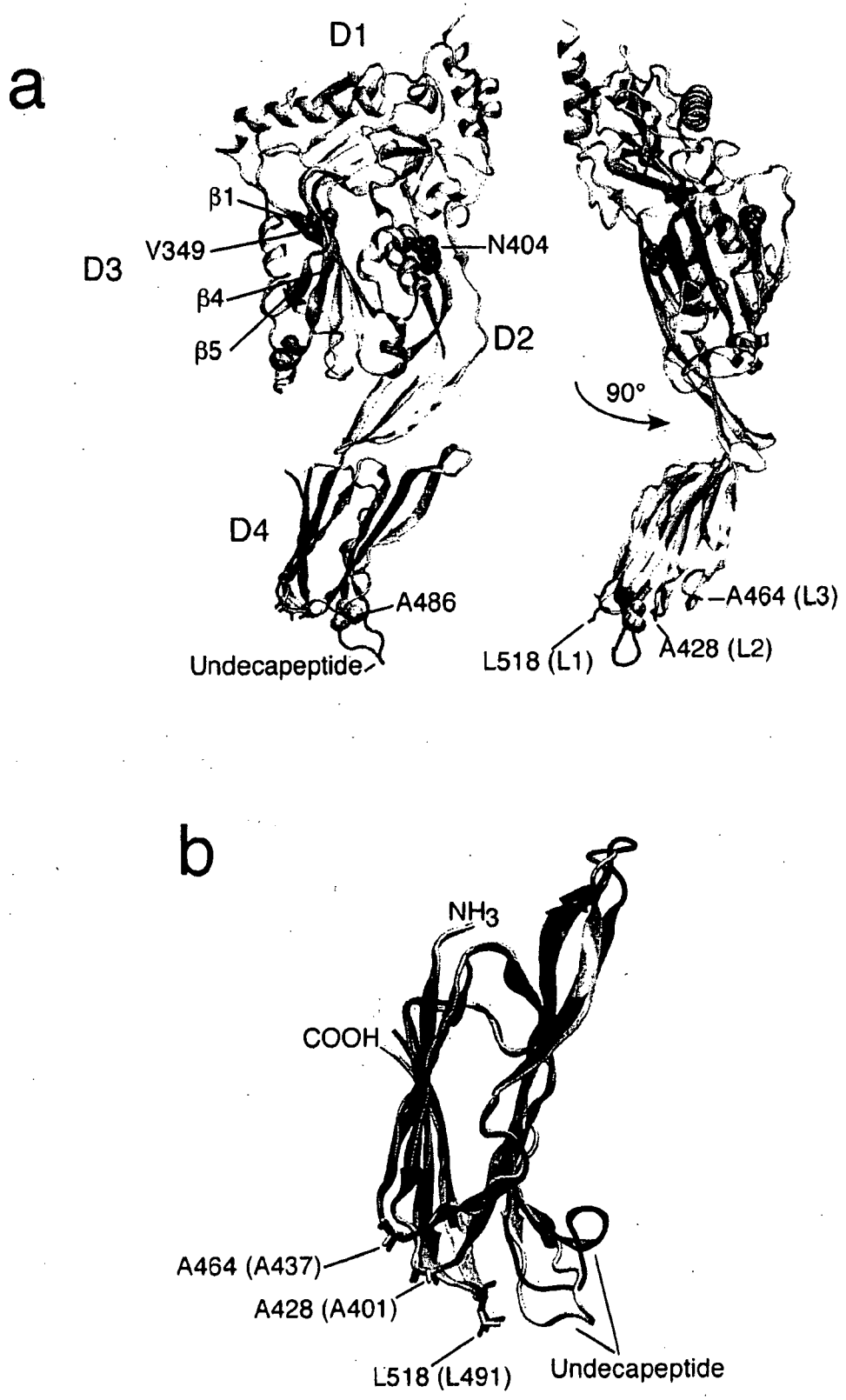

a
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β1
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N404
D3
β4
β5
D2
90°
D4
A486
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Undecapeptide
L518 (L1)
A428 (L2)
b
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A428 (A401)
L518 (L491)
Undecapeptide

