

(43) International Publication Date
26 April 2012 (26.04.2012)(10) International Publication Number
WO 2012/054879 A1

(51) International Patent Classification:

A61K 39/02 (2006.01) A61K 39/07 (2006.01)

(21) International Application Number:

PCT/US2011/057360

(22) International Filing Date:

21 October 2011 (21.10.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/405,710 22 October 2010 (22.10.2010) US

(71) Applicant (for all designated States except US): **DUKE UNIVERSITY** [US/US]; 2812 Erwin Road, Suite 306, Box 90083, Durham, North Carolina 27705 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **ST. GEME, Joseph W., III** [US/US]; 120 Muir Ln., Chapel Hill, North Carolina 27514 (US).(74) Agents: **BREEN, John, P.** et al.; Barnes & Thornburg LLP, Indianapolis, Illinois 46204 (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, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, 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, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

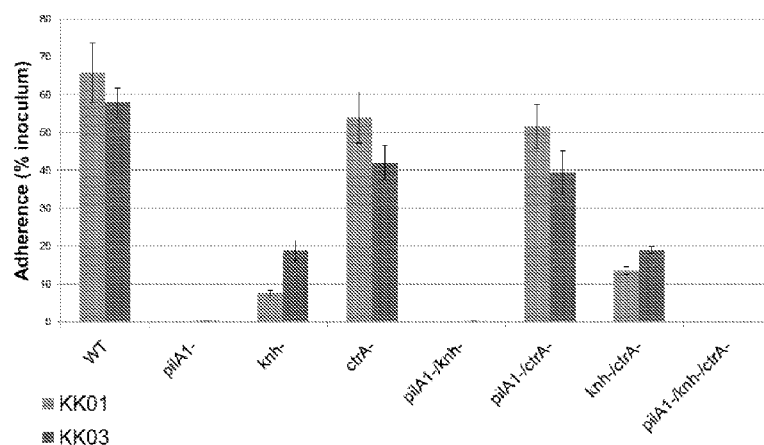
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU,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

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF SEPTIC ARTHRITIS, OSTEOMYELITIS, AND BACTEREMIA

Fig. 6



(57) Abstract: Compositions and methods for treating infections from gram negative bacteria are provided wherein the composition comprises a *Kingella* NhhA homolog (Knh) peptide or a *Kingella* capsular polysaccharide. These composition are used to induce an immune response in a subject to inhibit the establishment or maintenance of a pathogenic infection by gram negative bacterium including for example *Kingella* kingae. Methods are also provided for preventing or interfering with the ability of gram negative bacterium including for example *Kingella* kingae to adhere to cell and tissue surfaces.

COMPOSITIONS AND METHODS FOR THE TREATMENT OF SEPTIC ARTHRITIS, OSTEOMYELITIS, AND BACTEREMIA

CROSS REFERENCE TO RELATED APPLICATIONS:

This application claims priority to United States Provisional Patent Application
5 No. 61/405,710 filed on October 22, 2010, the complete disclosure of which is
incorporated herein by reference.

BACKGROUND

Gram-negative bacteria have become the leading cause of fatal bacterial
10 infections in hospital patients and are also an important etiology of community-acquired
disease. The mortality rate for patients suffering from gram-negative bacteremia has
approached forty percent in the past twenty years, despite the use of antibiotics and
aggressive support techniques. These bacteria are distinguished by a membrane that is
relatively impermeable to drugs as well as the presence of lipopolysaccharide (LPS,
15 endotoxin), which can be lethally toxic, even after the bacterial cells have been killed.
The lethality of gram-negative infections thus is due both to uncontrolled growth of the
viable bacteria and to the release of LPS.

LPS is unique to gram-negative bacteria and is a component of the outer
membrane. The LPS molecules from different bacterial species share structural
20 similarity.

Kingella kingae is a gram-negative bacterium that is being recognized
increasingly as an important cause of septic arthritis, osteomyelitis, and bacteremia in
young children. The pathogenesis of *K. kingae* disease begins with colonization of the
upper respiratory tract (posterior pharynx), a process that presumably requires bacterial
25 adherence to respiratory epithelium. Previous work has shown that type IV pili are
essential for adherence to respiratory epithelial cells. Densely piliated and sparsely
piliated variants of *K. kingae* isolates have been identified, as shown in Table 1 below:

5 Table I: Type IV pili expression in *K. kingae* strains

<u>Strain Designation</u>	<u>Pili Density</u>
KK01 variant of 269-492	Low
269-492	Intermediate
KK03 variant of 269-492	High

10 The *K. kingae* genome encodes other factors potentially involved in modulating adherence to the host, including a trimeric autotransporter, Knh, as well as genes involved in capsular polysaccharide export. Capsular polysaccharides have been shown to interfere with adhesive activity in other bacterial species, and capsular polysaccharides from *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* have been incorporated into vaccines that are highly effective against these pathogens.

15 A need exists for compositions and methods for the treatment or prophylaxis of gram-negative bacterial infection, which are effective against both the underlying infection and the widespread disorders that accompany the disease.

20 SUMMARY

One embodiment of the present disclosure is directed to compositions and methods for treating infections of subjects by gram negative bacteria. In accordance with one embodiment compositions and methods are provided for treating septic arthritis, osteomyelitis, and bacteremia caused by gram negative bacterial such as *Kingella kingae*.

25 In accordance with one embodiment a composition is provided that comprises a *Kingella NhhA homolog* (Knh) peptide. In one embodiment the Knh peptide comprises a contiguous 8, 10, 15, 18, 20, 25 or 30 amino acid fragment of SEQ ID NO: 1. In some 30 embodiments the amino acid fragment is selected from amino acids 45-1783 of SEQ ID NO: 1. In one embodiment the Knh peptide comprises an 8, 10, 15, 18, 20, 25 or 30

contiguous amino acid sequence, wherein said sequence has greater than 80%, 85%, 90%, 95% or 98% sequence identity to a 8, 10, 15, 18, 20, 25 or 30 contiguous amino acid sequence of SEQ ID NO: 1. In another embodiment the Knh peptide comprises amino acids 45- 1783, 85-485, 567-746, 834-1263, 1310-1405, or 1531-1633 of SEQ ID NO: 1, or a combination of two or more of said fragments. In one embodiment the composition comprises the *Kingella NhhA homolog* (Knh) protein (i.e., SEQ ID NO: 1).

In accordance with one embodiment a composition comprising a Knh peptide is used to prevent or inhibit the establishment or maintenance of a pathogenic infection by gram negative bacterium, including for example *Kingella kingae*. Accordingly, the composition can be used to treat septic arthritis, osteomyelitis, and bacteremia caused by gram negative bacterial such as *Kingella kingae*. In accordance with one embodiment a composition is provided for inducing an immune response in a subject wherein the immune response prevents or interferes with the ability of gram negative bacterium, that express Knh, including for example *Kingella kingae*, to adhere to cell and tissue surfaces or prevents, or inhibits proliferation or maintenance of *Kingella kingae* within the host.

In one embodiment a composition comprising a purified *Kingella* capsular polysaccharide is provided. In one embodiment the capsular polysaccharide is purified in accordance with the procedure of Example 2, and in a further embodiment the capsular polysaccharide is purified from *Kingella kingae*. In one embodiment the purified polysaccharide is conjugated to an immunogenic carrier protein.

In accordance with one embodiment a composition comprising a capsular polysaccharide conjugated to an immunogenic carrier protein is used to prevent or inhibit the establishment or maintenance of a pathogenic infection by gram negative bacterium, including for example *Kingella kingae*. Accordingly, the composition can be used to treat septic arthritis, osteomyelitis, and bacteremia caused by gram negative bacterial such as *Kingella kingae*. In accordance with one embodiment the capsular polysaccharide containing composition is used to induce an immune response in a subject wherein the immune response prevents or interferes with the ability of gram negative bacterium, that express Knh, including for example *Kingella kingae*, to adhere to cell and tissue surfaces, or prevents or inhibits proliferation or maintenance of *Kingella kingae* within the host.

In accordance with one embodiment a pharmaceutical composition is provided comprising a compound selected from the group consisting of an antigenic *Kingella* NhhA homolog peptide, a *Kingella* capsular polysaccharide and a *Kingella* type IV pilus peptide and a pharmaceutically acceptable carrier. In one embodiment the composition
5 comprises two or more components selected from the group consisting of an antigenic *Kingella* NhhA homolog peptide, a *Kingella* capsular polysaccharide and a *Kingella* type IV pilus peptide, and in one embodiment the composition comprises all three components. In one embodiment the antigenic *Kingella* NhhA homolog peptide comprises the polypeptide of SEQ ID NO: 2 or antigenic fragment thereof, and the
10 *Kingella* capsular polysaccharide and *Kingella* type IV pilus peptide are components purified from *Kingella kingae*. In a further embodiment any of the above listed compositions further comprises an adjuvant. In a further embodiment, the compositions disclosed herein further comprise an immunogenic carrier protein conjugated to the capsular polysaccharide.

15 In accordance with one embodiment antibodies specific for a *Kingella* NhhA homolog peptide, a *Kingella* capsular polysaccharide and a *Kingella* type IV pilus peptide are provided. In one embodiment the antibodies are specific for the *Kingella* NhhA homolog peptide of SEQ ID NO: 1, the *Kingella kingae* capsular polysaccharide or a *Kingella kingae* type IV pilus peptide. In accordance with one embodiment a
20 composition is provided comprising purified antibodies specific for a *Kingella* NhhA homolog protein, a *Kingella* capsular polysaccharide or a *Kingella* type IV pilus peptide. Alternatively, the composition may comprise two or more different antibodies (either a combination of monoclonal antibodies or comprising polyclonal antibodies) that have specificity for two or three of the components selected from the group consisting of a
25 *Kingella* NhhA homolog protein, a *Kingella* capsular polysaccharide or a *Kingella* type IV pilus peptide.

In one embodiment a method for preventing or inhibiting the adhesion of a gram negative bacterium to eukaryotic cells is provided, wherein the bacterium expresses *Kingella* NhhA. The method comprises contacting said gram negative bacteria with a
30 composition that interferes with *Kingella* NhhA homolog protein activity. In one embodiment the gram negative bacterium is a member of the genus *Kingella* and in a

further embodiment the gram negative bacterium is *Kingella kingae*. In one embodiment the method comprises the administration of a composition comprising one, two or three components selected from the group consisting of an antigenic *Kingella* NhhA homolog peptide, a *Kingella* capsular polysaccharide and a *Kingella* type IV pilus peptide and a pharmaceutically acceptable carrier. In one embodiment the composition comprises one, two or three components selected from the group consisting of the antigenic *Kingella* NhhA homolog protein of SEQ ID NO: 2, or antigenic fragment thereof, the *Kingella kingae* purified capsular polysaccharide and the *Kingella kingae* purified type IV pilus peptide. Each of these compositions may further comprise adjuvants and/or antimicrobial agents.

In a further embodiment a method for treating a gram negative bacterial infection, wherein said bacterium expresses Knh is provided. The method comprises administering an antigenic *Kingella* NhhA homolog peptide comprising the polypeptide of SEQ ID NO: 2 or antigenic fragment thereof in an amount sufficient to produce an immune response.

In a further embodiment the composition comprises a *Kingella* capsular polysaccharide and/or *Kingella* type IV pilus peptide. The composition can further include an antimicrobial agent.

