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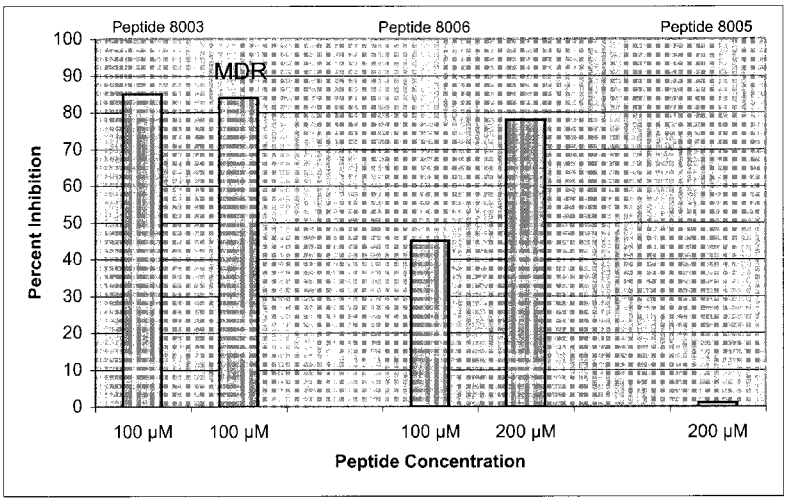
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(54) Title: PEPTIDE INHIBITORS OF TYPE III SECRETION

FIGURE 1C



(57) Abstract: The application relates to peptides that inhibit type HI secretion in Gram negative bacteria, compositions comprising said peptide inhibitors, and includes kits, methods, and uses thereof Specifically, the application relates to peptides that comprise interactive domains of translocator proteins, and particularly to peptide inhibitors comprising interactive domains of the translocator proteins PopD of *Pseudomonas aeruginosa* These interactive domains mediate binding between PopD and PopB, another translocator protein Peptides comprising these interactive domains disrupt the growth of Gram negative bacteria, inhibit subsequent infection of cells, reduce infection from Gram negative bacteria in an animal model, and are not cytotoxic

Title: Peptide inhibitors of Type III secretion

[0001] The present disclosure relates to novel peptide inhibitors and methods and uses thereof.

BACKGROUND OF THE DISCLOSURE

[0002] A number of Gram negative bacteria cause diseases in humans. Examples of Gram negative bacteria include *E. coli* 0157, *Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitca*, *Chlamydia pneumoniae* and *Chlamydia trachomatis*. All of these bacteria have in common a protein secretion apparatus, called type III secretion system (T3SS), which allows the bacteria to inject a family of effector proteins into susceptible host cells. The T3SS is a virulence factor required for infection of host cells, and for host cell invasion. The T3SS or injectosome is composed of approximately 25 proteins that form the secretion apparatus which resembles a hypodermic syringe. Some of the structural proteins of the injectosome are genetically conserved across the different bacteria and these conserved proteins perform essential functions. Two of these conserved proteins are called translocator proteins which form a pore or translocon in the host cell membrane through which the effector proteins from the bacteria are secreted. These two translocator proteins (i.e. PopB and PopD in Pseudomonas, SipB/C in Shigella, CopB/D in Chlamydia) interact in a specific way to form a hexameric protein complex or pore in the host cell membrane. However, the specific regions of the translocator proteins and the manner in which these regions interact with each other is not known.

SUMMARY OF THE DISCLOSURE

[0003] The present inventor investigated the binding regions or interactive domains of translocator proteins, which are part of the type III secretion system (T3SS) in Gram negative bacteria. Specifically, the present inventor investigated and mapped the interactive domains of the T3SS translocator proteins PopD and PopB of *Pseudomonas aeruginosa.* The inventor then developed and synthesized novel peptide inhibitors of type III secretion using amino acid sequence data from the interactive domains of

type III secretion translocator proteins. The inventor further determined that the novel peptide inhibitors inhibited type III secretion in Gram negative bacteria, inhibited subsequent infection of cells, and are not cytotoxic. The novel peptide inhibitors are used to inhibit and prevent type III secretion, to inhibit infection from Gram negative bacteria, to inhibit cell invasion of Gram negative bacteria and to treat or prevent infection from Gram negative bacteria.

[0004] Accordingly, the present disclosure includes novel isolated peptide inhibitors of type III secretion in Gram negative bacteria.

[0005] In one embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises the amino acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1) or a variant thereof.

[0006] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2) or a variant thereof.

[0007] In another aspect of the present disclosure, the peptide inhibitor of type III secretion in Gram negative bacteria comprises a fragment of the PopD translocator sequence: MIDTQYSLAATQAAIPSEPIAPGAAGRSVGTPQAAADLPQVPAARADRVELN APRQVLDPVRMEAAGSELDSSVELLLILFRIAQKARELGVLQRDNENQSIIHA QKAQVDEMRSGATLMIAMAVIAGVGALASAVVGSLGALKNGKAISQEKTLQ KNIDGRNELIDAKMQALGKTSDEDRKIVGKVWAADQVQDSVALRAAGRAFE SRNGALQVANTVIQSFVQMANASVQVRQGESQASAREGEVNATIGQSQKQ KVEDQMSFDAGFMKDVLQLIQQYTQSHNQAWRAAAGVV (SEQ ID NO: 3) or a variant thereof wherein the fragment or variant inhibits type III secretion.

[0008] Another aspect of the present disclosure is a composition comprising one or more peptide inhibitors disclosed herein.

[0009] Another aspect of the present disclosure is a kit comprising the peptide inhibitors or compositions comprising one or more of the peptide inhibitors disclosed herein.

[0010] Another aspect of the present disclosure is a method of inhibiting or preventing type III secretion in Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof. The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit or prevent type III secretion in Gram negative bacteria in a cell or subject in need thereof.

[0011] An additional aspect of the present disclosure is a method of inhibiting infection from Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof. The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit infection from Gram negative bacteria in a cell or subject in need thereof.

[0012] Another aspect of the present disclosure is a method of inhibiting cell invasion of Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof. The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit cell invasion of Gram negative bacteria in a cell or subject in need thereof.

[0013] An additional aspect of the present disclosure is a method of treating or preventing infection from Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof. The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to treat or prevent infection from Gram negative bacteria in a cell or subject in need thereof.

[0014] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the disclosure are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The disclosure will now be described in relation to the drawings in which:

[0016] Figure 1A is a Pepscan analysis of PopD peptide library and soluble PopB binding.

[0017] Figure 1B is a graph demonstrating that Peptide 8003 inhibits *Pseudomonas aeruginosa* infection of HeLa cells.

[0018] Figure 1C is a graph demonstrating inhibition of *Pseudomonas aeruginosa* by various peptides, and inhibition of a multi-drug resistant (MDR) *Pseudomonas aeruginosa* by Peptide 8003.

[0019] Figure 2 is a micrograph demonstrating that Peptide 8003 inhibits *Chlamydia trachomatis* infection of HeLa cells.

[0020] Figure 3 is a micrograph demonstrating that Peptide 8003 inhibits *Chlamydia pneumoniae* infection of HeLa cells.

[0021] Figure 4A is a diagram showing the sequence alignment of Peptide 8003 and *Chlamydia pneumoniae* CopD1.

[0022] Figure 4B is a diagram showing the sequence alignment of Peptide 8003 and *Chlamydia pneumoniae* Cop D2.

[0023] Figure 5 is a graph demonstrating that Peptide 8003 reduces the number of *Chlamydia trachomatis* bacteria in the genital tract of mice.

[0024] Figure 6 is a graph demonstrating that Peptide 8003 is not cytotoxic to HeLa cells.

DETAILED DESCRIPTION OF THE DISCLOSURE

I. PEPTIDE INHIBITORS, METHODS OF IDENTIFYING PEPTIDE INHIBITORS, COMPOSITIONS, AND KITS OF THE DISCLOSURE

PEPTIDE INHIBITORS

[0025] As mentioned above, the present inventor investigated the binding regions or interactive domains of translocator proteins in the type III secretion system (T3SS) of Gram negative bacteria. Specifically, the present disclosure describes the investigation and mapping of the binding regions or interactive domains on the T3SS translocator proteins PopD (SEQ ID NO: 3) and PopB of *Pseudomonas aeruginosa*. The present disclosure includes novel peptide inhibitors of type III secretion developed using the amino acid sequence data from the interactive domains of T3SS translocator proteins. The present disclosure also demonstrated that the novel peptide inhibitors inhibited type III secretion in Gram negative bacteria, inhibited subsequent infection of cells as shown in Figures 1-3, and are not cytotoxic (Figure 6). The present disclosure also demonstrated that the novel peptide inhibitors reduced infection from Gram negative bacteria of cells in an animal model (Figure 5). As described more fully below, the novel peptide inhibitors included in the present disclosure are used to inhibit and prevent type III secretion, to inhibit infection from Gram negative bacteria, to inhibit cell

invasion of Gram negative bacteria and to treat or prevent infection from Gram negative bacteria.

[0026] Accordingly, the present disclosure includes novel isolated peptide inhibitors of type III secretion in Gram negative bacteria. The present disclosure also includes a composition comprising one or more of the peptide inhibitors.

[0027] The present disclosure describes the development and synthesis of two distinct peptide inhibitors. These are designated as Peptide 8003 and Peptide 8006 in the present disclosure.

[0028] Using an overlapping peptide library, binding regions or interactive domains on the type III secretion system translocator proteins PopD and PopB of *Pseudomonas aeruginosa* were mapped and peptide inhibitors were developed. Peptide 8003 comprises the amino acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1) and Peptide 8006 comprises the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2).

[0029] Accordingly, the present disclosure includes an isolated peptide inhibitor of type III secretion in Gram negative bacteria.

