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(54) **Titre : MODULATION DE POLYPEPTIDES BACTERIENS MAM DANS UNE MALADIE PATHOGENE**  
(54) **Title: MODULATING BACTERIAL MAM POLYPEPTIDES IN PATHOGENIC DISEASE**

(57) **Abrégé/Abstract:**

The disclosure relates to methods and compositions for preventing or inhibiting pathogenic bacterial infections in a subject caused by pathogenic bacteria expressing a multivalent adhesion molecule (MAM) polypeptide by administering to a subject a composition comprising a MAM polypeptide or a non-pathogenic bacterium expressing a MAM polypeptide, or a combination thereof.

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(54) **Title:** MODULATING BACTERIAL MAM POLYPEPTIDES IN PATHOGENIC DISEASE

(57) **Abstract:** The disclosure relates to methods and compositions for preventing or inhibiting pathogenic bacterial infections in a subject caused by pathogenic bacteria expressing a multivalent adhesion molecule (MAM) polypeptide by administering to a subject a composition comprising a MAM polypeptide or a non-pathogenic bacterium expressing a MAM polypeptide, or a combination thereof.



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**DESCRIPTION****MODULATING BACTERIAL MAM POLYPEPTIDES IN PATHOGENIC DISEASE**

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**BACKGROUND OF THE INVENTION**

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**1. Field of the Invention**

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The present invention relates to fields of microbiology and medicine. More particularly, the present invention relates to a bacterial protein designated as MAM and methods and compositions for exploiting its use in treating pathogenic bacterial infections.

**2. Description of Related Art**

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Bacterial pathogens possess a large repertoire of virulence factors that target and manipulate the host cellular machinery to enable infection. Delivery of effector proteins to the host cytosol by type III, type IV and type VI secretion systems as well as delivery of extracellular toxins is a common strategy used by bacterial pathogens to abrogate the host immune response and alter cellular pathways to the pathogen's advantage (Alouf, 2000; Galan, 2009). Since the secretion of effector and toxin proteins is contact-dependent, the bacteria need to establish tight binding to the host to successfully start an infection. If one could establish the existence of a common virulence factor across species that enables a wide range of pathogen to establish strong initial host binding, which is required for the activation and secretion of other virulence factors, it would be possible to design a broadly effective therapeutic strategy that focuses on inhibition of this factor.

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**SUMMARY OF THE INVENTION**

Thus, in accordance with the present invention, there is provided a method of preventing or inhibiting a pathogenic bacterial infection in a subject caused by a pathogenic bacterium expressing a MAM polypeptide, comprising administering to said subject a

composition comprising a non-pathogenic bacterium expressing a MAM polypeptide comprising at least 5 mce repeat regions. The subject may be a mammal, such as a human or a non-human mammal. The pathogenic bacterium may be a Gram-negative, such as selected from the group consisting of *Vibrio parahaemolyticus*, *Vibrio cholera*, *Yersinia*  
5 *pseutotuberculosis*, and enteropathogenic *Escherichia coli*. The composition may be administered more than once.

The non-pathogenic bacterium may express a native MAM polypeptide, or a MAM polypeptide heterologous to the bacterium. The non-pathogenic bacterium may be *E. coli* strain BL21 or *E. coli* strain HS. The non-pathogenic bacterium may comprise a gene  
10 encoding a MAM polypeptide in an extrachromosomal self-replicating vector. The non-pathogenic bacterium may comprise a gene encoding a MAM polypeptide integrated into a chromosome of said non-pathogenic bacterium. The MAM polypeptide of said non-pathogenic bacterium may comprise 5, 6 or 7 mce repeat regions, or more than 7 mce repeat regions.

The composition may further comprise a probiotic, and/or may be administered orally, and/or may be selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, pet food, a tablet, a liquid bacterial suspension, dried oral supplement and wet oral supplement. The composition may also be  
15 administered topically, such as in the case where a subject has suffered a burn injury. The topical composition may be formulated as a cream, a gel, a salve, an ointment or a powder. The topical composition may be comprised in or disposed on a wound dressing. The composition may be comprised in or disposed on a surgical mesh or implantable device.

In another embodiment, there is provided a method of preventing or inhibiting a pathogenic bacterial infection in a subject caused by a pathogenic bacterium expressing a MAM polypeptide, comprising administering to said subject a composition comprising a MAM peptide or protein comprising at least 5 mce repeat regions. The subject may be a mammal, such as a human or non-human mammal. The pathogenic bacterium may be Gram-negative, such as selected from the group consisting of *Vibrio parahaemolyticus*,  
25 *Vibrio cholera*, *Yersinia pseutotuberculosis*, and enteropathogenic *Escherichia coli*.

The composition may be delivered orally. The composition may be administered more than once. The composition may be administered topically, such as to a subject that has suffered a burn injury. The topical composition may be formulated as a cream, a gel, a salve,



an ointment or a powder. The composition may be comprised in or disposed on a wound dressing, surgical mesh or implantable device.

The MAM peptide or protein may comprise 5, 6 or 7 mce repeat regions, or more than 7 mce repeat regions. The MAM protein or peptide may be coupled to a particle or bead, such as a particle or bead comprised of a polymer, a metal, or a lipid. The particle or bead may in particular be made of polystyrene, latex, a metal oxide, or polylactic-coglycolic acid (PLGA). The particle or bead may be a microsphere, a liposome, an nanoparticle, or a quantum dot.

Also provided is a method for identifying or quantifying phosphatidic acid on a membrane comprising contacting a membrane with a peptide or polypeptide comprising MAM mce repeat region. The peptide may comprise 1, 2, 3, 4 or 5 mce repeat regions. The peptide or polypeptide may be labeled, such as with a fluorescent label, a chemiluminescent label, and enzyme label, a dye, a colorimetric label, a quantum dot, or a ligand. The membrane may be part of an intact cell, such as a fixed cell or a living cell. The living cell may have been treated with an agent, and may further have, prior to treatment with said agent, been contacted with a MAM peptide or polypeptide to identify or quantify phosphatidic acid on said cell's membrane.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions, modifications, additions and/or rearrangements.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIGS. 1A-H.** MAM7 is an outer membrane protein involved in host cell adhesion. (FIG. 1A) Classification of mce-containing proteins using PFAM (Finn *et al.*, 2009). Western Blotting of subcellular fractions of (FIG. 1B) *V. parahaemolyticus* POR2, (FIG. 1C) *E. coli* BL21 expressing MAM7-myc and (FIG. 1D) *E. coli* BL21 expressing MAM7 $\Delta$ N1-44-myc. (-) total lysate before induction, (+) total lysate after induction, sup: culture supernatant, OM: outer membrane, peri: periplasm, IM: inner membrane, cyto: cytoplasm. (FIG. 1E) Detection profile of MAM7 and MAM7 $\Delta$ N1-44 after papain degradation (FIG. 1F) Detection profile of N1-44-TEV-MAM7myc after TEV treatment. P: cell pellet, S: supernatant. (FIG. 1G) Attachment of *V. parahaemolyticus* POR2, POR2 $\Delta$ MAM7 and POR2 $\Delta$ MAM7 complemented with plasmid-borne MAM7 (pMAM7) to HeLa, RAW264.7, Caco-2 and 3T3 fibroblast cells. (FIG. 1H) Attachment of *E. coli* BL21 expressing MAM7 or MAM7 $\Delta$ N1-44.

**FIGS. 2A-F.** Impact of MAM7-mediated adhesion on the outcome of *V. parahaemolyticus* infection. Lysis of (FIG. 2A) 3T3 fibroblasts, (FIG. 2B) Caco-2 epithelial cells, (FIG. 2C) RAW264.7 macrophages or (FIG. 2D) HeLa epithelial cells with POR1 (●), POR1 $\Delta$ MAM7 (o) or POR1 $\Delta$ MAM7+pMAM7 (∇). Cytotoxicity of 3T3 fibroblasts after infection with POR1 (black bar), POR1 $\Delta$ MAM7 (white bar) or POR1 $\Delta$ MAM7+pMAM7 (gray bar) and treatment with (FIG. 2E) gentamycin or (FIG. 2F) TEV protease.

**FIGS. 3A-C.** MAM7 adhesin is an important virulence factor for *V. parahaemolyticus* pathogenicity in the nematode *C. elegans*. (FIG. 3A) Lethality assays were performed using the germline deficient *C. elegans* strain SS104 glp-4(bn2) fed on RIMD 2210633, POR1, POR1 $\Delta$ MAM7 or a POR1 $\Delta$ MAM7 complemented strain. Dead worms were scored and data were analyzed using the Kaplan-Meier method. Morphology of worms fed on (FIG. 3B) HB101 or (FIG. 3C) POR1 for 48 hours and pictured using Nomarski optics.

**FIGS. 4A-D. Relationship between mce domain number, strength of binding and competitiveness.** (FIG. 4A) Saturation binding experiments with cultivated HeLa cells and purified, Alexa-fluor-labeled MBP-MAM1, -MAM2, -MAM6 and -MAM7 proteins. Equilibrium dissociation constants (KD values) were determined as  $14.9 \pm 3.3$ ,  $4.4 \pm 0.8$ ,  $0.6 \pm 0.1$  and  $0.2 \pm 0.1$   $\mu\text{M}$  for MAM1, MAM2, MAM6 and MAM7, respectively. (FIG. 4B) Indirect determination of binding affinities using MAM proteins to block the cell surface prior to attachment of *E. coli* BL21 expressing MAM7. KD values were  $49.4 \pm 12.2$ ,  $36.0 \pm 7.3$ ,  $5.4 \pm 2.2$  and  $1.5 \pm 0.5$   $\mu\text{M}$  for MAM1, MAM2, MAM6 and MAM7, respectively. (FIG. 4C) HeLa cell attachment of BL21+MAM1, BL21+MAM6 and BL21+MAM7 $\Delta$ N1-44 compared to BL21+MAM7 are determined as competitive indices (C.I.). (FIG. 4D) *E. coli* BL21 expressing either MAM1, MAM6, MAM7 or MAM7 $\Delta$ N1-44 were analyzed for their ability to block POR1 attachment to host cells and thus POR1-mediated cytotoxicity.

**FIGS. 5A-G. MAM7 attaches to host cells via fibronectin- and phospholipid-interactions.** Pulldown of fibronectin from human plasma with (FIG. 5A) GST-tagged MAM7 or (FIG. 5B) GST-tag only. (FIG. 5C) Saturation binding experiment with fluorescently labeled MBP-MAM1 and MBP-MAM7 on immobilized fibronectin. MBP-MAM7 binds to fibronectin with an affinity of  $15 \pm 4$   $\mu\text{M}$ , while no binding was detected with MAM1. Lipid overlay assays with (FIG. 5D) MBP and (FIG. 5E) MBP-MAM7. (FIG. 5F) Key for lipid strip. (FIG. 5G) Liposome association assays with MBP-tag, MBP-MAM1 and MBPMAM7. Proteins were incubated with liposomes containing only phosphatidylcholine (lane 1) or containing mixtures of PC and increasing mol% of phosphatidic acid (PA), lanes 2-9). Supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE.

**FIGS. 6A-J. Importance of MAM7 in *V. parahaemolyticus*, *Y. pseudotuberculosis*, *V. cholerae* and EPEC infection.** HeLa cells were infected with (FIG. 6A) *V. parahaemolyticus* RIMD, (FIG. 6B) *V. parahaemolyticus* POR1, (FIG. 6C) *V. cholerae*, (FIG. 6D) *Y. pseudotuberculosis* or (FIG. 6E) EPEC. P: addition of pathogen, C: competition of pathogen with BL21-MAM7, C<sup>m</sup>: competition of pathogen with BL21-MAM7 $\Delta$ N1-44. (FIG. 6F) Attachment of BL21-MAM7 (left), *V. parahaemolyticus* MAM7 $\Delta$ TM, and MAM7 homologs from *Y. pseudotuberculosis* (Yp), *V. cholerae* (Vc) or EPEC to 3T3 fibroblasts. (FIG. 6G) Attachment of wild-type (WT),  $\Delta$ MAM7 ( $\Delta$ ) and complemented  $\Delta$ MAM7 (C) strains of Yp, Vc an EPEC

to 3T3 fibroblasts. (FIG. 6H) Cytotoxicity (LDH release) of Yp wild-type (WT),  $\Delta MAM7$  ( $\Delta$ ) and complemented  $\Delta MAM7$  (C) strains towards 3T3 fibroblasts over time. (FIG. 6I) Cytotoxicity of Vc wild-type (Vc WT),  $\Delta MAM7$  (vc  $\Delta MAM7$ ) and complemented  $\Delta MAM7$  (Vc  $\Delta MAM7 + MAM7$ ) strains towards 3T3 cells. (FIG. 6J) Pedestal formation of EPEC wild-type,  $\Delta MAM7$  (checkered) and complemented  $\Delta MAM7$  (striped) strains on 3T3 cells over time.

**FIGS. 7A-B.** (FIG. 7A) Mce-domain boundaries as predicted for *V. parahaemolyticus* MAM7 are indicated in very light grey (mce1), light grey (mce2), dark grey (mce3), very dark grey (mce4), grey (mce5), very light grey (mce6) and light grey (mce7), respectively. (FIG. 7B) Alignments of MAM7 mce domains from *V. parahaemolyticus* MAM7 and MAM7 homologs from *Vibrio parahaemolyticus* (GI:28898385), *Vibrio cholerae* (GI:15641510), *Yersinia pseudotuberculosis* (GI:170024018) and EPEC (*Escherichia coli* O127:H6, GI:215487047) were created using ClustalW.

**FIG. 8.** Phylogenetic relationship among MAM7 adhesins in selected Gram-negative species. Evolutionary distances were calculated using the neighbor joining method. Branch lengths are proportional to the evolutionary distance.

**FIG. 9.** Expression of MAM7 in *V. parahaemolyticus* strains used in this study. RNA was extracted from POR1, POR2 and derivative strains and either reverse-transcribed into cDNA (left) prior to PCR or directly used for PCR (right) with MAM7- (upper panels) or ribosomal protein 14-specific primers (rP14, positive control, lower panels). POR1, POR2 and complemented  $\Delta MAM7$  strains show expression of MAM7. No products were observed with RNA only controls.

**FIGS. 10A-B.** Analysis of attachment and TEV-induced cleavage of bacteria expressing N<sub>1-44</sub>-TEV-MAM7. (FIG. 10A) Attachment of POR2 and derivative strains to 3T3 fibroblasts in the presence or absence of TEV protease. (FIG. 10B) Subcellular fractionation of POR2 $\Delta MAM7$  expressing N<sub>1-44</sub>-TEV-MAM7-myc from a plasmid. Lys: total lysate, sup: culture supernatant, OM: outer membrane, peri: periplasm, IM: inner membrane, cytop: cytoplasm.

**FIGS. 11A-B.** Subcellular localization and adhesive properties of MAM mutants. (FIG. 11A) Myc-tagged MAM7, MAM6 and MAM1 were expressed in *E. coli* BL21 and bacteria were subjected to subcellular fractionation. All three constructs were exclusively detected in the outer membrane fraction after Western Blotting with myc-

specific antibody. (-): uninduced lysate, (+): induced lysate, sup: culture supernatant, OM: outer membrane, peri: periplasm, IM: inner membrane, cyto: cytoplasm. (FIG. 11B) Bacterial attachment to HeLa cells was determined for *E. coli* BL21 expressing either MAM7 $\Delta$ N<sub>1-44</sub>, MAM7, MAM6 or MAM1.

5 **FIGS. 12A-C. Lipid-binding properties of MAM1.** Lipid overlay assays were carried out with (FIG. 12A) MBP only or (FIG. 12B) MBP-MAM1. Mce1, but not the tag alone binds phosphatidic acid (PA). (FIG. 12C) Key for lipid strip.

**FIGS. 13A-R. *E. coli* B21 expressing MAM7 protects against *V. parahaemolyticus*, *Y. pseudotuberculosis*, *V. cholerae*.** Phenotypes of (FIG. 13A) uninfected HeLa cells, (FIG. 13B) HeLa cells treated with BL21 or (FIG. 13C) BL21 expressing MAM7 as used for competition experiments shown in FIGS. 13D-R. (FIGS. 13D-R) HeLa cells with either no-preincubation (left panels), preincubation with BL21 expressing MAM7 $\Delta$ N<sub>1-44</sub> (middle) or MAM7 (right panels) were infected with (FIGS. 13D-F) *V. parahaemolyticus* RIMD 2210633, (FIGS. 13G-I) *V. parahaemolyticus* POR1, (FIGS. 13J-L) *V. cholerae* El Tor N16961, (FIGS. 13M-O) *Y. pseudotuberculosis* YP126 or (FIGS. 13P-R) EPEC O127:H6 E2348/69. Cells were stained with Hoechst DNA stain (blue) and rhodamine-phalloidin actin stain (red). Scalebar, 20  $\mu$ m.

**FIGS. 14A-D. Inhibition of *Pseudomonas aeruginosa*-mediated cytotoxicity using bead-immobilized MAM7.** Hela epithelial cells (80% confluency) were pre-incubated with bead-immobilized GST (FIG. 14A) or GST-MAM7 (FIG. 14B) for 30 minutes prior to infection with *P. aeruginosa* strain PAO1 at a multiplicity of infection of 20 for four hours. Cells were fixed and stained for DNA (dark grey) and actin (grey). Attached beads, light grey. Scale bar, 20  $\mu$ m. Attached beads per cell were determined by counting (FIG. 14C, black) and cytotoxicity was determined by measuring LDH released into the culture medium (FIG. 14D, dark grey). Error bars indicate s.e.m. (n $\geq$ 9). Cloning of expression constructs for GST and GST-MAM7 fusion protein as well as protein purifications have been described above. Purified proteins were immobilized on 1  $\mu$ m fluorescent orange latex beads (Sigma) as described by El Shazly *et al.* 2007). For inhibition experiments, a total amount of 7.5  $\mu$ g protein/10<sup>6</sup> beads/well in PBS were used.

**FIGS. 15A-D. MAM7 binding to fibronectin is required for rapid attachment to host cells.** Attachment of bacteria expressing MAM7 (BL21-MAM7, FIGS. 15A and 15C) or Alexa488-labeled MAM7 protein or labeled MBP control (FIGS. 15B and 15D) to

host cells treated with  $\alpha$ -fibronectin antibody (FIG. 15A), trypsin (FIG. 15B) or phospholipase C (FIGS. 15C and 15D).

**FIGS. 16A-F. The N-terminal 30 kDa fragment of fibronectin is sufficient for MAM7**

**binding.** (FIG. 16A) Fibronectin subunit (220 kDa) consisting of type I, type II and type III repeats. Each subunit contains an N-terminal region (I<sub>1-5</sub>, 30 kDa fragment) required for fibrin- and heparin binding (HBD), followed by a 45 kDa collagen binding domain (CBD, I<sub>6</sub>, II<sub>1-2</sub>, I<sub>7-9</sub>). MAM7 contains an N-terminal transmembrane region, which was excluded from recombinant proteins, followed by 7 consecutive mce domains. GST-MAM7 and MAM1 constructs used for pull-downs are depicted. Pull-down assays with GST-MAM7 and intact Fn (FIG. 16B), Fn30kDa (FIG. 16C) or Fn45kDa (FIG. 16D) or GST-MAM1 and Fn30kDa (FIG. 16E). Heparin inhibits attachment of BL21-MAM7 to host cells (FIG. 16F). M7: BL21-MAM7, M7Δ: BL21-MAM7ΔTM.

**FIGS. 17A-E. A minimum of five mce domains is required for stable binding of**

**fibronectin to MAM.** Pull-down experiments were performed using intact fibronectin and GST-MAM1, -MAM2, -MAM4, -MAM5, -MAM6 or -MAM7. Load and eluate fractions were analyzed by SDS-PAGE and visualized by Coomassie Staining (FIG. 17A). Binding of labeled MBP-MAM1, -MAM2, -MAM6 and -MAM7 to Fn-coated plates was determined using fluorescence saturation binding assays (FIG. 17B). Pull-down assays with GST-tagged individual mce domains (mce1 to mce7) and fibronectin. Load and eluate fractions were analyzed by SDS-PAGE and visualized by Coomassie Staining (FIG. 17C). Pull-down assays with GST-tagged mce1-5, 2-6 and 3-7 proteins and fibronectin (FIG. 17D), or GST-tagged (mce1)<sub>3</sub>, (mce1)<sub>5</sub> and (mce1)<sub>7</sub> concatemers and fibronectin (FIG. 17E).

**FIGS. 18A-D. Analysis of phosphatidic acid binding by MAM7 mce domains.**

Liposome association assays with individual mce domains and liposomes prepared from PC and PA and containing increasing concentrations of PA as indicated (0-80 mol%). Supernatant and pellet fractions were analyzed by SDS-PAGE and visualized by Coomassie staining (data not shown). % bound protein were determined by densitometry of gels and used to compare the affinities of mce1-7 constructs (FIG. 18A). Densitometry of mce6 (weakest binding to PA) compared to mce2 (tightest binding) and three mce6 point mutants (FIG. 18B). Pull-down assay of liposomes containing 50mol% PA and 50mol% PC on immobilized GST-mce domains (FIG.

18C). Sequence alignment of mce2 and mce6 (FIG. 18D). Positions of point mutations in mce6 are shown in blue (S646K), dark purple (Q664K) and pink (H703R), respectively.

**FIGS. 19A-F. MAM7, fibronectin and phosphatidic acid form a tripartite complex.**

5 Pull-down assays using GST-MAM7 or -MAM1, fibronectin and liposomes (with or without PA). GST-MAM proteins were incubated with fibronectin followed by liposomes (FIGS. 19A, 19C) or liposomes followed by fibronectin (FIGS. 19B, 19D). Bound proteins were analyzed by SDS-PAGE and Coomassie staining (FIGS. 9A, 19B). Liposomes in flowthrough and eluate fractions were quantitated using a molybdophosphoric acid assay and expressed as fractions of loaded lipid (FIGS. 19C, 10 FIGS. 19D). Alexa 488-labeled MAM7 was tested for binding to Fn-coated plates in the presence of increasing concentrations of liposomes prepared from PC (●) or a mixture containing 20:80mol % PC:PA (○) (FIG. 19E). Samples incubated with the highest concentration of liposomes (arrow) were analyzed for bound liposomes using 15 the molybdophosphoric acid assay and compared to plate assays carried out with MBP control (FIG. 19F).