In accordance with one embodiment a method of inducing an immune response in a subject is provided. The method comprises administering to the subject a composition comprising one, two or three components selected from the group consisting of an antigenic *Kingella* NhhA homolog peptide, a *Kingella* capsular polysaccharide and a *Kingella* type IV pilus peptide in an amount sufficient to induce an immune response. In one embodiment a vaccine for immunizing a subject against disease caused by a pathogenic bacterium is provided wherein the vaccine comprises, consists of, or consists essentially of at least one component of Knh. As used herein one component of Knh is intended to encompass any antigenic fragment of the *Kingella* NhhA homolog peptide (Knh) of SEQ ID NO: 1. In certain embodiments, the vaccine further comprises, consists of, or consists essentially of at least one component selected from the group consisting of capsular polysaccharide, type IV pili, and combinations thereof. In one embodiment the vaccine comprises one, two or three components selected from the group consisting of the antigenic *Kingella* NhhA homolog protein of SEQ ID NO: 2, or antigenic fragment

thereof, the *Kingella kingae* purified capsular polysaccharide and the *Kingella kingae* purified type IV pilus peptide

Another aspect of the present disclosure provides an immunogenic composition comprising, consisting of, or consisting essentially of at least one component of Knh and
5 a pharmaceutically acceptable adjuvant. In certain embodiments, the immunogenic composition further comprises, consists of, or consists essentially of at least one component selected from the group consisting of capsular polysaccharide, type IV pili, and combinations thereof. In yet another aspect, the present disclosure provides a pharmaceutical composition comprising, consisting of, or consisting essentially of at least
10 one component of Knh and a pharmaceutically acceptable carrier. In certain embodiments, the pharmaceutical composition further comprises, consists of, or consists essentially of at least one component selected from the group consisting of capsular polysaccharide, type IV pili, and combinations thereof.

Another aspect of the present disclosure provides a method for preventing
15 infection with a bacterium that expresses Knh comprising, consisting of, or consisting essentially of administering an immunologically effective dose of a vaccine or composition described herein. Another aspect of the present disclosure provides a method for treating infection with a bacterium that expresses Knh comprising, consisting of, or consisting essentially of administering a therapeutically effective dose of a
20 pharmaceutical composition described herein. In one embodiment the infection is caused by a gram negative bacterium of the *Kingella* genus, and in one embodiment the gram negative bacterium is *Kingella kingae*.

Another aspect of the present disclosure provides a method of inducing an immune response in a subject which has been exposed to or infected with a bacterium
25 comprising, consisting of, or consisting essentially of administering to the subject an amount of the pharmaceutical composition described herein, thereby inducing an immune response.

The present disclosure further encompasses the use of kits for administering at least one compound of the invention to a subject in need thereof.

Other aspects and advantages will become apparent to those skilled in the art from a review of the following description that proceeds with reference to the following illustrative drawings.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: presents a bar graph showing the adherence phenotypes of wild type 269-492 *K. kingae*, and *knh* and *pilA1* *K. kingae* mutants, to multiple host cell types.

Fig. 2: is a schematic representation of the *Knh* peptide structure showing the various domains of the peptide..

10 Fig. 3: is photo of a Western blot wherein antiserum to a recombinant 42kDa N-terminal fragment of *knh* was generated and used to probe *K. kingae* outer membrane preparations of *K. kingae* wild type strains KK01, KK02 and 269-492 relative to mutant strains *pilA1*⁻, KK01/*knh*⁻, KK02/*knh*⁻ and 269-492/*knh*⁻. *Knh* can be detected in the *K. kingae* outer membrane preparations including those from the non-adherent *PilA1* mutant
15 (*pilA1*⁻).

Fig. 4A & 4B: Disruption of the capsule export results in the loss of mucoid phenotype and surface polysaccharide. To determine if *K. kingae* expresses a capsular polysaccharide, the capsule export was disrupted by insertionally inactivating *ctrA*, encoding the putative capsule export outer membrane pore. Disruption of capsule export
20 leads to a loss of the mucoid growth phenotype commonly observed in encapsulated bacteria (Fig. 4A). As shown in Fig. 4B, capsule can be extracted from the bacterial surface with heat (H) or mild acid (A) treatment and detected with the cationic dye, Alcian blue, following SDS-PAGE separation. Capsule is not detected in the *ctrA* mutant. Type b encapsulated *H. influenzae* (C54 b+) and an isogenic capsule mutant
25 (C54 b-) are included as controls.

Fig. 5. *K. kingae* capsule contains sialic acid. Polysaccharide was extracted from the bacterial surface with heat as described in Fig 4 and hydrolyzed with sulfuric acid. The hydrolyzed capsule was subsequently reacted with thiobarbituric acid (TBA), which has been previously shown to react specifically with sialic acid. The chromogenic
30 reaction was then quantitated by reading the absorbance at 549nm. The *H. influenzae*

type b capsule was included as a negative control, as this capsule does not contain sialic acid.

Fig. 6 To determine the individual contributions of the three *K. kingae* surface factors (capsular polysaccharide, Knh and type IV pili), single, double, and triple mutants were generated and assayed for adherence to Chang cells. Disruption of capsule export in a *pilA1* mutant restored adherence to wild-type levels. Further disruption of *knh* in the *pilA1/ctrA* mutant background eliminated adherence.

DETAILED DESCRIPTION

DEFINITIONS

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Articles “a” and “an” are used herein to refer to one or to more than one (*i.e.* at least one) of the grammatical object of the article. By way of example, “an element” means at least one element and can include more than one element.

As used herein, an “antimicrobial” is a substance that kills, or inhibits the growth or the ability of a microbe (such as bacteria, fungi, or viruses) to infect or maintain an infection in its host cell/organism.

As used herein, the term “pharmaceutically acceptable carrier” includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

As used herein the term “pharmaceutically acceptable salt” refers to salts of compounds that retain the biological activity of the parent compound, and which are not biologically or otherwise undesirable. Many of the compounds disclosed herein are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

As used herein, the term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. For example, as used herein the term "treating an infection" will refer in general to decreasing the number of infectious agents present in a tissue or cell relative to a pretreatment status or relative to an untreated control infected with the relevant pathogen.

A "prophylactic" treatment is a treatment administered to a subject, who either does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology associated with the disease.

As used herein an "effective" amount or a "therapeutically effective amount" refers to a nontoxic but sufficient amount of a bioactive agent to provide the desired effect. The term "effective amount" is used interchangeably with "effective concentration" herein. The amount that is "effective" will vary from subject to subject, depending on the age and general condition of the individual, mode of administration, and the like. Thus, it is not always possible to specify an exact "effective amount." However, an appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

The term, "parenteral" means not through the alimentary canal but by some other route such as subcutaneous, intramuscular, intraspinal, or intravenous.

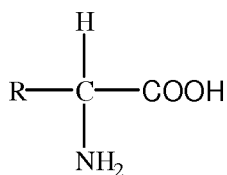
The term "about," as used herein, means approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. For example, in one aspect, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

A disease or disorder is "alleviated" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, are reduced.

As used herein, "amino acids" are represented by the full name thereof, by the three letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following table:

	<u>Full Name</u>	<u>Three-Letter Code</u>	<u>One-Letter Code</u>
	Aspartic Acid	Asp	D
	Glutamic Acid	Glu	E
5	Lysine	Lys	K
	Arginine	Arg	R
	Histidine	His	H
	Tyrosine	Tyr	Y
	Cysteine	Cys	C
10	Asparagine	Asn	N
	Glutamine	Gln	Q
	Serine	Ser	S
	Threonine	Thr	T
	Glycine	Gly	G
15	Alanine	Ala	A
	Valine	Val	V
	Leucine	Leu	L
	Isoleucine	Ile	I
	Methionine	Met	M
20	Proline	Pro	P
	Phenylalanine	Phe	F
	Tryptophan	Trp	W

25 The expression “amino acid” as used herein is meant to include compounds having the following general structure:



30 wherein R represents hydrogen or a hydrocarbon side chain, and includes both natural and synthetic amino acids, and both D and L amino acids. “Standard amino acid”

means any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid residue" means any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or derived from a natural source. As used herein, "synthetic amino acid" also encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and substitutions. Amino acids contained within the peptides of the present invention, and particularly at the carboxy- or amino-terminus, can be modified by methylation, amidation, acetylation or substitution with other chemical groups which can change the peptide's circulating half-life without adversely affecting their activity. Additionally, a disulfide linkage may be present or absent in the peptides of the invention.

As used herein an amino acid "substitution" refers to the replacement of one amino acid residue by a different amino acid residue.

As used herein, the term "conservative amino acid substitution" is defined herein as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly;

II. Polar, negatively charged residues and their amides:

Asp, Asn, Glu, Gln, cysteic acid and homocysteic acid;

III. Polar, positively charged residues:

His, Arg, Lys; Ornithine (Orn)

IV. Large, aliphatic, nonpolar residues:

Met, Leu, Ile, Val, Cys, Norleucine (Nle), homocysteine

V. Large, aromatic residues:

Phe, Tyr, Trp, acetyl phenylalanine

The term "identity" as used herein relates to the similarity between two or more sequences. Identity is measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100 to achieve a percentage. Thus, two copies of exactly the same sequence have 100% identity, whereas two sequences that have amino acid deletions, additions, or substitutions relative to one another have a lower

degree of identity. Those skilled in the art will recognize that several computer programs, such as those that employ algorithms such as BLAST (Basic Local Alignment Search Tool, Altschul et al. (1993) J. Mol. Biol. 215:403-410) are available for determining sequence identity.

5 The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope. Antibodies can be derived from natural sources or from recombinant sources and may be intact immunoglobulins, or immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a
10 variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂ fragments, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA
15 85:5879-5883; Bird et al., 1988, Science 242:423-426).

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule
20 encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

An "antibody combining site" is that structural portion of an antibody molecule
25 comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen. The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

The term "adjuvant" refers to a compound or mixture that enhances the immune
30 response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the

immune response (Hood et al., Immunology, Second Ed., 1984, Benjamin/Cummings: Menlo Park, Calif., p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response.

Adjuvant include, but are not limited to, complete Freund's adjuvant, incomplete Freund's
5 adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvant such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Preferably, the adjuvant is pharmaceutically acceptable.

10 The term "inhibit," as used herein, refers to the ability of a compound of the invention to reduce or impede a described function. In one embodiment, inhibition is at least 10%, at least 25%, at least 50%, at least 75% of the activity obtained in the absence of the inhibiting agent.

The phrase "inhibit infection", as used herein, refers to both direct and indirect
15 inhibition of infection, regardless of the mechanism.

The term "inhibit a protein", as used herein, refers to any method or technique which inhibits protein synthesis, levels, activity, or function, as well as methods of inhibiting the induction or stimulation of synthesis, levels, activity, or function of the protein of interest. The term also refers to any metabolic or regulatory pathway which
20 can regulate the synthesis, levels, activity, or function of the protein of interest. The term includes binding with other molecules and complex formation. Therefore, the term "protein inhibitor" refers to any agent or compound, the application of which results in the inhibition of protein function or protein pathway function. However, the term does not imply that each and every one of these functions must be inhibited at the same time.

25 As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the peptide of the invention in the kit for effecting alleviation of the various diseases or disorders recited herein. Optionally, or alternately, the instructional material may describe one or more methods of alleviating the diseases or disorders in a cell or a
30 tissue of a mammal.

As used herein, the term “purified” and the like terms relate to the isolation of a molecule or compound in a form that is substantially free (at least 60% free, 75% free, or 90% free) from other components normally associated with the molecule or compound in a native environment.

5 As used herein, the term "subject" and "patient" are used interchangeably herein and refer to both human and nonhuman animals. The term "nonhuman animals" of the disclosure includes all warm blooded vertebrates (*e.g.*, mammals, including for example, but not limited to livestock, horses, cats, dogs and other pets). In one embodiment the subject is a human..

10 As used herein, the term “gram-negative bacteria” refers to those bacteria that possess an outer membrane containing lipopolysaccharide (LPS) outside the peptidoglycan layer. Suitable gram-negative bacteria include, but are not limited to, *Kingella kingae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Serratia marcescens*,
15 *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Acinetobacter calcoaceticus*, *Alcaligenes xylosoxidans*, *Flavobacterium meningosepticum*, *Providencia stuartii* and *Citrobacter freundii*, community lung infections such as those caused by *Haemophilus influenzae*, *Legionella species*, *Moraxella catarrhalis*, *Branhamella catarrhalis*, *Enterobacter species*, *Acinetobacter species*, *Klebsiella species*, and *Proteus species*, and infections caused by other bacterial
20 species such as *Neisseria species*, *Shigella species*, *Salmonella species*, *Helicobacter pylori*, *Vibrionaceae* and *Bordetella species* as well as the infections is caused by a *Brucella species*, *Francisella tularensis* and/or *Yersinia pestis*. In one embodiment, the gram-negative bacteria is *K. kingae*.