[0030] As used herein the term “peptide” refers to a sequence of amino acids. The term is used interchangeably with “oligopeptide”, “polypeptide” and "protein". The peptide inhibitor described herein may comprise a sequence of approximately 10 to 300 amino acids in length, or 10 to 100 amino acids in length or suitably 20 to 50 amino acids in length. The term “amino acid” includes all of the naturally occurring amino acids as well as modified amino acids. The term “peptide inhibitor” or “peptide inhibitors” includes those produced from chemical synthesis and also includes peptide inhibitors produced synthetically to have the same primary structure or composition and the same function and/or activity of the peptide inhibitors disclosed herein. “Produced synthetically” includes for example, peptide inhibitors produced from recombinant DNA technology.

[0031] The term "isolated" as used herein means a macromolecule such as a peptide that has been identified, separated, recovered from its natural environment, and/or has been produced synthetically. The term "isolated" includes a peptide inhibitor, which is substantially free of chemical precursors, or other chemicals when produced synthetically from chemical synthesis, and also includes a peptide inhibitor, which is substantially free of cellular material or culture media when produced synthetically from recombinant techniques.

[0032] The term "type III secretion" refers to a function or activity that is part of the type III secretion system (T3SS), which is a protein secretion apparatus present in Gram negative bacteria responsible for virulence of cells, including cell invasion and/or infection of cells.

[0033] The term "Gram negative bacteria" as used herein includes all types of Gram negative bacteria, including, without limitation, *Pseudomonas aeruginosa, Chlamydia trachomatis*, *Chlamydia pneumoniae, Salmonella, Shigella, E. coli* O157, Salmonella, Shigella, Listeria, and/or Yersinia. The term "Gram negative bacteria" also includes Gram negative bacteria that are multi-drug resistant. As used herein, the term "multi-drug resistant" or "antibiotic resistant bacteria" or "resistant bacteria" includes, for example, types and strains of Gram negative bacteria that do not respond to treatment with antibiotics.

[0034] Another embodiment of the present disclosure is an isolated peptide inhibitor of type III secretion in Gram negative bacteria, wherein the peptide inhibitor comprises interactive domains of translocator proteins of type III secretion in Gram negative bacteria.

[0035] The term "interactive domains" as used herein refers to binding regions of amino acid sequences in translocator proteins of the type III secretion system (T3SS) present in Gram negative bacteria that bind to and/or interact with each other to form a pore or translocon in a cell membrane.

[0036] The term "translocator proteins" as used herein refers to proteins of the type III secretion system (T3SS) present in Gram negative bacteria which interact with each other via interactive domains to form a pore or translocon in a cell membrane through which bacterial proteins are secreted into the cell. In one embodiment, "translocator proteins" include PopD and PopB of *Pseudomonas aeruginosa.*

[0037] Another embodiment of the present disclosure is an isolated peptide inhibitor of type III secretion in Gram negative bacteria, wherein the peptide inhibitor comprises interactive domains of translocator proteins of type III secretion in Gram negative bacteria and the translocator proteins are PopB and PopD of *Pseudomonas aeruginosa.*

[0038] In one embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises a fragment of the PopD translocator sequence:MIDTQYSLAATQAAIPSEPIAPGAAGRSVGTPQAAADLPQVPAAR ADRVELNAPRQVLDPVRMEAAGSELDSSVELLLILFRIAQKARELGVLQRDN ENQSIIHAQKAQVDEMRSGATLMIAMAVIAGVGALASAVVGSLGALKNGKAI SQEKTLQKNIDGRNELIDAKMQALGKTSDEDRKIVGKVWAADQVQDSVALR AAGRAFESRNGALQVANTVIQSFVQMANASVQVRQGESQASAREGEVNAT IGQSQKQKVEDQMSFDAGFMKDVLQLIQQYTQSHNQAWRAAAGVV (SEQ ID NO: 3) or a variant thereof wherein the fragment or variant inhibits type III secretion.

[0039] In another embodiment, the isolated peptide inhibitors of type III secretion in Gram negative bacteria disclosed herein include peptide inhibitors with amino acid sequences in SEQ ID NO:1 and/or SEQ ID NO:2 or variants thereof.

[0040] The term "variant" as used herein includes modifications, substitutions, additions, derivatives, analogs, fragments or chemical equivalents of the amino acid sequences disclosed herein that perform substantially the same function as the peptide inhibitors disclosed herein in substantially the same way. For instance, the variants have the same function of being useful to inhibit type III secretion in Gram negative bacteria, to

prevent type III secretion in Gram negative bacteria, to inhibit infection from Gram negative bacteria, to inhibit cell invasion of Gram negative bacteria and/or to treat or prevent infection from Gram negative bacteria.

[0041] Variants of the peptide inhibitors disclosed herein also include, without limitation, conservative amino acid substitutions. A "conservative amino acid substitution" as used herein, is one in which one amino acid residue is replaced with another amino acid residue without abolishing the desired function or activity of the peptide inhibitors disclosed herein. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Conserved amino acid substitutions involve replacing one or more amino acids of the polypeptides of the disclosure with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting variant should be functionally equivalent. Changes which result in production of a chemically equivalent or chemically similar amino acid sequence are included within the scope of the disclosure. If the peptide inhibitors of the present disclosure are made using recombinant DNA technology, variants of the peptide inhibitors may be made by using polypeptide engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine. The phrase “conservative substitution” also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite activity.

[0042] In one embodiment, a variant of the peptide inhibitor having the amino acid sequence in SEQ ID NO: 1 comprises one or more conservative amino acid substitutions of the amino acid sequence in SEQ ID NO: 1.

[0043] In another embodiment, a variant of the peptide inhibitor having the amino acid sequence in SEQ ID NO: 2 comprises one or more conservative amino acid substitutions of the amino acid sequence in SEQ ID NO: 2.

[0044] In another embodiment, a variant of the peptide inhibitor having the amino acid sequence in SEQ ID NO: 3 comprises one or more conservative amino acid substitutions of the amino acid sequence in SEQ ID NO: 3.

[0045] Variants of the peptide inhibitors of the present disclosure also include additions and deletions to the amino acid sequences disclosed herein.

[0046] Variants of the peptide inhibitors of the present disclosure also include analogs thereof. The term "analog" as used herein includes any active agent capable of performing the function of the peptide inhibitors disclosed herein, and may include peptide mimetics and the like. The term "active" refers to molecules in a conformation suitable for performing substantially the same functions as the peptide inhibitors disclosed herein in substantially the same way. Peptide mimetics include synthetic structures that may serve as substitutes for peptides in interactions between molecules (see Morgan and Gainor. (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but are designed to retain the desired structural and functional features and thus may be suitable substitutes of the peptide inhibitor analog disclosed in the present disclosure.

[0047] Peptide mimetics also include molecules incorporating peptides into larger molecules with other functional elements (e.g., as described in WO 99/25044). Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367), and peptide libraries containing

peptides of a designed length representing all possible sequences of amino acids corresponding to an isolated peptide of the disclosure. Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

[0048] Variant peptide inhibitors of the present disclosure also include derivatives thereof. The term "derivative" refers to a peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be

substituted for serine; and ornithine may be substituted for lysine. A derivative of a polypeptide also optionally includes polypeptides comprising forms of amino acids that are oxidized.

[0049] Variant peptide inhibitors of the present disclosure also include fragments thereof. The term "fragment" as used herein means a portion of a polypeptide that contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more of the entire length of the reference polypeptide.

[0050] In another embodiment, the isolated peptide inhibitors of type III secretion in Gram negative bacteria disclosed herein include peptide inhibitors with amino acid sequences that are substantially or essentially identical to the amino acid sequences of SEQ ID NO:1 and/or SEQ ID NO:2.

[0051] The term "substantially identical" or "essentially identical" as used herein means an amino acid sequence that, when optimally aligned, for example using the methods described herein, share at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with a second amino acid sequence.

[0052] The term “sequence identity” as used herein refers to the percentage of sequence identity between two polypeptide and/or nucleotide sequences.

[0053] To determine the percent identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical

overlapping positions/total number of positions.times.100%). In one embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g., for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present disclosure. BLAST protein searches can be performed with the XBLAST program parameters set, e.g., to score-50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) can be used (see, e.g., the NCBI website). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11-17. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

[0054] The percentage of identity between two polypeptide sequences, the amino acid sequences of such two sequences are aligned, for example using the Clustal W algorithm (Thompson, JD, Higgins DG, Gibson TJ, 1994, *Nucleic Acids Res.* 22(22): 4673-4680.), together with BLOSUM 62 scoring matrix (Henikoff S. and Henikoff J.G., 1992, *Proc. Natl. Acad. Sci.* USA 89: 10915–10919.) and a gap opening penalty of 10 and gap extension penalty of 0.1, so that the highest order match is obtained between two sequences wherein at least 50% of the total length of one of the sequences is involved in the alignment.

[0055] Other methods that may be used to align sequences are the alignment method of Needleman and Wunsch (Needleman and Wunsch. *J. Mol. Biol.,* 1970, 48:443), as revised by Smith and Waterman (Smith and Waterman. *Adv. Appl. Math.* 1981, 2:482) so that the highest order match is obtained between the two sequences and the number of identical amino acids is determined between the two sequences. Other methods to calculate the percentage identity between two amino acid sequences are generally art recognized and include, for example, those described by Carillo and Lipton (Carillo and Lipton *SIAM J. Applied Math.* 1988, 48:1073) and those described in Computational Molecular Biology (Computational Molecular Biology, Lesk, e.d. Oxford University Press, New York, 1988, *Biocomputing: Informatics and Genomics Projects*). Generally, computer programs will be employed for such calculations.

[0056] Accordingly, the peptide inhibitor of type III secretion in Gram negative bacteria comprising an amino acid sequence that is substantially or essentially identical to the amino acid sequence in SEQ ID NO: 1 may be determined by percent (%) sequence identity.

[0057] In one embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises 90% sequence identity with the amino acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1).

[0058] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises 95% sequence identity with the amino

acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1).

[0059] In a further embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises all or part of the amino acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1) and/or a variant thereof.

[0060] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises the amino acid sequence: RAFESRNGALQVANTVIQSFVQMANASVQVR (SEQ ID NO: 1).

[0061] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprising an amino acid sequence that is substantially or essentially identical to the amino acid sequence in SEQ ID NO: 2 may be determined by percent (%) sequence identity.