**FIGS. 20A-L. Host cell attachment and lysis by bacterial isolates.** Attachment (top) and cell lysis (LDH release), (middle) caused by five different isolates each of *A. baumannii* (FIGS. 20A, 20E), *P. aeruginosa* (FIGS. 20B, 20F), *K. pneumoniae* (FIGS. 20C, 20G) and *E. coli* (FIGS. 20D, 20H) tested on HeLa (blue) and 3T3 (red) 20 cells. Values given are means  $\pm$  standard error (n=3) from a representative experiment performed in triplicate. Correlation between attachment and cytotoxicity on HeLa (●) and 3T3 (○) cells across different isolates (FIGS. 20I-L).

**FIGS. 21A-D. Anti-adhesion treatment of HeLa cells infected with bacterial isolates.**

25 Cytotoxocity of *A. baumannii* (FIG. 21A), *P. aeruginosa* (FIG. 21B), *K. pneumoniae* (FIG. 21C) and *E. coli* (FIG. 21D) isolates was measured following infection of HeLa cells left untreated (black), BL21-MAM7 treated (light grey) or treated with bead-immobilized MAM7 (dark grey). Values given are means  $\pm$  standard error (n=3) from a representative experiment performed in triplicate. Data points marked by an asterisk 30 were chosen for visualization using confocal microscopy (FIGS. 23A-R).

**FIGS. 22A-D. Anti-adhesion treatment of 3T3 cells infected with bacterial isolates.**

Cytotoxocity of *A. baumannii* (FIG. 22A), *P. aeruginosa* (FIG. 22B), *K. pneumoniae* (FIG. 22C) and *E. coli* (FIG. 22D) isolates was measured following infection of 3T3 cells left untreated (black), BL21-MAM7 treated (light grey) or treated with bead-

immobilized MAM7 (dark grey). Values given are means  $\pm$  standard error (n=3) from a representative experiment performed in triplicate. Data points marked by an asterisk were chosen for visualization using confocal microscopy (FIGS. 24A-R).

**FIGS. 23A-R.** Visualization of anti-adhesion treatment of HeLa cells for representative examples from each group of bacterial isolate. HeLa cells were either left untreated (row 1), pre-incubated with BL21-MAM7 (row 2) or pre-treated with bead-immobilized MAM7 (row 3). Cells were then left uninfected (controls, FIGS. 23A, 23G, 23M) or infected with *A. baumannii* isolate #1 (FIGS. 23B, 23H, 23N), *P. aeruginosa* isolate #1 (FIGS. 23C, 23I, 23O), *P. aeruginosa* isolate #3 (FIGS. 23D, 23J, 23P), *K. pneumoniae* isolate #1 (FIGS. 23E, 23K, 23Q) or *E. coli* isolate #5 (FIGS. 23F, 23L, 23R) for four hours. Cells were stained for actin (phalloidin-Alexa488, light grey) and DNA (Hoechst stain). Fluorescent latex beads are shown in yellow. Scalebar, 20  $\mu$ m.

**FIGS. 24A-R.** Visualization of anti-adhesion treatment of 3T3 cells for representative examples from each group of bacterial isolate. 3T3 cells were either left untreated (row 1), pre-incubated with BL21-MAM7 (row 2) or pre-treated with bead-immobilized MAM7 (row 3). Cells were then left uninfected (controls, FIGS. 24A, 24G, 24M) or infected with *A. baumannii* isolate #1 (FIGS. 24B, 24H, 24N), *P. aeruginosa* isolate #1 (FIGS. 24C, 24I, 24O), *P. aeruginosa* isolate #3 (FIGS. 24D, 24J, 24P), *K. pneumoniae* isolate #1 (FIGS. 24E, 24K, 24Q) or *E. coli* isolate #5 (FIGS. 24F, 24L, 24R) for four hours. Cells were stained for actin (phalloidin-Alexa488, green) and DNA (Hoechst stain). Fluorescent latex beads are shown in yellow. Scalebar, 20  $\mu$ m.

## **DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

Using bioinformatics, the inventors discovered a predicted outer membrane molecule that the have designated multivalent adhesion molecule (MAM) that encodes a putative transmembrane motif followed by six (MAM6) or seven (MAM7) mammalian cell entry (mce) domains. Unexpectedly, they found that MAM6 or MAM7 is encoded in a wide range of Gram-negative animal pathogens, but not Gram-positive or plant pathogenic bacteria. In contrast, proteins containing a single mce domain are wide-spread. In *Mycobacterium ssp.* and some Gram-positive bacteria, such as *Rhodococcus ssp.* or *Streptomyces ssp.*, the mce domain occurs in conjunction with a second domain of unknown function (DUF3407)



(Arruda *et al.*, 1993; Chitale *et al.*, 2001). Proteins containing one mce domain and a C-terminal low complexity region are thought to represent an accessory component of ABC transporters occurring in algae, higher plants and bacteria.

The inventors have tested whether MAMs, which constitute a new class of predicted  
 5 outer membrane proteins from Gram-negative bacteria, are involved in cellular attachment. *V. parahaemolyticus*, a Gram-negative bacterium that occurs in marine and estuarine environments and can cause shellfish-borne food-poisoning, was used as the representative Gram-negative bacterium for analysis of MAM7s (Daniels *et al.*, 2000). As discussed below, MAM enables a wide range of Gram-negative pathogens to establish high affinity binding to  
 10 host cells during the early stage of infection by engaging both protein-protein and protein-lipid interactions with the host cell membrane. Exploiting this interaction, the inventors have demonstrated that non-pathogenic bacteria expressing MAM can protect host cells from pathogen-mediated cytotoxicity by preventing the binding of a range of Gram-negative pathogens to host cells. This work has been extended to the use of isolated recombinant  
 15 MAM proteins with positive results. These and other aspects of the invention are discussed further below.

## 1. MAM

The present invention relates to peptides and polypeptides of MAM. An exemplary  
 20 sequence for *V. parahaemolyticus* MAM7 is presented in SEQ ID NO:1.

### A. Polypeptides and Peptides

MAM polypeptides/peptides will comprise molecules of 90 to about 830 residues in length having the sequence of SEQ ID NO:1, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO:  
 25 21, or SEQ ID NO: 26. A particular preferred length may be less than 850 residues, less than 800 residues, less than 750 residues, less than 700 residues, less than 650 residues, less than 600 residues, less than 550 residues, less than 500 residues, less than 450 residues, less than 400 residues, less than 350 residues, less than 300 residues, 250 residues, less than 200 residues, less than 150 residues, less than 100 residues, less than 75 residues, or less than 50,  
 30 including all intervals in-between.

MAM polypeptides/peptides may also be defined by the number of mce domains. For example, MAM polypeptides/peptides may have more than 7 mce domains, such as 8, 9, 10, 11, 12, 13, 14, 15, 16 or 20 mce domains, including ranges of 8-10, 8-12, 8-14, 8-16, and 8-

20 domains. Alternatively, the number of domains may be 7 or fewer, including no more than 6, 5, 4, 3 or 2 mce domains.

The peptides may be generated synthetically or by recombinant techniques, and are purified according to known methods, such as precipitation (*e.g.*, ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

The peptides may be labeled using various molecules, such as fluorescent, chromogenic or colorimetric agents. The peptides may also be linked to other molecules. The links may be direct or through distinct linker molecules. The linker molecules in turn may be subject, *in vivo*, to cleavage, thereby releasing the agent from the peptide. Peptides may also be rendered multimeric by linking to larger, and possibly inert, carrier molecules.

## **B. Variants or Analogs of MAM**

### **i) Substitutional Variants**

It also is contemplated in the present invention that variants or analogs of MAM peptides may also inhibit bacterial infection. Polypeptide sequence variants of MAM, primarily making conservative amino acid substitutions to SEQ ID NO:1, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 26 may provide improved compositions.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The following is a discussion based upon changing of the amino acids of a peptide to create an equivalent, or even an improved, second-generation molecule. For example, certain

amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a peptide that defines that peptide's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a peptide with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences coding the peptide without appreciable loss of their biological utility or activity, as discussed below.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant peptide, which in turn defines the interaction of the peptide with other molecules.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a peptide with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101

states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-

0.5  $\pm$  1); alanine (-0.5); histidine \*-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide containing molecules that mimic elements of protein secondary structure (Johnson *et al*, 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used, in conjunction with the principles outline above, to engineer second generation molecules having many of the natural properties of MAM, but with altered and even improved characteristics.

## ii) Altered Amino Acids

The present invention may employ peptides that comprise modified, non-natural and/or unusual amino acids. A table of exemplary, but not limiting, modified, non-natural and/or unusual amino acids is provided herein below. Chemical synthesis may be employed to incorporated such amino acids into the peptides of interest.

**Table 1 – Modified, Non-Natural and Unusual Amino Acids**

<u>Abbr.</u>	<u>Amino Acid</u>	<u>Abbr.</u>	<u>Amino Acid</u>
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
BAad	3- Aminoadipic acid	Hyl	Hydroxylysine

**Table 1 – Modified, Non-Natural and Unusual Amino Acids**

<u>Abbr.</u>	<u>Amino Acid</u>	<u>Abbr.</u>	<u>Amino Acid</u>
BAla	beta-alanine, beta-Amino-propionic acid	AHyl	allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Aile	allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
BAib	3-Aminoisobutyric acid	Melle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

### iii) Mimetics

In addition to the variants discussed above, the present inventors also contemplate that structurally similar compounds may be formulated to mimic the key portions of peptide or polypeptides of the present invention. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and, hence, also are functional equivalents.

Certain mimetics that mimic elements of protein secondary and tertiary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and/or antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

Some successful applications of the peptide mimetic concept have focused on mimetics of  $\beta$ -turns within proteins, which are known to be highly antigenic. Likely  $\beta$ -turn

structure within a polypeptide can be predicted by computer-based algorithms, as discussed herein. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

5 Other approaches have focused on the use of small, multidisulfide-containing proteins as attractive structural templates for producing biologically active conformations that mimic the binding sites of large proteins (Vita *et al.*, 1998). A structural motif that appears to be evolutionarily conserved in certain toxins is small (30-40 amino acids), stable, and high permissive for mutation. This motif is composed of a beta sheet and an alpha helix bridged in  
10 the interior core by three disulfides.

Beta II turns have been mimicked successfully using cyclic L-pentapeptides and those with D-amino acids (Weisshoff *et al.*, 1999). Also, Johannesson *et al.* (1999) report on bicyclic tripeptides with reverse turn inducing properties.

Methods for generating specific structures have been disclosed in the art. For  
15 example, alpha-helix mimetics are disclosed in U.S. Patents 5,446,128; 5,710,245; 5,840,833; and 5,859,184. These structures render the peptide or protein more thermally stable, also increase resistance to proteolytic degradation. Six, seven, eleven, twelve, thirteen and fourteen membered ring structures are disclosed.

Methods for generating conformationally restricted beta turns and beta bulges are  
20 described, for example, in U.S. Patents 5,440,013; 5,618,914; and 5,670,155. Beta-turns permit changed side substituents without having changes in corresponding backbone conformation, and have appropriate termini for incorporation into peptides by standard synthesis procedures. Other types of mimetic turns include reverse and gamma turns. Reverse turn mimetics are disclosed in U.S. Patents 5,475,085 and 5,929,237, and gamma  
25 turn mimetics are described in U.S. Patents 5,672,681 and 5,674,976.

#### iv) Modifications

A useful modification for delivery of peptides and peptidomimetics is PEG-ylation. PEG-ylation is the process of covalent attachment of polyethylene glycol polymer chains to  
30 another molecule, normally a drug or therapeutic protein. PEG-ylation is routinely achieved by incubation of a reactive derivative of PEG with the target macromolecule. The covalent attachment of PEG to a drug or therapeutic protein can “mask” the agent from the host's immune system (reduced immunogenicity and antigenicity), increase the hydrodynamic size (size in solution) of the agent which prolongs its circulatory time by reducing renal clearance.

PEG-ylation can also provide water solubility to hydrophobic drugs and proteins. Exemplary PEG-ylation technologies are described in U.S. Patents 7,666,400, 7,610,156, 7,587,286, 6,552,170 and 6,420,339.

### 5           C.       Fusion Proteins

Another variant is a fusion protein. This molecule generally has all or a substantial portion of the original molecule, in this case a peptide comprising 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more MCE sequences, linked at the N- or C-terminus, to all or a portion of a second peptide or polypeptide, for example, linked at the N-terminal to glutathione S-transferase (GST), or linked at the C terminal to a myc tag.

### D.       Purification of Peptides Proteins

It may be desirable to purify MAMs, variants, peptide-mimics or analogs thereof. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term “purified protein or peptide” as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, “purified” will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term “substantially purified” is used, this designation will refer to a composition in which the protein or peptide forms the

major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a “-fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine



particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used

for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

### E. Peptide Synthesis

MAM-related peptides may be generated synthetically for use in various embodiments of the present invention. Because of their relatively small size, the peptides of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart & Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); Barany and Merrifield (1979).

Short peptide sequences, or libraries of overlapping peptides, usually from about 6 up to about 35 to 50 amino acids, which correspond to the selected regions described herein, can be readily synthesized and then screened in screening assays designed to identify reactive peptides. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

### 2. MAM Nucleic Acids

Important aspects of the present invention concern isolated DNA segments and recombinant vectors encoding MAM and portions thereof, the creation and use of recombinant host cells through the application of DNA technology, that express MAM or peptides thereof, and biologically functional equivalents thereof. Sequences for MAM nucleic acids include SEQ ID NO:1 and fragments thereof.

The present invention concerns DNA segments, isolatable from bacterial cells that are free from total genomic DNA and that encode a MAM polypeptide or peptide. As used herein, the term “DNA segment” refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding MAM refers to a DNA segment that contains wild-type, polymorphic or mutant MAM coding sequences yet is isolated away from, or purified free from, total mammalian genomic DNA. Included within the term “DNA segment” including DNA segments such recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

## 10           **A.     Homologs**

Certain aspects of the present invention concern isolated DNA segments and recombinant vectors incorporating DNA sequences that are homologous to a nucleic acid sequence encoding MAM and the portions thereof SEQ ID NO:1, which illustrates the MAM7 protein of *Vibrio parahaemolyticus* (GI:28898385). As used herein, the term “homologous” is defined as being substantially identical, sufficiently complementary, similar, or having a common ancestry or evolutionary origin to a MAM nucleic acid encoded by SEQ ID NO:1, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 26 or a fragment thereof. Homologs may be defined by percentage homology at either the protein or nucleic level. Alternatively, homologs are identified operationally by hybridization under various conditions.

MAM homologs were identified in *Shewanella oneidensis* (GI:24374148; SEQ ID NO:12), *Shewanella baltica* (GI:126174666; SEQ ID NO:13), *Azotobacter vinelandii* (GI:226946271; SEQ ID NO:14), *Pseudomonas aeruginosa* (GI:12698379; SEQ ID NO:15), *Photobacterium profundum* (GI:54309112; SEQ ID NO:16), *Aliivibrio salmonicida* (GI:209695044; SEQ ID NO:17), *Vibrio cholerae* (GI:15641510; SEQ ID NO:18), *Citrobacter koseri* (GI:157145397; SEQ ID NO:19), *Escherichia coli* O139:H28 (non-pathogenic) (GI:157157260; SEQ ID NO:20), *Escherichia coli* O127:H6 (pathogenic) (GI:215487047; SEQ ID NO:21), *Salmonella paratyphi* (GI:56413233; SEQ ID NO:22), *Salmonella typhimurium* (GI:16765190; SEQ ID NO:23), *Klebsiella pneumoniae* (GI:152970897; SEQ ID NO:24), *Yersinia bercovieri* (GI:238783417; SEQ ID NO:25), *Yersinia pseudotuberculosis* (GI:170024018; SEQ ID NO:26), *Serratia odorifera* (GI:270261529; SEQ ID NO:27), *Mannheimia haemolytica* (GI:254363206; SEQ ID NO:28), *Pasteurella (multocida) haemolytica* (GI:15602131; SEQ ID NO:29), *Haemophilus parasuis*

(GI:167854634; SEQ ID NO:30), and *Actinobacillus pleuropneumoniae* (GI:53729097; SEQ ID NO:31). The MAM homologs listed above are exemplary and are not limiting.

## B. Variants

5 In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a MAM, a peptide, peptide-mimic or a biologically functional equivalent of an entire MAM or an mce domain thereof. The term “biologically functional equivalent” is well understood in the art and is further defined in detail herein. Accordingly, protein or nucleic acid sequences that have about 70%,  
10 about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%, and any range derivable therein, such as, for example, about 70% to about 80%, and more particularly  
15 about 81% and about 90% or about 85% to about 99%; or even more particularly, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of, for example, SEQ ID NO:1, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 26. In particular embodiments, the biological activity of a MAM peptide, or a biologically functional equivalent, comprises binding to the MAM host cell receptor.

20 Another way of defining homology for nucleic acids is by hybridization conditions. For example, a nucleic acid will hybridize to sequences of greater or less homology based on the stringency of the hybridization conditions. For example, high stringency conditions may be exemplified by those including approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5  $\mu$ M MgCl<sub>2</sub>, at temperatures ranging from approximately 40 °C to about 72 °C.

25 It will also be understood that nucleic acid sequences may include those that encode additional residues, such as 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein, polypeptide or peptide activity where an amino acid sequence expression is concerned. The addition of terminal sequences  
30 particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

### 3. Bacterial Species

#### A. Pathogens

##### i. *Acinetobacter baumannii*

*Acinetobacter baumannii* is a Gram-negative bacterial pathogen that has rapidly emerged as a leading cause of infection world-wide. In fact, *A. baumannii* is now responsible for up to 20% of all intensive care unit infections in some regions of the world. This organism causes a range of diseases, with pneumonia being the most prevalent. As a result of its resistance to drug treatment, some estimates state the disease is killing tens of thousands of U.S. hospital patients each year.

*A. baumannii* forms opportunistic infections. There have been many reports of *A. baumannii* infections among American soldiers wounded in Iraq, earning it the nickname “Iraqibacter.” Multi-drug resistant *Acinetobacter baumannii* is abbreviated as MDRAB. Multidrug-resistant *Acinetobacter* is not a new phenomenon; it has always been inherently resistant to multiple antibiotics.

*Acinetobacter baumannii* is the most relevant human pathogen within the *Acinetobacter* genus. Most *A. baumannii* isolates are multiresistant, containing in their genome small, isolated islands of alien (meaning transmitted genetically from other organisms) DNA and other cytological and genetic material; this has led to more virulence. *Acinetobacter* have no flagellum; the name is Greek for “motionless.”

*Acinetobacter* enters into the body through open wounds, catheters, and breathing tubes. It usually infects those with compromised immune systems, such as the wounded, the elderly, children or those with immune diseases. Colonization poses no threat to people who aren't already ill, but colonized health care workers and hospital visitors can carry the bacteria into neighboring wards and other medical facilities. The number of nosocomial infections (hospital-acquired infections) caused by *A. baumannii* has increased in recent years; as have most other nosocomial pathogens (MRSA, VRSA, VRE, *etc.*).

The first military outbreaks of severe *A. baumannii* infections occurred in April, 2003 in American soldiers returning from Iraq. Early reports attributed the infections to the Iraqi soil. Later testing demonstrated widespread contamination of field hospitals, via transportation of personnel and equipment from previously contaminated European hospitals, as the most plausible vector.

Nosocomial *A. baumannii* bacteremia may cause severe clinical disease that is associated with an elevated mortality rate. This opportunistic pathogen expresses a myriad of

factors that could play a role in human pathogenesis. Among these factors are the attachment to and persistence on solid surfaces, the acquisition of essential nutrients such as iron, the adhesion to epithelial cells and their subsequent killing by apoptosis, and the production and/or secretion of enzymes and toxic products that damage host tissues. However, very little is known about the molecular nature of most of these processes and factors and almost nothing has been shown with regard to their role in bacterial virulence and the pathogenesis of serious infectious diseases. Fortunately, some of these gaps can now be filled by testing appropriate isogenic derivatives in relevant animal models that mimic the infections in humans, particularly the outcome of deadly pneumonia. Such an approach should provide new and relevant information on the virulence traits of this normally underestimated bacterial human pathogen.

Multidrug-resistant *A. baumannii* is a common problem in many hospitals in the U.S. and Europe. First line treatment is with a carbapenem antibiotic such as imipenem, but carbapenem resistance is increasingly common. Other treatment options include polymyxins, tigecycline and aminoglycosides. The institution of strict infection-control measures, such as monitored hand washing, can lower hospital infection rates. MDRAB infections are difficult and costly to treat. A study at a public teaching hospital found that the mean total hospital cost of patients who acquired MDRAB was \$98,575 higher than that of control patients who had identical burn severity of illness indices.

20

ii. *Staphylococcus aureus*

*Staphylococcus aureus* is a major human pathogen, causing a wide variety of illnesses ranging from mild skin and soft tissue infections and food poisoning to life-threatening illnesses such as deep post-surgical infections, septicaemia, endocarditis, necrotizing pneumonia, and toxic shock syndrome. These organisms have a remarkable ability to accumulate additional antibiotic resistance determinants, resulting in the formation of multiply-drug-resistant strains. Methicillin, being the first semi-synthetic penicillin to be developed, was introduced in 1959 to overcome the problem of penicillin-resistant *S. aureus* due to  $\beta$ -lactamase (penicillinase) production (Livermore, 2000). However, methicillin-resistant *S. aureus* (MRSA) strains were identified soon after the introduction of methicillin (Barber, 1961; Jevons, 1961).