25 As defined herein, "adhesion" means noncovalent binding of a bacterium to a eukaryotic cell, including for example a human cell, or to a cell secretion or matrix, wherein the binding is stable enough to withstand washing with physiological buffered aqueous solution (*e.g.* PBS).

EMBODIMENTS

One embodiment of the present disclosure is directed to compositions and methods for treating infections from gram negative bacteria. In accordance with one embodiment compositions and methods are provided for treating septic arthritis, osteomyelitis, and bacteremia caused by gram negative bacterial such as *Kingella kingae*. As disclosed herein such compositions may include one of three *K. kingae* surface factors: capsular polysaccharide, Knh and/or type IV pili.

In accordance with one embodiment a pharmaceutical composition is provided comprising a purified *Kingella* capsular polysaccharide and a pharmaceutically acceptable carrier. In one embodiment the *Kingella* capsular polysaccharide is purified from the bacteria using the procedure disclosed in Example 2. In one embodiment the capsule is purified from *Kingella kingae*. The compositions can be used to induce an immune response in subjects that will interfere or inhibit gram negative infections of the subject. In accordance with one embodiment the composition can be used to treat septic arthritis, osteomyelitis, and bacteremia caused by the gram-negative bacterium, *Kingella kingae*.

Polysaccharide based vaccines have been successfully used to combat bacterial infections and are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J. M., Chiu, S. S., Wong, V. K., et al. JAMA 275: 1499-1503, 1996). Accordingly, in one embodiment the purified *Kingella* capsular polysaccharide is conjugated to an immunogenic carrier protein. Examples of immunogenic carrier proteins which are currently used for coupling to polysaccharide or oligosaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, DT CRM197 and TT), Keyhole Limpet Haemocyanin (KLH), *Pseudomonas aeruginosa* exoprotein A (rEPA) and the purified protein derivative of Tuberculin (PPD), *Neisseria meningitidis* surface proteins, protein D from *Haemophilus influenzae*, pneumolysin or fragments of any of the above as well as additional compounds known to those skilled in the art. The immunogenic carrier protein can be conjugated to the capsular polysaccharide using standard techniques known to those skilled in the art.

The *Kingella* capsular polysaccharide composition in one embodiment further comprise a *Kingella* NhhA homolog peptide (Knh peptide). More particularly the

composition further comprises an antigenic fragment of SEQ ID NO: 1. In one embodiment the fragment comprises amino acids 85-485, 567-746, 834-1263, 1310-1405, or 1531-1633 of SEQ ID NO: 1. In one embodiment the antigenic fragment comprises a 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 contiguous amino acid sequence fragment of SEQ ID NO: 2. In a further embodiment the antigenic fragment comprises a 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 contiguous amino acid sequence that differs from a corresponding 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 contiguous amino acid sequence fragment of SEQ ID NO: 2 by 1 or 2 amino acid modifications (i.e., an amino acid substitution, deletion or insertion). In one embodiment the antigenic fragment comprises a 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 contiguous amino acid sequence that differs from a corresponding 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 contiguous amino acid sequence fragment of SEQ ID NO: 2 by 1 or 2 amino acid substitutions, typically conservative amino acid substitutions. In one embodiment the *Kingella* NhhA homolog peptide component comprises an 8, 10, 12, 15 or 20 amino acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7. In another embodiment the *Kingella* NhhA homolog peptide component comprises a 15 amino acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or a 15 amino acid sequence fragment that has at least 60%, 70%, 80% or 90% sequence identity with a 15 amino acid sequence selected from any of the sequences of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7. In one embodiment the composition comprises two or more distinct *Kingella* NhhA homolog peptides as disclosed herein.

In a further embodiment the *Kingella* capsular polysaccharide composition comprises a purified *Kingella* pili peptide. In one embodiment the *Kingella* pili peptide is purified from *Kingella kingae*. In one embodiment the *Kingella* capsular polysaccharide composition comprises a purified *Kingella* pili peptide and a *Kingella* NhhA homolog peptide as disclosed herein. In one embodiment the capsular polysaccharide is conjugated to an immunogenic carrier protein and in one embodiment the capsular polysaccharide is conjugated to a *Kingella* NhhA homolog peptide.

In accordance with one embodiment a pharmaceutical composition is provided comprising a *Kingella* NhhA homolog peptide and a pharmaceutically acceptable carrier. the *Kingella* NhhA homolog peptide any comprising any antigenic fragment of SEQ ID NO: 1 as disclosed herein. In one embodiment the *Kingella* NhhA homolog peptide comprises amino acids 85-485, 567-746, 834-1263, 1310-1405, or 1531-1633 of SEQ ID NO: 1, or an 8, 10, 15 or 20 amino acid sequence fragment of a sequence selected from amino acids 85-485, 567-746, 834-1263, 1310-1405, or 1531-1633 of SEQ ID NO: 1. In one embodiment the *Kingella* NhhA homolog peptide comprises a 10 amino acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7. In a further embodiment the *Kingella* NhhA homolog peptide comprises a 6, 8, 10, 12, 15 amino acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or a 6, 8, 10, 12, 15 amino acid sequence fragment that has at least 60%, 70%, 80% or 90% sequence identity with a corresponding 6, 8, 10, 12 or 15 amino acid sequence fragment selected from any of the sequences of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7. In one embodiment the *Kingella* NhhA homolog peptide comprises a 15 amino acid sequence fragment of SEQ ID NO: 2. in a further embodiment the composition comprises an adjuvant.

In one embodiment the composition comprising a *Kingella* NhhA homolog peptide further comprises a purified compound, or fragment thereof, selected from the group consisting of *Kingella* capsular polysaccharide and *Kingella* type IV pilus peptide.

In a further aspect, the present disclosure provides an immunogenic composition comprising at least one component of a *Kingella* NhhA homolog peptide (Knh). In a still further aspect, the present disclosure provides an immunogenic composition comprising at least one component Knh and at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof. In a particular aspect, the immunogenic compounds also comprise a pharmaceutically acceptable adjuvant.

The present disclosure also provides pharmaceutical compositions comprising at least one component of Knh or at least on component of the capsular polysaccharide, and

a pharmaceutically acceptable carrier. In one embodiment the Knh component comprises an 8, 10, 12 or 15 amino acid fragment of SEQ ID NO: 2, or any other antigenic fragment of SEQ ID NO: 1 as disclosed herein. In one embodiment the composition comprises 2 or 3 different amino acid fragment of SEQ ID NO: 2. In one embodiment the pharmaceutical composition comprises a purified capsular polysaccharide isolated from *Kingella kingae*. The disclosure also provides pharmaceutical compositions comprising at least one component of Knh and at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof, and a pharmaceutically acceptable carrier. Such pharmaceutical composition are used in accordance with one embodiment to prevent gram-negative bacterial attachment to mucosal surfaces.

In one embodiment composition are provided to interfering with the function of Knh or the capsular polysaccharide. In one embodiment the interference relates to preventing the expression of the protein, or in another embodiment the interference relates to inhibiting the assembly or binding properties of the compound. Examples of suitable compounds include, but are not limited to, small molecules, antibodies, antisense RNAs, iRNAs, cDNAs, antibodies, peptides, protein kinase inhibitors, combinations thereof, and the like. In certain embodiments, the compound is a small molecule. In such embodiments, the small molecules generally have a molecular weight of approximately 450 kDa or less and may include, but are not limited to, monosaccharides, oligosaccharides, amino acids, oligopeptides, nucleotides, olionucleotides, polypeptides and their derivatives. In some embodiments, the small molecules are capable of disrupting and/or inhibiting Knh and/or type IV pili function.

In accordance with one embodiment a composition is provided comprising antibodies to Knh and/or type IV pili and/or capsular polysaccharide. Blocking adherence using such antibodies blocks the initial step in infection thereby reducing colonization. This in turn decreases person-to-person transmission and prevents development of symptomatic disease. Furthermore, such antibodies can be used in diagnostic screens to detect *Kingella kingae* and/or determine the pathogenicity of the strain based on the detected surface antigens. In one embodiment the antibody binds to a Knh component comprising an 8, 10, 12 or 15 amino acid fragment of SEQ ID NO: 2, or

any other antigenic fragment of SEQ ID NO: 1 as disclosed herein. In one embodiment the composition comprises antibodies that bind to 2 or 3 different amino acid fragment of SEQ ID NO: 2. In one embodiment the pharmaceutical composition comprises an antibody that specifically binds to a purified capsular polysaccharide isolated from
5 *Kingella kingae*. The disclosure further provides a pharmaceutical composition comprising a combination of at least two antibodies, one directed to Knh and another directed to either type IV pili, capsular polysaccharide, or both, and a pharmaceutically acceptable carrier.

As used herein, the term “component” refers to any portion, fragment, and the like
10 of a protein/molecule that can (1) inhibit/disrupt the function of the protein/molecule (e.g., for Knh, inhibit or disrupt its ability to facilitate bacterial adherence) and/or (2) confer immunogenicity in a subject. In preferred embodiments, the component comprises both properties. For example, suitable components of the Knh molecule may include those domains highlighted in FIG 2, such as YadA-like head domains, Trp ring
15 domain, KG domain, neck domain, iSneck2 domain and the like. A suitable component for the capsular polysaccharide may include *ctrA*, the putative capsule export outer membrane pore. Components of the capsular polysaccharide have been used in vaccines to treat other infectious agents, such as *H. influenzae*, *N. meningitidis* and *S. pneumonia*. (see, e.g., Vicki, B. et al. (1996) *J. Pediatrics* 128(3): 363-365; Lionel, K.K. et al. (2010)
20 *N. Eng. J. Med.* 362:1511-1520; Janoff, E. et al. (1999) *J. Clin. Invest.* 104(8):1139-1147). Suitable components of type IV pili may include, but are not limited to, pilA1, pilA2, recJ and fimB (see, e.g., Kehl-Fie, T. et al. (2008) *J. Bacteriol.* 190:7157-7163).

The present disclosure further provides an antibody capable of specifically recognizing or binding to Knh and/or type IV pili and/or the *Kingella* capsular
25 polysaccharide. The antibody may be a monoclonal or polyclonal antibody. Further, the antibody may be labeled with a detectable marker that is either a radioactive, calorimetric, fluorescent, or a luminescent marker. The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody.

30 Antibodies against the isolated polypeptides of the present invention include naturally raised and recombinantly prepared antibodies. These may include both

polyclonal and monoclonal antibodies prepared by known genetic techniques, as well as bi-specific (chimeric) antibodies, and antibodies including other functionalities suiting them for diagnostic use. Such antibodies can be used in immunoassays to diagnose infection with a particular strain or species of bacteria. The antibodies can also be used for passive immunization to treat an infection with gram-negative bacteria. These antibodies may also be suitable for modulating bacterial adherence and/or invasion including but not limited to acting as competitive agents. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab, Fab', F(ab')₂ and F(v) fragments, and an Fab expression library. Fab and F(ab')₂ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Pat. No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody. Various procedures known in the art may be used for the production of polyclonal antibodies to polypeptide or derivatives or analogs thereof (see, e.g., Antibodies--A Laboratory Manual, Harlow and Lane, eds., Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., 1988). For the production of antibody, various host animals can be immunized by injection with the compound, an immunogenic

fragment thereof, or a derivative (e.g., fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the polypeptide can be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvant may be used to increase the immunological response, depending on the host species.

For preparation of monoclonal antibodies, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (see, e.g., *Antibodies--A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., 1988). These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495 497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77 96).

Monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). Human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc. Natl. Acad. Sci. U.S.A* 80:2026 2030) or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77 96). Techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *J. Bacteriol.* 159 870; Neuberger et al., 1984, *Nature* 312:604 608; Takeda et al., 1985, *Nature* 314:452 454) by splicing the genes from a mouse antibody molecule specific for a polypeptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this disclosure. Such human or humanized chimeric antibodies are typically used in therapy of human infections or diseases, since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al, 1989, *Science* 246:1275 1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for the polypeptide, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g., radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Antibodies can be labeled for detection *in vitro*, e.g., with labels such as enzymes, fluorophores, chromophores, radioisotopes, dyes, colloidal gold, latex particles, and chemiluminescent agents. Alternatively, the antibodies can be labeled for detection *in vivo*, e.g., with radioisotopes (preferably technetium or iodine); magnetic resonance shift reagents (such as gadolinium and manganese); or radio-opaque reagents. The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate. The polypeptide can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of

the currently available counting procedures. The preferred isotope may be selected from ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re .

Enzyme labels are likewise useful, and can be detected by any of the presently utilized calorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle-by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, β -glucuronidase, β -D-glucosidase, β -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S.

Pat. Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

The therapeutic possibilities that are raised by the existence of Knh in gram-negative bacteria derive from the fact that the Knh of the present disclosure has been found to play a role in adhesion of gram-negative bacteria, such as *K. kingae*. Thus, Knh is anticipated to be involved in or required for gram-negative bacterial adhesion to host cells, which is critical for bacterial survival and virulence in the human host.

In accordance with one embodiment, the present disclosure extends to vaccines based on the Knh protein (SEQ ID NO; 1) or the purified capsular polysaccharide of *Kingella kingae* described herein. The present invention provides a vaccine comprising at least one component of Knh or the purified capsular polysaccharide of *Kingella kingae* and a pharmaceutically acceptable adjuvant. In one embodiment the capsular polysaccharide is conjugated to an immunogenic carrier protein. The present invention further provides a vaccine comprising at least one component of Knh and at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof, and a pharmaceutically acceptable adjuvant.

The modes of administration of the vaccine or compositions of the present invention may comprise the use of any suitable means and/or methods for delivering the vaccine or composition to the host animal whereby they are immunostimulatively effective. Delivery modes may include, without limitation, parenteral administration methods, such as paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly,

intracranially and intratumorally. In one embodiment the antigenic composition disclosed herein are administered intramuscularly.

In one embodiment, since the desired result of vaccination is to elucidate an immune response to the antigen, and thereby to the pathogenic organism, administration directly, or by targeting or choice of a viral vector, indirectly, to lymphoid tissues, e.g., lymph nodes or spleen, is desirable. Since immune cells are continually replicating, they are ideal target for retroviral vector-based nucleic acid vaccines, since retroviruses require replicating cells. These vaccines and compositions can be used to immunize mammals, for example, by the intramuscular or parenteral routes, or by delivery to mucosal surfaces using microparticles, capsules, liposomes and targeting molecules, such as toxins and antibodies. The vaccines and immunogenic compositions may be administered to mucosal surfaces by, for example, the nasal or oral (intra-gastric) routes. Alternatively, other modes of administration including suppositories may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols and triglycerides. Oral formulations may include normally employed excipients, such as pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The antigenic compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 1 to 95% of the immunogenic compositions disclosed herein. The immunogenic compositions are administered in a manner compatible with the dosage formulation, and in such amount as to be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be immunized, including, for example, the capacity of the subject's immune system to synthesize antibodies, and if needed, to produce a cell-mediated, humoral or antibody-mediated immune response. Precise amounts of antigen and immunogenic composition to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by those skilled in the art and may be of the order of micrograms to milligrams. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and will vary according to the size of the host.

Passive immunity can be conferred to a subject suspected of suffering an infection with gram-negative bacteria by administering to the subject antiserum, polyclonal antibodies, or a neutralizing monoclonal antibody against Knh, or the *Kingella* capsular polysaccharide. Although passive immunity does not confer long-term protection, it can be a valuable tool for the treatment of a bacterial infection in a subject who has not been vaccinated. Passive immunity is particularly important for the treatment of antibiotic resistant strains of bacteria, since no other therapy may be available. Preferably, the antibodies administered for passive immune therapy are autologous antibodies. For example, if the subject is a human, preferably the antibodies are of human origin or have been "humanized," in order to minimize the possibility of an immune response against the antibodies. The active or passive vaccines of the invention can be used to protect an animal subject from infection by gram-negative bacteria, particularly *K. kingae*.

Typical doses for a vaccine composed of a protein antigen are in the range of 2.5-50 µg of total protein per dose. Typical doses for a polysaccharide-protein conjugate vaccine are 7.5-25 µg of polysaccharide and 1.25-250 µg of carrier protein. These types of vaccines are typically given intramuscularly. Dosing schedules of a vaccine can be readily determined by the skilled artisan, particularly by comparison of similar vaccines. If used as a universal vaccine, the vaccine would be integrated into the routine immunization schedule. Most similar vaccines require a primary series of immunizations (usually 2 or 3 doses at 2 month intervals beginning at 1 or 2 months of age) and a single booster at 12-18 months of age. A smaller number of doses or a single dose may be adequate in older children (over a year of age). For immunization of pregnant women, an exemplary immunization schedule would be a single dose given in the second or early third trimester. For immunization of non-pregnant adults, a single dose would probably be used. The requirement for subsequent booster doses in adults is difficult to predict--this would be based on the immunogenicity of the vaccine and ongoing surveillance of vaccine efficacy.

In a further embodiment of the present disclosure a method for the prevention or treatment of mammals to control the amount or activity of a gram-negative bacterium is provided, so as to treat or prevent the adverse consequences of invasive, spontaneous, or idiopathic pathological states. The disclosure provides a method for preventing infection

with a gram-negative bacterium that expresses Knh, comprising administering an immunogenically effective dose of a vaccine comprising at least one component of Knh to a subject. In certain embodiments, the vaccine further comprises at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof. The present disclosure is directed to a method for treating infection with a gram-negative bacterium that expresses Knh comprising administering a therapeutically effective dose of a pharmaceutical composition comprising at least one component of Knh and a pharmaceutically acceptable carrier to a subject. In certain embodiments, the pharmaceutical composition further comprises at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof.

In a further embodiment, the disclosure provides a method of inducing an immune response in a subject which has been exposed to or infected with a gram-negative bacterium comprising administering to the subject an amount of the pharmaceutical composition comprising at least one component of Knh and/or a *Kingella* capsular polysaccharide, and a pharmaceutically acceptable carrier, thereby inducing an immune response. In certain embodiments, the pharmaceutical composition comprises an 8 or 15 amino acid fragment of SEQ ID NO: 2 and at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof.

The disclosure still further provides a method for preventing infection by a gram-negative bacterium in a subject comprising administering to the subject an amount of a pharmaceutical composition comprising at least one component of Knh and a pharmaceutically acceptable carrier or diluent, thereby preventing infection by a gram-negative bacterium. In certain embodiments, the pharmaceutical composition further comprises at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof.

Also encompassed by the present disclosure is a method of inhibiting colonization of host cells in a subject who has been exposed to or infected with a gram-negative bacterium comprising administering to the subject an amount of a pharmaceutical composition comprising at least one component of Knh, thereby inducing an immune response. The therapeutic peptide that blocks colonization is delivered by the respiratory

mucosal. In certain embodiments, the pharmaceutical composition further comprises at least one component selected from the group consisting of type IV pili, capsular polysaccharide, and combinations thereof.

In accordance with one embodiment the pharmaceutical compositions disclosed
5 herein comprise therapeutically effective amounts of polypeptide products or antibodies of the disclosure together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers useful in therapy against bacterial infection or in inducing an immune response. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration
10 regimen. In one embodiment the pharmaceutical compositions can be liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-
15 oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid,
20 hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the polypeptides of the present invention. The choice of compositions will depend on the physical and chemical properties of the polypeptide. Controlled or sustained release
25 compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the polypeptides of the present invention coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the
30 invention incorporate particulate forms, protective coatings, protease inhibitors or

permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

In one embodiment the pharmaceutically acceptable carrier may include, but is not limited to, 0.01 0.1M and more typically 0.05M phosphate buffer or 0.8% saline.

5 Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles
10 include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

15 In accordance with one embodiment a therapeutically effective amount is an amount sufficient to prevent, or reduce by at least about 30, 50 or 90 percent, a clinically significant infection by a gram negative bacterium such as *Kingella kingae*.

Alternatively, in the case of a vaccine or immunogenic composition, a therapeutically effective amount is used herein to mean an amount sufficient and suitable to elicit an
20 immune response and antibody response in an individual, and particularly to provide a response sufficient to prevent, or reduce by at least about 30, 50 or 90 percent, a clinically significant infection by gram-negative bacterium. In one embodiment a sufficient amount may include but is not limited to from about 1 µg/kg to about 1000 mg/kg. The amount may be 10 mg/kg. In the context of the present invention, a deficit
25 in the response of the host is evidenced by continuing or spreading bacterial infection. An improvement in a clinically significant condition in the host includes a decrease in bacterial load, clearance of bacteria from colonized host cells, reduction in fever or inflammation associated with infection, or a reduction in any symptom associated with the bacterial infection. Accordingly a therapeutically effective amount is an amount
30 sufficient to cause an improvement in a clinically significant condition in the subject.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or
5 coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

When administered, compounds are often cleared rapidly from mucosal surfaces
10 or the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent administrations of relatively large doses of bioactive compounds may be required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran,
15 polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the
20 compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

As noted above, the present disclosure provides therapeutic compositions
25 comprising pharmaceutical compositions comprising vaccines, polypeptides, nucleic acids and antibodies, anti-antibodies, and agents, to compete with the Gram-negative bacterium for pathogenic activities, such as adherence to host cells. The preparation of therapeutic compositions which contain an active component is well understood in the art. Typically, such compositions are prepared as an aerosol of the polypeptide delivered
30 to the nasopharynx or as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be

prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

An active component can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

According to the disclosure, the component or components of a therapeutic composition of the invention may be introduced parenterally, transmucosally, e.g., orally, nasally, pulmonarailly, or rectally, or transdermally. In one embodiment, administration is parenteral, e.g., via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration. Oral or pulmonary delivery may be preferred to activate mucosal immunity; since Gram-negative bacteria generally colonize the nasopharyngeal and pulmonary mucosa, particularly that of neonates, mucosal immunity may be a particularly effective preventive treatment. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527 1533 (1990); Treat et al., in

Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353 365 (1989); Lopez-Berestein, *ibid.*, pp. 317 327; see generally *ibid.*

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507. (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al, *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115 138 (1984)). Typically, a controlled release device is introduced into a subject in proximity of the site of inappropriate immune activation or a tumor. Other controlled release systems are discussed in the review by Langer (*Science* 249:1527 1533 (1990)).

The subject in whom administration of an active component as set forth above is an effective therapeutic regimen for a bacterial infection is preferably a human, but can be any animal. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., i.e., for veterinary medical use.

A therapeutically effective dosage can be determined by the ordinary skilled medical worker based on patient characteristics (age, weight, sex, condition, complications, other diseases, etc.), as is well known in the art. Generally, for intravenous injection or infusion, dosage may be lower than for intraperitoneal, intramuscular, or other routes of administration. The dosing schedule may vary, depending on the circulation half-life, and the formulation used. The compositions are administered in a manner compatible with the dosage formulation in the therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

For treatment of a bacterial infection, one may administer the present active component in conjunction with one or more pharmaceutical compositions used for treating bacterial infection, including but not limited to (1) antibiotics; (2) soluble carbohydrate inhibitors of bacterial adhesin; (3) other small molecule inhibitors of bacterial adhesin; (4) inhibitors of bacterial metabolism, transport, or transformation; (5) stimulators of bacterial lysis, or (6) anti-bacterial antibodies or vaccines directed at other bacterial antigens. Other potential active components include anti-inflammatory agents, such as steroids and non-steroidal anti-inflammatory drugs. Administration may be simultaneous (for example, administration of a mixture of the present active component and an antibiotic), or may be in seriatim. Thus, in a specific instance where it is desired to reduce or inhibit the infection resulting from a bacterium mediated binding of bacteria to a host cell, or an antibody thereto, or a ligand thereof or an antibody to that ligand, the polypeptide is introduced to block the interaction of the bacteria with the host cell.