[0062] In one embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises 90% sequence identity with the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2).

[0063] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises 95% sequence identity with the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2).

[0064] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises all or part of the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2) and/or a variant thereof.

[0065] In another embodiment, the peptide inhibitor of type III secretion in Gram negative bacteria comprises the amino acid sequence: QMSFDAGFMKDVLQLIQQYTQSHNQAWRAAA (SEQ ID NO: 2).

[0066] The inventor has compared the sequence of Peptide 8003 (SEQ ID NO: 1) to that of PopD sequences from other gram negative bacteria. In

particular, the sequence similarity between the C-terminal PopD 8003 peptide (Peptide 8003) and the C-terminus of CopD1 and CopD2 from *Chlamydia pneumoniae* was determined and is shown in Figs 4A and 4B. The sequence alignment of Peptide 8003 with: 1) the C'-terminus of *Chlamydia pneumoniae* CopD1 (Cpn1020); and 2) the C'-terminus of *Chlamydia pneumoniae* CopD2 (Cpn1019); are presented in Figures 4A (SEQ ID NO: 4) and 4B (SEQ ID NO: 5), respectively. The data indicates that these sequences are highly conserved. These conserved areas are 100% helical.

[0067] Accordingly, the disclosure includes a peptide inhibitor of type III secretion comprising a fragment of a sequence shown in Figures 4A and/or 4B (SEQ ID NOS: 4-5) or a variant thereof.

[0068] Another aspect of the present disclosure is an isolated nucleotide encoding a peptide inhibitor and/or variant disclosed herein. A nucleotide sequence that encodes a peptide inhibitor and/or variant of the present disclosure may be deduced by a person skilled in the art using computer nucleic acid prediction programs and/or mRNA codon- amino acid tables that are readily available in the art.

[0069] The term "isolated nucleotide" as used herein refers to a nucleotide or nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated nucleic acid" is also substantially free of sequences which naturally flank the nucleic acid (i.e. sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded. The nucleic acid sequences contemplated by the present disclosure include isolated nucleotide sequences encoding a peptide inhibitor and/or variant disclosed herein.

METHODS OF IDENTIFYING INHIBITORS

[0070] In addition to the peptide inhibitors described above, other peptide inhibitors may be developed and identified using the methods described herein. For example, peptide inhibitors may be developed by mapping binding regions or interactive domains of translocator proteins, identifying candidate peptide inhibitors, and producing candidate peptide inhibitors. In one embodiment, the interactive domains of translocator proteins are mapped using an overlapping peptide library map. In another embodiment, the interactive domains of translocator proteins are mapped using Pepscan analysis. In another embodiment, candidate peptide inhibitors are identified or selected by evaluating the degree of binding or interaction of various amino acid regions within the interactive binding domains on translocator proteins. In another embodiment, the amino acid sequence of candidate peptide inhibitors is extended to include an approximate length of 30 to 35 amino acids to improve binding affinity. In another embodiment, for candidate proteins, any proteins that are known to interact to form bi- or tri-molecular complexes that are functionally important either as an holoenzyme or scaffolding complex would be candidate proteins for the development of peptide inhibitors. In one embodiment, candidate peptide inhibitors may be produced synthetically. In another embodiment, candidate peptide inhibitors may be produced synthetically using solid-phase chemistry or solid phase chemical synthesis. In another embodiment, candidate peptide inhibitors may be produced synthetically using recombinant protein molecules to act as a carrier molecule for the peptide inhibitor. In one embodiment, a "fusion protein" (carrier molecule plus peptide inhibitor at one end) may be produced as a recombinant protein in, for example, without limitation, yeast or bacteria. In another embodiment, the yeast or bacteria may be produced in large fermenters. In another embodiment, the peptide inhibitor may be produced as a GST (glutathione S-transferase) peptide construct. In another embodiment, the peptide inhibitor may be produced by TEV cleavage from a recombinant GST protein or other protein construct with purification of the cleaved peptide.

[0071] Accordingly, another embodiment of the present disclosure is a peptide inhibitor obtained by: mapping interactive domains of translocator proteins, identifying a candidate peptide inhibitor and producing the candidate peptide inhibitor.

[0072] The term "identifying" or "identified" means candidate peptide inhibitors that are selected on the basis of the degree of binding or interaction of amino acid regions on translocator proteins. The term "candidate" as used herein in reference to a peptide inhibitor includes peptide inhibitors that comprise interactive domains of translocator proteins of the type III secretion system present in Gram negative bacteria.

COMPOSITIONS

[0073] Another aspect of the present disclosure includes peptide inhibitors disclosed herein suitably formulated into pharmaceutical compositions for administration to mammals in a biologically compatible form suitable for administration *in vivo*. The term "biologically compatible form suitable for administration *in vivo*" means a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects.

[0074] Accordingly, another embodiment of the present disclosure includes a composition comprising one or more peptide inhibitors and a pharmaceutically acceptable carrier or diluent, or mixtures thereof. In one embodiment, the composition comprises Peptide 8003 and a pharmaceutically acceptable carrier or diluent, or mixtures thereof. In another embodiment, the composition comprises Peptide 8006 and a pharmaceutically acceptable carrier or diluent, or mixtures thereof. In another embodiment, the composition comprises a peptide inhibitor comprising a fragment of the amino acid sequence of SEQ ID NO: 3 or variant thereof and a pharmaceutically acceptable carrier or diluent, or mixtures thereof. In another embodiment, the composition comprises: Peptide 8003, Peptide 8006

and a pharmaceutically acceptable carrier or diluent, or mixtures thereof. In another embodiment, the composition comprises: Peptide 8003, Peptide 8006, a peptide inhibitor comprising a fragment of the amino acid sequence of SEQ ID NO: 3 or variant thereof and a pharmaceutically acceptable carrier or diluent, or mixtures thereof.

[0075] The compositions containing the peptide inhibitors described herein can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle, including for example, a carrier or diluent. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (2003 – 20th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999. On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles, carriers or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

[0076] Pharmaceutical compositions include, without limitation, lyophilized powders or aqueous or non-aqueous sterile injectable solutions or suspensions, which may further contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially compatible with the tissues or the blood of an intended recipient. Other components that may be present in such compositions include water, surfactants (such as Tween), alcohols, polyols, glycerin and vegetable oils, for example. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, tablets, or concentrated solutions or suspensions. The pharmaceutical composition may be supplied, for example but not by way of limitation, as a lyophilized powder which is reconstituted with sterile water or saline prior to administration to the subject.

[0077] Compositions of the present disclosure may comprise a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable

carriers include essentially chemically inert and nontoxic compositions that do not interfere with the effectiveness of the biological activity of the pharmaceutical composition. Examples of suitable pharmaceutical carriers include, but are not limited to, water, saline solutions, glycerol solutions, ethanol, N-(1(2,3-dioleyloxy)propyl)N,N,N-trimethylammonium chloride (DOTMA), diolesylphosphotidyl-ethanolamine (DOPE), and liposomes. Such compositions should contain a therapeutically effective amount of the compound, together with a suitable amount of carrier so as to provide the form for direct administration to the subject.

[0078] The composition may be in the form of a pharmaceutically acceptable salt which includes, without limitation, those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylarnino ethanol, histidine, procaine, etc.

[0079] In accordance with the methods and uses of the present disclosure, the peptide inhibitors or compositions comprising one or more of the peptide inhibitors, may be administered to a subject in a variety of forms depending on the selected route of administration, as will be understood by those skilled in the art. The peptide inhibitors or compositions comprising one or more of the peptide inhibitors disclosed in the present disclosure may be administered for example, by parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, transepithelial, intrapulmonary, aerosol, topical, transdermal, buccal, nasal, rectal, intrathecal, sublingual, or oral administration, and the pharmaceutical compositions may be formulated accordingly. Parenteral administration may occur by continuous infusion over a selected period of time.

KITS

[0080] Another aspect of the present disclosure is a kit comprising one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein and instructions for use thereof.

[0081] The kit can also include ancillary agents. For example, the kit can include an instrument for injecting the peptide inhibitors or compositions of the present disclosure into a subject, such as a syringe; a vessel for storing or transporting the peptide inhibitors or compositions; and/or pharmaceutically acceptable carriers or diluents, or mixtures thereof.

II. METHODS AND USES OF THE DISCLOSURE

[0082] Another aspect of the present disclosure is a method of inhibiting type III secretion in Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof.

[0083] The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit type III secretion in Gram negative bacteria in a cell or subject in need thereof. Another aspect of the disclosure includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for the manufacture of a medicament to inhibit type III secretion in Gram negative bacteria in a cell or subject in need thereof. A further aspect of the present disclosure includes one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for use in inhibiting type III secretion in Gram negative bacteria in a cell or subject in need thereof.

[0084] As used herein, "inhibit" or "inhibiting" a function or activity, such as type III secretion in Gram negative bacteria, infection from Gram negative bacteria, or cell invasion from Gram negative bacteria, is to reduce the function or activity when compared to otherwise same conditions except for a condition or parameter of interest. The terms "inhibitor" and "inhibition", in the context of the present disclosure, are intended to have a broad meaning and include peptide inhibitors which directly or indirectly act on type III secretion and/or the T3SS in Gram negative bacteria, infection from Gram negative bacteria or cell invasion from Gram negative bacteria and/or reduce function or activity of type III secretion and/or T3SS in Gram negative bacteria, or infection from Gram negative bacteria or cell invasion from Gram negative bacteria. The phrase "inhibiting type III secretion" or "inhibit type III secretion" means reducing type III secretion as compared to otherwise same conditions, and includes reduction accomplished by directly or indirectly acting on the type III secretion system in Gram negative bacteria.