Since their first identification, strains of MRSA have spread and become established as major nosocomial (hospital-acquired (HA)-MRSA) pathogens worldwide (Ayliffe, 1997;

Crossley *et al.*, 1979; Panlilio *et al.*, 1992; Voss *et al.*, 1994). Recently, these organisms have evolved and emerged as a major cause of community-acquired infections (CA-MRSA) in healthy individuals lacking traditional risk factors for infection, and are causing community-outbreaks, which pose a significant threat to public health (Begier *et al.*, 2004; Beilman *et al.*, 2005; Conly *et al.*, 2005; Gilbert *et al.*, 2006; Gilbert *et al.*, 2005; Harbarth *et al.*, 2005; Holmes *et al.*, 2005; Issartel *et al.*, 2005; Mulvey *et al.*, 2005; Robert *et al.*, 2005; Said-Salim *et al.*, 2005; Vandenesch *et al.*, 2003; Vourli *et al.*, 2005; Wannet *et al.*, 2005; Wannet *et al.*, 2004; Witte *et al.*, 2005; Wylie & Nowicki, 2005).

10                   iii.     *Acinetobacter spp.*

*Acinetobacter spp.* other than *A. baumannii* include *A. calcoaceticus*, *A. lwoffii*, *A. junii*, *A. anitratus*, *A. baumannii-calcoaceticus* complex. *Acinetobacter* is a Gram-negative genus of bacteria belonging to the *Gammaproteobacteria*. Non-motile, *Acinetobacter* species are oxidase-negative, and occur in pairs under magnification. They are important soil organisms where they contribute to the mineralisation of, for example, aromatic compounds. *Acinetobacter* are a key source of infection in debilitated patients in the hospital. Different species of bacteria in this genus can be identified using Fluorescence-Lactose-Denitrification medium (FLN) to find the amount of acid produced by metabolism of glucose.

Species of the genus *Acinetobacter* are strictly aerobic, nonfermentative, Gram-negative bacilli. They show preponderantly a coccobacillary morphology on nonselective agar. Rods predominate in fluid media, especially during early growth. The morphology of *Acinetobacter spp.* can be quite variable in Gram stained human clinical specimens, and cannot be used to differentiate *Acinetobacter* from other common causes of infection.

Most strains of *Acinetobacter*, except some of the *A. lwoffii* strains, grow well on MacConkey agar (without salt). Although officially classified as non-lactose fermenting, they are often partially lactose fermenting when grown on MacConkey agar. They are oxidase negative, nonmotile and usually nitrate negative.

*Acinetobacter* species are generally considered nonpathogenic to healthy individuals. However, several species persist in hospital environments and cause severe, life-threatening infections in compromised patients. The spectrum of antibiotic resistances of these organisms together with their survival capabilities make them a threat to hospitals as documented by recurring outbreaks both in highly developed countries and elsewhere. An important factor for their pathogenic potential is probably an efficient means of horizontal gene transfer even though such a mechanism has so far only been observed and analyzed in *Acinetobacter*

*baylyi*, a species that lives in the soil and has never been associated with infections. *Acinetobacter* is frequently isolated in nosocomial infections and is especially prevalent in intensive care units, where both sporadic cases as well as epidemic and endemic occurrence is common. *A. lwoffii* is responsible for most cases of *Acinetobacter meningitis*.

5        *Acinetobacter* species are innately resistant to many classes of antibiotics, including penicillin, chloramphenicol, and often aminoglycosides. Resistance to fluoroquinolones has been reported during therapy and this has also resulted in increased resistance to other drug classes mediated through active drug efflux. A dramatic increase in antibiotic resistance in *Acinetobacter* strains has been reported by the CDC and the carbapenems are recognized as  
10 the gold-standard and treatment of last resort. *Acinetobacter* species are unusual in that they are sensitive to sulbactam; sulbactam is most commonly used to inhibit bacterial beta-lactamase, but this is an example of the antibacterial property of sulbactam itself.

#### iv. *Pseudomonas aeruginosa*

15        *Pseudomonas aeruginosa* is a common bacterium which can cause disease in animals and humans. It is found in soil, water, skin flora and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and has thus colonised many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged  
20 tissues or people with reduced immunity. The symptoms of such infections are generalised inflammation and sepsis. If such colonisations occur in critical body organs such as the lungs, the urinary tract, and kidneys, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment including catheters, causing cross infections in hospitals and clinics. It is implicated in hot-tub rash.

25        It is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. An opportunistic human pathogen, *P. aeruginosa* is also an opportunistic pathogen of plants. *P. aeruginosa* is the type species of the genus *Pseudomonas* (Migula).

*P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green and fluorescent, now also known as pyoverdin), and pyorubin (red-  
30 brown). King, Ward, and Raney developed *Pseudomonas* Agar P (aka King A media) for enhancing pyocyanin and pyorubin production and *Pseudomonas* Agar F (aka King B media) for enhancing fluorescein production.

*P. aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like or tortilla-like odor *in vitro*. Definitive clinical identification of *P. aeruginosa*



often includes identifying the production of both pyocyanin and fluorescein, as well as its ability to grow at 42°C. *P. aeruginosa* is capable of growth in diesel and jet fuel, where it is known as a hydrocarbon-utilizing microorganism, causing microbial corrosion. It creates dark gellish mats sometimes improperly called “algae” because of their appearance.

5           Although classified as an aerobic organism, *P. aeruginosa* is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate-level phosphorylation. Adaptation to microaerobic or anaerobic environments is essential for  
10 certain lifestyles of *P. aeruginosa*, for example, during lung infection in cystic fibrosis patients, where thick layers of alginate surrounding bacterial mucoid cells can limit the diffusion of oxygen.

          The G+C-rich *Pseudomonas aeruginosa* chromosome consists of a conserved core and a variable accessory part. The core genomes of *P. aeruginosa* strains are largely  
15 collinear, exhibit a low rate of sequence polymorphism, and contain few loci of high sequence diversity, notably the pyoverdine locus, the flagellar regulon, pilA, and the O-antigen biosynthesis locus. Variable segments are scattered throughout the genome, of which about one-third are immediately adjacent to tRNA or tmRNA genes. The three known hot spots of genomic diversity are caused by the integration of genomic islands of the  
20 pKLC102/PAGI-2 family into tRNA<sup>Lys</sup> or tRNA<sup>Gly</sup> genes. The individual islands differ in their repertoire of metabolic genes, but share a set of syntenic genes which confer their horizontal spread to other clones and species. Colonization of atypical disease habitats predisposes to deletions, genome rearrangements, and accumulation of loss-of-function mutations in the *P. aeruginosa* chromosome. The *P. aeruginosa* population is characterized  
25 by a few dominant clones widespread in disease and environmental habitats. The genome is made up of clone-typical segments in core and accessory genome and of blocks in the core genome with unrestricted gene flow in the population.

          Cell-surface polysaccharides play diverse roles in the bacterial lifestyle. They serve as a barrier between the cell wall and the environment, mediate host-pathogen interactions, and  
30 form structural components of biofilms. These polysaccharides are synthesized from nucleotide-activated precursors, and, in most cases, all the enzymes necessary for biosynthesis, assembly, and transport of the completed polymer are encoded by genes organized in dedicated clusters within the genome of the organism. Lipopolysaccharide is one of the most important cell-surface polysaccharides, as it plays a key structural role in outer

membrane integrity, as well as being an important mediator of host-pathogen interactions. The genetics for the biosynthesis of the so-called A-band (homopolymeric) and B-band (heteropolymeric) O antigens have been clearly defined, and much progress has been made toward understanding the biochemical pathways of their biosynthesis. The exopolysaccharide alginate is a linear copolymer of  $\beta$ -1,4-linked D-mannuronic acid and L-glucuronic acid residues, and is responsible for the mucoid phenotype of late-stage cystic fibrosis disease. The *pel* and *psl* loci are two recently-discovered gene clusters which also encode exopolysaccharides found to be important for biofilm formation. Rhamnolipid is a biosurfactant whose production is tightly regulated at the transcriptional level, but the precise role that it plays in disease is not well understood at present. Protein glycosylation, particularly of pilin and flagellin, is a recent focus of research by several groups, and it has been shown to be important for adhesion and invasion during bacterial infection.

An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections.

It is the most common cause of infections of burn injuries and of the external ear (otitis externa), and is the most frequent colonizer of medical devices (e.g., catheters). *Pseudomonas* can, in rare circumstances, cause community-acquired pneumonias, as well as ventilator-associated pneumonias, being one of the most common agents isolated in several studies. Pyocyanin is a virulence factor of the bacteria and has been known to cause death in *C. elegans* by oxidative stress. However, research indicates that salicylic acid can inhibit pyocyanin production. One in ten hospital-acquired infections are from *Pseudomonas*. Cystic fibrosis patients are also predisposed to *P. aeruginosa* infection of the lungs. *P. aeruginosa* may also be a common cause of “hot-tub rash” (dermatitis), caused by lack of proper, periodic attention to water quality. The most common cause of burn infections is *P. aeruginosa*. *Pseudomonas* is also a common cause of post-operative infection in radial keratotomy surgery patients. The organism is also associated with the skin lesion ecthyma gangrenosum. *Pseudomonas aeruginosa* is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with *P. aeruginosa* via the foam padding found in tennis shoes.

Depending on the nature of infection, an appropriate specimen is collected and sent to a bacteriology laboratory for identification. First, a Gram stain is performed, which should show Gram negative rods with no particular arrangement. Then, if the specimen is pure, the organism is grown on MacConkey agar plate to produce colorless colonies (as it does not

ferment lactose); but, if the specimen is not pure, then the use of a selective plate is essential. Cetrimide agar has been traditionally used for this purpose. When grown on it, *P. aeruginosa* may express the exopigment pyocyanin, which is blue-green in color, and the colonies will appear flat, large, and oval. It also has a characteristic fruity smell. *P. aeruginosa* is  
5 catalase+, oxidase+, nitrase+, and lipase+. When grown on TSI medium, it has a K/K/g-/H<sub>2</sub>S-profile, meaning that the medium will not change color. Finally, serology could help, which is based on H & O antigens.

*P. aeruginosa* is frequently isolated from non-sterile sites (mouth swabs, sputum, and so forth), and, under these circumstances, it often represents colonisation and not infection.  
10 The isolation of *P. aeruginosa* from non-sterile specimens should, therefore, be interpreted cautiously, and the advice of a microbiologist or infectious diseases physician/pharmacist should be sought prior to starting treatment. Often no treatment is needed.

When *P. aeruginosa* is isolated from a sterile site (blood, bone, deep collections), it should be taken seriously, and almost always requires treatment.

15 *P. aeruginosa* is naturally resistant to a large range of antibiotics and may demonstrate additional resistance after unsuccessful treatment, particularly through modification of a porin. It should usually be possible to guide treatment according to laboratory sensitivities, rather than choosing an antibiotic empirically. If antibiotics are started empirically, then every effort should be made to obtain cultures, and the choice of  
20 antibiotic used should be reviewed when the culture results are available.

Antibiotics that have activity against *P. aeruginosa* include: aminoglycosides (gentamicin, amikacin, tobramycin); quinolones (ciprofloxacin, levofloxacin, and moxifloxacin); cephalosporins (ceftazidime, cefepime, cefoperazone, ceftazidime, but not cefuroxime, ceftriaxone, cefotaxime); ureidopenicillins and carboxypenicillins (piperacillin, ticarcillin; *P. aeruginosa* is intrinsically resistant to all other penicillins); carbapenems  
25 (meropenem, imipenem, doripenem, but not ertapenem); polymyxins (polymyxin B and colistin); and monobactams (aztreonam). These antibiotics must all be given by injection, with the exception of fluoroquinolones and of aerosolized tobramycin. For this reason, in some hospitals, fluoroquinolone use is severely restricted in order to avoid the development  
30 of resistant strains of *P. aeruginosa*. In the rare occasions where infection is superficial and limited (for example, ear infections or nail infections), topical gentamicin or colistin may be used. Phage therapy against ear infections caused by *Pseudomonas aeruginosa* was reported in the journal Clinical Otolaryngology in August 2009.

*Pseudomonas aeruginosa* is a highly relevant opportunistic pathogen. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility. This low susceptibility is attributable to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes (*e.g.*, *mexAB*, *mexXY*, *etc.*) and the low permeability of the bacterial cellular envelopes. In addition to this intrinsic resistance, *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally-encoded genes or by the horizontal gene transfer of antibiotic resistance determinants. Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic events including acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. Hypermutation favors the selection of mutation-driven antibiotic resistance in *P. aeruginosa* strains producing chronic infections, whereas the clustering of several different antibiotic resistance genes in integrons favors the concerted acquisition of antibiotic resistance determinants. Some recent studies have shown that phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants may be important in the response of *P. aeruginosa* populations to antibiotics treatment.

v. *Burkholderia spp.*

*Burkholderia spp.* (*B. cepacia*, *B. cenocepacia*, *B. cepacia* complex) are members of a genus of proteobacteria probably best-known for its pathogenic members *Burkholderia mallei* (responsible for glanders, a disease that occurs mostly in horses and related animals), *Burkholderia pseudomallei* (causative agent of melioidosis), and *Burkholderia cepacia* (an important pathogen of pulmonary infections in people with cystic fibrosis).

The *Burkholderia* (previously part of *Pseudomonas*) genus name refers to a group of virtually ubiquitous gram-negative, motile, obligately aerobic rod-shaped bacteria including both animal/human and plant pathogens as well as some environmentally-important species. In particular, *B. xenovorans* (previously named *Pseudomonas cepacia* then *B. cepacia* and *B. fungorum*) is renowned for its ability to degrade chlororganic pesticides and polychlorinated biphenyls (PCBs). Due to their antibiotic resistance and the high mortality rate from their associated diseases, *Burkholderia mallei* and *Burkholderia pseudomallei* are considered to be potential biological warfare agents, targeting livestock and humans.

vi. *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the

mouth, skin, and intestines. It is clinically the most important member of the *Klebsiella* genus of Enterobacteriaceae; it is closely related to *K. oxytoca* from which it is distinguished by being indole-negative and by its ability to grow on both melezitose and 3-hydroxybutyrate. It naturally occurs in the soil and about 30% of strains can fix nitrogen in anaerobic conditions.

5 As a free-living diazotroph, its nitrogen fixation system has been much studied.

Members of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first, O antigen, is a lipopolysaccharide of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for subtyping.

10 Research has implicated molecular mimicry between HLA-B27 and two molecules in *Klebsiella* microbes as the cause of ankylosing spondylitis. As a general rule, *Klebsiella* infections tend to occur in people with a weakened immune system from improper diet (alcoholics and diabetics). Many of these infections are obtained when a person is in the hospital for some other reason (a nosocomial infection). The most common infection caused  
15 by *Klebsiella* bacteria outside the hospital is pneumonia.

New antibiotic resistant strains of *K. pneumoniae* are appearing, and it is increasingly found as a nosocomial infection. *Klebsiella* ranks second to *E. coli* for urinary tract infections in older persons. It is also an opportunistic pathogen for patients with chronic pulmonary disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoscleroma. Feces  
20 are the most significant source of patient infection, followed by contact with contaminated instruments.

Multiply-resistant *Klebsiella pneumoniae* have been killed *in vivo* via intraperitoneal, intravenous or intranasal administration of phages in laboratory tests.

25                   vii. *Stenotrophomonas maltophilia*

*Stenotrophomonas maltophilia* is an aerobic, nonfermentative, Gram-negative bacterium. It is an uncommon bacteria and it is difficult to treat infections in humans. Initially classified as *Pseudomonas maltophilia*, *S. maltophilia* was also grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in  
30 1993.

*S. maltophilia* are slightly smaller ( $0.7\text{--}1.8 \times 0.4\text{--}0.7$  micrometers) than other members of the genus. They are motile due to polar flagella and grow well on MacConkey agar producing pigmented colonies. *S. maltophilia* are catalase-positive, oxidase-negative

(which distinguishes them from most other members of the genus) and have a positive reaction for extracellular DNase.

*S. maltophilia* is ubiquitous in aqueous environments, soil and plants, including water, urine, or respiratory secretions; it has also been used in biotechnology applications. In immunocompromised patients, *S. maltophilia* can lead to nosocomial infections.

*S. maltophilia* frequently colonizes breathing tubes such as endotracheal or tracheostomy tubes, the respiratory tract and indwelling urinary catheters. Infection is usually facilitated by the presence of prosthetic material (plastic or metal), and the most effective treatment is removal of the prosthetic material (usually a central venous catheter or similar device). The growth of *S. maltophilia* in microbiological cultures of respiratory or urinary specimens is therefore sometimes difficult to interpret and not a proof of infection. If, however, it is grown from sites which would be normally sterile (e.g., blood), then it usually represents true infection.

In immunocompetent individuals, *S. maltophilia* is a relatively unusual cause of pneumonia, urinary tract infection, or blood stream infection; in immunocompromised patients, however, *S. maltophilia* is a growing source of latent pulmonary infections. *S. maltophilia* colonization rates in individuals with cystic fibrosis have been increasing.

*S. maltophilia* is naturally resistant to many broad-spectrum antibiotics (including all carbapenems) and is thus often difficult to eradicate. Many strains of *S. maltophilia* are sensitive to co-trimoxazole and ticarcillin, though resistance has been increasing. It is not usually sensitive to piperacillin, and sensitivity to ceftazidime is variable.

#### viii. *Haemophilus influenzae*

*Haemophilus influenzae*, formerly called Pfeiffer's bacillus or *Bacillus influenzae*, is a non-motile Gram-negative rod-shaped bacterium first described in 1892 during an influenza pandemic. A member of the *Pasteurellaceae* family, it is generally aerobic, but can grow as a facultative anaerobe. *H. influenzae* was mistakenly considered to be the cause of influenza until 1933, when the viral etiology of the flu became apparent. Still, *H. influenzae* is responsible for a wide range of clinical diseases.

In 1930, 2 major categories of *H. influenzae* were defined: the unencapsulated strains and the encapsulated strains. Encapsulated strains were classified on the basis of their distinct capsular antigens. There are six generally recognized types of encapsulated *H. influenzae*: a, b, c, d, e, and f. Genetic diversity among unencapsulated strains is greater than within the encapsulated group. Unencapsulated strains are termed nontypable (NTHi) because they lack

capsular serotypes, however they can be classified by multi-locus sequence typing. The pathogenesis of *H. influenzae* infections is not completely understood, although the presence of the capsule in encapsulated type b (Hib), a serotype causing conditions such as epiglottitis, is known to be a major factor in virulence. Their capsule allows them to resist phagocytosis and complement-mediated lysis in the non-immune host. The unencapsulated strains are almost always less invasive, however they can produce an inflammatory response in humans which can lead to many symptoms. Vaccination with Hib conjugate vaccine is effective in preventing Hib infection. Several vaccines are now available for routine use against Hib, however vaccines are not yet available against NTHi.

Most strains of *H. influenzae* are opportunistic pathogens - that is, they usually live in their host without causing disease, but cause problems only when other factors (such as a viral infection or reduced immune function) create an opportunity.

Naturally-acquired disease caused by *H. influenzae* seems to occur in humans only. In infants and young children, *H. influenzae* type b (Hib) causes bacteremia, pneumonia, and acute bacterial meningitis. Occasionally, it causes cellulitis, osteomyelitis, epiglottitis, and infectious arthritis. Due to routine use of the Hib conjugate vaccine in the U.S. since 1990, the incidence of invasive Hib disease has decreased to 1.3/100,000 in children. However, Hib remains a major cause of lower respiratory tract infections in infants and children in developing countries where vaccine is not widely used. Unencapsulated *H. influenzae* causes ear infections (otitis media), eye infections (conjunctivitis), and sinusitis in children and is associated with pneumonia.

Clinical diagnosis of *H. influenzae* is typically performed by bacterial culture or latex particle agglutination. Diagnosis is considered confirmed when the organism is isolated from a sterile body site. In this respect, *H. influenzae* cultured from the nasopharyngeal cavity or sputum would not indicate *H. influenzae* disease because these sites are colonized in disease free individuals. However, *H. influenzae* isolated from cerebrospinal fluid or blood would indicate a *H. influenzae* infection.

Bacterial culture of *H. influenzae* is performed on agar plates, preferably Chocolate agar, plate with added X(Hemin) & V(NAD) factors at 37 °C in an enriched CO2 incubator. Blood agar growth is only achieved as a satellite phenomenon around other bacteria. Colonies of *H. influenzae* appear as convex, smooth, pale, grey or transparent colonies. Gram-stained and microscopic observation of a specimen of *H. influenzae* will show Gram-negative, coccobacilli, with no specific arrangement. The cultured organism can be further characterized using catalase and oxidase tests, both of which should be positive. Further

serological is necessary to distinguish the capsular polysaccharide and differentiate between *H. influenzae* b and non-encapsulated species.

Although highly specific, bacterial culture of *H. influenzae* lacks in sensitivity. Use of antibiotics prior to sample collection greatly reduces the isolation rate by killing the bacteria before identification is possible. Beyond this, *H. influenzae* is a finicky bacterium to culture, and any modification of culture procedures can greatly reduce isolation rates. Poor quality of laboratories in developing countries has resulted in poor isolation rates of *H. influenzae*.

*H. influenzae* will grow in the hemolytic zone of *Staphylococcus aureus* on Blood Agar plates. The hemolysis of cells by *S. aureus* releases nutrients vital to the growth of *H. influenzae*. *H. influenzae* will not grow outside the hemolytic zone of *S. aureus* due to the lack of nutrients in these areas.

*Haemophilus influenzae* produces beta lactamases, and it is also able to modify its penicillin binding protein, so it has gained resistance to the penicillin family of antibiotics. In severe cases cefotaxime and ceftriaxone are the elected antibiotics, delivered directly into the bloodstream, and for the less severe cases an association of ampicillin and sulbactam, cephalosporins of the second and third generation, or fluoroquinolones.