Figure 2

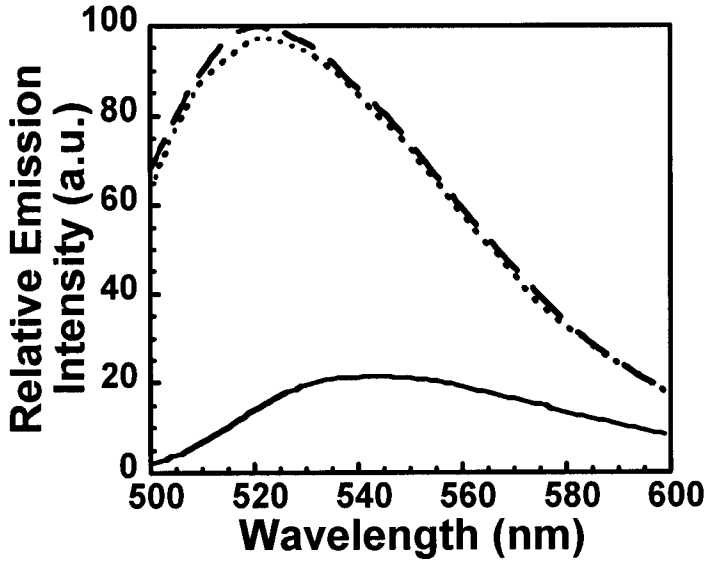


Figure 3

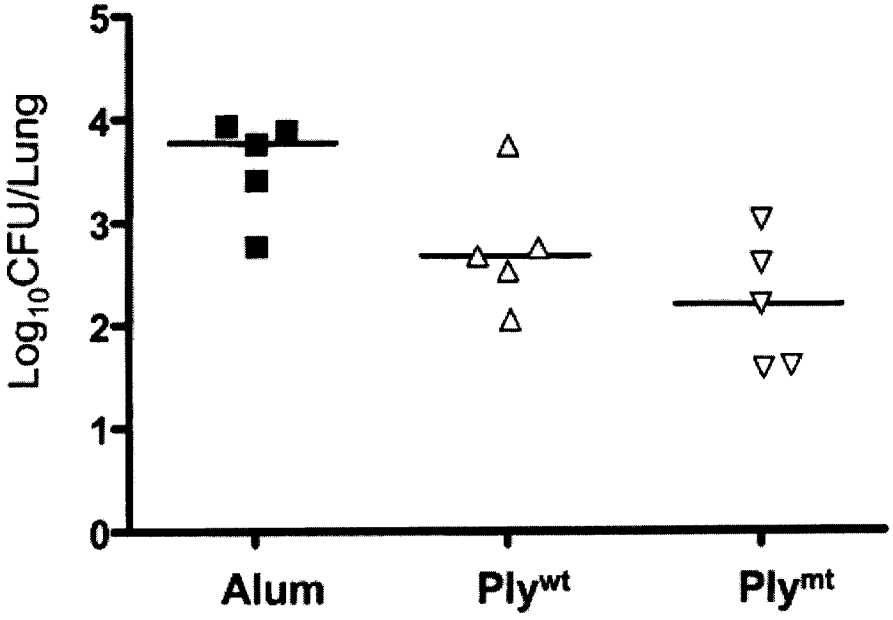


Figure 7