[0085] The term "subject" as used herein refers to any member of the animal kingdom, preferably avian or mammal. In one embodiment, the mammal is a cow, sheep, horse, swine, pig, goat or koala bear. In another embodiment, the avian is a bird susceptible to infections with Gram negative bacteria. In another embodiment, the mammal is a human. In one embodiment, the human is a subject that is immunocompromised. The term "immunocompromised" means a subject who has an immunodeficiency, and thus has an immune system that is impaired by disease or treatment of a disease as compared to a subject who is not immunocompromised. In another embodiment, an immunocompromised subject includes a subject having chronic obstructive pulmonary disease (COPD), a lung infection, or is infected with HIV.

[0086] The term "therapeutically effective amount", "effective amount" or "sufficient amount" means a quantity sufficient to, when administered to the subject, including a mammal, for example a human, achieve a desired result, for example an amount effect to inhibit type III secretion in a subject. Effective

amounts of therapeutic may vary according to factors such as the disease state, age, sex, weight of the subject. Dosage or treatment regime may be adjusted to provide the optimum therapeutic response. In addition, a "treatment" regime of a subject with a therapeutically effective amount may consist of a single administration, or alternatively comprise a series of applications. The length of the treatment period depends on a variety of factors, such as the severity of the infection or disease, the age of the subject, the concentration and the activity of the peptide inhibitors, or a combination thereof. It will also be appreciated that the effective dosage of the peptide inhibitors used for the treatment or prevention may increase or decrease over the course of a particular treatment or prevention regime. Changes in dosage may result and become apparent by standard diagnostic assays known in the art. The peptide inhibitors of the present disclosure may be administered before, during or after exposure to the bacteria.

[0087] A further aspect of the present disclosure is a method of preventing type III secretion in Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof.

[0088] The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to prevent type III secretion in Gram negative bacteria in a cell or subject in need thereof. Another aspect of the disclosure includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for the manufacture of a medicament to prevent type III secretion in Gram negative bacteria in a cell or subject in need thereof. A further aspect of the present disclosure includes one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for use in preventing type III secretion in Gram negative bacteria in a cell or subject in need thereof.

[0089] The term "preventing type III secretion" or "prevent type III secretion" as used herein means blocking virulence of cells via blockage and/or interference of the type III secretion system in Gram negative bacteria as compared to otherwise same conditions.

[0090] An additional aspect of the present disclosure is a method of inhibiting infection from Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof.

[0091] The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit infection from Gram negative bacteria in a cell or subject in need thereof. Another aspect of the disclosure includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for the manufacture of a medicament to inhibit infection from Gram negative bacteria in a cell or subject in need thereof. A further aspect of the present disclosure includes one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for use in inhibiting infection from Gram negative bacteria in a cell or subject in need thereof.

[0092] The term "inhibiting infection" or "inhibit infection" as used herein means reducing infection from Gram negative bacteria of cells as compared to otherwise same conditions. The inhibition of infection may be assessed using assays known to those skilled in the art. For example, inhibition of infection may be assessed by measuring bacterial counts, colonization, and replication of Gram negative bacteria and comparing to otherwise same conditions. Inhibition of infection may also be assessed for example, by evaluating symptoms of infection, including for example, inflammation, redness, heat and/or pain as compared to otherwise same conditions.

[0093] Another aspect of the present disclosure is a method of inhibiting cell invasion of Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof.

[0094] The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to inhibit cell invasion from Gram negative bacteria in a cell or subject in need thereof. Another aspect of the disclosure includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for the manufacture of a medicament to inhibit cell invasion from Gram negative bacteria in a cell or subject in need thereof. A further aspect of the present disclosure includes one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for use in inhibiting cell invasion from Gram negative bacteria in a cell or subject in need thereof.

[0095] The term "inhibiting cell invasion" or "inhibit cell invasion" as used herein means reducing migration of Gram negative bacteria into cells as compared to otherwise same conditions. Inhibition of cell invasion may be assessed using assays known to those skilled in the art including, but not limited to, *in vitro* invasion assays.

[0096] An additional aspect of the present disclosure is a method of treating or preventing infection from Gram negative bacteria comprising administering an effective amount of one or more of the peptide inhibitors disclosed herein or the compositions comprising one or more of the peptide inhibitors disclosed herein to a cell or subject in need thereof.

[0097] The present disclosure also includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein to treat or prevent infection from Gram negative bacteria in a cell or subject in need thereof. Another aspect of the

disclosure includes uses of one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for the manufacture of a medicament to treat or prevent infection from Gram negative bacteria in a cell or subject in need thereof. A further aspect of the present disclosure includes one or more of the peptide inhibitors disclosed herein or a composition comprising one or more of the peptide inhibitors disclosed herein for use in treating or preventing infection from Gram negative bacteria in a cell or subject in need thereof.

[0098] As used herein, and as well understood in the art, "treatment" or "prevention" are approaches for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. "Palliating" a disease or disorder means that the extent and/or undesirable clinical manifestations of a disorder or a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not treating the disorder.

[0099] The phrase "treating or preventing infection from Gram negative bacteria" includes treating infection from Gram negative bacteria, preventing infection from Gram negative bacteria, decreasing the severity of infection from Gram negative bacteria, inhibiting Gram negative bacteria colonization, reducing shedding of Gram negative bacteria, and preventing Gram negative bacteria colonization or improving signs and symptoms related to infection from Gram negative bacteria. The present disclosure also includes the treatment or prevention of any disease that is associated with an infection from Gram negative bacteria.

[00100] In understanding the scope of the present disclosure, the term "comprising" and its derivatives, as used herein, are intended to be open ended terms that specify the presence of the stated features, elements, components, groups, integers, and/or steps, but do not exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The foregoing also applies to words having similar meanings such as the terms, "including", "having" and their derivatives. Finally, terms of degree such as "substantially", "about" and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a deviation of at least ±5% of the modified term if this deviation would not negate the meaning of the word it modifies.

[00101] The above generally describes the present disclosure. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the disclosure. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

[00102] The following non-limiting examples are illustrative of the present disclosure:

IV. EXAMPLES

Summary

[00103] Prior to the present disclosure, the manner in which the two translocator proteins of *Pseudomonas aeruginosa*, namely, PopB and PopD interacted *viz*. which amino acids of the first protein interact with which amino acids of the second protein, was not known. By using an established method called Pepscan analysis, the inventor mapped the binding regions or interactive domains of PopB and PopD proteins and then synthesized cognate

peptides that encompass the interacting domains of these translocator proteins. The peptides were then assessed for their ability to prevent type III secretion system (T3SS) and subsequent infection of HeLa cells *in vitro*.

Materials and Methods:

Materials

[00104] HeLa cells, *Pseudomonas aeruginosa*, *C. pneumonaie* and other bacteria used in the study are obtained commercially from the American Type Culture Collection (ATCC). Antibody for staining *C. pneumoniae* is obtained commercially from one of several suppliers. Peptides were synthesized by the APTC Peptide Synthesis Facility at The Hospital for Sick Children, Toronto. All other common reagents were purchased from Sigma Chemicals in St. Louis.

Methods

[00105] ***Pepscan analysis***: Pepscan analysis was performed as described by Timmerman et al. (*Journal of Molecular Recognition* 2007, 20: 283-299). An overlapping peptide library for PopD consisting of 15 amino acid residues with a register change every 3 amino acids was synthesized and immobilized onto a polypropylene membrane. Soluble recombinant PopB protein (1 or 10 ug/mL) containing a C-terminal 6 Histidine tag was added and allowed to interact with immobilized peptides. Detection of bound PopB protein was determined by enzyme linked immunoassay using anti-His antibody conjugated to horse radish peroxidase and a suitable substrate.

[00106] ***Selection of candidate peptides***: Candidate peptides were selected from the results of the Pepscan analysis based on signal strength obtained in the enzyme immunoassay. Signals greater than three-fold above background with readings above 300 fluorescent units were deemed to be significant. Readings generated for over 3000 individual peptides were aligned with the peptide sequence and the amino acid sequence with the highest weighted signal was selected as a candidate peptide.

[00107] ***Solid phase synthesis of peptides***: Solid phase peptide synthesis (SSPS) using F-moc blocking groups is commonly used by commercial suppliers of synthetic peptides (Carpino L.A. "1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive". *J. Am. Chem. Soc.* 1993, 115(10): 4397–4398). The C-terminal amino acid was attached to a cross-linked polystyrene resin via an acid labile bond with a linker molecule. This resin was insoluble in the solvents used for synthesis, making it relatively simple and fast to wash away excess reagents and by-products. The N-terminus was protected with the Fmoc group, which is stable in acid, but removable by base. Any side chain functional groups were protected with base stable, acid labile groups.

[00108] ***Invasion assay***: An *in vitro* invasion assay for *Pseudomonas aeruginosa* was performed using standard methods as described by Ha and Jin (*Infect. Immun.* 2001, 69: 4398-4406) and for *Chlamydia pneumoniae* as described by Coombes and Mahony (*Cell. Microbiol.* 2002, 4:447-460). Preformed monolayers of confluent HeLa cells were used for invasion with *Pseudomonas aeruginosa* and *Chlamydia pneumoniae.* Briefly, an overnight culture of *Pseudomonas aeruginosa* was subcultured 1:50 and after 3 hours cells were collected and a dilution of 1:100 prepared. An aliquot of cells was added to the monolayer and allowed to infect for 30 minutes. Bacteria were washed off and any remained bacteria were killed with gentimicin (1 hr). Monolayers were extensively washed with phosphate buffered saline and HeLa cells were lysed with 1% Triton X detergent. Viable intracellular bacteria were plated onto Lauria-Bertani medium in agar plates and colonies of bacteria were counted after 30 hours incubation at 37°C. The *in vitro* invasion assay for C. pneumoniae is performed by inoculating *C. pneumoniae* onto preformed monolayers of HeLa cells and allowing infection to proceed for 72 hours at which time the number of infected cells is determined by staining cytoplasmic inclusions containing *C. pneumoniae* with antibodies that are specific for *C. pneumoniae* and a second fluorescent-labelled anti immunoglobulin antibody and reading using a fluorescent microscope.