#### ix. *Streptococcus pneumoniae*

*Streptococcus pneumoniae* is a gram-positive, alpha-hemolytic, bile soluble aerotolerant anaerobe and a member of the genus *Streptococcus*. A significant human pathogenic bacterium, *S. pneumoniae* was recognized as a major cause of pneumonia in the late 19th century and is the subject of many humoral immunity studies.

Despite the name, the organism causes many types of pneumococcal infection other than pneumonia, including acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is the most common cause of bacterial meningitis in adults and children, and is one of the top two isolates found in ear infection, otitis media. Pneumococcal pneumonia is more common in the very young and the very old.

*S. pneumoniae* can be differentiated from *S. viridans*, some of which are also alpha hemolytic, using an optochin test, as *S. pneumoniae* is optochin sensitive. *S. pneumoniae* can also be distinguished based on its sensitivity to lysis by bile. The encapsulated, gram-positive coccoid bacteria have a distinctive morphology on gram stain, the so-called, "lancet shape." It has a polysaccharide capsule that acts as a virulence factor for the organism; more than 90



different serotypes are known, and these types differ in virulence, prevalence, and extent of drug resistance.

*S. pneumoniae* is part of the normal upper respiratory tract flora but as with many natural flora, it can become pathogenic under the right conditions (*e.g.*, if the immune system of the host is suppressed). Invasins such as Pneumolysin, an anti-phagocytic capsule, various  
5 adhesins and immunogenic cell wall components are all major virulence factors.

Both *H. influenzae* and *S. pneumoniae* can be found in the human upper respiratory system. A study of competition in a laboratory revealed that, in a petri dish, *S. pneumoniae* always overpowered *H. influenzae* by attacking it with hydrogen peroxide. When both  
10 bacteria are placed together into a nasal cavity, within 2 weeks, only *S. pneumoniae* survives. When both are placed separately into a nasal cavity, each one survives. Upon examining the upper respiratory tissue from mice exposed to both bacteria, an extraordinarily large number of neutrophil immune cells were found. In mice exposed to only one bacteria, the cells were not present. Lab tests show that neutrophils that were exposed to already dead *H. influenzae*  
15 were more aggressive in attacking *S. pneumoniae* than unexposed neutrophils. Exposure to killed *H. influenzae* had no effect on live *H. influenzae*.

#### x. *Escherichia coli*

*Escherichia coli*, commonly abbreviated as *E. coli*, is a Gram-negative, rod-shaped,  
20 facultative anaerobic and non-sporulating bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for product recalls.

Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and  
25 neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and Gram-negative pneumonia.

Certain strains of *E. coli*, such as O157:H7, O121 and O104:H21, produce potentially lethal toxins. Food poisoning caused by *E. coli* is usually caused by eating unwashed vegetables or undercooked meat. *E. coli* infection outbreaks in the United States have also  
30 occurred even when eating shelled nuts, including Hazelnuts. O157:H7 is also notorious for causing serious and even life-threatening complications such as Hemolytic-uremic syndrome. This particular strain is linked to the 2006 United States *E. coli* outbreak due to fresh spinach. Severity of the illness varies considerably; it can be fatal, particularly to young children, the elderly or the immunocompromised, but is more often mild. Earlier, poor hygienic methods

of preparing meat in Scotland killed seven people in 1996 due to *E. coli* poisoning, and left hundreds more infected.

If *E. coli* bacteria escape the intestinal tract through a perforation (for example from an ulcer, a ruptured appendix, or due to a surgical error) and enter the abdomen, they usually cause peritonitis that can be fatal without prompt treatment. However, *E. coli* are extremely sensitive to such antibiotics as streptomycin or gentamicin. Unfortunately, it has been suggested that *E. coli* quickly acquires drug resistance and the treatment with antibiotics does not improve the outcome of the disease, but may in fact significantly increase the chance of developing haemolytic-uremic syndrome.

Intestinal mucosa-associated *E. coli* are observed in increased numbers in the inflammatory bowel diseases, Crohn's disease and ulcerative colitis. Invasive strains of *E. coli* exist in high numbers in the inflamed tissue, and the number of bacteria in the inflamed regions correlates to the severity of the bowel inflammation. Enteric *E. coli* are classified on the basis of serological characteristics and virulence properties. Virotypes include: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), and Enterohemorrhagic *E. coli* (EAEC).

Among these virotypes, EPEC causes diarrhea in humans, rabbits, dogs, cats and horses. EPEC cells utilize an adhesin known as intimin to bind host intestinal cells. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately invasive and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" is likely the prime cause of diarrhoea in those afflicted with EPEC.

Transmission of pathogenic *E. coli* often occurs via faecal-oral transmission. Common routes of transmission include: unhygienic food preparation, farm contamination due to manure fertilization, irrigation of crops with contaminated greywater or raw sewage, feral pigs on cropland, or direct consumption of sewage-contaminated water. Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7, and they can carry it asymptotically and shed it in their faeces. Food products associated with *E. coli* outbreaks include raw ground beef, raw seed sprouts or spinach, raw milk, unpasteurized juice, unpasteurized cheese and foods contaminated by infected food workers via faecal-oral route.

Certain strains of *E. coli*, specifically serotype O157:H7, have also been transmitted by flies, as well as direct contact with farm animals, petting zoo animals, and airborne particles found in animal-rearing environments.

xi. *Vibrio cholerae*

*Vibrio cholera*, also known as *Kommabacillus*, is a gram negative comma-shaped bacterium with a polar flagellum that causes cholera in humans. There are two major biotypes of *Vibrio cholerae* identified by hemagglutination testing, classical and El Tor, and numerous serogroups. The classical biotype is found only in Bangladesh, whereas the El Tor is found throughout the world.

*Vibrio cholerae* pathogenicity genes code for proteins directly or indirectly involved in the virulence of the bacteria. Because of their same transcriptional regulation and their implication in the same pathway, pathogenicity genes are generally organized in operons and/or gene clusters. In *Vibrio cholerae*, most of virulence genes are located in two pathogenicity plasmids, which are organized as prophages: CTX (Cholera ToXins) plasmid and TCP (Toxin-Coregulated Pilus) plasmid, also named as *Vibrio cholerae* Pathogenicity Island (VPI). Virulent and epidemic strains of *Vibrio cholerae* require these two genetic elements to cause infections.

xii. *Vibrio parahaemolyticus*

*Vibrio parahaemolyticus* is a curved, rod-shaped, Gram-negative bacterium found in brackish saltwater that causes gastrointestinal illness in humans, when ingested. *V. parahaemolyticus* is oxidase positive, facultatively aerobic, and does not form spores. Like other members of the genus *Vibrio*, this species is motile, with a single, polar flagellum.

While infection of *Vibrio parahaemolyticus* can occur via the fecal-oral route, the predominant cause of the acute gastroenteritis caused by *Vibrio parahaemolyticus* is through ingestion of bacteria in raw or undercooked seafood, usually oysters. Wound infections also occur, but are less common than seafood-borne disease. The disease mechanism of *Vibrio parahaemolyticus* infections has not been fully elucidated.

Outbreaks tend to be concentrated along coastal regions during the summer and early fall when higher water temperatures favor higher levels of bacteria. Seafood most often implicated includes squid, mackerel, tuna, sardines, crab, shrimp, and bivalves like oysters and clams. The incubation period of ~24 hours is followed by explosive, watery diarrhea accompanied by nausea, vomiting, abdominal cramps, and sometimes fever. *Vibrio parahaemolyticus* symptoms typically resolve within 72 hours, but can persist for up to 10 days in immunocompromised individuals. As the vast majority of cases of *Vibrio parahaemolyticus* food infection are self-limiting, treatment is not typically necessary. In severe cases, fluid and electrolyte replacement is indicated.

Additionally, swimming or working in affected areas can lead to infections of the eyes or ears and open cuts and wounds. Following Hurricane Katrina, there were 3 wound infections caused by *Vibrio parahaemolyticus* and 2 of these led to death.

5                   xiii. *Yersinia pseudotuberculosis*

*Yersinia pseudotuberculosis* is a Gram-negative bacterium which primarily causes Pseudotuberculosis (Yersinia) disease in animals; humans occasionally get infected zoonotically, most often through the food-borne route.

10           In animals, *Yersinia pseudotuberculosis* can cause tuberculosis-like symptoms, including localized tissue necrosis and granulomas in the spleen, liver, and lymph node.

          In humans, symptoms of Pseudotuberculosis (Yersinia) include fever and right-sided abdominal pain, but the diarrheal component is often absent, which sometimes makes the resulting condition difficult to diagnose. *Yersinia pseudotuberculosis* infections can mimic appendicitis, especially in children and younger adults, and, in rare cases the disease may  
15           cause skin complaints (erythema nodosum), joint stiffness and pain (reactive arthritis), or spread of bacteria to the blood (bacteremia).

          Pseudotuberculosis (Yersinia) usually becomes apparent 5–10 days after exposure and typically lasts 1–3 weeks without treatment. In complex cases or those involving immunocompromised patients, antibiotics may be necessary for treatment; ampicillin,  
20           aminoglycosides, tetracycline, chloramphenicol, or a cephalosporin may all be effective.

          The recently described syndrome Izumi-fever has also been linked to infection with *Y.pseudotuberculosis*.

          This bacterium possesses many virulence factors to facilitate attachment, invasion, and colonization of its host. Superantigens, bacterial adhesions, and the actions of Yops  
25           (which are bacterial proteins once thought to be “Yersinia outer membrane proteins”) that are encoded on the “[plasmid] for Yersinia virulence” – commonly known as the pYV – cause host pathogenesis and allow the bacteria to live parasitically.

*Yersinia pseudotuberculosis* adheres strongly to intestinal cells via chromosomally encoded proteins so that Yop secretion may occur, to avoid being removed by peristalsis, and  
30           to invade target host cells.

          Certain strains of *Yersinia pseudotuberculosis* express a superantigenic exotoxin, YPM, or the *Yersinia pseudotuberculosis* -derived mitogen, from the chromosomal ypm gene. Strains which carry the exotoxin gene are rare in Western countries where the disease, when at all apparent, manifests itself largely with minor symptoms, whereas more than 95%

of strains from Far Eastern countries contain ypm and are correlated with Izumi fever and Kawasaki disease.

Although the superantigen poses the greatest threat to host health, all virulence factors contribute to *Yersinia pseudotuberculosis* viability *in vivo* and define the bacterium's pathogenic characteristics. *Yersinia pseudotuberculosis* can live extracellularly due to its formidable mechanisms of phagocytosis and opsonisation resistance; yet, by limited pYV action, it can populate host cells, especially macrophages, intracellularly to further evade immune responses and be disseminated throughout the body.

#### xiv. *Salmonella*

*Salmonella* is a genus of rod-shaped, Gram-negative, non-spore-forming, predominantly motile enterobacteria with flagella which grade in all directions (*i.e.* peritrichous). They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide, which can readily be detected by growing them on media containing ferrous sulfate, such as TSI. Most isolates exist in two phases: a motile phase I and a nonmotile phase II.

*Salmonella* is closely related to the *Escherichia* genus and are found worldwide in cold- and warm-blooded animals, including humans, and in the environment. They cause illnesses like typhoid fever, paratyphoid fever, and the foodborne illness. *Salmonella* infections are zoonotic and can be transferred between humans and nonhuman animals. Many infections are due to ingestion of contaminated food. Typhoid/paratyphoid *Salmonella* is distinguished from enteritis *Salmonella* because of the possession of a special virulence factor and a capsule protein (virulence antigen), which can cause serious illness, such as *Salmonella enterica subsp. enterica* serovar Typhi. *Salmonella typhi*. is adapted to humans and does not occur in animals.

Enteritis *Salmonellosis* or Food Poisoning *Salmonella* is a group consisting of potentially all other serotypes (over a thousand) of the *Salmonella* bacterium, most of which have never been found in humans. These are encountered in various *Salmonella* species, most having never been linked to a specific host, and can also infect humans. The organism enters through the digestive tract and must be ingested in large numbers to cause disease in healthy adults. Gastric acidity is responsible for the destruction of the majority of ingested bacteria. The infection usually occurs as a result of massive ingestion of foods in which the bacteria are highly concentrated similarly to a culture medium. However, infants and young

children are much more susceptible to infection, easily achieved by ingesting a small number of bacteria. It has been shown that, in infants, the contamination could be through inhalation of bacteria-laden dust.

After a short incubation period of a few hours to one day, the germ multiplies in the intestinal lumen causing an intestinal inflammation with diarrhoea that is often mucopurulent and bloody. In infants, dehydration can cause a state of severe toxicosis. The symptoms are usually mild. There is normally no sepsis, but it can occur exceptionally as a complication in weakened elderly patients (Hodgkin's disease, for example). Extraintestinal localizations are possible, especially *Salmonella* meningitis in children, osteitis, etc. Enteritis *Salmonella*, e.g., *Salmonella enterica* subsp. *enterica* serovar *enteritidis*, can cause diarrhoea, which usually does not require antibiotic treatment. However, in people at risk such as infants, small children, the elderly, *Salmonella* infections can become very serious, leading to complications. If these are not treated, HIV patients and those with suppressed immunity can become seriously ill. Children with sickle cell anaemia who are infected with *Salmonella* may develop osteomyelitis.

In Germany, *Salmonella* infections must be reported. Between 1990 and 2005, the number of officially recorded cases decreased from approximately 200,000 cases to approximately 50,000. It is estimated that every fifth person in Germany is a carrier of *Salmonella*. In the USA, there are approximately 40,000 cases of *Salmonella* infection reported each year. According to the World Health Organization, over 16 million people worldwide are infected with typhoid fever each year, with 500,000 to 600,000 fatal cases.

*Salmonella* can survive for weeks outside a living body. They have been found in dried excrement after more than 2.5 years. *Salmonella* are not destroyed by freezing. Ultraviolet radiation and heat accelerate their demise; they perish after being heated to 55 °C (131 °F) for one hour, or to 60 °C (140 °F) for half an hour. To protect against *Salmonella* infection, it is recommended that food be heated for at least ten minutes at 75 °C (167 °F) so that the centre of the food reaches this temperature.

## **B. Non-Pathogenic Bacteria**

In accordance with the present invention, it will be useful to provide non-pathogenic bacteria into which recombinant mce-containing constructs can be introduced. Such non-pathogenic bacteria are defined simply as those that do not produce overt disease and/or those that produce acceptable/clinically manageable side effects.

i. *Escherichia coli*

As described *supra*, *Escherichia coli*, commonly abbreviated as *E. coli*, is a Gram-negative rod-shaped bacterium. While some strains of *E. coli* are pathogenic, most and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic bacteria within the intestine.

*E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population.

*E. coli* normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or with the individuals handling the child. In the bowel, it adheres to the mucus of the large intestine. It is the primary facultative anaerobe of the human gastrointestinal tract. Facultative anaerobes are organisms that can grow in either the presence or absence of oxygen. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals.

Nonpathogenic *Escherichia coli* strain Nissle 1917 also known as Mutaflor is used as a probiotic agent in medicine, mainly for the treatment of various gastroenterological diseases, including inflammatory bowel disease

Because of its long history of laboratory culture and ease of manipulation, *E. coli* also plays an important role in modern biological engineering and industrial microbiology.

Considered a very versatile host for the production of heterologous proteins, researchers can introduce genes into the microbes using plasmids, allowing for the mass production of proteins in industrial fermentation processes. Genetic systems have also been developed which allow the production of recombinant proteins using *E. coli*. Modified *E. coli* have been used in vaccine development, bioremediation, and production of immobilised enzymes. *E. coli* cannot, however, be used to produce some of the more large, complex proteins which contain multiple disulfide bonds and, in particular, unpaired thiols, or proteins that also require post-translational modification for activity.

*E. coli* is frequently used as a model organism in microbiology studies. Cultivated strains (e.g., *E. coli* K12) are well-adapted to the laboratory environment, and, unlike wild-type strains, have lost their ability to thrive in the intestine.

BL21 strain of *E. coli* has been widely used as a host for the expression of recombinant proteins. As an *E. coli* B strain, it lacks the major protease, encoded by the lon

gene, catalyzing the endoproteolytic cleavage of damaged and recombinant proteins in the cell. (GE Healthcare life sciences).

*E. coli* HS strain is a human commensal isolate that was originally isolated from a laboratory scientist at the Walter Reed Army Institute of Research. In human challenge experiments, strain HS colonized the human gastrointestinal tract with no overt signs of disease. The genome sequence of strain HS represents the genomic baseline for colonization of the human gastrointestinal tract. This isolate is serotype O9, phylogroup A, motile, competent and amenable to genetic manipulation.

#### 4. Engineering Cells to Express MAM Proteins

In certain embodiments, the present invention involves either the production of MAM peptides or the transfer of a MAM nucleic acid to a host cell. Such methods both rely upon expression constructs containing a MAM coding region and the means for its expression, plus elements that permit replication of the constructs. Within certain embodiments, expression vectors are employed to express the MAM polypeptide product or variants thereof. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

Throughout this application, the term “expression construct” is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

The term “vector” is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be “exogenous,” which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the



cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Sambrook *et al.* (1989) and Ausubel *et al.* (1994).

The term “expression vector” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

A “promoter” is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally-associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as “endogenous.” Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not

“naturally-occurring,” *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in  
5 connection with the compositions disclosed herein.

Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively  
10 directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to  
15 direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

## 5. Methods of Treating Bacterial Infections

20 The present invention contemplates, in one embodiment, the treatment of subjects suffering from bacterial infections or at risk of the same due to various medical or environmental conditions. A variety of medical situations lend themselves to risk of infections. For example, patients on chronic antibiotic therapy, immunosuppressed patients, patients having had surgery, and patients with traumatic wounds including burns all are at  
25 risk of developing bacterial infections.

Administration of compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such  
30 compositions would normally be administered as pharmaceutically acceptable compositions. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

### A. Cell-Based Therapies

Specifically, the present inventors intend to provide a non-pathogenic bacterial cell that expresses a MAM peptide or polypeptide.

5 Non-pathogenic bacterial cells provided herein include bacteria that are harmless to the host, as well as probiotic bacteria that are beneficial to the host. Throughout the application, the term “probiotic” refers to a live microorganism beneficial to the host organism. These probiotic microorganisms provide a variety of benefits, including immune system support, cancer prevention and irritable bowel syndrome relief. Probiotics are  
10 commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements. Probiotic microorganisms include, but not limited to, certain bacteria, yeasts and bacilli. The most common types of probiotic bacteria are lactic acid bacteria and bifidobacteria.

In certain embodiments, nonpathogenic bacterial cells used for treatment of  
15 pathogenic bacterial infection express a native MAM polypeptide. The MAM peptide expressed therein has one or more mce repeat regions, preferably 6 or more mce repeat regions, more preferably, 6 or 7 mce repeat regions. Non-pathogenic bacterial cells expressing a native MAM polypeptide.

20 In further embodiments, non-pathogenic bacterial cells used for treatment of pathogenic bacterial infection do not express a native MAM polypeptide and are engineered to express a MAM polypeptide heterologous to that bacterium. The heterologous MAM peptide expressed therein has one or more mce repeat regions, preferably 5 or more, or 6 or more mce repeat regions, more preferably, 6 or 7 mce repeat regions. Non-pathogenic  
25 bacterial cells used for expressing a heterologous MAM peptide include, but not limited to, certain harmless *E. coli* strains, such as BL21 or HS. The methods of engineering cells to express a MAM polypeptide has been extensively discussed *supra*, and are incorporated into this section by its entirety.

In still further embodiments, non-pathogenic bacterial cells used for treatment of  
30 pathogenic bacterial infection express a native MAM polypeptide and are further engineered to express a heterologous MAM polypeptide. The native MAM polypeptide and the heterologous MAM polypeptide expressed therein may contain the same number of mce repeat regions or different number of mce repeat regions. The native MAM polypeptide and

the heterologous MAM polypeptide expressed therein may originate from the same bacterial strain or different bacterial strains.

The present invention contemplates the use of nonpathogenic bacterial cells provided herein, as well as compositions that include such cells, to treat pathogenic bacterial infection  
5 in a subject. Such compositions may further comprise other probiotic microorganisms, including, but not limited to, certain strains of bacteria, such as lactic acid bacteria and bifidobacteria, yeasts and bacilli.

In another aspect of the present invention, the nonpathogenic bacterial cells and the compositions provided herein are administered to a subject, preferably a mammal, such as a  
10 human, a horse, a cow, a dog, a cat, more preferably, a human.

The present invention contemplates the treatment of subjects suffering from diseases caused by pathogenic bacterial infections or at risk of the same due to various medical or environmental conditions. Such diseases and conditions include, for example, pneumonia, deep post-surgical infections, septicaemia, endocarditis, necrotizing pneumonia, toxic shock  
15 syndrome, inflammation and sepsis pneumonia, urinary tract infections, blood stream infection bacteremia, acute bacterial meningitis, pneumococcal infections, including acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess astroenteritis, neonatal meningitis haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia, Gram-negative  
20 pneumonia, cholera, gastrointestinal illness, tuberculosis-like symptoms, including localized tissue necrosis and granulomas in the spleen, liver, and lymph node, typhoid fever, paratyphoid fever, diarrhea, ear infections, inflammatory bowel diseases, Crohn's disease, ulcerative colitis, and foodborne illnesses.

It is also contemplated that the non-pathogenic bacterial cells administered to a  
25 subject to prevent or inhibit a pathogenic bacterial infection may selectively or preferably localize or accumulate in the host's organism infected by said pathogenic bacteria. For example, certain virulent *E. coli* strains may mainly infect urinary tract and gastrointestinal tract. The nonpathogenic bacteria which is prone to reside or proliferate in those locations are optionally engineered and administered to said subject. The administered non-pathogenic  
30 bacteria can survive, replicate, proliferate and accumulate at those locations. The selective accumulation mainly attributes to the proliferative properties of administered nonpathogenic bacteria and the condition or the environment of the infection site. In some embodiments, the selective accumulation of the non-pathogenic bacterial cells at a target site may be enhanced through the incorporation of a targeting moiety into the cells. The administered non-

pathogenic bacteria normally do not cause any harmful effects to the body of the subject and are usually cleared from the body by the subject's immune system.