Also contemplated herein is pulmonary delivery of an inhibitor of the polypeptide of the present disclosure having which acts as adhesin inhibitory agent (or derivatives thereof). The adhesin inhibitory agent (or derivative) is delivered to the lungs of a mammal, where it can interfere with bacterial binding to host cells. Other reports of preparation of proteins for pulmonary delivery are found in the art [Adjei et al.(1990) Pharmaceutical Research, 7:565 569; Adjei et al. (1990) International Journal of Pharmaceutics, 63:135 144 (leuprolide acetate); Braquet et al (1989), Journal of Cardiovascular Pharmacology, 13(suppl. 5):143 146 (endothelin-1); Hubbard et al(1989) Annals of Internal Medicine, Vol. III, pp. 206 212 (.alpha.1-antitrypsin); Smith et al. (1989) J. Clin. Invest. 84:1145 1146 (.alpha.-1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colo., March, (1990) (recombinant human growth hormone); Debs et al. (1988) J. Immunol. 140:3482 3488 (interferon-.gamma. and tumor necrosis factor alpha); Platz et al., U.S. Pat. No. 5,284,656 (granulocyte colony stimulating factor)]. A method and composition for pulmonary delivery of drugs is described in U.S. Pat. No. 5,451,569, issued Sep. 19, 1995 to Wong et al.

All such devices require the use of formulations suitable for the dispensing of adhesin inhibitory agent (or derivative). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvant and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified adhesin inhibitory agent may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise adhesin inhibitory agent (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active adhesin inhibitory agent per ml of solution. The formulation may also include a buffer and a simple sugar (e.g., for adhesin inhibitory agent stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced

aggregation of the adhesin inhibitory agent caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the adhesin inhibitory agent (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

The liquid aerosol formulations contain adhesin inhibitory agent and a dispersing agent in a physiologically acceptable diluent. The dry powder aerosol formulations of the present invention consist of a finely divided solid form of adhesin inhibitory agent and a dispersing agent. With either the liquid or dry powder aerosol formulation, the formulation must be aerosolized. That is, it must be broken down into liquid or solid particles in order to ensure that the aerosolized dose actually reaches the mucous membranes of the nasal passages or the lung. The term "aerosol particle" is used herein to describe the liquid or solid particle suitable for nasal or pulmonary administration, i.e., that will reach the mucous membranes. Other considerations, such as construction of the delivery device, additional components in the formulation, and particle characteristics are important. These aspects of pulmonary administration of a drug are well known in the art, and manipulation of formulations, aerosolization means and construction of a delivery device require at most routine experimentation by one of ordinary skill in the art. In a particular embodiment, the mass median dynamic diameter will be 5 micrometers or less in order to ensure that the drug particles reach the lung alveoli [Wearley, L. L. (1991) Crit. Rev. in Ther. Drug Carrier Systems 8:333].

Systems of aerosol delivery, such as the pressurized metered dose inhaler and the dry powder inhaler are disclosed in Newman, S. P., Aerosols and the Lung, Clarke, S. W. and Davia, D. editors, pp. 197 22 and can be used in connection with the present disclosure. In a further embodiment, as discussed in detail infra, an aerosol formulation of the present disclosure can include other therapeutically or pharmacologically active

ingredients in addition to adhesin inhibitory agent, such as but not limited to an antibiotic, a steroid, a non-steroidal anti-inflammatory drug, etc.

The present disclosure provides aerosol formulations and dosage forms for use in treating subjects suffering from bacterial, e.g., gram-negative bacteria, infection. In general such dosage forms contain adhesin inhibitory agent in a pharmaceutically acceptable diluent. Pharmaceutically acceptable diluents include but are not limited to sterile water, saline, buffered saline, dextrose solution, and the like. In a specific embodiment, a diluent that may be used in the present invention or the pharmaceutical formulation of the present invention is phosphate buffered saline, or a buffered saline solution generally between the pH 7.0 8.0 range, or water. The liquid aerosol formulation of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, surfactants and excipients. The formulation may include a carrier. The carrier is a macromolecule which is soluble in the circulatory system and which is physiologically acceptable where physiological acceptance means that those of skill in the art would accept injection of said carrier into a patient as part of a therapeutic regime. The carrier preferably is relatively stable in the circulatory system with an acceptable plasma half life for clearance. Such macromolecules include but are not limited to Soya lecithin, oleic acid and sorbitan trioleate, with sorbitan trioleate preferred.

The formulations of the present embodiment may also include other agents useful for pH maintenance, solution stabilization, or for the regulation of osmotic pressure. Examples of the agents include but are not limited to salts, such as sodium chloride, or potassium chloride, and carbohydrates, such as glucose, galactose or mannose, and the like.

The present disclosure further contemplates liquid aerosol formulations comprising adhesin inhibitory agent and another therapeutically effective drug, such as an antibiotic, a steroid, a non-steroidal anti-inflammatory drug, etc.

It is also contemplated that the present aerosol formulation can be prepared as a dry powder formulation comprising a finely divided powder form of adhesin inhibitory agent and a dispersant. Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing adhesin inhibitory agent (or derivative)

and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation. The adhesin inhibitory agent (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 mm (or microns), most preferably 0.5 to 5 mm, for most effective delivery to the distal lung. In another embodiment, the dry powder formulation can comprise a finely divided dry powder containing adhesin inhibitory agent, a dispersing agent and also a bulking agent. Bulking agents useful in conjunction with the present formulation include such agents as lactose, sorbitol, sucrose, or mannitol, in amounts that facilitate the dispersal of the powder from the device.

The present disclosure further contemplates dry powder formulations comprising adhesin inhibitory agent and another therapeutically effective drug, such as an antibiotic, a steroid, a non-steroidal anti-inflammatory drug, etc. Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Pat. No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Pat. No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: Modern Pharmaceutics Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the component or components (or chemically modified forms thereof) and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatized component or components. The component or components may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the component molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood

stream from the stomach or intestine. Also desired is the increase in overall stability of the component or components and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski and Davis, 1981, "Soluble Polymer-Enzyme Adducts" In: Enzymes as Drugs, Hocenberg and Roberts, eds., Wiley-Interscience, New York, N.Y., pp. 367 383; Newmark, et al. (1982) J. Appl. Biochem. 4:185 189. Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the component (or derivative) the location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the protein (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine. To ensure full gastric resistance a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used. The peptide therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression. Colorants and

flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents. One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, a-lactose, anhydrous lactose, cellulose, sucrose, modified dextran and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch, including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants. Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the polypeptide (or derivative) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Also contemplated herein is pulmonary delivery of the present polypeptide (or derivatives thereof). The polypeptide (or derivative) is delivered to the lungs of a mammal while inhaling and coats the mucosal surface of the alveoli. Other reports of this include Adjei et al. (1990) *Pharmaceutical Research* 7:565 569; Adjei et al. (1990) *International Journal of Pharmaceutics* 63:135 144 (leuprolide acetate); Braquet et al. (1989) *Journal of Cardiovascular Pharmacology*, 13 (suppl. 5):143 146 (endothelin-1); Hubbard et al. (1989) *Annals of Internal Medicine*, Vol. III, pp. 206 212 (a1-antitrypsin); Smith et al (1989) *J. Clin. Invest.* 84:1145 1146 (a-1-proteinase); Oswein et al. (1990) "Aerosolization of Proteins", *Proceedings of Symposium on Respiratory Drug Delivery II*, Keystone, Colo., March, (recombinant human growth hormone); Debs et al. (1988) *J. Immunol.* 140:3482 3488 (interferon-g and tumor necrosis factor alpha) and Platz et al., U.S. Pat. No. 5,284,656 (granulocyte colony stimulating factor). A method and composition for pulmonary delivery of drugs for systemic effect is described in U.S. Pat. No. 5,451,569, issued Sep. 19, 1995 to Wong et al.

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including

but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise polypeptide (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution.

- 5 The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a
10 finely divided powder containing the polypeptide (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or
15 combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing polypeptide (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal
20 of the powder from the device, e.g., 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 mm (or microns), most preferably 0.5 to 5 mm, for most effective delivery to the distal lung.

Nasal or nasopharyngeal delivery of the polypeptide (or derivative) is also
25 contemplated. Nasal delivery allows the passage of the polypeptide directly over the upper respiratory tract mucosal after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran tide nomenclature, J. Biol. Chem., 243:3552-59 (1969), abbreviations for amino acid.

30 In accordance with one embodiment a kit is provided for administering the vaccine formulations disclosed herein. The kit may include a *Kingella* NhhA homolog protein as

disclosed herein and/or a *Kingella* capsular polysaccharide and/or a *Kingella* type IV pili peptide. In one embodiment the kit further comprises a device (e.g., a syringe or nebulizer) for administering the composition of the kit. The kit may further include instructional material which describes administering the kit active ingredients to a
5 subject. As used herein, an “instructional material” includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions disclosed herein for its designated use. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the composition or be shipped together with a container which contains the
10 composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the composition be used cooperatively by the recipient.

The disclosure may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the disclosure. The following
15 examples are presented in order to more fully illustrate the preferred embodiments of the disclosure and should in no way be construed, however, as limiting the scope of the disclosed embodiments.

EXAMPLE 1

20 Identification of components involved in *K. kingae* Adhesion

Methods: A series of molecular techniques including Mariner transposon mutagenesis, gene disruptions, Western blotting, capsule staining, genome analysis and quantitative adherence assays were utilized.

Results: Screening of a Mariner transposon library for mutants deficient in
25 adherence identified a predicted trimeric autotransporter protein designated the *Kingella* *NhhA* *homolog* (Knh) that was required for full-level adherence. A *K. kingae* mutant deficient in type IV pilus expression but still expressing surface Knh was unable to adhere to host epithelium, indicating that type IV pili are necessary for Knh-mediated adherence. It was hypothesized that another surface factor was interfering with Knh-
30 mediated adherence and that type IV pili are essential to overcome this interference.

Examination of the *K. kingae* genome revealed the presence of a capsule export

operon, and India ink staining suggested the presence of a *K. kingae* surface capsule. To investigate the possibility that capsule is the factor responsible for blocking Knh-mediated adherence, the *ctrA* gene was disrupted in wild-type, pili-deficient, and Knh-deficient *K. kingae* backgrounds and the resulting strains assayed for adherence. It was discovered that disruption of capsule export in a non-piliated background restored adherence to wild-type levels. To determine if Knh is responsible for mediating the adherence observed in the non-encapsulated/non-piliated mutant, the *knh* gene was disrupted in this background to create a capsule-/pili-/Knh- triple mutant and this mutant exhibited a complete loss of adherence.

1. *Type IV pili and Knh are required for full-level adherence.* As shown in Fig. 1, elimination of Knh leads to reduction of adherence to respiratory epithelial cells (Chang and A549) and synovial cells (Hig-82 and SW982), while elimination of the major type IV pilus subunit, PilA1, abrogates adherence to all four cell lines. Negative staining TEM analysis revealed the Knh mutant to have wild-type levels of pili and the PilA1 mutant was non-piliated. These data indicate that Knh is incapable of promoting adherence in the absence of type IV pili. As shown in Fig. 2, *knh* is a 1783 amino acid trimeric autotransporter that possesses three main structural features common to autotransporters: (1) an N-terminal signal peptide that targets the preprotein to the Sec pathway (AAs 1-45); (2) an internal passenger domain that is presented on the bacterial surface (AAs 45-1695); and (c) a C-terminal beta-barrel that serves to anchor the protein in the outer surface.