[00109] ***Animal model:*** An animal model of *Chlamydia trachomatis* in the genital tract of mice was used to evaluate peptide inhibitors of the present disclosure. Two concentrations of Peptide 8003 (low,100 µM and high, 200 µM) were mixed with 107 ifu of *C. trachomatis* serovar D in 30 µL PBS and injected into the vagina of female Balb/c mice on day 0 and genital tract swabs were collected on days 5, 7, 10, and 13. Swabs were rolled onto glass slides, fixed and stained for *C. trachomatis* using an FITC-conjugated monoclonal antibody. Direct fluorescent antibody (DFA) scores are the mean counts (+/- SEM) of bacteria per high power field.

[00110] ***Adenylate kinase activity assay:*** HeLa cells were treated with various concentrations of Peptide 8003 for 30 minutes and culture supernatants (0.05 mL) were collected and tested for adenylate kinase activity measured as relative light units (RLU) using the ToxiLight™ assay (Lonza) according to the manufacturer's instructions. The detergent lysis aliquot shows maximum enzyme activity when cells are completely lysed by detergent.

Results:

[00111] Pepscan analysis was performed with 3000 peptides to map the interactive domains of *Pseudomonas aeruginosa* PopB and PopD. The Pepscan analysis results show the enzyme immunoassay absorbance results for soluble recombinant PopB protein binding to immobilized overlapping peptides (3,000) of the PopD library (Fig 1A). Candidate peptides from the interactive domains were synthesized by solid phase chemistry and tested for their ability to block HeLa cell invasion using an *in vitro* invasion assay. Two peptides that form the interactive domains of PopB and PopD were evaluated extensively in an *in vitro* cell invasion assay. One peptide designated as Peptide 8003 inhibits invasion of *Pseudomonas aeruginosa* in a dose-dependent fashion (Fig 1B). Inhibition is seen at a peptide concentration as low as 25 uM and complete inhibition is seen at 200 uM. A second peptide, designated as Peptide 8006 which is also part of the interactive domain gave partial inhibition (45%) at 100 uM, and 78% inhibition at 200uM while an

irrelevant peptide based on influenza A, designated as Peptide 8005, gave no inhibition (Fig 1C). Peptide 8003 also inhibited a multi-drug resistant (MDR) *Pseudomonas aeruginosa* bacteria isolated from a patient with pneumonia by 90% (Fig 1C).

[00112] Since the translocator proteins are genetically conserved and share some amino acid sequences, Peptide 8003 was tested for its ability to inhibit other Gram negative bacteria. *Chlamydia trachomatis* and *Chlamydia pneumoniae* were also both inhibited by Peptide 8003 in a dose-dependent fashion with 96% inhibition observed at 100 uM (Figs 2 and 3). Peptide 8006 and an irrelevant influenza peptide did not inhibit Chlamydia from invading cells. The sequence similarity between the C-terminal PopD 8003 peptide (Peptide 8003) and the C-terminus of *Chlamydia pneumoniae* translocator proteins was determined and is shown in Figs 4A and 4B. The sequence alignment of Peptide 8003 with: 1) the C'-terminus of *Chlamydia pneumoniae* CopD1 (Cpn1020) and 2) the C-terminus of *Chlamydia pneumoniae* CopD2 (Cpn1019) is presented in Figures 4A and 4B, respectively. These conserved areas are 100% helical.

[00113] Peptide 8003 was tested in a *Chlamydia trachomatis* mouse model. Swabs from infected mice were examined under high power microscopy and counts of bacteria were determined. Peptide 8003 reduced infection in animals at a concentration of 100 µM as soon as 5 days following infection by approximately 25%. This 25% reduction in infection remained steady for up to 13 days post-infection. A dose of 200 µM of peptide did not impact infection until 13 days post infection, at which point an approximate 50% reduction in infection was observed (Fig 5).

[00114] In order to evaluate the cytotoxicity of Peptide 8003, HeLa cells were treated with increasing concentrations and cell lysis was evaluated using an adenylate kinase assay. Peptide concentrations up to 1.5 mM were not cytotoxic (Fig 6).

Discussion and Conclusion:

[00115] The present disclosure describes the discovery of peptide inhibitors, such as Peptide 8003 and Peptide 8006, which inhibited type III secretion likely by disrupting or preventing the formation of the T3SS translocon. Peptide 8003 inhibited three different Gram negative bacteria including *Pseudomonas aeruginosa, Chlamydia trachomatis* and *Chlamydia pneumoniae*, inhibited all three bacteria from invading epithelial cells, and was not cytotoxic even at high concentrations. Peptide 8003 also reduced infection from *Chlamydia trachomatis* of cells in a mouse model. The peptide inhibitors may also inhibit additional Gram negative bacteria that have a conserved T3SS and thus block the infection process. The peptide inhibitors may be used in clinical medicine for the treatment of infections due to Gram negative bacteria. For bacteria such as *Pseudomonas aeruginosa* that are multi-drug resistant, the peptides described could provide an important adjunctive therapy for strains of *P. aeruginosa* that are resistant to antibiotics currently in use.

[00116] The approach employed in the present disclosure aimed at treating Gram negative bacteria by targeting or blocking T3SS has for example at least four apparent advantages. First, the peptides were useful for several different types of Gram negative bacteria as demonstrated in the present disclosure. Second, the peptides were useful for Gram negative antibiotic resistant bacteria as demonstrated above. Third, the peptide should not induce or select for resistant bacteria as commonly occurs with antibiotics that are small molecules because the peptide interacts with a long stretch of amino acid residues within the interactive domain of the target protein. Fourth, the peptides were not cytotoxic. Without wishing to be bound by any particular theory, the peptide inhibitors described herein may inhibit or block the function of the T3SS by preventing the formation of the translocon protein complex, or by disrupting an already formed translocon protein complex via competitive inhibition.

[00117] There is some sequence similarity between Peptide 8003 and the C-terminus of *Chlamydia pneumoniae* CopD2 (Cpn1019) and the C'-terminus of *Chlamydia pneumoniae* CopD1 (Cpn1020). These results indicate that this sequence is conserved and functionally important. These conserved areas are 100% helical further suggesting a conserved role in protein interaction. Since the Pseudomonas translocators PopB and PopD, are genetically more similar to Salmonella and Shigella than they are to either *C. trachomatis* or *C. pneumoniae,* Peptide 8003 will likely also inhibit Salmonella and Shigella.

[00118] While the present disclosure has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the disclosure is not limited to the disclosed examples. To the contrary, the disclosure is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

[00119] All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Claims:

1. An isolated peptide inhibitor of type III secretion in Gram negative bacteria.

2. The peptide inhibitor of claim 1 comprising interactive domains of translocator proteins of type III secretion in Gram negative bacteria.

3. The peptide inhibitor of claim 1 or 2, wherein the translocator proteins are PopB and/or PopD of *Pseudomonas aeruginosa*.

4. The peptide inhibitor of any one of claims 1 to 3, comprising the amino acid sequence in SEQ ID NO: 1 or a variant thereof.

5. The peptide inhibitor of any one of claims 1 to 3, comprising all or part of the amino acid sequence in SEQ ID NO: 1 or a variant thereof.

6. The peptide inhibitor of any one of claims 1 to 3, comprising an amino acid sequence that is substantially or essentially identical to the amino acid sequence in SEQ ID NO: 1.

7. The peptide inhibitor of claim 6, wherein the peptide inhibitor has a percent sequence identity of 90% with the amino acid sequence in SEQ ID NO: 1.

8. The peptide inhibitor of claim 7, wherein the percent sequence identity is 95%.

9. The peptide inhibitor of any one of claims 1 to 3, comprising the amino acid sequence in SEQ ID NO: 1.

10. The peptide inhibitor of any one of claims 1 to 3, comprising the amino acid sequence in SEQ ID NO: 2 or a variant thereof.

11. The peptide inhibitor of any one of claims 1 to 3, comprising all or part of the amino acid sequence in SEQ ID NO: 2 or a variant thereof.

12. The peptide inhibitor of any one of claims 1 to 3, comprising an amino acid sequence that is substantially or essentially identical to the amino acid sequence in SEQ ID NO: 2.

13. The peptide inhibitor of claim 12, wherein the peptide inhibitor has a percent sequence identity of 90% with the amino acid sequence in SEQ ID NO: 2.

14. The peptide inhibitor of claim 13, wherein the percent sequence identity is 95%.

15. The peptide inhibitor of any one of claims 1 to 3, comprising the amino acid sequence in SEQ ID NO: 2.

16. The peptide inhibitor of any one of claims 1 to 3, wherein the peptide inhibitor comprises a fragment of the amino acid sequence of SEQ ID NO: 3 or a variant thereof.

17. The peptide inhibitor of any one of claims 1 to 3, obtained by mapping interactive domains of translocator proteins, identifying a candidate peptide inhibitor and producing the candidate peptide inhibitor.

18. A composition comprising one or more of the peptide inhibitors of any one of claims 1 to 17 and a pharmaceutically acceptable carrier or diluent, or mixtures thereof.

19. A kit comprising the peptide inhibitors of any one of claims 1 to 17 or the composition of claim 18 and instructions for the use thereof.

20. Use of the peptide inhibitors of any one of claims 1 to 17 to inhibit type III secretion in Gram negative bacteria in a cell or subject in need thereof.

21. Use of the peptide inhibitors of any one of claims 1 to 17 to prevent type III secretion in Gram negative bacteria in a cell or subject in need thereof.

22. Use of the peptide inhibitors of any one of claims 1 to 17 to inhibit infection from Gram negative bacteria in a cell or subject in need thereof.

23. Use of the peptide inhibitors of any one of claims 1 to 17 to inhibit cell invasion of Gram negative bacteria in a cell or subject in need thereof.

24. Use of the peptide inhibitors of any one of claims 1 to 17 to treat or prevent infection from Gram negative bacteria in a cell or subject in need thereof.

25. Use of the composition of claim 18 to inhibit type III secretion in Gram negative bacteria in a cell or subject in need thereof.

26. Use of the composition of claim 18 to prevent type III secretion in Gram negative bacteria in a cell or subject in need thereof.

27. Use of the composition of claim 18 to inhibit infection from Gram negative bacteria in a cell or subject in need thereof.