### **B. Peptide/Protein Therapy**

Another therapy approach is the provision, to a subject, of MAM peptides, synthetic or recombinant, or variants, mimetics or analogs thereof. Formulations would be selected based on the route of administration and purpose including, but not limited to, parenteral formulations, topical formulations, liposomal formulations and classic pharmaceutical preparations for oral administration.

In another aspect of delivery, the peptides and polypeptides of the present invention can be delivered by encapsulating or embedding in a delivery vehicle. For example, liposomes, which are artificially prepared vesicles made of lipid bilayers have been used to delivery a variety of drugs. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine) or other surfactants. In particular, liposomes containing cationic or neutral lipids have been used in the formulation of drugs. Liposomes should not be confused with micelles and reverse micelles composed of monolayers, which also can be used for delivery.

Nanoparticles are generally considered to be particulate substances having a diameter of 100 nm or less. Microparticles may be large, such as those in the micrometer range. In contrast to liposomes, which are hollow, nanoparticles tend to be solid. Thus, the drug will be less entrapped and more either embedded in or coated on the nanoparticle. Nanoparticles can be made of metals including oxides, silica, polymers such as polymethyl methacrylate, and ceramics. Similarly, nanoshells are somewhat larger and encase the delivered substances with these same materials. Either nanoparticles or nanoshells permit sustained or controlled release of the peptide or polypeptide, and can stabilize it to the effects of *in vivo* environment.

The present invention contemplates that all types of materials and structures, including inorganic and organic materials, can be used for the nanoparticles of the present invention. Non-limiting examples of these materials and structures include polymersomes, liposomes, polyplexes and conjugates described *infra*. Additional non-limiting materials include poly(orthoesters), poly(anhydrides), poly(phosphoesters), poly(phosphazenes) and others. Preferably, the material is the biodegradable polymer poly(lactic-co-glycolic acid) (PLGA). PLGA is a well-studied polymer for drug delivery and is FDA-approved for a number of *in vivo* applications. Other non-limiting materials include, for example, polyesters (such as poly(lactic acid), poly(L-lysine), poly(glycolic acid) and poly(lactic-co-glycolic

acid)), poly(lactic acid-co-lysine), poly(lactic acid-graft-lysine), polyanhydrides (such as poly(fatty acid dimer), poly(fumaric acid), poly(sebacic acid), poly(carboxyphenoxy propane), poly(carboxyphenoxy hexane), copolymers of these monomers and the like), poly(anhydride-co-imides), poly(amides), poly(ortho esters), poly(iminocarbonates),  
5 poly(urethanes), poly(organophosphazenes), poly(phosphates), poly(ethylene vinyl acetate) and other acyl substituted cellulose acetates and derivatives thereof, poly(caprolactone), poly(carbonates), poly(amino acids), poly(acrylates), polyacetals, poly(cyanoacrylates), poly(styrenes), poly(vinyl chloride), poly(vinyl fluoride), poly(vinyl imidazole), chlorosulfonated polyolefins, polyethylene oxide, copolymers, polystyrene, and blends or co-  
10 polymers thereof. In another aspects, the nano-particles include hydroxypropyl cellulose (HPC), N-isopropylacrylamide (NIPA), polyethylene glycol, polyvinyl alcohol (PVA), polyethylenimine, chitosan, chitin, dextran sulfate, heparin, chondroitin sulfate, gelatin, etc. and their derivatives, co-polymers, and mixtures thereof. In particular, one type of nanoparticles is semiconductor quantum dots with a size smaller than 10 nm. Quantum dots  
15 are inorganic nanocrystals possessing fluorescent properties. Peptides can be coated onto quantum dots using a thiol-exchange reaction. The quantum dots can be further coupled with a substance, such as polyethylene glycol (PEG), that reduces aggregation of the quantum dots, minimize non-specific binding and maintains solubility in aqueous solvents (Kerman *et al.*, 2002). A non-limiting method for making nano-particles is described in U.S. Patent  
20 Publication 2003/0138490.

In a further aspect of the present invention, the peptides and polypeptides of the present invention can be delivered by nanoparticles or nanoclusters comprising a plurality of nanoparticles.

Nanoparticles/nanoclusters can associate or conjugate with nucleic acids, proteins,  
25 peptides, polypeptides, drugs, vaccine and virus vectors, and other therapeutic agents, diagnostic agents, and a combination thereof. These conjugates may be entangled, embedded, incorporated, encapsulated, bound to the surface through covalent and/or non-covalent bonds, with nanoparticles. Nanoparticles may associate with multiple conjugates. For example, nanoparticles may contain a first conjugate on its surface, a second conjugate  
30 encapsulated within nanoparticles, and a third conjugate incorporated into the material of nanoparticles.

It is contemplated that nanoparticles or nanoclusters release conjugates in a given environment in an immediate release or a sustained release manner, or after a given period of time in a controlled matter. It is further contemplated that nanoparticles or nanoclusters

associated with multiple conjugates may release different conjugates at the same time, or at different time in a controlled manner or in response to a specific trigger event. Non-limiting examples of trigger events include a selected pH range, a selected temperature range, an electric current, a selected ion strength, pressure, the presence of certain liquid, the presence  
5 of a specific enzyme, protein, chemical.

In certain embodiments of the present invention, nanoparticles/nanoclusters conjugate with MAM peptides, synthetic or recombinant, or variants, mimetics or analogs thereof. The MAM peptide has one or more mce repeat regions, preferably 6 or more mce repeat regions, more particularly, 6 or 7 mce repeat regions. Nanoparticles/nanoclusters may conjugate with  
10 several different MAM peptides comprising different number of mce repeat regions, or MAM peptides of different species.

In further embodiments of the present invention, nanoparticles/nanoclusters further comprise a moiety or an ingredient which directs or assists conjugated nanoparticles/nanoclusters to a target site in a body, for example, gastrointestinal tract.

15 It is contemplated that after conjugated nanoparticles/nanoclusters are administered into the body of a subject, the conjugated MAM peptides are released in an immediate and/or sustained release manner, preferably at the target site.

The present invention contemplates the treatment of subjects suffering from diseases caused by pathogenic bacterial infections or at risk of the same due to various medical or  
20 environmental conditions by administering a composition comprising MAM peptides. Such diseases and conditions include, for example, pneumonia, deep post-surgical infections, septicaemia, endocarditis, necrotizing pneumonia, toxic shock syndrome, inflammation and sepsis pneumonia, urinary tract infections, blood stream infection bacteremia, acute bacterial meningitis, pneumococcal infections, including acute sinusitis, otitis media, meningitis,  
25 bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess astroenteritis, neonatal meningitis haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia, Gram-negative pneumonia, cholera, gastrointestinal illness, tuberculosis-like symptoms, including localized tissue necrosis and granulomas in the spleen, liver, and lymph node, typhoid fever, paratyphoid fever, diarrhea, ear infections,  
30 inflammatory bowel diseases, Crohn's disease, ulcerative colitis, and foodborne illness.

In another aspect of the present invention, a composition comprising both MAM peptides and non-pathogenic bacteria expressing MAM peptides is administered to a subject to prevent or inhibit a pathogenic bacterial infection.

### C. Combined Therapy

In order to increase the effectiveness of an MAM-expressing non-pathogenic bacterium, protein or peptide therapy, it may be desirable to combine these compositions with another agent effective in the treatment of bacterial infections. The terms “contacted” and “exposed,” when applied to a cell, tissue or organism, are used herein to describe the process by which an MAM-expressing non-pathogenic bacterium, protein or peptide and another anti-bacterial agent are delivered to a target cell, tissue or organism or are placed in direct juxtaposition with the target cell, tissue or organism.

The MAM-expressing non-pathogenic bacterium, protein or peptide may precede, be co-current with and/or follow the other agent(s) by intervals ranging from minutes to weeks. In embodiments where the MAM-expressing non-pathogenic bacterium, protein or peptide and other agent(s) are applied separately to a cell, tissue or organism, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the MAM-expressing non-pathogenic bacterium, protein or peptide and other agent(s) would still be able to exert an advantageously combined effect on the cell, tissue or organism. For example, in such instances, it is contemplated that one may contact the cell, tissue or organism with two, three, four or more modalities substantially simultaneously (*i.e.*, within less than about a minute) as the MAM-expressing non-pathogenic bacterium, protein or peptide. In other aspects, one or more agents may be administered within of from substantially simultaneously, about 1 minute, about 5 minutes, about 10 minutes, about 20 minutes about 30 minutes, about 45 minutes, about 60 minutes, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 18 hours, about 24 hours, about 36 hours, about 48 hours, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, about 10 days, about 11 days, about 12 days, about 13 days, about 14 days, about 21 days, about 4 weeks, about 5 weeks, about 6 weeks, about 7 week or about 8 weeks or more, and any range derivable therein, prior to and/or after administering the MAM-expressing non-pathogenic bacterium, protein or peptide.

Various combination regimens of the MAM-expressing non-pathogenic bacterium, protein or peptide treatment and one or more other anti-bacterial agents may be employed. Non-limiting examples of such combinations are shown below, wherein an MAM-expressing non-pathogenic bacterium, protein or peptide composition is “A” and the other anti-bacterial agent is “B”:



A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B  
 B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A  
 B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

5

Administration of the MAM-expressing non-pathogenic bacterium, protein or peptide to a cell, tissue or organism may follow general protocols for the administration of pharmaceuticals, taking into account the toxicity, if any. It is expected that the treatment cycles would be repeated as necessary. In particular embodiments, it is contemplated that various additional agents may be applied in any combination with the present invention.

Antibiotics that may be employed include the aminoglycosides (Amikacin (IV), Gentamycin (IV), Kanamycin, Neomycin, Netilmicin, Paromomycin, Streptomycin (IM), Tobramycin (IV)), the carbapenems (Ertapenem (IV/IM), Imipenem (IV), Meropenem (IV)), Chloramphenicol (IV/PO), the fluoroquinolones (Ciprofloxacin (IV/PO), Gatifloxacin (IV/PO), Gemifloxacin (PO), Grepafloxacin (PO), Levofloxacin (IV/PO), Lomefloxacin, Moxifloxacin (IV/PO), Norfloxacin, Ofloxacin (IV/PO), Sparfloxacin (PO), Trovafloxacin (IV/PO)), the glycopeptides (Vancomycin (IV), the lincosamides (Clindamycin (IV/PO), macrolides/ketolides (Azithromycin (IV/PO), Clarithromycin (PO), Dirithromycin, Erythromycin (IV/PO), Telithromycin), the cephalosporins (Cefadroxil (PO), Cefazolin (IV), Cephalexin (PO), Cephalothin, Cephapirin, Cephadrine, Cefaclor (PO), Cefamandole (IV), Cefonicid, Cefotetan (IV), Cefoxitin (IV), Cefprozil (PO), Cefuroxime (IV/PO), Loracarbef (PO), Cefdinir (PO), Cefditoren (PO), Cefixime (PO), Cefoperazone (IV), Cefotaxime (IV), Cefpodoxime (PO), Ceftazidime (IV), Cefibuten (PO), Ceftizoxime (IV), Ceftriaxone (IV), Cefepime (IV)), monobactams (Aztreonam (IV)), nitroimidazoles (Metronidazole (IV/PO)), oxazolidinones (Linezolid (IV/PO)), penicillins (Amoxicillin (PO), Amoxicillin/Clavulanate (PO), Ampicillin (IV/PO), Ampicillin/Sulbactam (IV), Bacampicillin (PO), Carbenicillin (PO), Cloxacillin, Dicloxacillin, Methicillin, Mezlocillin (IV), Nafcillin (IV), Oxacillin (IV), Penicillin G (IV), Penicillin V (PO), Piperacillin (IV), Piperacillin/Tazobactam (IV), Ticarcillin (IV), Ticarcillin/Clavulanate (IV)), streptogramins (Quinupristin/Dalfopristin (IV), sulfonamide/folate antagonists (Sulfamethoxazole/Trimethoprim (IV/PO)), tetracyclines (Demeclocycline, Doxycycline (IV/PO), Minocycline (IV/PO), Tetracycline (PO)), azole antifungals (Clotrimazole, Fluconazole (IV/PO), Itraconazole (IV/PO), Ketoconazole (PO), Miconazole, Voriconazole (IV/PO)), polyene antifungals (Amphotericin B (IV), Nystatin),

echinocandin antifungals (Caspofungin (IV), Micafungin), and other antifungals (Ciclopirox, Flucytosine (PO), Griseofulvin (PO), Terbinafine (PO)).

#### **D. Pharmaceutical Formulations**

5        Pharmaceutical formulations of the present invention comprise an effective amount of an MAM-expressing non-pathogenic bacterium, protein or peptide dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases “pharmaceutical or pharmacologically acceptable” refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The  
10       preparation of such pharmaceutical compositions are known to those of skill in the art in light of the present disclosure, as exemplified by Remington’s Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological  
15       Standards.

As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents,  
20       flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art. Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

The pharmaceuticals of the present invention may comprise different types of carriers  
25       depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intramuscularly, intraperitoneally,  
30       subcutaneously, subconjunctival, intravesicularly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, inhalation (*e.g.*, aerosol), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter,

via a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art.

The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

The pharmaceuticals may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can

also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine.

In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, *etc.*), lipids (*e.g.*, triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof.

In certain embodiments, the compositions are prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (*e.g.*, hard or soft shelled gelatin capsules), sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with the food of the diet. Preferred carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention, the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

In certain preferred embodiments an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, *etc.*; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid

carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both.

5 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of  
10 preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated,  
15 where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

The present invention also provides kits, such as therapeutic kits, as well as kits for preparing expression constructs encoding MAM polypeptide. For example, a kit may comprise one or more pharmaceutical composition as described herein and optionally  
20 instructions for their use. Kits may also comprise one or more devices for accomplishing administration of such compositions. For example, a subject kit may comprise a pharmaceutical composition and device for accomplishing intramuscular injection of the pharmaceutical composition comprising the expression constructs encoding MAM peptide into a human subject, and bacterial cells carrying the same.

25 Kits may comprise a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container may hold a composition which includes an expression constructs that is effective for therapeutic or non-therapeutic applications, such as described above. The label on the container may indicate that the composition is used for a specific  
30 therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above. The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

## 6. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Materials and Methods

**Bioinformatic analysis.** Classification of mce domain-containing proteins was supported by analysis of domain architecture using PFAM (Protein Families Database, world-wide-web at pfam.sanger.ac.uk/) and manual identification of mce domains using multiple sequence alignments generated with ClustalW (world-wide-web at ebi.ac.uk/clustalw/) (Finn *et al.*, 2000). PSORTb v3.0.2 (world-wide-web at psort.org/psortb/, (Yu *et al.*, 2010) and the TMHMM server v2.0 (world-wide-web at cbs.dtu.dk/services/TMHMM/) were used to predict subcellular localization and transmembrane regions within MAM7. A neighbor-joining tree of MAM7 proteins was based on a multiple sequence alignment from ClustalW and generated using the programs quicktree and newicktops from the mobyle portal (mobyle.pasteur.fr/cgi-bin/portal.py, (Neron *et al.*, 2009; Howe *et al.*, 2002).

**Recombinant DNA.** MAM7-(myc) constructs for expression in *V. parahaemolyticus* were generated by amplification of MAM7 including the endogenous promoter region from POR1 genomic DNA and cloning of the resulting fragment into NcoI/EcoRI sites of plasmid pBAD/Myc-His (Invitrogen) in which the ampicillin resistance gene was replaced by a kanamycin resistance gene to allow selection in *V. parahaemolyticus*. The same method was used to generate complementation vectors for EPEC, *Y. pseudotuberculosis* and *V. cholerae* MAM7 knockout strains, except that the *V. cholerae* MAM7 construct was cloned into SacI/KpnI sites. MAM7-(myc), MAM7 $\Delta$ N<sub>1-44</sub>(myc), MAM1-(myc) and MAM6-(myc) for expression in *E. coli* were also amplified from POR1 genomic DNA and cloned into NcoI/EcoRI sites of pBAD/Myc-His K<sup>n</sup> but excluded the promoter region and their

expression was driven by the *araBAD* promoter. N<sub>1-44</sub>-TEV-MAM7-(myc) constructs for expression in *V. parahaemolyticus* or *E. coli* were generated by introducing a TEV (tobacco etch virus protease)-cleavage site into existing MAM7-(myc) constructs through site directed mutagenesis. MBP (maltose binding protein)-Cys-mce constructs containing zero (control), one, two, six or seven mce domains were amplified from POR1 genomic DNA and cloned into BamHI and NotI sites of pET28b-MBP (6xHis-tag, MBP-tag). GST (glutathione S-transferase-tag)-MAM7 construct was produced by cloning the MAM7 into BamHI/NotI sites of pGEX-4T-1 (GE Healthcare).

**Construction of EPEC, *V. parahaemolyticus*, *V. cholerae* and *Y. pseudotuberculosis*  $\Delta$ MAM7 deletion strains.** An EPEC  $\Delta$ MAM7 strain was generated using Gene doctoring as previously described (Lee *et al.*, 2009). Deletion strains of *V. parahaemolyticus*, *Y. pseudotuberculosis* and *V. cholerae* were generated essentially as described (Milton *et al.*, 1996; Garbom *et al.*, 2004). Briefly, regions 1 kb upstream and downstream of VP1611 (*MAM7*) were cloned into the suicide vector pDM4 using BglII, SpeI and SalI sites. The construct was transferred into *E. coli* SM10 cells by electroporation and SM10 cells were used as donor strain for triparental mating with *V. parahaemolyticus* POR1 and POR2. After mating, positive clones were selected by replating on MMM (marine minimal medium) containing 25  $\mu$ g/ml chloramphenicol. Individual colonies were streaked on MMM plates containing 15 % sucrose to cure the plasmid. Individual colonies were screened for loss of the plasmid and deletion of VP1611 was confirmed by transcriptional analysis and sequencing. For transcriptional analysis, RNA was isolated from POR strains, deletion strains and complemented strains using the RNeasy kit (QIAGEN). RNA was treated with DNase I (QIAGEN) and used as template for reverse transcription (M-MLT reverse transcriptase, Promega). Both cDNA and original RNA (control) were used as template for PCR with both VP1611-specific and ribosomal protein 14 -specific (control) primers.

**Subcellular fractionation.** Cells expressing myc-tagged MAM proteins were pelleted and resuspended in 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 20 % w/v sucrose and 0.1 mg/ml lysozyme to give an OD<sub>600</sub> of 6.0. Samples were left at 22 °C for 30 minutes and subsequently pelleted again (5 min, 10000 $\times$ g, 4 °C). The supernatant (periplasmic and outer membrane fractions) was transferred to a fresh tube and centrifuged at 100000 $\times$ g, 4 °C for one hour to separate periplasmic (soluble) and outer membrane (pellet) fractions. To recover the cytoplasmic and inner membrane fractions the residual pellet after the first incubation and centrifugation step was resuspended to an OD<sub>600</sub> of 6.0 in 10 mM Tris-HCl pH 7.5, 5 mM

EDTA and left at 22 °C for 10 minutes. Lysed spheroblasts were separated in inner membrane (pellet) and cytoplasmic (soluble) fractions by centrifugation (100000×g, 4 °C for one hour). Pellet fractions were resuspended to a volume equal to the supernatant and equal volumes of samples were separated by SDS-PAGE. Myc-tagged proteins were detected using

5 Western Blotting.

**Western Blotting.** Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were blocked with 5 % skim milk powder in TBS-T (Tris-buffered saline containing 0.05 % Tween 20) for one hour at 22 °C. Membranes were probed with anti-myc antibody 9E10 (Santa Cruz) diluted 1:1000 into blocking buffer for 1  
10 hour at 22 °C. After three washes with TBS-T, membranes were incubated with anti-mouse HRP (horseradish peroxidase)-conjugated secondary antibody (GE Healthcare) diluted 1:5000 into blocking buffer for 1 hour at 22 °C. Membranes were washed three more times with TBS-T and proteins were detected using the ECL plus detection system (GE Healthcare). To ensure successful fractionation, the inventors probed with antibodies specific  
15 to the outer membrane protein OmpA (a gift from the Silhavy lab, 1:300,000, 1h at 22°C), the periplasmic protein PhoA (Acris, 1:1000, 1h at 22°C), the inner membrane protein SecE (a gift from the Collinson group, 1:10000, 1h at 22°C) or the cytoplasmic protein RNA-polymerase (Acris, 1:1000, 1h at 22°C).

**Protease protection experiments.** Cells expressing myc-tagged MAM constructs  
20 were resuspended in PBS to give an OD<sub>600</sub> of 1. Papain was added to final concentrations of 0, 0.1, 1, 10, 100 and 500 µg/ml and cells were incubated at 22 °C for 10 minutes. Reactions were stopped by adding Complete Protease Inhibitor Cocktail (Roche), 5xSDS loading buffer and then boiled for 10 minutes. Samples were separated by SDS-PAGE and myc-tagged proteins were detected by Western Blotting.

**Attachment assays.** Tissue culture cells were washed with PBS (phosphate-buffered saline) prior to the addition of bacteria in tissue culture medium without antibiotics. Bacteria were added to give a multiplicity of infection (MOI) of 10. To determine the exact amount of input, bacteria were added to empty wells. Plates were centrifuged (1000×g, 22 °C, 5 minutes) prior to incubation at 37 °C for 30 minutes to 1 hour. Cells were washed three times  
30 with PBS and lysed by adding 0.5 % Triton X-100 in PBS. Input samples and lysates were serially diluted, plated on either MLB (*V. parahaemolyticus*) or LB (*E. coli*, *Y. pseudotuberculosis*, *V. cholerae*) plates and enumerated by colony counting. For competition experiments, tissue culture cells were incubated with anti-Fn or anti-mouse IgG antibodies



(50 µg/ml in PBS) for 30 minutes prior to attachment experiments. For experiments with phospholipase C (PLC), tissue culture cells were treated with either 10 or 50 µg/ml phospholipase C (Sigma) in PBS for 15 minutes prior to infection. In other cases, bacterial cells were pre-incubated with fibronectin from human plasma (200 µg/ml in PBS) or PBS-  
5 buffered liposomes prepared from 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) or a mixture containing 20 mol% of PC and 80 mol% of 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA) (both Avanti Polar Lipids Inc.) as described below.