As shown in Fig. 3, Knh can be detected in the *K. kingae* outer membrane. Antiserum to a recombinant 42kDa N-terminal fragment of *knh* was generated and used to probe *K. kingae* outer membrane preparations. The predicted molecular mass of the Knh monomer is 180kDa. Formic acid denaturation of outer membranes results in two reactive bands: one at the predicted monomer size and a much higher molecular mass band representing the trimer.

Conclusion: Expression of Knh in the non-adherent PilA1 mutant provides evidence that Knh alone cannot mediate adherence of *K. kingae* to host epithelial or synovial cells. Based on the above data, it is hypothesized that another surface factor may block Knh-mediated adherence and the presence of functional type IV pili are able

to overcome this adherence-blocking phenotype.

2. *K. kingae* genome encodes homologs involved in capsular polysaccharide expression. As shown in Table 2, the *K. kingae* genome contains homologs to many genes involved in capsule expression in the related pathogen, *Neisseria meningitidis*, including the *ctrABCD* ABC-type transporter operon implicated in capsule export. In addition, *K. kingae* encodes *lipA* and *lipB* homologs that have been demonstrated to be involved in capsule export in a variety of gram-negative bacteria including *N. meningitidis*, *E. coli* and *H. influenzae*.

Table 2: Putative capsule genes in *K. kingae*

<i>K. kingae</i> gene product	Homology to <i>Neisseria meningitidis</i> gene product	Function
CtrA	51% identity/ 71% similarity	Capsule export
CtrB	59% identity/ 76% similarity	Capsule export
CtrC	75% identity/ 87% similarity	Capsule export
CtrD	81% identity/ 91% similarity	Capsule export
LipB	57% identity/ 70% similarity	Capsule export
LipA	50% identity/ 66% similarity	Capsule export

K. kingae expresses a capsular polysaccharide. As shown in Fig. 4, disruption of the capsule export results in the loss of mucoid phenotype and surface polysaccharide. To determine if *K. kingae* expresses a capsular polysaccharide, the capsule export was disrupted by insertionally inactivating *ctrA*, encoding the putative capsule export outer membrane pore (Fig. 4A). Disruption of capsule export leads to a loss of the mucoid growth phenotype commonly observed in encapsulated bacteria. As shown in Fig. 4B, capsule can be extracted from the bacterial surface with heat (H) or mild acid (A) treatment and detected with the cationic dye, Alcian blue, following SDS-PAGE separation. Capsule is not detected in the *ctrA* mutant. Type b encapsulated *H. influenzae* (C54 b+) and an isogenic capsule mutant (C54 b-) are included as controls.

Direct detection of *K. kingae* surface capsule was identified by cell staining. Strain 269-492 and 269-492*ctrA* mutant bacteria were first stained with cationic ferritin, which has been shown previously to bind bacterial capsules due to the highly acid nature of the various polysaccharides. The stained bacteria were then processed for thin section

TEM analysis. Strain 269-492 was found to have a thick layer of stained capsule on the bacterial surface, while the *ctrA* mutant lacks this distinctive staining.

As shown in Fig. 5, *K. kingae* capsule contains sialic acid. Polysaccharide was extracted from the bacterial surface with heat as described in Fig. 4 and hydrolyzed with sulfuric acid. The hydrolyzed capsule was subsequently reacted with thiobarbituric acid (TBA), which has been previously shown to react specifically with sialic acid. The chromogenic reaction was then quantitated by reading the absorbance at 549nm. The *H. influenzae* type b capsule was included as a negative control, as this capsule does not contain sialic acid.

3. Capsule blocks Knh-mediated adherence. To determine the individual contributions of these three *K. kingae* surface factors (*knh*, capsule and type IV pili), single, double, and triple mutants were generated and assayed for adherence to Chang cells. Disruption of capsule export in a *pilA1* mutant restored adherence to wild-type levels. Further disruption of *knh* in the *pilA1/ctrA* mutant background eliminated adherence. These data indicate that *K. kingae* expresses a capsular polysaccharide that contains sialic acid and is capable of blocking Knh-mediated adherence in the absence of type IV pili. The presence of functional type IV pili are able to overcome the inhibitory phenotype of capsule.

Conclusions: *K. kingae* type IV pili and a novel trimeric autotransporter, Knh, are necessary for full level adherence to respiratory epithelial and synovial cells. The *K. kingae* genome contains genes with homology to those involved in capsular polysaccharide expression in *N. meningitidis*. *K. kingae* expresses a sialic acid-containing capsular polysaccharide that can be visualized on the bacterial surface as well as extracted from the surface. The capsule interferes with Knh-mediated adherence in the absence of type IV pili. Pili are able to overcome the adherence-blocking phenotype, presumably by extending beyond the capsule. Disruption of *knh* in the adherent *pilA1/ctrA* double mutant background eliminates adherence, suggesting that Knh is an adhesin.

Variations and modifications of the herein described systems, apparatuses, methods and other applications will undoubtedly suggest themselves to those skilled in the art. Accordingly, the foregoing description should be taken as illustrative and not in a

limiting sense.

EXAMPLE 2

Capsule purification and composition analysis.

5 *Kingella kingae* strain 269-492 was cultured on chocolate agar for 18 hours at 37°C with 5% CO₂, and bacterial growth was suspended in 1ml of brain-heart infusion broth. Using a sterile swab, the suspension was spread over the surface of 10 x 10cm chocolate agar plates. After overnight incubation under the same culture conditions, the bacterial growth was scraped from the agar surface with a sterile swab and suspended in 10 80mL 1x phosphate buffered saline pH 7.4 (PBS) with 1% formaldehyde. The bacterial suspension was centrifuged at 2500 x g, and the resulting pellet was resuspended in 80mL 50mM Tris-acetate pH 5.0 and shaken for 30 min to release the polysaccharide from the bacterial surface. Bacteria were removed by centrifugation at 10,000x g for 20 min, and the supernatant was filtered through a 0.22 µm pore filter. The 80mL volume 15 was concentrated to 2mL using Amicon 100,000 molecular weight cut-off (MWCO) concentrator tubes.

Following concentration, 220µl 10x Roche Rapid DNase buffer, 5ug Roche Rapid DNase, and 5ug Fermentas RNase A were added to the concentrated extract to digest any contaminating DNA or RNA. After overnight incubation at 37°C, 10µg of 20 Roche Proteinase K was added and incubated at 55°C for 2hrs followed by 4hrs at 37°C to digest away any contaminating protein in addition to the DNase and RNase. The sample was extracted with an equal volume Tris-saturated phenol followed by an equal volume chloroform to separate the polysaccharide from the Proteinase K and any remaining protein. The sample was then dialyzed in H₂O in 4 x 5L volumes over a 25 period of 24hrs and subsequently concentrated to a final 1mL volume. The sample was flash frozen in a dry ice-ethanol bath and lyophilized. An aliquot of the lyophilized polysaccharide extract was sent to the University of Georgia Complex Carbohydrate Research Center (CCRC), Athens, GA, for composition analysis using the following protocol:

30

300ug of sample was placed into a test tube. 20ug inositol was then added as the internal standard. The sample was then frozen and lyophilized. Methyl glycosides were then prepared from the dry sample by methanolysis in 1 M HCl in methanol at 80°C (17 hours), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for
5 detection of amino sugars). The sample was then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 hours). These procedures were carried out as previously described in Merkle and Poppe (1994) *Methods Enzymol.* 230: 1-15; York, et al. (1985) *Methods Enzymol.* 118:3-40. GC/MS analysis of the TMS methyl glycosides was performed on an Agilent 6890N GC interfaced to a 5975B MSD, using a Supelco
10 EC-1 fused silica capillary column (30m × 0.25 mm ID).

The composition analysis revealed approximately equimolar concentrations of galactose (27.1%), N-Acetyl galactosamine (28.5%), and ketodeoxyoctonate (KDO) (28.8%), with small relative abundances of heptose, xylose, glucose, and N-Acetyl glucosamine (all <5%). We plan to use the CCRC in the future for linkage and NMR
15 analysis to identify the order and linkages of the sugar units in the capsular polysaccharide.

EXAMPLE 3

Generation of antiserum against *K. kingae* capsular polysaccharide.

20 Experiments are currently underway to produce guinea pig and rabbit antiserum to the *K. kingae* polysaccharide using a polysaccharide-carrier protein conjugate as the antigen. In initial experiments, we plan to conjugate the purified polysaccharide described above to ovalbumin using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) chemistry. Briefly, 1mg CDAP dissolved in 100μL
25 acetonitrile will slowly be added to 1mg purified polysaccharide dissolved in 100μL H₂O. After 30sec at 25°C, 100μl of aqueous 0.2M triethylamine was added followed by 1mg ovalbumin dissolved in 100μL 0.25M HEPES buffer pH 8.2 and incubated at 25°C for 16hrs. The polysaccharide-protein conjugate will then be buffer exchanged with 0.9% NaCl using a 100,000 MWCO concentration filter which will also remove
30 unconjugated ovalbumin. The resulting conjugate will be analyzed using Bradford assay and SDS-PAGE followed by Coomassie blue staining to confirm the presence of protein

in the high molecular mass range. SDS-PAGE followed by alcian blue staining will also be used to confirm the presence of high molecular mass polysaccharide.

Once the conjugate is in-hand, we will ship it to Cocalico Biologicals Inc., Reamstown, PA, for injection into a guinea pig and rabbit following a standard antiserum production protocol. Serum from the conjugate-injected animals will be examined for the presence of antibody reactivity to purified polysaccharide and whole *K. kingae* organisms. If this initial attempt to generate antiserum is unsuccessful, we plan to repeat the conjugation procedure with the Diphtheria toxin mutant CRM197 (Phenex Inc., San Diego, CA), a well characterized carrier protein currently in use in human vaccines, in place of ovalbumin as carrier protein to achieve a stronger antibody response to the conjugate. Future experiments will utilize the polysaccharide conjugates developed as described above as potential immunogens once an animal model of *K. kingae* disease is established.

To test the viability of *K. kingae* polysaccharide as a protective antigen, an animal model of *K. kingae* disease will be developed. Initial experiments were undertaken to develop a murine model of *K. kingae* septic arthritis based on the methods developed for *Staphylococcus aureus* that recapitulates much of the human septic arthritis pathology in the mouse. The following 5 strains were chosen: Swiss Webster, Balb/c, C3H/HeJ, C3H/HeN, and C57BL. In pilot experiments, 10^{10} CFU *K. kingae* strain 269-492 was inoculated into 4 mice per strain via the lateral tail vein. The mice were followed for a total of 30 days and were examined daily for days 1-14 and every other day for days 15-30. Mice were examined visually for signs of illness, including ruffling of coat and weight loss and characteristic symptoms of septic arthritis, including swelling and redness of joints and pain on weight bearing. Mice that developed symptoms of septic arthritis were subjected to necropsy and joint tissue procurement for histology studies. Swiss Webster and Balb/c mice developed an abnormal gait, dragging their hind limbs, suggesting bone or joint involvement. Histopathologic analysis of the hind limbs revealed a modest neutrophilic infiltrate in the hips, without over purulent fluid.

Future studies that expand on our pilot experiments are in the planning stage for development of a suitable animal model of *K. kingae* disease. We will pursue two different avenues. First, the Swiss Webster and Balb/c mouse strains that showed signs

of disease will be inoculated via the interperitoneal route instead of the tail vein, with the reasoning that route of infection may influence the ability of *K. kingae* to cause disease and result in more fulminant infection. Along similar lines, B-cell deficient, neutrophil deficient, and complement deficient will be examined, with the idea that

5 immunodeficient mice may develop more overt *K. kingae* disease. Second, we will explore the possibility that neonatal rats to determine if they are a better host species for a *K. kingae* infection. Using the animal model, we will explore the ability of active immunization with purified *K. kingae* capsule and passive immunization with antiserum against the *K. kingae* capsule to protect against challenge with *K. kingae*.