28. Use of the composition of claim 18 to inhibit cell invasion of Gram negative bacteria in a cell or subject in need thereof.

29. Use of the composition of claim 18 to treat or prevent infection from Gram negative bacteria in a cell or subject in need thereof.

30. The use of any one of claims 20 to 29 wherein the Gram negative bacteria is *Pseudomonas aeruginosa, Chlamydia trachomatis*, *Chlamydia pneumoniae, Salmonella, Shigella, E. coli* O157, Salmonella, Shigella, Listeria, and/or Yersinia.

31. The use of any one of claims 20 to 30 wherein the Gram negative bacteria are multi-drug resistant.

32. The use of any one of claims 20 to 31 wherein the subject is immunocompromised.

33. The use of claim 32 wherein the immunocompromised subject has chronic obstructive pulmonary disease (COPD), a lung infection or is infected with HIV.

34. The use of any one of claims 20 to 33 wherein the subject is a mammal.

35. The use of claim 34 wherein the mammal is a human.

FIGURE 1A

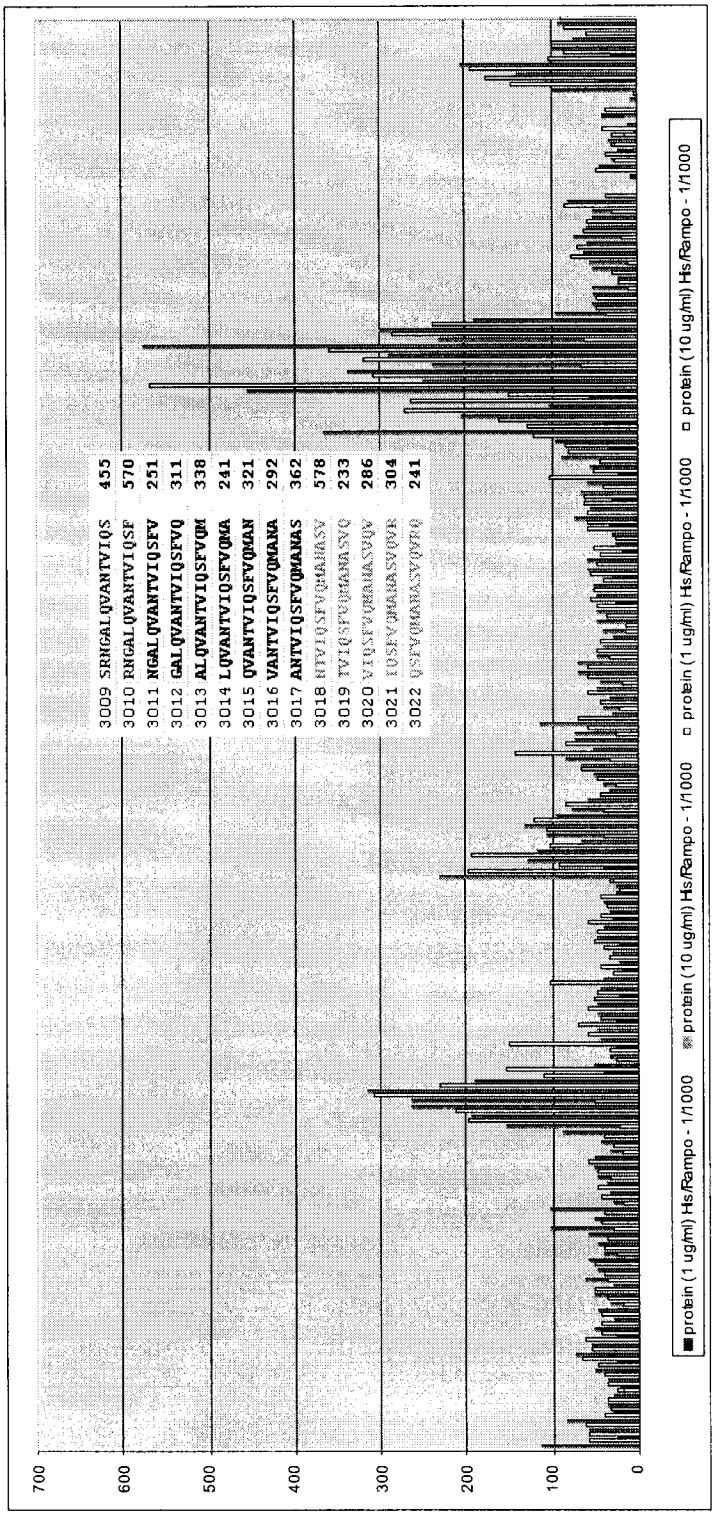

3009 SRNGALQVANTVIQS 455
3010 RNGALQVANTVIQSF 570
3011 NGALQVANTVIQSFV 251
3012 GALQVANTVIQSFVQ 311
3013 ALQVANTVIQSFVQM 338
3014 LQVANTVIQSFVQMA 241
3015 QVANTVIQSFVQMAN 321
3016 VANTVIQSFVQMANA 292
3017 ANTVIQSFVQMANAS 362
3018 NTVIQSFVQMANASV 578
3019 TVIQSFVQMANASVQ 233
3020 VIQSFVQMANASVQV 286
3021 IQSFVQMANASVQVR 304
3022 QSFVQMANASVQVRQ 241
700
600
500
400
300
200
100
0
protein (1 ug/ml) Hs/Rampo - 1/1000
protein (10 ug/ml) Hs/Rampo - 1/1000
protein (1 ug/ml) Hs/Rampo - 1/1000
protein (10 ug/ml) Hs/Rampo - 1/1000

FIGURE 1B

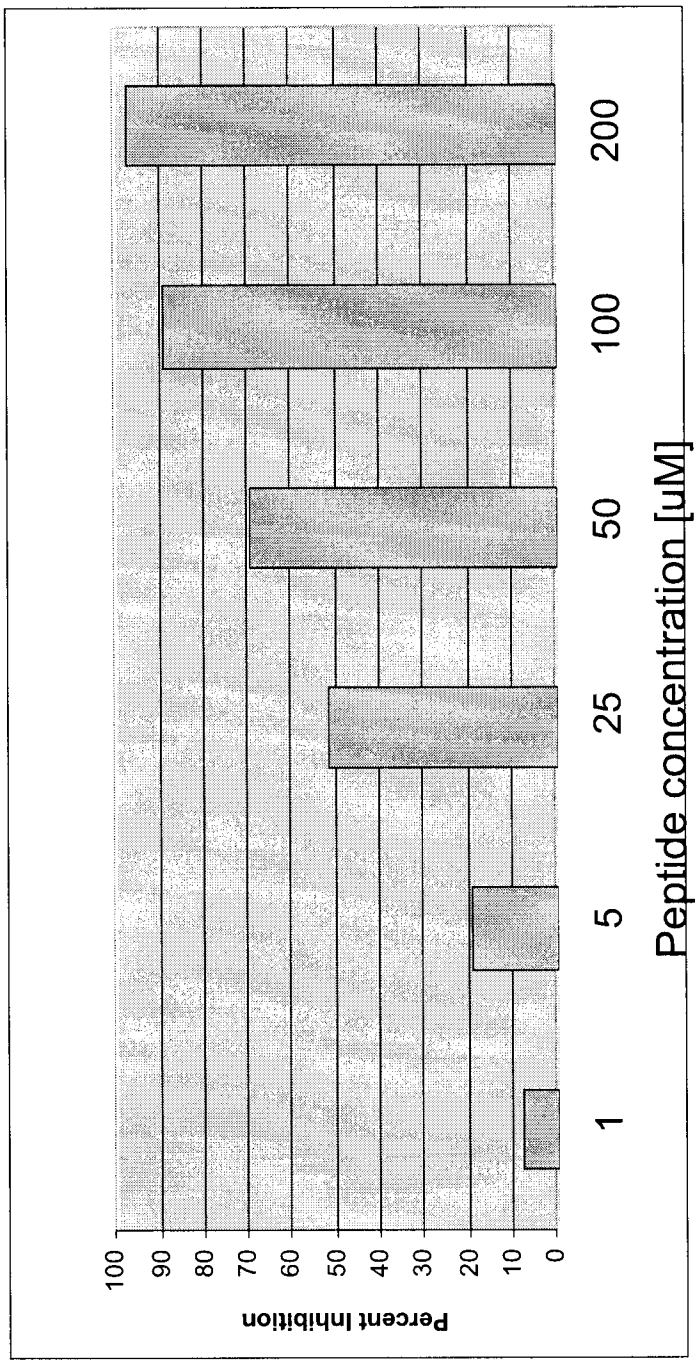

Percent Inhibition
100
90
80
70
60
50
40
30
20
10
0
1
5
25
50
100
200
Peptide concentration [uM]