**Cytotoxicity assays.** Tissue culture cells were washed with PBS prior to the addition of bacteria in tissue culture medium without antibiotics at an MOI of 10 (*Vibrio*) or an MOI  
10 of 100 (*Yersinia*, EPEC). Infections were started by centrifugation of plates (1000×g, 22 °C, 5 minutes) prior to incubation at 37 °C. 200 µl of supernatant was removed in triplicate from each well at timepoints as indicated, centrifuged (1000×g, 22 °C, 5 minutes), and 100 µl of the supernatant transferred to a fresh 96well plate for assays. To quantitate cell lysis, the inventors measured the amount of lactate dehydrogenase (LDH) released into the culture  
15 medium using the LDH cytotoxicity detection kit (Takara) according to the manufacturer's protocol.

**Gentamycin-protection and TEV-cleavage assays.** Tissue culture cells washed with PBS were incubated with bacteria diluted into DMEM to give an MOI of 10. For gentamycin protection experiments, gentamycin was added to a final concentration of 150 µg/ml at  
20 indicated time points. For TEV-cleavage assays, TEV protease was added to a final concentration of 8 µg/ml at time points as indicated. For 0 minutes time points, gentamycin or TEV protease were added prior to the centrifugation step. Cytotoxicity was determined 4 hours after infection using LDH release assays.

**Expression and purification of recombinant MAM proteins.** *E. coli* BL21 cells  
25 transformed with the appropriate expression construct were grown in LB containing appropriate antibiotics for 16 hours. Fresh LB containing antibiotics as indicated were inoculated with pre-culture at a ratio of 1:100 and grown at 37 °C shaking at 120 rpm until the culture reached an OD<sub>600</sub> of 0.6. Protein production was induced by adding IPTG to a final concentration of 0.4 mM and cells were grown for a further four hours at 37 °C. Cells  
30 were harvested by centrifugation and the pellet resuspended in 5 volumes of binding buffer (20 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM imidazole for MBP-MAM constructs or 20 mM Tris-HCl pH 7.5, 150 mM NaCl for GST-MAM7). PMSF (phenylmethylsulfonyl fluoride), lysozyme and MgCl<sub>2</sub> were added to concentrations of 1 mM, 2 mg/ml and 5 mM,

respectively, and after 30 minutes of incubation on ice the suspension was sonicated on ice for a total time of 2 minutes (3 sec. on, 7 sec. off, 70 % output) using a Sonicator3000 (Misonix) and centrifuged (30 min., 8000×g, 4 °C). For MBP-MAM constructs, the supernatant was loaded onto a 5 ml HisTrap HP column (GE Healthcare) charged with 3 column volumes (c.v.) of 50 mM NiSO<sub>4</sub> and equilibrated with 3 c.v. of binding buffer. The column was washed with binding buffer at a flow rate of 1.5 ml/min until the absorbance of the eluate went back to background levels before bound protein was eluted using a linear gradient of 5-500 mM imidazole over 10 c.v. Fractions were analyzed by SDS-PAGE and those containing the protein of interest were pooled and dialyzed against gel-filtration buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl). Dialyzed fractions were further purified by gel-filtration on a Superdex S75 HL 16/60 column (GE Healthcare) equilibrated with 2 c.v. of gel-filtration buffer using a flow rate of 1 ml/min. Fractions containing pure protein of interest were pooled, concentrated to 100 μM and buffer-exchanged against 20 mM Hepes pH 7.0.

**Fluorescent labeling of recombinant MAM proteins.** MBP-MAM proteins were labeled on a single cysteine residue introduced between the MBP tag and MAM protein sequence using Alexa Fluor® 488 C<sub>5</sub> maleimide (Invitrogen). For labeling, proteins were incubated in 20 mM Hepes pH 7.0 with a 10-fold molar excess of TCEP (tris(2-carboxyethyl)phosphine) at 4°C for 2 hours, followed by a 5-fold molar excess of Alexa Fluor® 488 C<sub>5</sub> maleimide at 37 °C for one hour. The reaction was quenched by adding an excess of beta-mercaptoethanol. Excess dye was removed and protein was buffer-exchanged into PBS using centrifugal filters (Millipore). Protein labeling was determined to be more than 95 % efficient using mass spectrometry.

GST-MAM7 lysate was prepared as described above, but using 20 mM Tris-HCl pH 7.5, 150 mM NaCl as resuspension buffer. The cleared lysate was purified using 1ml glutathione-sepharose beads (GE Healthcare) equilibrated in binding buffer. Bound protein was washed with 40 c.v. of binding buffer and eluted with 5 c.v. of 20 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.1 % beta-mercaptoethanol, 0.3 % glutathione. After buffer exchanging, the protein was further purified by gel-filtration as described for MBP-MAM proteins.

**Fluorescence attachment assays.** HeLa cells were cultured in sterilized Corning costar 96 well plates (15 000 cells/well) for 16 hours prior to experiments. Alexa-labeled proteins were diluted into PBS to give final concentrations of 0.1-100 μM and incubated with cells washed with PBS for 1 hour at 37 °C. Initial fluorescence and fluorescence output were

measured on a plate reader ( $\lambda_{\text{excitation}}$  485 nm,  $\lambda_{\text{emission}}$  520 nm) prior to and following three washes with PBS, respectively. Data was expressed as % bound fluorescence and corrected for values determined for MBP alone. Levels of bound protein were blotted as a function of protein concentration and data were fit to a single-site binding model using Sigma Plot. For fluorescence attachment assays on trypsinized cells, cells were detached from the culture dish by trypsinization, which was stopped by adding DMEM after 5 minutes. Cells were adjusted to 600,000/ml in PBS and incubated with 100  $\mu$ M labeled MBP-MAM7 or MBP in PBS for 30 minutes at 37 °C. Fluorescence of the cell suspension was measured before and after extensive washing with PBS as described above.

**Competitive binding experiments.** MBP-MAM proteins were prepared in DMEM at concentrations between 0 and 300  $\mu$ M, added to HeLa cells cultured at a density of 150,000 cells/ml. After incubation at 37 °C for one hour, *E. coli* BL21 cells expressing MAM7 were added to an MOI of 1, centrifuged to enable attachment and incubated a further hour at 37 °C. Cells were washed three times with PBS and lysed by adding PBS containing 0.5 % Triton X-100. Lysates were serially diluted and the number of attached bacteria was determined by plating and counting. The number of attached bacteria was expressed as percentage of input (also quantified by dilution plating).

**Determination of competitive index.** Bacterial cultures of *E. coli* BL21 expressing MAM7 $\Delta$ N<sub>1-44</sub>, MAM6 and MAM1 were mixed with a strain expressing MAM7 and empty pGEX plasmid, which was used as an additional ampicillin resistance marker, and incubated with cultured 3T3 fibroblasts at an MOI of 10 for each strain at 37 °C for one hour. Cells were washed three times with PBS, lysed and bacteria were quantitated by dilution plating. Lysates were double plated on both LB containing kanamycin and LB containing kanamycin and ampicillin. Competitive indices were calculated as follows:

$$\text{C.I.} = \text{cfu of mutant/cfu of wild-type bacteria} = \frac{[(\text{cfu on kan}) - (\text{cfu on kan+amp})]/(\text{cfu on kan+amp})}{\text{cfu on kan+amp}}$$

**Competition experiments with BL21 and pathogenic bacteria.** HeLa cells were cultured on cover slips at 150,000 cells/ml and incubated with *E. coli* BL21 expressing MAM7 or MAM7 $\Delta$ N<sub>1-44</sub> (MOI 100) for one hour at 37 °C. Cells were washed three times with PBS and infected with pathogenic bacteria for four hours. After four hours, replicate wells of cells were either lysed or washed with PBS and fixed with 3.2 % paraformaldehyde in PBS for 15 minutes. The culture supernatants from lysed cells were used to determine cytotoxicity using LDH-release assays. Fixed cells were permeabilized with 0.1 % Triton X-

100 in PBS for 3 minutes and treated with Hoechst (Sigma) and rhodamine-phalloidin (Molecular Probes) for 10 minutes to stain for DNA and actin, respectively. Cover slips were mounted onto 10 % (w/v) glycerol and 0.7 % (w/v) propyl gallate in PBS, sealed with nail polish and viewed using a Zeiss LSM510 META Laser Scanning Confocal Microscope.

5 Images were processed using ImageJ and Photoshop software.

**Pull-down and plate assays with fibronectin.** For pull-down experiments, equimolar mixtures of purified fibronectin derived from human plasma (Fisher Scientific) and either GST-MAM7 or GST (control) were incubated in 10 mM phosphate buffer pH 7.5 containing 150 mM NaCl at 22 °C for 30 minutes. 10 % of the sample volume were retained as loading control, while the residual sample was incubated with 0.2 volumes of glutathione-sepharose beads equilibrated in assay buffer at 22 °C for one hour. Beads were washed three times with 0.9 volumes of assay buffer prior to boiling in 0.9 volumes of SDS loading buffer. Equal volumes of loading control, washes and eluate were analyzed by SDS-PAGE and Coomassie staining. For plate assays, Alexa Fluor® 488-labeled MBP-MAM proteins were diluted into 10 PBS to give final concentrations between 0.1 and 100 µM. Proteins were incubated in human fibronectin coated 96-well plates (R&D systems) at 22 °C for one hour. Fluorescence levels were measured prior and after washing plates three times with PBS. Data were analyzed as described for fluorescence attachment assays.

**Lipid overlay assays.** Pre-spotted PIPstrips™ (Echelon Biosciences Inc.) were incubated in T-TBS containing 5 % dry milk powder for one hour at 22 °C. MBP-MAM proteins were diluted into blocking buffer to give a final concentration of 10 µM and incubated with the PIP strip for one hour at 22 °C. Strips were washed with T-TBS three times for 10 minutes each and incubated with His antibody (QIAGEN) diluted 1:1000 into blocking buffer at 22 °C for one hour. Strips were washed three times with T-TBS and incubated with anti-mouse HRP-conjugated secondary antibody (GE Healthcare) diluted 1:5000 into blocking buffer for 1 hour at 22 °C. After three more washes, bound proteins were detected using the ECL plus detection system (GE Healthcare).

**Liposome association experiments.** PBS-buffered liposomes were prepared from 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) or mixtures of PC and 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA) (both Avanti Polar Lipids Inc.) as described previously (Selyunin *et al.*, 2011), but using PBS as buffer. 300 µg liposomes were incubated with 100 µg MBP-MAM protein in PBS for one hour at 22 °C. Mixtures were centrifuged at 100 000×g, 4 °C

for one hour and both pellet and supernatant fractions were separated by SDS-PAGE and proteins were detected by Coomassie staining.

**Nematode lethality assays.** 150 µl bacterial culture were spotted on NGM (nematode growth medium) plates and incubated at 30 °C for 16 hours prior to transfer of 20 to 30  
5 synchronized L4 stage worms onto the bacterial lawn. *C. elegans* germline deficient mutant SS104 *glp-4(bn2)* strain was used for all experiments. Prior to seeding, worms were maintained at 15 °C on nematode growth medium (NGM) agar plates and fed on *E. coli* HB101. The worms were washed with M9 buffer twice to remove surface-bound bacteria prior to plating. Plates containing worms were maintained at 25 °C to inhibit egg laying  
10 during the experiment and scored for dead worms every 24 hours. Worms were considered dead when they no longer responded to touch. For complementation experiments, kanamycin was added to plates prior to spotting bacterial cultures to maintain the plasmid. Data was analyzed using the Kaplan-Meier method and survival curves were compared using the logrank test. For microscopy, worms were fed on test strains for 48 hours, mounted on 2 %  
15 agarose pads and observed under Nomarski optics using a Zeiss Axioplan microscope.

**Statistical analysis.** Statistical significance was analyzed using the unpaired two-tailed t-test. All experiments were done at least three times in triplicate, except for attachment experiment, which were done three times in duplicate and attachment experiment with recombinant proteins, which were done twice in duplicate. All values are given as means ±  
20 standard deviation, unless otherwise indicated. For nematode lethality assays, data were plotted according to the Kaplan-Meier method and survival curves were compared using the logrank test. Statistical significance was set at  $P < 0.005$ . RIMD ( $P < 0.0001$ ), POR1 ( $P < 0.0001$ ), POR1ΔMAM7 ( $P = 0.99$ ), POR1ΔMAM7 +pMAM7 ( $P = 0.0002$ ) were compared with HB101.

## 25 Results

Using bioinformatics, the inventors searched the genome of *Vibrio parahaemolyticus*, a Gram-negative bacterium that occurs in marine and estuarine environments and can cause shellfish-borne food-poisoning, for a constitutively expressed protein that might be involved in the initial binding of bacteria to a host cell (Daniels *et al.*, 2000). The inventors discovered  
30 a predicted outer membrane molecule that the inventors have called multivalent adhesion molecule (MAM) that includes a putative transmembrane motif followed by six (MAM6) or seven (MAM7) mammalian cell entry (mce) domains (FIG. 1A, FIG 7A). Unexpectedly, the inventors found that MAM6 or MAM7 is encoded in a wide range of Gram-negative animal

pathogens but not Gram-positive or plant pathogenic bacteria (FIG. 1A, FIG 7B, FIG. 8). In contrast, proteins containing a single mce domain are wide-spread (FIG. 1A). In *Mycobacterium ssp.* and some Gram-positive bacteria, such as *Rhodococcus ssp.* or *Streptomyces ssp.*, the mce domain occurs in conjunction with a second domain of unknown function (DUF3407) (Arruda *et al.*, 1993; Chitale *et al.*, 2001). Proteins containing one mce domain and a C-terminal low complexity region are thought to represent an accessory component of ABC transporters occurring in algae, higher plants and bacteria (Awai *et al.*, 2005). Herein, the inventors tested whether MAMs, which constitute a novel class of predicted outer membrane proteins from Gram-negative bacteria, are involved in cellular attachment. Initially, the inventors used *V. parahaemolyticus* as the representative Gram-negative bacterium for analysis of MAM7s and followed these studies with analyses on the role of MAMs in attachment for other Gram-negative pathogens (Daniels *et al.*, 2000).

**MAM7 is a novel outer membrane protein mediating host cell attachment.** The inventors analyzed the intracellular localization of *V. parahaemolyticus* MAM7 (VP1611) by replacing the endogenous gene with a plasmid-borne, C-terminally myc-tagged version of MAM7 under the control of its endogenous promoter using a non-cytotoxic strain of *V. parahaemolyticus* [POR2 (Park *et al.*, 2004)], (FIG. 9). MAM7-myc was constitutively transcribed when the strain was grown in marine LB (MLB, FIG. 9) and, based on subcellular fractionation, localizes exclusively to the outer membrane (FIG. 1B). Outer membrane localization was also observed after arabinose-induced, heterologous expression of *V. parahaemolyticus* MAM7-myc in *E. coli* strain BL21 (FIG. 1C), which does not contain a MAM7 ortholog. The first 44 N-terminal amino acids of MAM7 contain a stretch of hydrophobic residues (aa. 21-40) predicted to form a transmembrane helix. Deletion of the N-terminal 44 amino acids (MAM7 $\Delta$ N<sub>1-44</sub>-myc) lead to cytoplasmic retention of the protein (FIG. 1D). To assess whether MAM7 was on the surface of the bacteria, BL21-MAM7 and BL21-MAM7 $\Delta$ N<sub>1-44</sub>-myc were tested for protease sensitivity. Treatment of these strains with increasing concentrations of papain lead to a gradual loss of the epitope tag on cells expressing MAM7-myc, but not MAM7 $\Delta$ N<sub>1-44</sub>-myc, further supporting the localization of the C-terminal epitope to the extracellular space (FIG. 1E). To explore if the N-terminal sequence is embedded in the outer membrane, the inventors introduced a TEV protease-cleavable peptide between the hydrophobic N-terminal sequence (res. 1-44) and the mce domains (N<sub>1-44</sub>-TEV-MAM7-myc). The protein expressed from this construct was correctly localized and successfully cleaved by TEV protease incubated with intact bacteria (FIG. 1F),

demonstrating that the N-terminal peptide contains the information necessary for outer membrane targeting and membrane anchored of MAM7, while the rest of the protein is exposed extracellularly.

Having established that MAM7 is an outer membrane protein, the inventors next tested whether MAM7 is important for early attachment of *V. parahaemolyticus* to host cells. Using *V. parahaemolyticus* POR2 (Park *et al.*, 2004) and the POR2 $\Delta$ MAM7 derivative, the inventors observed that in the absence of MAM7, attachment of *V. parahaemolyticus* was decreased from approximately 80% to ~35-40% for all tested host cell lines, including HeLa and Caco-2 epithelial cells, RAW264.7 macrophages and 3T3 fibroblasts. The attachment of POR2 MAM7 was recovered by a plasmid expressing MAM7 with or without a TEV cleavage site or myc tag (FIG. 1G, FIGS. 10A-B). The non-adherent BL21 strain was converted to an adherent strain by inducing expression of *V. parahaemolyticus* MAM7, but not by the mutant MAM7 $\Delta$ N<sub>1-44</sub> (FIG. 1H). The inventors conclude that MAM7 contributes to the attachment of *V. parahaemolyticus* to a broad range of mammalian cells and is sufficient to mediate efficient cellular attachment of a Gram-negative strain in the absence of other adhesion proteins.

**MAM7-mediated attachment augments type-III-mediated cell death.** The translocation of effector proteins to manipulate host signaling pathways is a key step in the pathogenesis of many Gram-negative organisms. *V. parahaemolyticus* features two type III secretion systems (T3SS) that translocate at least eight different effector proteins into the host cytosol with the aim to alter the cellular response to infection to the pathogen's advantage (Burdette *et al.*, 2008; Broberg *et al.*, 2010). The inventors hypothesized that intimate association that can be mediated through a variety of adhesion mechanisms between pathogen and host is a prerequisite for successful T3SS-effector translocation during infection. POR1, a *V. parahaemolyticus* strain containing both T3SSs but lacking the thermostable direct hemolysins *tdhA* and *tdhS*, causes T3SS-dependent cell lysis within 2-3 hours after infection (Park *et al.*, 2004; Burdette *et al.*, 2008). To test the contribution of MAM7 in POR1-mediated cell lysis, the inventors created a POR1 strain deleted for MAM7 (POR1 $\Delta$ MAM7, FIG. 9) and a POR1 $\Delta$ MAM7 strain complemented with MAM7 containing a TEV-cleavable sequence inserted between residues 44 and 45 and a C-terminal myc tag (N<sub>1-44</sub>-TEV-MAM7-myc). The N<sub>1-44</sub>-TEV-MAM7-myc protein was confirmed to localize to the outer membrane (FIG. 10B). In addition, treatment of the POR2 $\Delta$ MAM7+N<sub>1-44</sub>-TEV-MAM7-myc strain with TEV-protease for five minutes resulted in a strain that displayed a decrease in attachment

comparable to that observed for POR2 $\Delta$ MAM7 (FIG. 10A). The inventors next used the POR1 strains to assess the contribution of MAM7 attachment to host cell cytotoxicity during *V. parahaemolyticus* infections.

Gentamycin protection assays were used to assess how long bacteria must remain attached to induce 100% lysis. *V. parahaemolyticus* strains were used to infect 3T3 cells and at various time points during the infection gentamycin was added. After four hours of infection, the cells were tested for cell lysis using an LDH release assay. When gentamycin was added at the start of the infection (0 minutes) minimal lysis was observed after 4 hours, whereas if no gentamycin was added almost 100% lysis was observed after the same time. When infected cells were treated at 10, 30, 60 or 90 minutes after infection with gentamycin, minimal lysis was observed. These results support the hypothesis that *V. parahaemolyticus* has to remain associated with 3T3 fibroblasts for at least 90 minutes to efficiently mediate T3SS-induced cell lysis (FIG. 2A). To test if MAM7-mediated attachment contributes to host cell binding through this initial phase of infection, the inventors performed infections using the POR1 $\Delta$ MAM7+N<sub>1-44</sub>-TEV-MAM7 strain and treated cells with TEV protease at various time points during the infection. Cell lysis was measured at four hours after infection. As predicted, cells infected with either POR1 strain or the POR1 $\Delta$ MAM7+N<sub>1-44</sub>-TEV-MAM7 strain, but not the POR1 $\Delta$ MAM7 strain, displayed 100% cell lysis. However, POR1 $\Delta$ MAM7+N<sub>1-44</sub>-TEV-MAM7 infected cells treated with TEV protease immediately, 10 or 30 minutes after the start of infection showed a decrease of ~ 40% cytotoxicity (FIG. 2B). This level of toxicity is comparable to that observed with the POR1 $\Delta$ MAM7 strain, supporting the hypothesis that MAM7 is playing an important role in attachment during the early stages of infection.

To further investigate the role of MAM7 in adhesion and cytotoxicity, the inventors performed time course infection studies on a range of mammalian cell lines using POR1, POR1 $\Delta$ MAM7 and POR1 $\Delta$ MAM7 complemented strains. The POR1 $\Delta$ MAM7 strain lysed 3T3 fibroblasts and RAW264.7 macrophages less efficiently than either POR1 or POR1 $\Delta$ MAM7+ MAM7 (FIGS. 2C-D). In addition, the onset of lysis induced by the POR1 $\Delta$ MAM7 strain was delayed by 30-40 minutes (FIGS. 2C-D). In contrast, no significant difference was observed between POR1 and POR1 $\Delta$ MAM7 in either Caco-2 or HeLa epithelial cells (FIGS. 2E-F). In total these results support the hypothesis that, with some cell lines, MAM7 mediated attachment plays an important role in mediating the initial attachment of bacteria to host cells during the early stages of infection. It also supports the hypothesis



that other molecules, some of which might be host cell type dependent, are playing a role in attachment during later stages of infection with *V. parahaemolyticus*.