EXAMPLE 4

Elucidating the adhesive properties of the Knh trimeric autotransporter

Research Design

Analysis of Knh using the daTAA tool available from the Max-Planck Institute
15 reveals the characteristic C terminus of trimeric autotransporters, a series of 19 YadA-like head (Ylhead) domains with similarity to the β -roll that has been implicated in the adhesive activity of the Yersinia YadA trimeric autotransporter, and a series of 7 Trp ring domains with similarity to the Trp ring domains that have been implicated in adhesive activity of the 2 binding domains in the *H. influenzae* Hia and Hsf trimeric
20 autotransporters when present adjacent to an iSneck domain (see Fig. 2). We anticipate that Knh has adhesive activity and higher affinity for respiratory epithelial cells and synovial cells than type IV pili, explaining the observation that Knh is essential for full-level adherence and mediates a higher level of adherence than do pili. To explore this hypothesis, we will: 1) examine the ability of Knh to bind directly to respiratory
25 epithelial cells and synovial cells; 2) localize the Knh binding domain(s); and 3) determine the binding affinity of the Knh binding domain(s).

To examine the ability of Knh to bind directly to respiratory epithelial and synovial cells and establish that Knh functions directly as an adhesin, we will express the knh gene in a nonadherent host strain and assess the ability to confer capacity for
30 adherence to Chang respiratory epithelial cells and SW982 synovial cells. Initially we will introduce knh into *E. coli* DH5 α , a nonadherent laboratory strain. We have had

success in expressing the *H. influenzae* Hia and Hsf trimeric autotransporters in DH5 α but have found that the *Haemophilus* cryptic genospecies Cha trimeric autotransporter is not stably localized to the outer membrane in DH5 α . If necessary, we will consider alternative nonadherent laboratory host strains, including other laboratory *E. coli* strains, *H. influenzae* strain DB117, *Kingella oralis* ATCC 51147, and *Kingella denitrificans* ATCC 33394.

To complement these experiments using whole bacteria, we will generate a GST fusion protein containing the full-length passenger domain (amino acids 56-1694) using the plasmid pGEX-6P-1. Ultimately we will examine the purified GST-Knh56-1694 protein for the ability to bind directly to Chang and SW982 cells as assessed by immunofluorescence microscopy using an anti-GST antibody and a Cy2-conjugated secondary antibody. As a positive control, we will use purified GST-HiaBD1, a GST fusion protein that contains the high-affinity binding domain in Hia. As a negative control, we will use purified GST-Hia221-698, a GST fusion protein that contains a structural fragment of Hia and lacks adhesive activity. In addition, we will examine the ability of GST-Knh56-1694 or Knh56-1694 by itself after cleavage of the GST moiety using PreScission Protease to block bacterial adherence by *K. kingae* strain 269-492ctrA/pilA1 (expressing Knh and capable of high level pilus-independent adherence). Given our preliminary data demonstrating that insertional inactivation of knh results in reduced *K. kingae* adherence, that Knh is localized to the outer membrane and migrates as an oligomer, and that Knh has predicted structural domains characteristic of a trimeric autotransporter, we anticipate that Knh will have direct adhesive activity.

In an effort to localize the Knh binding domain, we will generate a series of GST fusion proteins corresponding to fragments of the passenger domain, an approach that we have used successfully to characterize the *H. influenzae* Hia, Hsf, and Hap autotransporters. One possibility is that the Knh binding domain resides at the N-terminus in the region containing some or all of the 19 Ylhead domains (residues 85-485), similar to observations with the *Yersinia* YadA trimeric autotransporter.

Alternatively, it is possible that the Knh binding domain resides in a region containing Trp ring domains (residues 567-746 correspond to two Trp ring domains; residues 834-1263 correspond to one KG domain and three Trp ring domains; residues

1310-1405 correspond to one KG domain and one Trp ring domain; residues 1531-1633 correspond to one KG domain and one Trp ring domain), similar to observations with the *H. influenzae* Hia and Hsf proteins, which also require an adjacent iSneck domain. It is also possible that Knh contains multiple binding domains in different regions of the passenger domain, corresponding to the N-terminal region containing the 19 Ylhead domains and the C-terminal region containing the 7 Trp ring domains, potentially representing a new model for multivalent binding. GST fusion proteins will be purified and examined for the ability to bind directly to Chang and SW982 cells as assessed by immunofluorescence microscopy using an anti-GST antibody and a Cy2-conjugated secondary antibody. In addition, we will examine the ability of the GST fusion proteins to block bacterial adherence by *K. kingae* strain 269-492ctrA/pilA1 (expressing Knh and capable of high level pilus-independent adherence). In the event that we encounter difficulties generating GST fusion proteins, we will consider 6xHis tagged proteins or MBP fusion proteins.

In related experiments, we will delete fragments from the knh gene and will examine the resulting constructs in our nonadherent laboratory host strain (*E. coli*, *H. influenzae*, *K. oralis*, or *K. denitrificans*) or in *K. kingae* strain 269-492ctrA/pilA1, aiming to confirm that deletion of the region implicated in adherence with GST fusion proteins eliminates adhesive activity. In order to make derivatives in *K. kingae* strain 269-492ctrA/pilA1, we will generate a plasmid that contains the knh gene and upstream and downstream flanking sequence, including a tetracycline cassette in the upstream sequence. Following deletion of the relevant sequence, the modified gene will be recombined into the chromosome of strain 269-492ctrA/pilA1 using the tetracycline cassette as a selectable marker and sequencing transformants to confirm the intended sequence.

We anticipate that Knh may have multiple binding domains, corresponding to the series of Ylhead domains and/or the series of Trp ring domains. To determine the binding affinity of the Knh binding domain(s), we will use a cell-based ELISA employing Chang respiratory epithelial cells and SW982 synovial cells. The region of Knh that is implicated in adherence will be purified as a GST fusion protein and will be added to monolayers. Monolayers will be washed with PBS, incubated with anti-GST

antibody, and then incubated with a horseradish peroxidase conjugated secondary antibody, and protein binding will be detected using ABTS. Equilibrium dissociation constants (K_d values) will be determined using GraphPad PRISM software. If there are multiple Knh binding domains, we will perform studies of all the binding domains. We
5 anticipate that the K_d will be lower for the Knh binding domain(s) than for PilC1 or PilC2, indicating a higher affinity.

Claims:

1. A pharmaceutical composition comprising
a purified *Kingella* capsular polysaccharide; and
5 a pharmaceutically acceptable carrier.
2. The composition of claim 1 wherein the capsular polysaccharide is conjugated to
an immunogenic carrier protein.
- 10 3. The composition of claim 2 further comprising a *Kingella* NhhA homolog
peptide.
4. The composition of claim 3 wherein the *Kingella* NhhA homolog peptide
comprises a 10 amino acid sequence fragment of a peptide selected from the group
15 consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID
NO: 6, SEQ ID NO: 7.
5. The composition of claim 3 wherein the *Kingella* NhhA homolog peptide
comprises a 15 amino acid sequence fragment of a peptide selected from the group
20 consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID
NO: 7, or a 15 amino acid sequence fragment that has at least 90% sequence identity with
a 15 amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID
NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.
- 25 6. The composition of claim 3 further comprising a *Kingella* pili peptide.
7. The composition of claim 2 wherein the immunogenic carrier protein is selected
from the group consisting of Diphtheria toxoids, Tetanus toxoids and Keyhole Limpet
Haemocyanin.

8. The composition of claim 3 wherein the *Kingella* NhhA homolog peptide is conjugated to the capsular polysaccharide.

9. A pharmaceutical composition comprising
5 a *Kingella* NhhA homolog peptide; and
a pharmaceutically acceptable carrier.

10. The composition of claim 9 wherein the *Kingella* NhhA homolog peptide comprises a 10 amino acid sequence fragment of a peptide selected from the group
10 consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

11. The composition of claim 9 wherein the *Kingella* NhhA homolog peptide comprises a 15 amino acid sequence fragment of a peptide selected from the group
15 consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or a 15 amino acid sequence fragment that has at least 90% sequence identity with a 15 amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

20 12. The composition of claim 9 wherein the *Kingella* NhhA homolog peptide comprises a 15 amino acid sequence fragment of SEQ ID NO: 2.

13. The composition of claim 9 further comprising an adjuvant.

25 14. The composition of claim 10 further comprising a purified compound, or fragment thereof, selected from the group consisting of *Kingella* capsular polysaccharide and *Kingella* type IV pilus peptide.

15. The composition of claim 11 wherein the composition further comprises
30 a *Kingella* capsular polysaccharide; and
a *Kingella* type IV pilus peptide.

16. A composition comprising purified antibodies specific for *Kingella* NhhA homolog protein or a *Kingella* capsular polysaccharide.

5 17. A method for inhibiting the adhesion of a gram negative bacterium to eukaryotic cells, wherein said bacterium expresses *Kingella* NhhA, said method comprising contacting said gram negative bacteria with a composition that interferes with *Kingella* NhhA homolog protein activity.

10 18. The method of claim 17 wherein the composition further comprises an antimicrobial agent.

19. The method of claim 17 wherein the method comprises the step of contacting said gram negative bacteria with antibodies specific for *Kingella* NhhA homolog protein.

15 20. A method of inducing an immune response in a subject, said method comprising administering to the subject an amount of the pharmaceutical composition of claim 2, thereby inducing an immune response.

20 21. The method of claim 20 wherein said composition further comprises a 10 amino acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

22. The method of claim 21 wherein the composition further comprises a *Kingella*
25 type IV pilus peptide.

23. A method of inducing an immune response in a subject, said method comprising administering to the subject an amount of the pharmaceutical composition of claim 9, thereby inducing an immune response.

24. A method for treating septic arthritis, osteomyelitis, and bacteremia, said method comprising administering an immunologically effective dose of a composition of claim 2.

25. The method of claim 24 wherein said composition further comprises a 10 amino
5 acid sequence fragment of a peptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