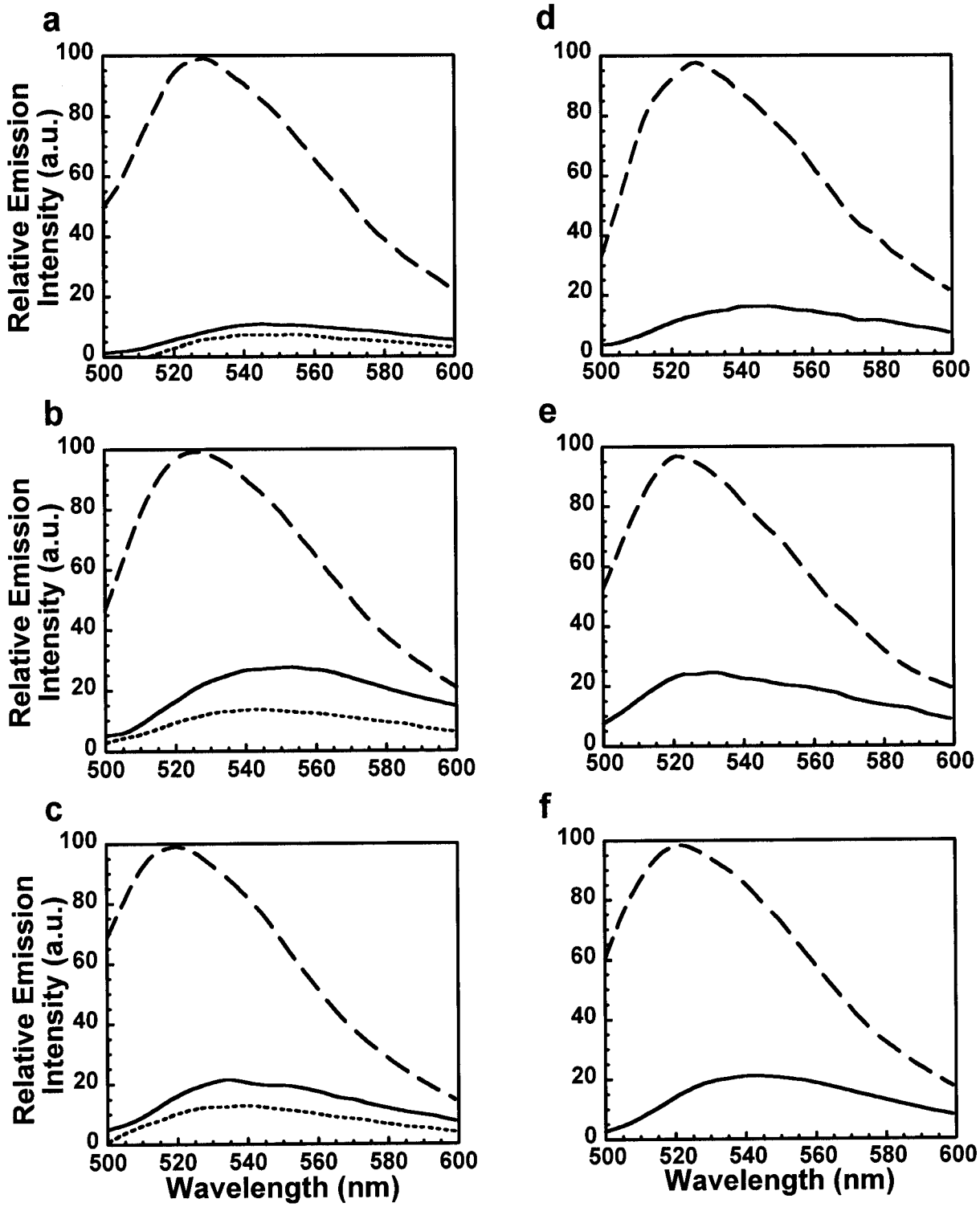

a
b
c
d
e
f
Relative Emission Intensity (a.u.)
Wavelength (nm)
0
20
40
60
80
100
500
520
540
560
580
600