**MAM7 is required for *V. parahaemolyticus*-induced pathogenicity in *C. elegans*.**

The nematode *C. elegans* has been used as a model host for a variety of bacterial pathogens, including *Vibrio cholerae* and *Vibrio vulnificus* (Vaitkevicius *et al.*, 2006; Dhakal *et al.*, 2006). In the absence of a relevant animal model for *V. parahaemolyticus*, the inventors tested whether MAM7 mediated adhesion plays a role during infection of nematodes. Synchronized germline-deficient L4 stage worms were fed with either RIMD 2210633, POR1, POR1 $\Delta$ MAM7 or POR1 $\Delta$ MAM7+ MAM7 strains. Worms fed either non-pathogenic *E. coli* HB101 or POR1 $\Delta$ MAM7 exhibited a normal life expectancy profile (FIG. 3A). RIMD 2210633 and POR1-infected worms displayed severe phenotypic changes by day 2 of feeding including growth retardation, and increased frequency in distention of the intestinal tract leading to abdominal rupture, and died at a much faster rate than worms fed either HB101 or POR1 $\Delta$ MAM7 (FIGS. 3A-C). Both RIMD 2210633 and POR1 killed the worms within 13 days, supporting the hypothesis that most of the lethality is mediated by T3SS effectors rather than the thermostable hemolysins (Park *et al.*, 2004). Full virulence was reconstituted in the POR1 $\Delta$ MAM7 by reconstituting the strain with plasmid-encoded MAM7 (FIG. 3A). Analysis of worms for abdominal rupture at day seven revealed a significant increase in injury of the RIMD 2210633 and POR1 fed worms (FIG. 3D). Overall, the results support the hypothesis that MAM7 adhesion plays an important role during pathogenicity in the nematode infection model.

**Multiple mce-domains are required to mediate stable attachment of MAM to host cells.** The number of tandem mce domains in pathogenic Gram-negative bacteria is strikingly constant (always 6-7 domains). The inventors therefore hypothesized that 6-7 mce domains are the minimum number of domains required for stable host cell attachment and thus explored the relation between domain number and host cell affinity. The inventors produced recombinant proteins containing one, two, six or seven mce domains in tandem with a maltose binding protein (MBP)-tag and a single cysteine residue between mce-domains and MBP-tag to allow labeling of the proteins with a single fluorophore. The amount of protein bound to host cells was measured using fluorescence spectroscopy and the affinities of individual constructs was determined using saturation binding experiments. The affinity increased non-linearly with the number of mce-domains, with equilibrium dissociation constants ranging from  $15 \pm 3 \mu\text{M}$  for one mce domain to  $0.2 \pm 0.1 \mu\text{M}$  for seven

domains in tandem (FIG. 4A). The affinities of MAM proteins for host cells were also determined indirectly, using unlabeled MAM proteins to block the host cell surface prior to measuring residual binding of *E. coli* BL21 expressing MAM7. Affinities determined by this method were consistent with those determined by fluorescence assays (FIG. 4B).

5 Having established the binding affinities for proteins containing varying numbers of mce domains, the inventors analyzed whether strains expressing one mce domain were able to compete for host adhesion with strains expressing six or seven domains in tandem. All MAM constructs used were correctly localized to the outer membrane, as shown by subcellular fractionation and attachment experiments (FIGS. 11A-B). *E. coli* BL21 strains  
10 expressing MAM1, MAM6 or MAM7 $\Delta$ N<sub>1-44</sub> were mixed with a strain expressing MAM7 and the ratio of attachment to host cells was determined as competitive index between the strains. While MAM6 and MAM7 were similarly effective in conferring adhesive properties on *E. coli* BL21 (C.I. of 0.8), MAM1 and MAM7 $\Delta$ N<sub>1-44</sub> were far less competitive (C.I. of 0.27 and 0.19, respectively) (FIG. 4C). Next, the inventors investigated the ability of *E. coli* BL21  
15 expressing MAM1, MAM6 or MAM7 to inhibit attachment of and cytolysis by *V. parahaemolyticus* POR1. When POR1 infections were performed in the presence of *E. coli* BL21 expressing MAM7 or MAM6 at an identical multiplicity of infection, the attachment of POR1 and thus pathogen-mediated cytotoxicity was significantly decreased. Addition of BL21 expressing MAM1 or MAM7 $\Delta$ N<sub>1-44</sub>, however, had little or no effect on the outcome of  
20 a POR1 infection (FIG. 4D). These data demonstrate how the requirement for high affinity attachment to host cells necessitates the presence of a high number of mce domains. While the inventors observed low affinity binding with one or two mce domains, the expression of six or seven domains in tandem results in a steep increase in affinity enabling the bacterial strain expressing these constructs to successfully compete for host binding. Due to problems  
25 with misfolding and insolubility, the inventors could not study constructs containing between three to five mce repeats, and hypothesize that such proteins might not occur in nature for similar reasons.

**MAM7 establishes both protein-protein and protein-lipid interactions with host cells.** The secondary structure of MAM7 is predicted to be rich in beta-strands connected by  
30 flexible loop regions, a composition similar to that of fibronectin-binding proteins from Gram-positive bacteria (Schwarz-Linek *et al.*, 2003). The inventors therefore tested if MAM7 could also bind fibronectin. Immobilized GST-MAM7 but not GST alone was able to pull-down purified fibronectin from human plasma (FIGS. 5A-B). Titrations of fluorophore-

labeled MBP-MAM7 against immobilized Fn showed the interaction between hFn and MAM7 to be of moderate affinity ( $K_D$  of  $15 \pm 4 \mu\text{M}$ ), while no measurable interaction was detected between fibronectin and MAM1 (FIG. 5C). To further explore the possibility that MAM7 binds fibronectin on cells, the inventors treated the cells with trypsin to degrade  
 5 extracellular proteins and then assessed whether MAM7 could bind to cells. Cells treated with trypsin reduced the number of MAM7 molecules on cells by over 100-fold (FIG. 5D). Furthermore, the specificity of binding by MAM7 to fibronectin on cells was shown by either blocking MAM7 binding to cells with an anti-fibronectin antibody or by competing with soluble fibronectin (FIG. 5E).

10 *Arabidopsis* Tgd2 is the substrate-binding component of a chloroplast lipid transporter which is involved in phosphatidic acid (PA) trafficking and necessary for biogenesis of thylakoid membrane lipids (Awai *et al.*, 2006). Tgd2 contains a single mce domain which was shown to display weak binding to phosphatidic acid (Lu and Benning, 2009). Using lipid overlay assays, the inventors showed that both MAM1 and MAM7 bound to PA, while no  
 15 binding was observed with the MBP-tag alone (FIGS. 5F-H, FIGS. 12A-C). The inventors compared the binding affinities for MAM1 and MAM7 to PA using liposome association assays (FIG. 5I). While MAM7 showed stoichiometric binding to PA when present in the liposomes at concentrations as low as 1 mol%, MAM1 only bound to liposomes containing at least 3 mol% PA. To assess whether MAM7 binding to PA occurs *in vivo*, the inventors  
 20 analyzed binding of MAM7 to 3T3 cells incubated without and with prior phospholipase C (PLC) treatment. In the presence of PLC, PA is converted to diacylglycerol and the inventors observed that binding of BL21 expressing MAM7 to cells is compromised (FIG. 5J). To further assess whether MAM7 is binding to PA on cells, the inventors pre-incubated BL21-MAM7 with phospholipids and assessed attachment of the bacteria to 3T3 cells. BL21-MAM7  
 25 could bind to cells after incubation with liposomes containing 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) but not after incubation with liposomes containing 20 mol% of PC and 80 mol% PA (FIG. 5K). These observations further support the model that MAM7 binds PA on the surface of cells.

**Non-pathogenic *E. coli* heterologously expressing MAM7 ameliorates the effects**  
 30 **of infection by Gram-negative pathogens.** Since the inventors successfully used non-pathogenic *E. coli* BL21 expressing MAM7 to inhibit infection by *V. parahaemolyticus* POR1 (FIG. 4D and FIG. 6B, FIGS. 13G-I), the inventors hypothesized that pre-incubation of host cells with BL21 expressing MAM7 would ameliorate infections caused by a broad range of pathogenic Gram-negative strains that are predicted to encode MAM7. The inventors

investigated the protective effect of *E. coli* BL21 + MAM7 upon infection with *V. parahaemolyticus* RIMD 2210633 strain that is equivalent to POR1 but features two thermostable direct hemolysins that are thought to contribute to cell lysis (Nishibuchi *et al.*, 1992) (FIG. 6A, FIGS. 13D-F), *V. cholerae* El Tor N16961 (FIG. 6C, FIGS. 13J-L), *Y. pseudotuberculosis* YP126 (FIG. 6D, FIGS. 13M-O) and the enteropathogenic *E. coli* (EPEC) strain O127:H6 E2348/69 (FIG. 6E, FIGS. 23P-R). For all infections the inventors performed, the inventors observed a drastic decrease in pathogenicity as manifested either by decreased cytotoxicity (*V. parahaemolyticus*, *V. cholerae* and *Y. pseudotuberculosis*) or decreased actin pedestal formation (EPEC). Pre-incubation with *E. coli* BL21-MAM7 alone did not induce any phenotypic changes in host cells (FIGS. 13A-C), and therefore, residual cytotoxicity was presumably due to soluble toxins being secreted into the extracellular medium. Based on these results the inventors propose that MAM7 expressed on the non-pathogenic *E. coli* strain is masking sites needed by the pathogenic bacteria to initiate binding with the host cells.

We hypothesized that MAM7 from the various species are functionally redundant in that all mediate binding to host cells. To test this, the inventors cloned and expressed the MAM7 from *Y. pseudotuberculosis*, *V. cholerae* or EPEC into non-adhesive *E. coli* BL21. Each of these MAM7 homologues enabled BL21 cells to attach to 3T3 cells at a level similar to that observed for BL21 expressing *V. parahaemolyticus* MAM7 (FIG. 6F). To assess whether MAM7 from *Y. pseudotuberculosis*, *V. cholerae* or EPEC plays a role in host cell adhesion during infection, the inventors created MAM7 deletion strains for each of these pathogens (*YpΔMAM7*, *VcΔMAM7*, *EPECΔMAM7*) and then reconstituted the MAM7 deletion strains with a wild-type copy of MAM7 (*YpΔMAM7*+MAM7, *VcΔMAM7*+MAM7, *EPECΔMAM7*+MAM7). After incubating these various strains with 3T3 cells, the inventors observed that attachment is compromised for the MAM7 deletion strains, but not for the wild-type or reconstituted deletion strains (FIG. 6G). The inventors next tested whether the absence of MAM7 might attenuate cell culture cytotoxicity induced by *Y. pseudotuberculosis* and *V. cholerae* or reduce pedestal formation by EPEC. 3T3 cells infected with either *YpΔMAM7* or *VcΔMAM7* displayed reduced cytotoxicity over time when compared to wild-type or reconstituted *Y. pseudotuberculosis* and *V. cholerae* strains (FIGS. 6H, 6I). When 3T3 cells were infected with *EPECΔMAM7*, a reduced number of pedestals was observed over time when compared to wild-type or reconstituted EPEC. Interestingly, the phenotype induced by the pathogen appears only to be attenuated during the early time points in the

infection (FIG. 6J). In agreement with findings from others, the attachment at the later times of infection is mediated by adherence molecules induced during infection, such as *Yersinia* invasin, *Enterococcus ace*, or type IV pili (Lebreton *et al.*, 2009; Herooven and Dersch, 2006; Boekema *et al.*, 2004).

5           One of the pathogens the inventors focus on is *Pseudomonas aeruginosa*. *P. aeruginosa* thrives in most environments, including water, soil and on human skin. In immunocompromised patients, it can cause catheter-associated lung and urinary tract infections, but it is also a major burden for cystic fibrosis patients and can cause persistent wound infections, for example in burn patients (Hoiby, 2011; Branski *et al.*, 2009). Due to its  
10 clinical importance, the inventors studied if *P. aeruginosa*-mediated cytotoxicity could be attenuated by MAM7 in a tissue culture model of infection (FIGS. 14A-D). The second focus of the inventors' current studies in MAM7-based inhibitors is dedicated to finding alternative modes of delivery. While non-pathogenic bacteria expressing surface-bound MAM7 may be a suitable vehicle for gastrointestinal delivery of MAM7 to prevent or combat enteric  
15 pathogens, their use on an open wound would most likely exacerbate inflammatory responses and could therefore have adverse effects on wound healing. The inventors are therefore studying alternative modes of delivery for MAM7 to the site of infection. One such approach is to immobilize recombinant MAM7 on the surface of inert polymer beads, which are similar in size to the bacteria the inventors have previously used (1  $\mu$ m). The inventors tested the  
20 efficacy of bead-immobilized MAM7 against *P. aeruginosa* infection of epithelial cells and compared it to control beads displaying GST, which do not bind to host cells (FIGS. 1A-D). In each case, the inventors counted the number of bound beads per cell (fluorescent beads were used for ease of visualization) and determined the cytotoxic effect of *P. aeruginosa* using lactate dehydrogenase (LDH) release assays. Upon infection, host cells lyse and release  
25 LDH into the culture medium, which can be detected colorimetrically and compared to a standard of detergent-lysed cells (100 % lysis). GST-beads did not show any significant attachment to host cells and failed to inhibit infection (FIGS. 1A, 1C). In contrast, MAM7 beads bound to host cells ( $17.1 \pm 0.9$  beads/cell) and, as a consequence, attenuated *P. aeruginosa*-mediated cell killing (cytotoxicity decreased from 76% to 4%).

30           These studies demonstrate that MAM7-based inhibition may potentially be developed as a tool to attenuate not only enteric pathogens but also hospital-acquired and wound-associated infections, such as those caused by *P. aeruginosa*. The adhesin can be expressed at the surface of non-pathogenic bacteria but may also be delivered by alternative routes, such as immobilized on beads, which may aid in future applications in decreasing risks associated

with the introduction of live bacteria into a living organism. In the future, the inventors hope to be able to extend the application of MAM7 to include other clinically relevant Gram-negative pathogens and develop tools for its efficient delivery to the site of potential infection.

5

### Discussion

Although many bacterial adhesins are known, they are usually species-specific and many are induced during infection (Boland *et al.*, 2000; Kline *et al.*, 2009). Herein, the inventors have described a novel adherence factor, MAM7, used by a wide range of Gram-negative bacterial pathogens to mediate an initial, high-affinity interaction with host cells. While the N-terminal hydrophobic sequence of MAM7 is necessary for outer membrane localization and anchoring of the protein, no dedicated system is necessary for protein transport or membrane insertion, since MAM7 can be heterologously expressed and correctly localized by *E. coli* BL21. MAM7 is used for bacterial adhesion to a variety of mammalian cells and is a crucial factor contributing to host cell infection, as was observed in a tissue culture model for Gram-negative pathogens, including *V. parahaemolyticus*, *Y. pseudotuberculosis*, *V. cholerae* or EPEC. By means of multivalent interactions with the host cell surface, MAM7 contributes to the adherence of bacterial pathogens during the early stages of infection thereby facilitating injection of T3SS effectors into the host cytoplasm. However, specificity of a pathogen for a certain cell type must be mediated by other, strain-specific adhesion molecules, which are most likely strengthened by the initial MAM7 interaction with host cells. The other adhesion factors are likely to dominate attachment during later phases of infection, where MAM7 binding becomes dispensable (FIGS. 2A-F).

The binding of the outer membrane adhesion factor MAM7 to host cells is mediated by a multivalent protein-protein interaction between the adhesin and the extracellular matrix component fibronectin. By contrast, the protein-lipid interaction between MAM7 and membrane-bound phosphatidic acid can be mediated by a single mce domain, albeit at a lower apparent affinity. Therefore, while mce domains have been integrated into a diverse range of proteins, the number of mce domains plays an important role in determining ligand binding affinities and thus the functionality of mce containing proteins: Mce-proteins involved in lipid transport require a low affinity transient interaction with their ligand and contain only one mce domain (*e.g.*, Tgd2). In contrast, constitutively expressed proteins with multiple mce domains appear to mediate an early, high affinity interaction with the host cell surface. The inventors show that this not only increased lipid-binding affinity but also gives

rise to a new functionality (fibronectin-binding) which further strengthens the interaction between MAM7 and host cells. While interactions with extracellular matrix components such as fibronectin are a common strategy employed by both Gram-positive (FnBPs of *Staphylococcus* spp.) and Gram-negative (e.g., *Yersinia* invasin YadA, *Salmonella* MisL or *Campylobacter* CadF) pathogens to achieve host cell adhesion, the direct binding of an adhesin to membrane phosphatidic acid is, to the inventors' knowledge, a novel mechanism for host-pathogen interaction (Froman *et al.*, 1987; Terti *et al.*, 1992; Dorsey *et al.*, 2005; Konkel *et al.*, 1997; Henderson *et al.*, 2011). Although at 2-3 mol% phosphatidic acid is only a minor component of eukaryotic membranes, the inventors showed that these concentrations are sufficient to mediate stable attachment of MAM7. The strategies for protein and lipid interactions are combined in MAM7-mediated adhesion to achieve efficient binding of Gram-negative pathogens to their host.

The inventors show that non-pathogenic BL21 expressing MAM7 can be used to prevent binding of a range of Gram-negative pathogens to host cells, thus offering protection against pathogen-mediated cytotoxicity. Based on bioinformatic analysis, it appears that a large number of Gram-negative pathogens contain a MAM7 (or MAM6) and are predicted to use this protein to mediate the initial, high affinity attachment to host cells. The inventors' studies show that MAM7 molecules from several Gram-negative pathogens, including *V. parahaemolyticus*, *Y. pseudotuberculosis*, *V. cholerae* or EPEC, mediate early attachment of the pathogen to host cells. Based on these studies with aforementioned pathogens, the inventors propose that MAM7 could play an important role during the initial phase of infection for many Gram-negative pathogens that express this adhesion molecule.

The identification and initial characterization of this broadly expressed adhesion factor is important for understanding molecular interactions between Gram-negative pathogens and their target host cells. The adhesion factor MAM7 appears to be constitutively expressed, allowing the bacteria to be primed for immediate attachment when encountering a host cell. The MAM7-mediated attachment appears to be important during the initial phases of infection to allow for the production or presentation of other factors that might be involved in later stages of infection (FIGS. 2A-F, FIGS. 6A-J). These initial studies demonstrate how bacteria expressing MAM7 have an advantage over other bacteria that do not have MAM7 on their cell surface (FIGS. 4A-D). In addition, the multivalent interaction of the mce repeats in MAM7 allows for binding of substrates not recognized by a single repeat. Finally, future microbial and biochemical studies will address the possibility of countering Gram-negative pathogens with their own outer membrane adhesion factor, MAM7, to attenuate infection.

## EXAMPLE 2

### Materials & Methods

**Construction of plasmids.** Cloning of MAM7 for expression in BL21, MBP-MAM7 for fluorophore labeling and GST-MAM7 has been described elsewhere (Krachler *et al.*, 2011). Constructs for GST-MAM6, -mce1-5, -mce2-6, -mce3-7 as well as GST-mce1 to -mce7 were all amplified from *V. parahaemolyticus* POR1 genomic DNA and cloned into the plasmid pGEX-rTEV using BamHI and NotI sites. GST-mce2 point mutants were generated by whole plasmid mutagenesis using GST-mce2 as template. GST-mce1 concatemers containing 3, 5 or 7 mce1 domains were generated by amplifying mce 1 fragments containing the following restriction sites: BamHI/XbaI, XbaI/HindIII, HindIII/XhoI, XhoI/EcoRI, EcoRI/PstI, PstI/NcoI and NcoI/NotI for mce 1 fragments 1-7, respectively, and cloning into plasmid pGEX-rTEV.

**Protein purification.** MBP-His-tagged and GST-tagged proteins were purified using Ni-NTA and glutathione agarose beads, respectively, followed by gel filtration as described previously (Krachler *et al.*, 2011).

**Attachment assays.** Attachment assays with live bacteria or purified labeled protein were carried out as described (Krachler *et al.*, 2011). To determine if attachment was fibronectin- and phosphatidic acid-dependent, tissue culture cells were incubated with anti-Fn antibody (50 µg/ml in PBS, Sigma) or treated with 50 µg/ml phospholipase C (Sigma) in PBS for 15 minutes prior to infection. For attachment of labeled protein in the absence of fibronectin, the inventors used trypsinized cells as described previously (Krachler *et al.*, 2011). To test if bacterial attachment could be abolished using heparin, cells were pre-incubated with heparin at concentrations between 10-500 µM in DMEM for 30 minutes prior to attachment assays.

**Fn pull-down assays.** A detailed protocol for pull-down assays with GST-MAM constructs and fibronectin can be found elsewhere (Krachler *et al.*, 2011). Variations of this protocol included the use of proteolytic fibronectin fragments (30 kDa N-terminal heparin binding domain and 45 ka gelatin binding domain, both from Sigma) or an additional incubation step with an equimolar amount of liposomes (30 min at 22°C). PBS-buffered liposomes were prepared from 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) or mixtures of PC and 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA), (both Avanti Polar Lipids Inc.) as described previously (Selyunin *et al.*, 2011).



**Quantitation of phospholipids.** Liposomes in load, flowthrough and eluate fractions were quantified using the method of Worth and Wright (1977). Briefly, samples were extracted with a mixture of chloroform and methanol, centrifuged and molybdophosphoric acid (Sigma) was added to the organic phase. Samples were centrifuged and the aqueous phase removed. Metol and sodium bisulfate were added to reduce the organic phase and the aqueous phase was removed again following centrifugation. The amount of phospholipid was determined by measuring absorbance at 680 nm and expressed as fraction of the amount detected in loaded fractions.