26. The method of claim 25 wherein the composition further comprises an
antimicrobial agent.

10

15

Fig. 1

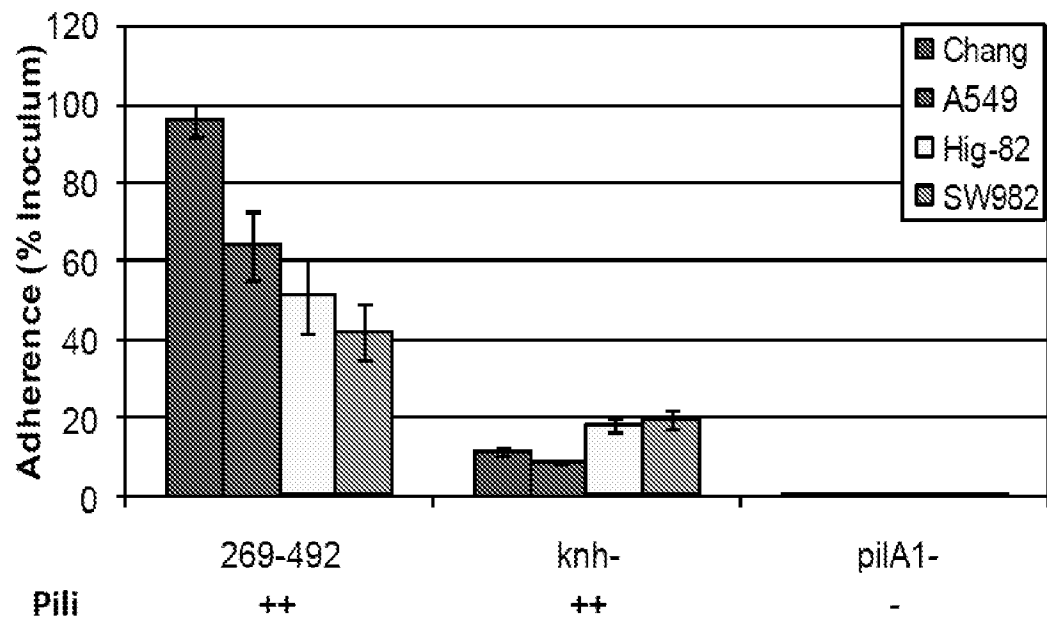


Fig. 2

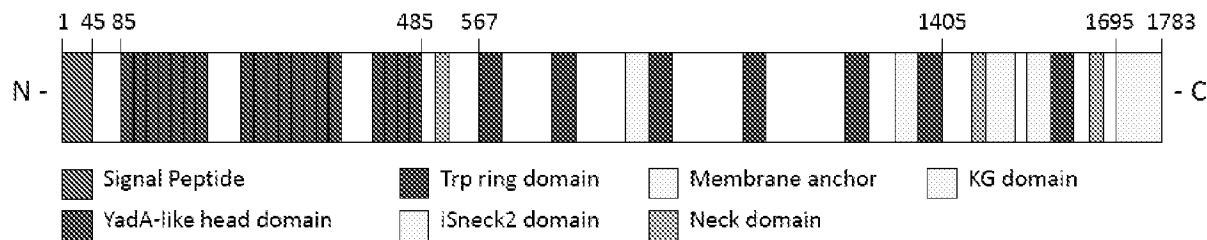


Fig. 3

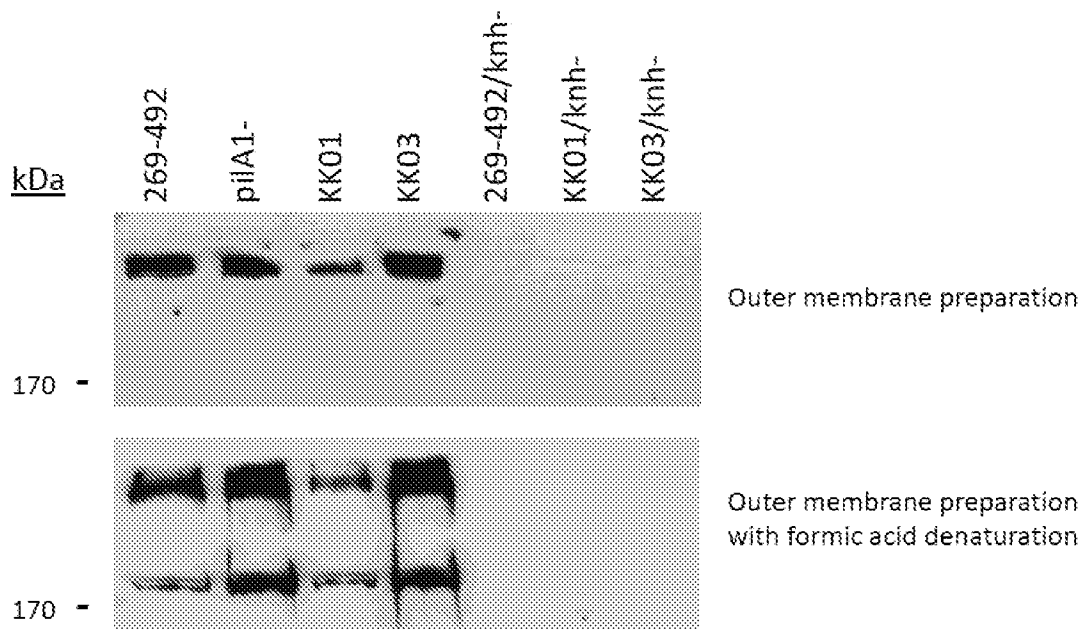


Fig 4

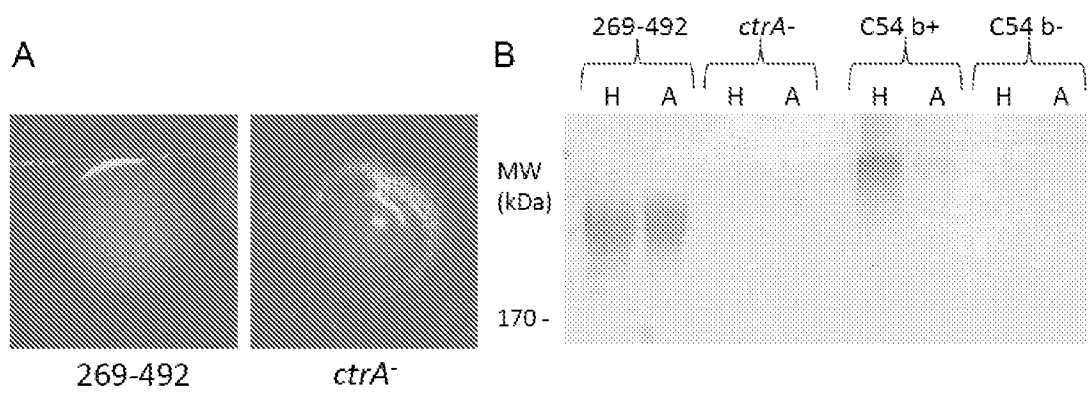


Fig. 5

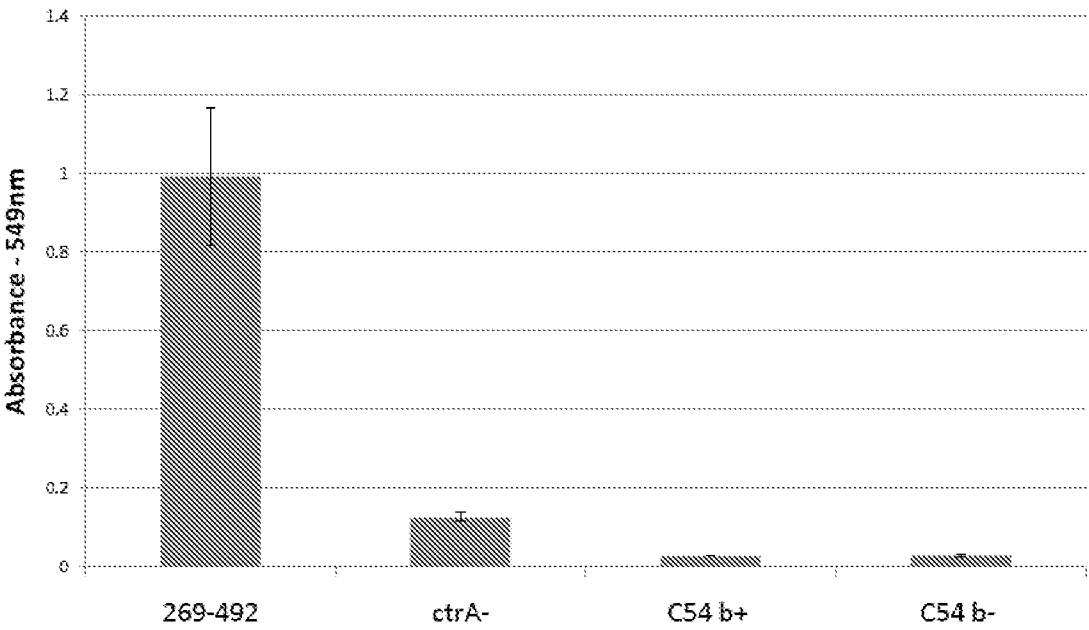
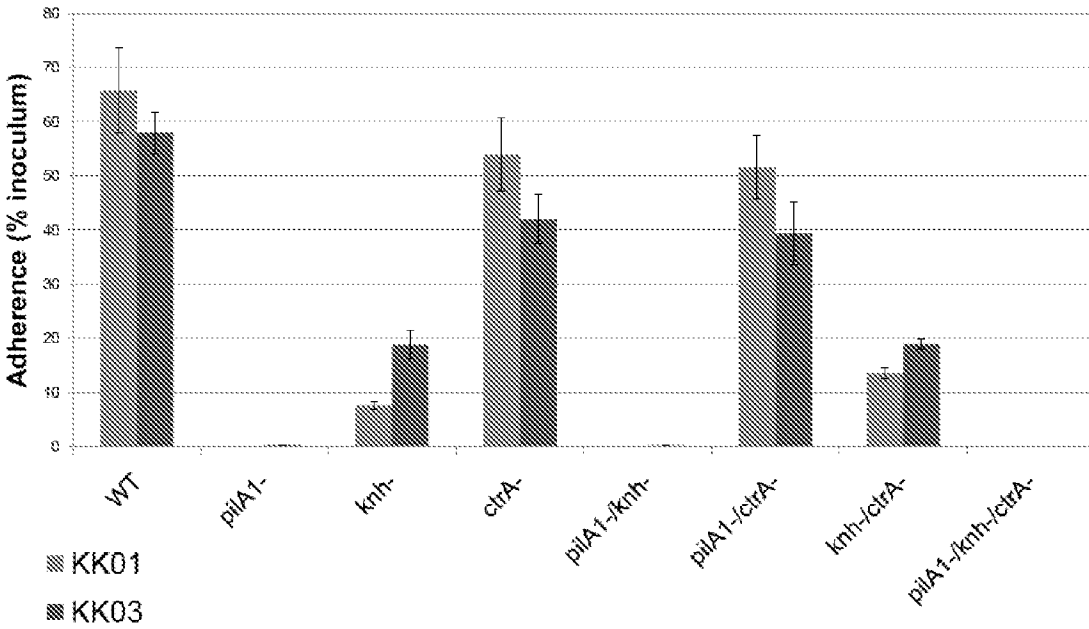


Fig. 6



INTERNATIONAL SEARCH REPORT

2011/057360 02-03-2012

International application No.

PCT/US 11/57360

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 39/02, 39/07 (2012.01) USPC - 424/190.1, 424/246.1 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) IPC(8) - A61K 39/02, 39/07 (2012.01) USPC - 424/190.1, 424/246.1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC - 424/184.1 (Text Search) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PubWEST (PGPB, USPT, USOC, EPAB, JPAB) and Google Scholar. Search Terms: kingella, vaccin\$, polysaccharide, LPS, antigens\$, Neisseria hia/hsf homologue, Nhha, Neisseria, carrier, capsul\$, pili, knh, adher\$, adhe\$, pilus, hsf, antibody, passive immunity, mening\$, hia.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	US 2006/0034854 A1 (BERTHET et al.) 16 February 2006 (16.02.2006) claim 64; para [0020], [0030], [0042], [0050], [0051], [0059], [0116], [0119], [0120], [0122]-[0124], [0133]	1-4, 6-10, 13-14, 20-26 ----- 5, 11-12, 15
Y --- A	US 2006/0257852 A1 (RAPPUOLI et al.) 16 November 2006 (16.11.2006) para [0727]	1-4, 6-8, 14, 16, 20-22, 24-26 ----- 5
Y	WEINSTOCK, G. et al. Uniprot Direct Submission Accession C4GMS0 [online]. 07 July 2009 [Retrieved on 22 February 2012]. Retrieved from the Internet: <URL: http://www.uniprot.org/uniprot/C4GMS0.txt?version=1.2 >, pages 1-2	3-4, 6, 8-10, 13-14, 17-19, 21-23, 25 and 26 ----- 5, 11-12, 15
Y	KEHL-FIE, T.E. et al. Kingella kingae Expresses Type IV Pili That Mediate Adherence to Respiratory Epithelial and Synovial Cells. Journal of Bacteriology. November 2008, Vol 190(21), pages 7157-7163: abstract; pg 7163, col 1, para 2	6, 17-19, 22 and 24-26
Y	US 2002/0197260 A1 (GRANOFF et al.) 26 December 2002 (26.12.2002) claim 1; para [0114]	16
Y	US 2002/0160016 A1 (PEAK et al.) 31 October 2002 (31.10.2002) para [0260]-[0263]	17-19
A,P	MUZYNY, D. et al. Uniprot Direct Submission Accession F5S9C5 [online]. 27 July 2011 [retrieved on 22 February 2012]. Retrieved from the Internet: < http://www.uniprot.org/uniprot/F5S9C5.txt?version=1.2 >, pages 1-2.	5, 11-12 and 15
☐ Further documents are listed in the continuation of Box C. ☐		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 23 February 2012 (23.02.2012)		Date of mailing of the international search report 02 MAR 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774