FIGURE 1C

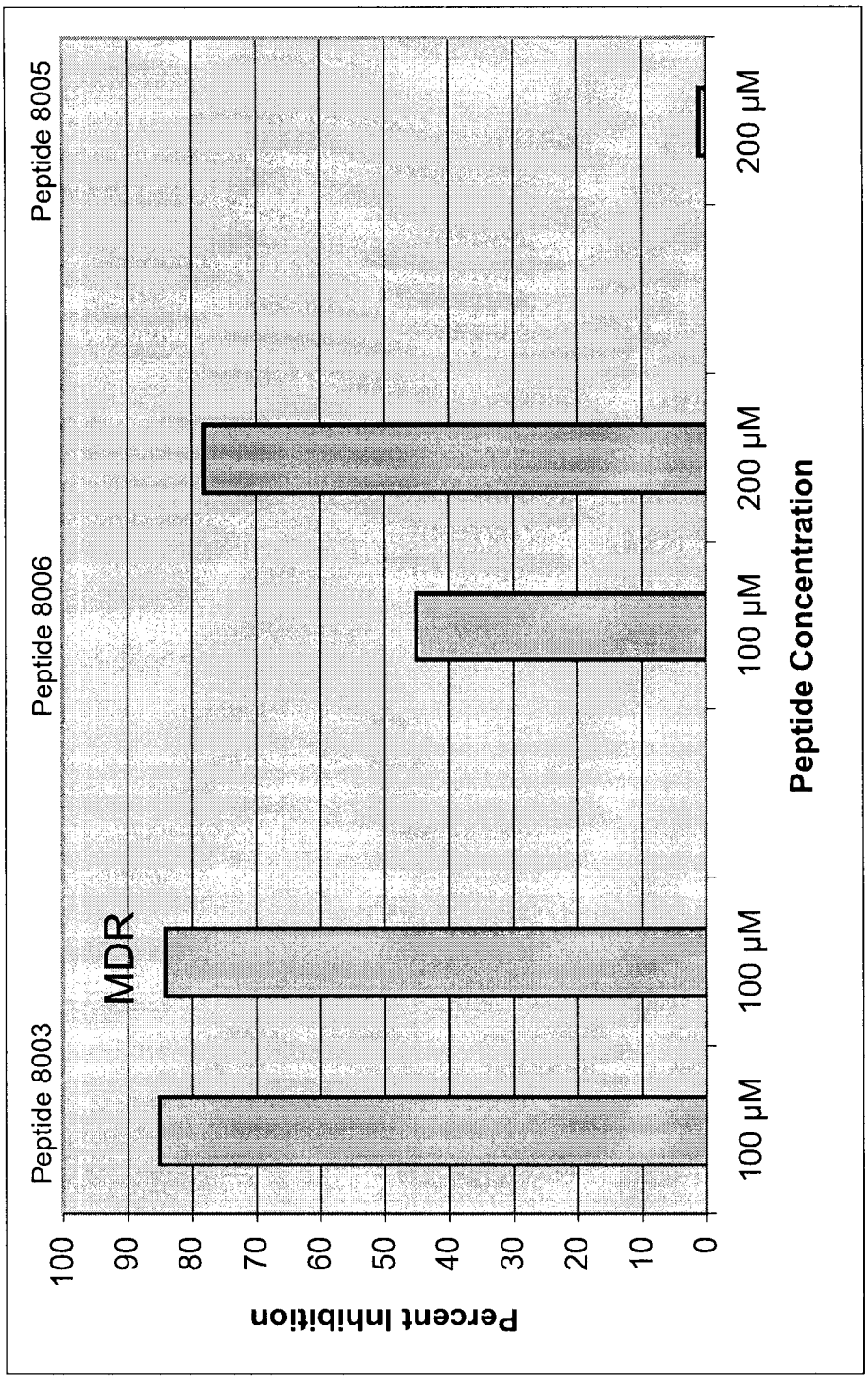

MDR
Peptide 8003
Peptide 8006
Peptide 8005
100 µM
100 µM
100 µM
200 µM
200 µM
Peptide Concentration
Percent Inhibition
0
10
20
30
40
50
60
70
80
90
100

FIGURE 2

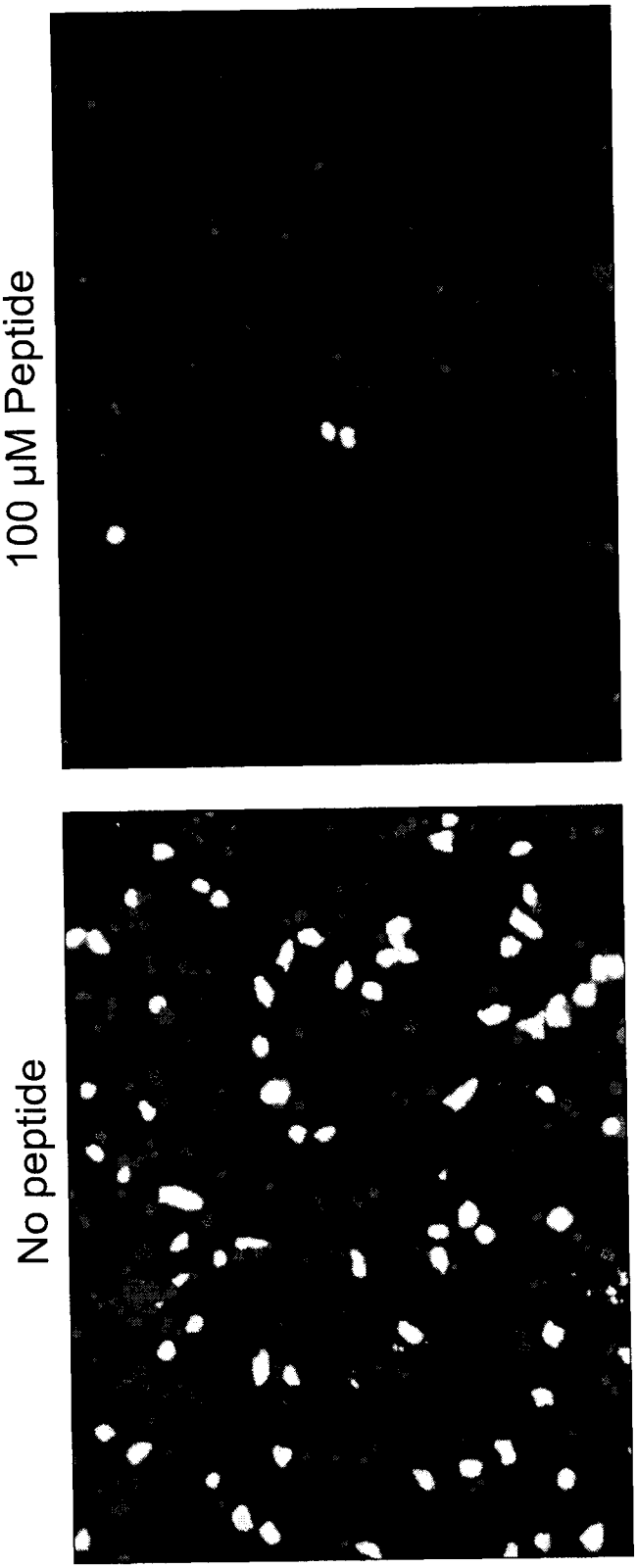


FIGURE 3

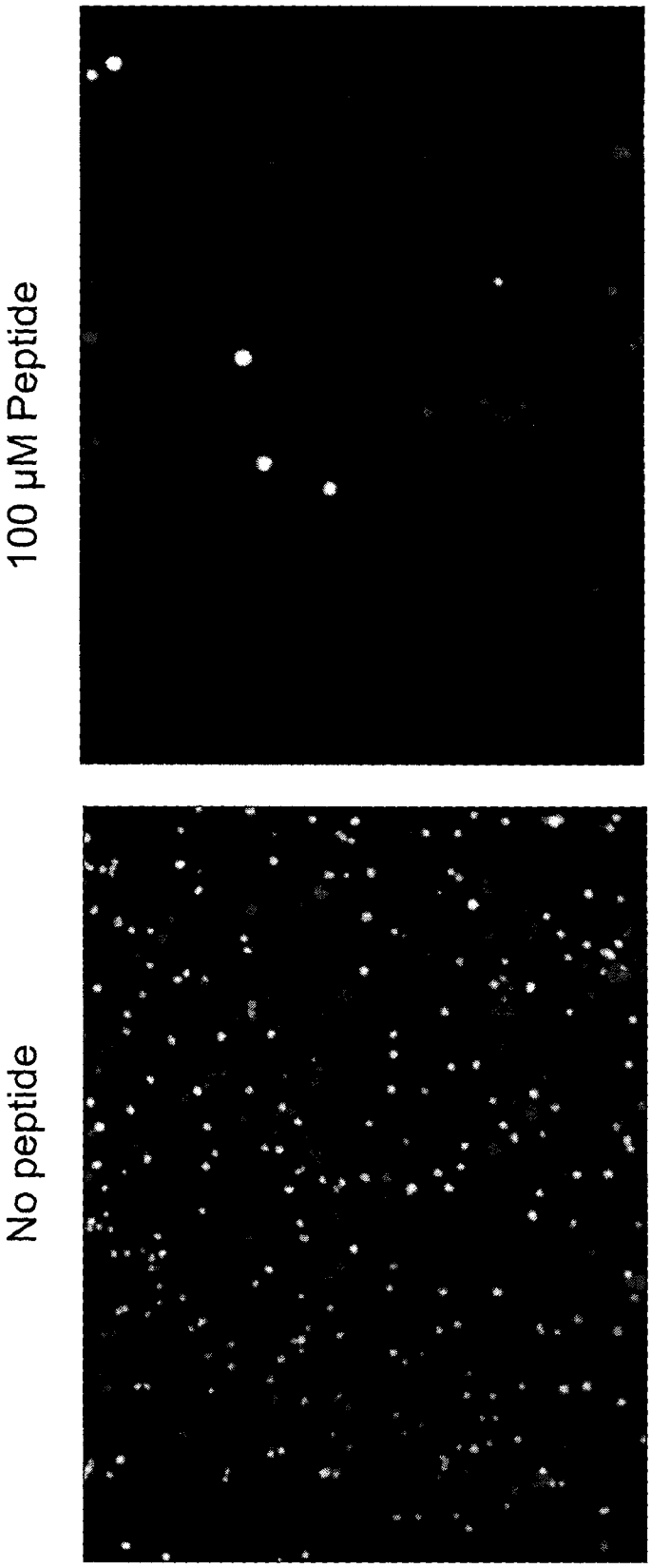


FIGURE 4A

Chlamydia pneumoniae CopD1 sequence:

```
                10         20         30         40         50         60
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   MSSWLSQASE VLLNQDPYIP DAPRSQESSV PKISYSITVA PQEAQKSLPK FFTQKFQSQC
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

                70         80         90        100        110        120
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   KSEPPITHHK TFIIATPRER ILRFGSSFES QLHNTSQAQT SSPWNLFSQK NSTEASKALM
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               130        140        150        160        170        180
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   QELTMPKSPE KTSEKALDKN LSSKQEGSCK NFDTLHLQQH LKLFGTVDSL YSQSLDSEQQ
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   ELLQSRREER SETYANQQSS EKKIETKVQI KDLCKDLFSQ DQDSNQKQKK SPFQQDTSRK
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               250        260        270        280        290        300
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   NRIAKAAQAV PVIPPPSIGV FTLSYLLTKQ GILSDFSSYG CHKDSVESTQ RELDALHEKR
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               310        320        330        340        350        360
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   IETIKVSIEK EKRERLWGSL SDIIGWLAPF VSIGIGIVAI LSGGGIFAFA GFFAGLISLV
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               370        380        390        400        410        420
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   IKCLEKLKFW DWLEKHLPIK NEELRRKIIT IIQWVVYLTP VILSICTLKV ENLGFSPIIE
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1020   GAIKGIQPAI ESTMAALRCA ILFSQAEIYK LKGKLTKIQL DIELKSFDRD DHYERSQELL
peptide   ---------- ---------- ---------- ---------- ----RAFE-- ---SR-----

               490        500        510
          ....|....| ....|....| ....|....|
Cpn1020   DNMESSFEAL SRILNYMREL DQVYLHSLRG
peptide   ---NGALQVA NTVIQSFVQM ANASVQ-VR-
```