Figure 4

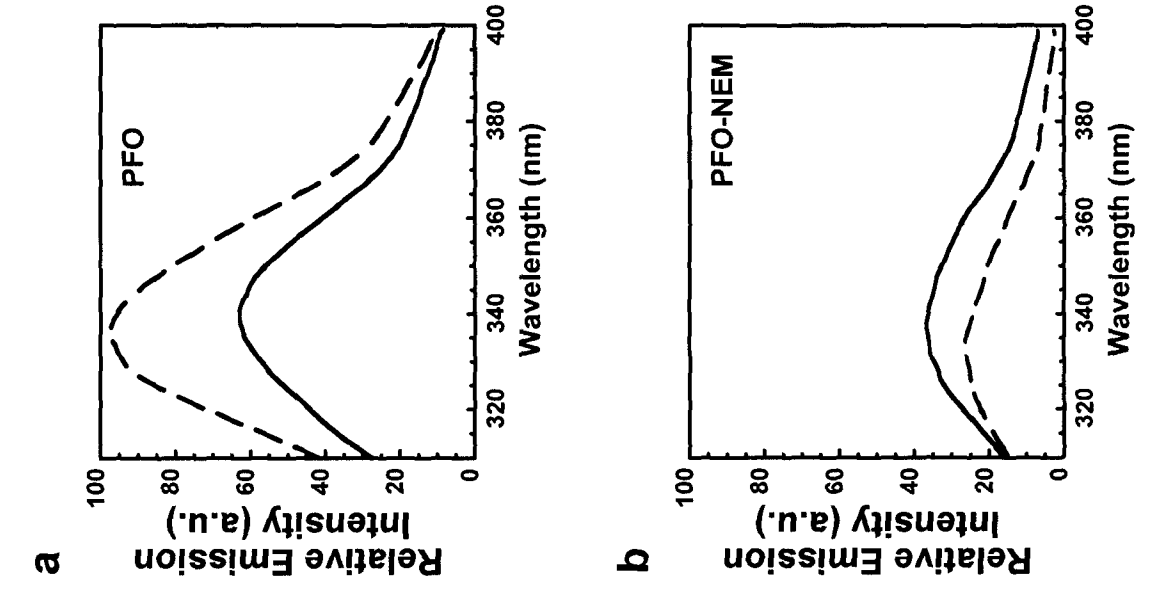


Figure 6

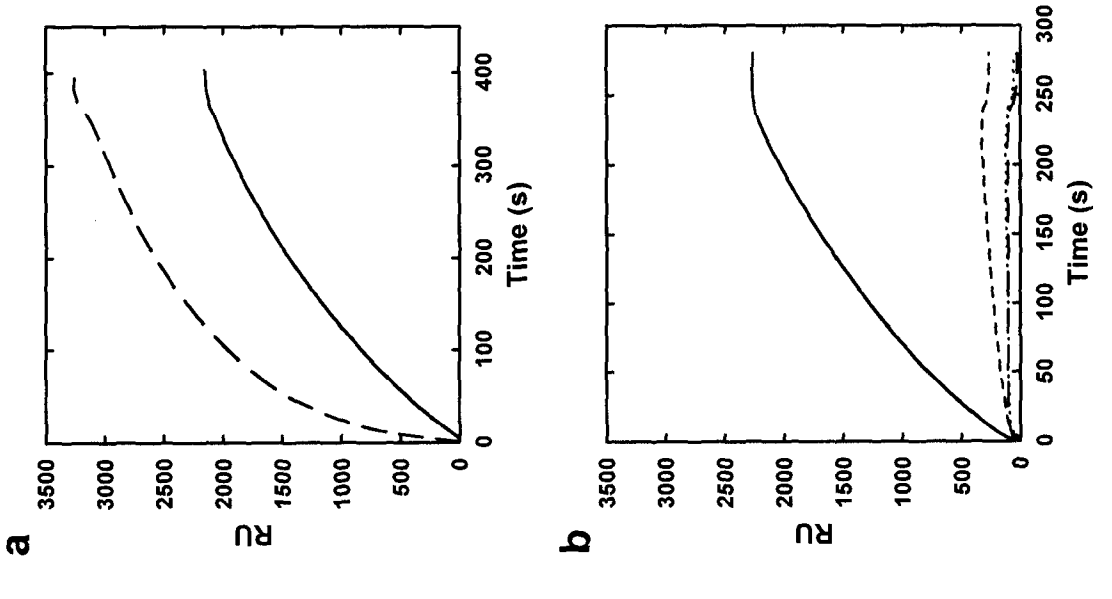


Figure 5