FIGURE 4B

Chlamydia pneumoniae CopD2 sequence:

```
                10         20         30         40         50         60
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   MTVSYQSIST PPPEGEFDIF VDGNATEEAV VAAEVQVALP AGEQYAMLRA TSELCFGILT
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

                70         80         90        100        110        120
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   QSECALTQAL PPKEKPLQEE QFLVKNGILM RSTSLPNLKP GQSQQTSLAS HRNPLAQQST
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               130        140        150        160        170        180
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   SSNSTGKAST ETTSSSFPFF SCKAPEGDSS VDKTFTVSVQ TPKAQEQQEA SASQSQAQFH
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   VRSYSSSTIK EHSAKEKVSQ STKSAETQKH TQTKSDATLS PMSLYSTLHK EVPQALSSTK
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               250        260        270        280        290        300
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   SQQKDEEHRD QRQQEGYEQE QEQEEGKKKT PWCTVESLQQ TSSSNQVYES YTPIIPDPIV
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               310        320        330        340        350        360
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   EFALSESQLS VLAGKRVTNL DVLRICTELM KLMLKSRAND TMTRLEEREL MEREAHELAA
Peptide   ---------- ---------- ---------- ---------- ---------- ----------

               370        380        390        400        410        420
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   SYSRQAKYAR WLGIATATLG ILGAIAPMVG EISGDSILGF VQRISGRFKD ATAKTFFKGI
Peptide   ---------- ---------- ---------- -------RAF ESR-NGALQV AN--------

               430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Cpn1019   GKVFTSLSQL TEAASKVHEL SESAVRAVAE YRKEVFRMRQ DEVTRTIEEV KDNWKSMDNF
Peptide   -TVIQSFVQM ANASVQVR-- ---------- ---------- ---------- ----------

               490
          ....|....| ....|
Cpn1019   LLNILQTEHD AARSLYQ
Peptide   ---------- -------
```

FIGURE 5

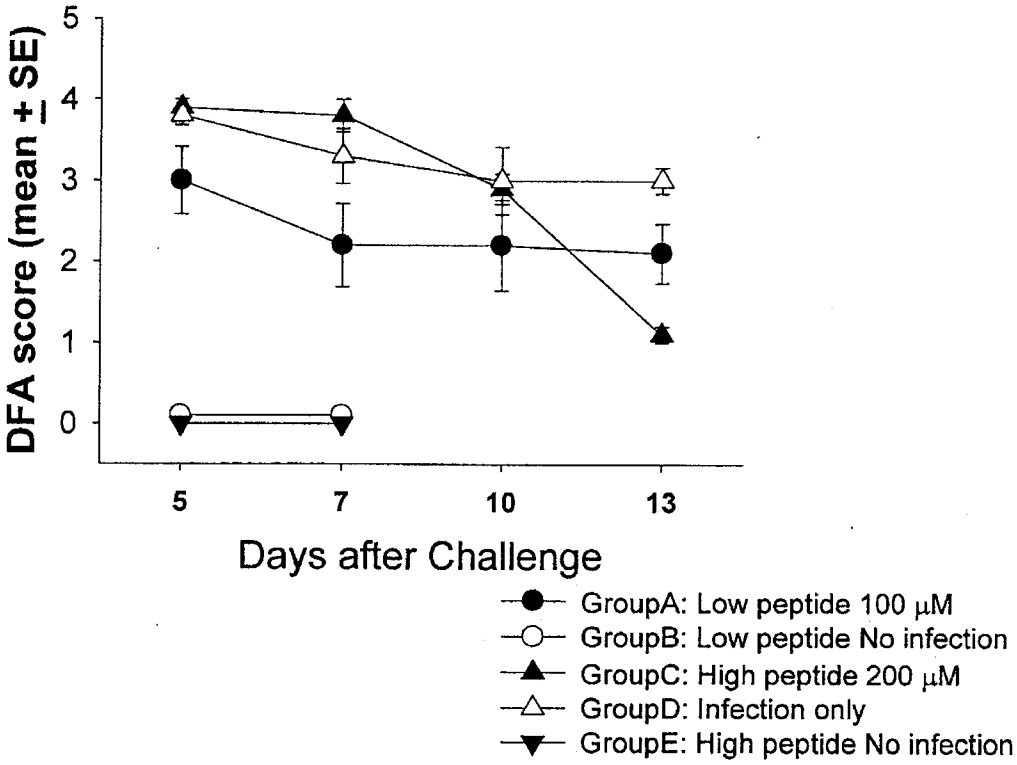
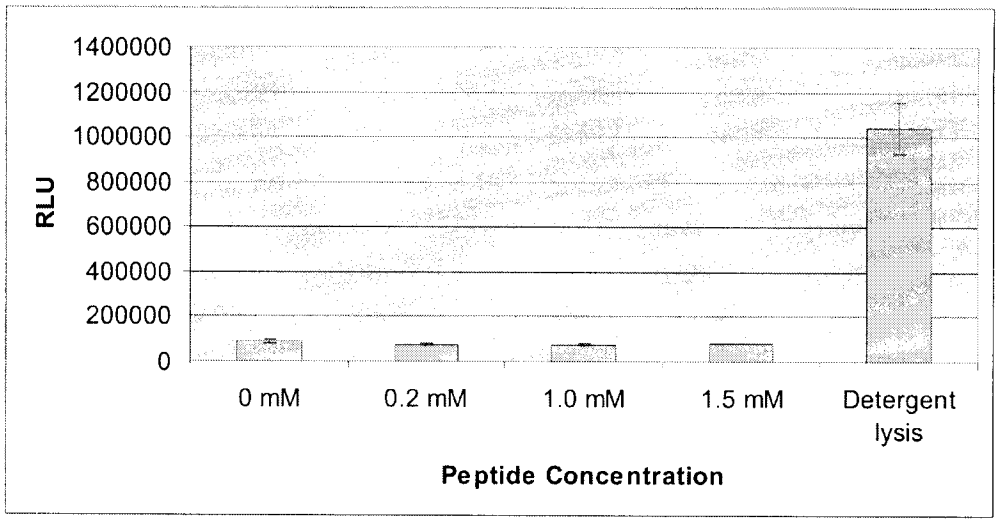

DFA score (mean ± SE)
5
4
3
2
1
0
5
7
10
13
Days after Challenge
GroupA: Low peptide 100 μM
GroupB: Low peptide No infection
GroupC: High peptide 200 μM
GroupD: Infection only
GroupE: High peptide No infection

FIGURE 6


1400000
1200000
1000000
800000
600000
400000
200000
0
RLU
0 mM
0.2 mM
1.0 mM
1.5 mM
Detergent lysis
Peptide Concentration

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001473

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07K 14/21*** (2006.01) , ***A61K 38/16*** (2006.01) , ***A61P 31/04*** (2006.01) , ***C07K 14/295*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/21 (2006.01) , ***A61K 38/16*** (2006.01) , ***A61P 31/04*** (2006.01) , ***C07K 14/295*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

GenomeQuest, Scopus, Delphion, PubMed, Google Scholar, Canadian Patent Database

Search Terms: SEQ ID NOs: 1-3, type III secretion, PopB, PopD, inhibit(or), translocator, Gram negative bacteria, Pseudomonas, Chlamydia Salmonella, Shigella, SipB, SipC, CopB, CopD, YopB, YopD, therapeutic, treatment, antibiotic, antimicrobial, antibacterial, Mahoney

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRANK, D.W. et al., Generation and characterization of a protective monoclonal antibody to *Pseudomonas aeruginosa* PcrV, The Journal of Infectious Diseases, 01 July 2002, Vol. 186, No. 1, pages 64-73 ISSN 0022-1899 *entire document	1, 18-35
X	WO 2004/019980 A1 (HILL, J. et al.) 11 March 2004 *entire document	1, 18-35
X	US 2006/0287252 A1 (OMURA, S. et al.) 21 December 2006 *paragraphs 1-4, 8, 58-77, and 91	1, 18-35
X	OCHOA, T.J. et al., Lactoferrin disruption of bacterial type III secretion systems, June 2004, BioMetals, Vol. 17, No. 3, pages 257-260 ISSN 0966-0844 *entire document	1, 18-35

[X] Further documents are listed in the continuation of Box

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 December 2009 (04-12-2009)	05 February 2010 (05-02-2010)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Jessica Smith (819) 994-6977

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

      [X] on paper

      [X] in electronic form

   b. (time)

      [X] in the international application as filed

      [X] together with the international application in electronic form

      [ ] subsequently to this Authority for the purposes of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant	Relevant to claim No.
P, X	CA 2648406 A1 (TRAN VAN NHIEU, G. et al.) 03 July 2009 *entire document	1, 2, 17-35
A	BROMS, J.E. et al, Dissection of homologous translocon operons reveals a distinct role for YopD in type III secretion by *Yersinia pseudotuberculosis*, Microbiology, September 2003, Vol. 149, No. 9, pages 2615-2626 ISSN 1350-0872 *page 2615	1-19

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2009/001473

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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