**Fn plate assay with labeled protein.** 96-well plates coated with 1  $\mu$ g fibronectin per well were incubated with MAM constructs prepared in PBS at concentrations between 0.1 and 100  $\mu$ M at 22°C for one hour. Initial fluorescence and fluorescence output were measured on a plate reader ( $\lambda_{\text{excitation}}$  485 nm,  $\lambda_{\text{emission}}$  520 nm) prior to and following three washes with PBS, respectively. Data was expressed as % bound fluorescence and corrected for values determined for MBP alone. Levels of bound protein were blotted as a function of protein concentration and data were fit to a single-site binding model using Sigma Plot.

**Liposome association assays.** Binding of GST and GST-mce constructs to liposomes were carried out as described in the literature (Krachler *et al.*, 2011). 300  $\mu$ g liposomes containing PC alone or 1-80 mol% PA were incubated with 100  $\mu$ g GST and GST-mce proteins in PBS for one hour at 22 °C. Mixtures were centrifuged at 100,000 $\times$ g, 4 °C for one hour and both pellet and supernatant fractions were separated by SDS-PAGE and proteins detected by Coomassie staining. Band intensities were determined using the gel analysis software UN-SCAN-IT (Silk Scientific Inc.) and intensities of pellet samples (% bound) were expressed as fraction of total intensities (supernatant and pellet samples combined).

## Results

**Phosphatidic acid is essential for MAM7 attachment to host cells, while fibronectin reduces the time required for binding.** The inventors' previous studies showed that two different types of host receptors, the extracellular matrix protein fibronectin and the membrane phospholipid phosphatidic acid, recognize MAM7 adhesin *in vitro* and contribute to attachment *in vivo* (Krachler *et al.*, 2011). When studying MAM7 binding to cells independent of fibronectin, the inventors observed that although no binding was detected without fibronectin after 30 minutes, attachment to cells would gradually take place

if the incubation time was extended. The inventors therefore performed a 3 hour time course experiment looking at binding of *E. coli* BL21 expressing *V. parahaemolyticus* MAM7 on their surface (BL21-MAM7) to Hela cells in the presence and absence of fibronectin (FIG. 15A). Attachment was compared to a negative control of BL21 expressing MAM7 $\Delta$ TM, a  
 5 version of MAM7 missing the N-terminal 44 amino acids containing the translocation and membrane anchoring signal (Krachler *et al.*, 2011).

In the presence of both host receptors, binding of BL21-MAM7 to host cells was highly efficient, with more than 40 % of bacteria binding within the first 10 minutes of incubation (FIG. 15A). Full binding capacity was reached after 30 minutes. When binding to  
 10 fibronectin was blocked with anti-fibronectin antibodies so that binding could be studied independent of the contribution of fibronectin, no bacterial attachment was observed within the first 30 minutes of the experiment and the binding was the same as the negative control (FIG. 15A). However, gradual binding was observed from 40 minutes onwards, and close to full binding capacity was reached after 60 minutes. Overall, binding in the absence of  
 15 fibronectin was delayed by approximately 30 minutes. When the inventors repeated the time course experiment using host cells that were treated with trypsin to degrade fibronectin and purified, fluorophore-labeled MAM7, they obtained similar results showing a delay in host cell binding of approximately one hour (FIG. 15B).

Next, the inventors studied the contribution of phosphatidic acid on host cells to  
 20 MAM7 attachment. They performed similar time course experiments as described for fibronectin, but instead phosphatidic acid was eliminated from the host surface by treatment with phospholipase C (PLC). In the absence of phosphatidic acid, binding of both BL21-MAM7 (FIG. 15C) and labeled MAM7 protein (FIG. 15D) was reduced to background binding for the duration of the experiment (3 hours), similar to that observed with BL21-MAM7 $\Delta$ TM and MBP controls, respectively. The inventors conclude that although both  
 25 fibronectin and phosphatidic acid act as host receptor for MAM7 attachment, the contribution of fibronectin to overall binding is dispensable when more time is allowed for attachment. While phosphatidic acid is essential for MAM7-mediated binding, fibronectin contributes by accelerating bacterial attachment.

30 **A 30 kDa N-terminal fragment of fibronectin is sufficient for binding to MAM7.**  
 Fibronectin is a dimeric 440 kDa glycoprotein involved in many vital processes including cell adhesion, migration, differentiation and wound healing (Grinnell, 1984; Pankov and Yamada, 2002). Each fibronectin molecule is composed of three types of domains denoted type I, type

II and type III repeats, which vary in terms of structural and functional properties (Pankov and Yamada, 2002) (FIG. 16A). To analyze which region of fibronectin is involved in MAM7 binding, the inventors performed pull-down experiments with GST-MAM7 and either full-length soluble fibronectin, a 30 kDa proteolytic fragment containing the N-terminal 5 type I repeats (I<sub>1-5</sub>, heparin binding region I) or a 45 kDa fragment containing repeat I<sub>6</sub>, II<sub>1-2</sub> and I<sub>7-9</sub> (gelatin and collagen binding region, FIG. 16A). Both full-length fibronectin and the 30 kDa fragment, but not the 45 kDa fragment, were pulled-down by GST-MAM7 (FIG. 16B-D). As negative control, the inventors used MAM1, which does not bind fibronectin (Krachler *et al.*, 2011) and no interaction with the 30 kDa fragment was observed (FIG. 16E). Since the 30 kDa fragment has been shown to bind heparin (Ingham *et al.*, 1990), the inventors tested whether attachment of BL21-MAM7 to Hela cells would be inhibited by adding heparin to the attachment assay. While in the absence of heparin ~80% of bacteria attached to the host cells within 30 minutes, the addition of increasing concentrations (10-500  $\mu$ M) of heparin from intestinal mucosa gradually blocked attachment, with only ~40% of BL21-MAM7 remaining attached at 500  $\mu$ M heparin (FIG. 16F). This demonstrates that the N-terminal region of fibronectin encompassing repeats I<sub>1-5</sub> mediates MAM7 attachment and that early binding of bacteria could be blocked by adding the competing fibronectin ligand heparin.

**At least 5 tandem mce domains are required for stable binding to fibronectin.**

As previously described, full-length MAM7 can stably bind to fibronectin, while no binding was detected with a construct containing only the first N-terminal mce domain of MAM7 (MAM1) (Krachler *et al.*, 2011). To further delineate the region of MAM7 required for fibronectin binding, the inventors performed pull-down experiments of fibronectin with GST-tagged proteins containing all seven mce domains (MAM7) or successive truncations of mce domains from the C-terminus (designated MAM6 to MAM1). Both load and eluates were analyzed by SDS-PAGE and Coomassie staining. Fibronectin was only pulled down by GST-MAM7, GST-MAM6 and GST-MAM5, while no appreciable interaction was observed with GST-MAM1 to -MAM4 (FIG. 17A). Affinity measurements using immobilized fibronectin and fluorophore-labeled MAM constructs showed that MBP-MAM7 bound fibronectin with a  $K_D$  of  $15 \pm 3 \mu$ M, while the affinity was decreased for MBP-MAM6 ( $K_D = 36 \pm 9 \mu$ M). No interaction could be detected with either MBP-MAM1 or MBP-MAM2 (FIG. 17B). The inventors could not determine affinities for MAM3-MAM5, as MBP-tagged constructs were unstable and selective thiol-labeling is unfeasible with GST-tagged proteins (GST itself contains cysteins). To determine if one mce domain specifically was responsible for

mediating the interaction with fibronectin, the inventors used GST-constructs of all seven individual mce domains for pull-down experiments. Individual mce domains were autonomously folded into a mixed  $\alpha/\beta$  structure, as determined by NMR (unpublished observation). This approach showed that none of the single mce domains was bound by  
5 fibronectin (FIG. 3C). However, several constructs containing stretches of five mce domains (mce 1-5, mce 3-6 and mce 2-7) efficiently interacted with fibronectin in pull-down assays (FIG. 17D). These data support the idea that all mce domains contributed to binding, but at least five domains in tandem were required to achieve detectable binding affinity. To further  
10 test this, the inventors constructed concatemers containing 3, 5 or 7 identical mce 1 domains and analyzed their interaction with fibronectin using pull-downs. Concatemers containing 5 or 7 mce 1 domains, but not the concatemer containing 3 mce 1 domains, were able to pull-down fibronectin (FIG. 17E). Taken together, these findings are consistent with the hypothesis that, in principle, all mce domains can contribute to fibronectin binding, but at least five domains together are required to achieve a high affinity interaction.

15       **Key basic residues modulate binding affinity of mce domains to phosphatidic acid.** As described above, phosphatidic acid is essential for stable binding of MAM7 to host cells. Although the inventors had previously shown that MAM1 is sufficient to bind to phosphatidic acid, it was unclear how the other mce domains contributed to phosphatidic acid binding in the absence of fibronectin. The inventors tested all seven individual mce domains  
20 (mce1-mce7) for phosphatidic acid binding using liposome association assays. Proteins were incubated with liposomes containing a mixture of PC and increasing amounts (1-80 mol%) of PA, followed by separation of liposome-bound and un-bound fractions by ultracentrifugation. All fractions were analyzed by SDS-PAGE (data not shown) followed by densitometry (FIG. 18A). In contrast to GST alone, which did not bind liposomes and was only found in  
25 supernatants (data not shown), all seven mce domains were bound by PA-containing liposomes. However, the inventors observed significant differences in their apparent affinities: While mce 1, 2, 3 and 4 bound equally well, binding was decreased ~ 6-fold for mce 5, ~ 20-fold for mce 7 and more than 100-fold for mce 6 (FIG. 18A). Although so far only few phosphatidic acid binding proteins have been characterized in detail, it has been  
30 demonstrated that basic residues are often key determinants of binding affinity (Stace and Ktistakis, 2006; Lu and Benning, 2009). When the inventors analyzed the mce domains of MAM7 for overall charge, they found only minor differences between individual domains. When they looked at charge distribution, however, the inventors observed that several basic residues that are otherwise well conserved between individual domains are mutated in mce 6.

These include two residues which are lysines in mce 2, the strongest binding mce domain, but a serine and glutamine in mce 6, respectively, and a well conserved arginine which is replaced by a histidine in mce 6 (FIG. 18D). To test if these residues contributed to phosphatidic acid binding, the inventors mutated the respective positions in mce 6 to the amino acids of the corresponding position in mce 2 (mce 6 S646K, Q664K and H703R) and tested the resulting proteins for PA binding in liposome association assays. The mce 6 S664K mutant, but not the Q664K or H703R mutants, showed a significant increase in association with liposomes, changing the binding affinity of mce 6 relative to high binding mce domains from 100 fold less to approximately 10 fold less. (FIG. 18B and data not shown). The inventors also used liposomes containing 50 mol% PA and 50 mol% PC to perform pull-down experiments, a composition of liposomes where some degree of binding was observed with all mce constructs. GST-mce proteins were immobilized and incubated with liposomes. Bound liposomes were quantified using a molybdophosphoric acid assay. In agreement with liposome assays, these experiments also showed strong binding of liposomes to mce 1,2,3,4 and 7 while mce 5 and 6 showed weaker binding (FIG. 18C). Mce 6 S646K displayed enhanced affinity compared to wild-type mce 6, while the other mutants had equally low affinities as mce 6.

**MAM7 forms a tripartite complex with fibronectin and phosphatidic acid.**

Although the inventors have dissected the individual interactions between MAM7 and fibronectin and PA, it remains unclear if MAM7 binds to both types of receptors simultaneously or binding is mutually exclusive. To analyze whether MAM7, fibronectin and phosphatidic acid is competitive, the inventors performed pull-down assays with equimolar amounts of MAM7, Fn and PA (in the form of liposomes consisting of a 1:1 mixture of PC and PA or, as negative control, PC only). First, the inventors pre-incubated GST-MAM7 with liposomes, followed by incubation with fibronectin (FIGS. 19A, 19C). Next, they pre-incubated GST-MAM7 with fibronectin first and competed with liposomes (FIGS. 19B, 19D). For both experiments, the inventors analyzed eluate and flow-through fractions after adding the competing molecule by SDS-PAGE to detect protein (FIGS. 19A, 19B and data not shown) and with a molybdophosphoric acid assay to detect liposome-containing fractions (FIGS. 19C-D). Both fibronectin and PA-containing liposomes were pulled down individually by MAM7 (FIGS. 19A-D, lane 1 and 2, respectively). Addition of an equimolar mix of both ligands also resulted in efficient pull down of both ligands by MAM7 (FIGS. 19A-D, lane 4). As a control, the inventors performed binding assays with liposomes containing only PC and the inventors did not observe binding of MAM7 to liposomes but did

observe binding to fibronectin (FIGS. 19A-D, lanes 5-7). Furthermore, no binding to MAM1 was observed with fibronectin or liposomes containing PC alone, but was observed with liposomes containing a 1:1 mixture of PC:PA (FIGS. 19A-D, lanes 8-10). To test for competition between fibronectin and PA binding, the inventors also performed plate assays using fibronectin-coated plates and fluorophore-labeled MBP-MAM7. MAM7-fluorescence was measured before and after incubation with liposomes containing either PC or a mixture of PC and 80 mol% PA. Incubation with increasing concentrations of liposomes did not replace fibronectin as MAM7 ligand, so that MAM7 remained bound to the plate and fluorescence levels did not decrease significantly (FIG. 19E). The inventors also analyzed samples from the plate assay after incubation with liposomes containing 100  $\mu$ M PA or PC only (FIG. 19E, arrow). Using the molybdophosphoric acid assay, they found that PA-containing liposomes remained associated with Fn-bound MAM7 after washing steps, while liposomes prepared from PC alone did not (FIG. 19F). In addition, the inventors did not detect any associated liposomes when Fn-coated plates were pre-incubated with MBP-tag alone (FIG. 19F). These results support the hypothesis that MAM7 can interact with both fibronectin and phosphatidic acid ligands simultaneously, forming a tripartite complex.

## Discussion

The inventors' previous work has identified MAM7 as a factor involved in the initial attachment of bacterial pathogens to host cells. They have also demonstrated that MAM7 expressed on the surface of non-pathogenic bacteria may be utilized as a potent inhibitor of pathogen infection in tissue culture. MAM7 is capable of binding both fibronectin and phosphatidic acid on the host cell surface and both interactions are required for bacterial adhesion to host cells (Krachler *et al.*, 2011). Upon further dissection of host cell binding into discrete binding events, either by masking fibronectin with an anti-Fn antibody or by degrading phosphatidic acid, the inventors found that in the absence of fibronectin, MAM7 is still able to attach to host cells but establishment of a stable interaction takes significantly longer than in the presence of fibronectin (FIGS. 15A-D). Since fibronectin is an abundant protein in the extracellular matrix surrounding cells *in vivo* as well as in tissue culture, they speculate that it facilitates rapid initial attachment of bacteria in the vicinity of the host cell membrane, thereby increasing the likelihood for the pathogen to establish a high affinity interaction with the host cell membrane via the second MAM7 ligand, phosphatidic acid.

The inventors found that the MAM7 binding site in fibronectin is located to the 30 kDa N-terminal region (FIGS. 16A-F). The same region is also exploited as receptor by other bacterial adhesins, including fibronectin-binding proteins (FnBPs) from the Gram-positive pathogens *Staphylococcus aureus* and *Streptococcus pyogenes* (Schwarz-Linek *et al.*, 2006). Thus, future experiments will be carried out to address whether MAM7 binding to host cells might be able to diminish adhesion and invasion of these pathogens. In contrast to FnBP-expressing pathogens utilizing the 30 kDa fragment for attachment, most of the MAM7-containing pathogens the inventors studied, such as *V. parahaemolyticus*, *V. cholerae*, *Y. pseudotuberculosis* and EPEC, remain extracellular during infection and are not internalized by host cells (Burdette *et al.*, 2009; Simonet *et al.*, 1990; Rosqvist *et al.*, 1988; Celli *et al.*, 2001). Equally, non-pathogenic BL21-MAM7 did not get internalized by host cells. Many factors could account for this difference in bacterial fate following fibronectin binding (Cossart and Sansonetti, 2004). FnBPs such as *S. pyogenes* F1 bind to fibronectin in a way that leaves the RGD motif required for integrin recruitment, which is necessary for internalization, exposed (Ensenberger *et al.*, 2004). It is possible that MAM7 binding renders fibronectin in a different conformation, which does not allow for integrin recruitment. It is also possible that fibronectin does not get recruited by MAM7 at a density sufficient to induce clustering of integrin receptors and thus activation of downstream pathways necessary

for cellular uptake (Tran Van Nhieu and isberg, 1993). Indeed, recent work analyzing the link between repeats within *S. aureus* FnBPA and cellular invasion has shown that only very high (subnanomolar) affinity interactions between FnBP repeats and fibronectin can lead to sufficient clustering of integrin receptors to promote cellular invasion (Edwards *et al.*, 2010).

5 It is thus possible that the interaction between MAM7 and fibronectin is not of high enough affinity to promote cellular uptake.

The 30 kDa fragment of fibronectin also contains features required for fibronectin cross-linking and fibrin binding (Hormann *et al.*, 1987; Vakonakis *et al.*, 2007). Thus, it is possible that binding of MAM7 interferes with these processes, as has been described for  
10 other adhesins interacting with the same region (Tomasini-Johansson *et al.*, 2001; Matsuka *et al.*, 2003). The inventors are currently conducting experiments to study the consequences of MAM7 binding on integrin signaling, fibrin-fibronectin cross-linking and fibrillogenesis. They detected no discernible binding with MAM constructs containing one to three mce domains, possibly due to very low affinity, and only weak binding with 4 mce domains. The  
15 inventors found that at least 5 mce domains are required for stable binding of MAM to fibronectin. The sharp increase in binding affinity between four and five mce domains (FIG. 17A) as well as the non-linear increase in affinity between six and seven mce domains observed in fluorescent saturation binding experiments (FIG. 17B) is in better agreement with a cooperative rather than a linear binding model. However, a clear distinction between the  
20 two would require fibronectin binding analyses with MBP-proteins containing three to five mce domains, which the inventors currently are unable to produce and purify. Since the 30 kDa region required for MAM binding contains five consecutive type I repeats, it is tempting to speculate that each of these repeats binds to one mce domain. However, determination of the exact stoichiometry of binding will be subject to further studies but might be difficult to  
25 determine in the context of lipid binding, which could modulate the affinity and stoichiometry of binding between MAM7 and fibronectin.

In contrast to fibronectin, which seems to contribute to fast binding but is dispensable for high affinity binding, phosphatidic acid is indispensable to establish stable binding of MAM7 to the host surface (FIGS. 15C, 15D). This underlines earlier findings showing that  
30 the relative binding affinity of MAM7 for fibronectin is relatively minor compared to its affinity for intact host cells (apparent  $K_D$  of 15  $\mu$ M and 200 nM for fibronectin and intact cells, respectively). No common motif mediating interactions between proteins and phosphatidic acid has been identified to date. However, many PA-binding motifs contain a high number of basic residues which are thought to establish electrostatic interactions with



the phosphate headgroup (Lu and Benning, 2009). Based on alignments of individual mce domains within MAM7 and their differences in affinity for PA, the inventors determined at least one basic residue (mce 2 Lys166) which is crucial for PA-binding. The position is well conserved between different mce domains in MAM7 with the exception of mce 6 and mce 5, both of which bound PA with lower affinity than other mce domains. Mutation of this position to a lysine in mce 6 lead to at least 20-fold increased affinity for PA. Mutation of other candidate residues (which were conserved as basic residues in the other mce domains) within mce 6 that were mutated to basic residues did not seem to have any effect on PA binding. Ultimately, structural studies on mce domains in their free and ligand-bound forms will be required to shed more light on the detailed mechanisms behind host cell binding.

In this study, the inventors dissected the interaction of the adhesin MAM7 with host cells into discrete binding events and analyzed their contribution to overall host cell binding. They analyzed MAM7 for features defining its ability to interact with fibronectin and phosphatidic acid, respectively, and defined the region of fibronectin required for its interaction with MAM7. They also studied whether MAM7 is capable of binding to fibronectin and phosphatidic acid simultaneously and found that the three molecules likely form a tripartite complex. These studies form an important basis for current and future efforts to develop MAM7-derived tools for the attenuation of Gram-negative bacterial infections.

### EXAMPLE 3

#### Materials & Methods

**Bacterial strains-isolation and growth conditions.** Bacterial isolates were acquired from patients treated at the San Antonio Military Medical Center within the period from 2006 to 2010. Cultures were obtained from sites as indicated in Table 1. All isolates were grown in LB or LB agar or in DMEM at 37 °C for attachment and infection experiments, unless otherwise indicated.

**Attachment assays.** Bacterial attachment to host cells was essentially tested for as previously described above. Briefly, mammalian cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HeLa cells) or 10% bovine calf serum (3T3 fibroblasts), (SAFC Biosciences), 5 mM sodium pyruvate and penicillin/streptomycin mix at 37 °C with 5% CO<sub>2</sub>. Cells were washed with PBS (phosphate-buffered saline) prior to the addition of bacteria in DMEM without antibiotics. Bacteria were added to give a multiplicity of infection (MOI) of 10. To determine the exact amount of

input, bacteria were added to empty wells. Plates were centrifuged (1000×g, 22 °C, 5 minutes) prior to incubation at 37 °C for 1 hour. Cells were washed three times with PBS and lysed by adding 0.5 % Triton X-100 in PBS. Input samples and Triton lysates were serially diluted, plated on LB agar and enumerated by colony counting.

5       **Lactate dehydrogenase (LDH) release assays.** To measure cytotoxicity, tissue culture cells were washed with PBS prior to the addition of bacteria in DMEM without antibiotics at an MOI of 10. Infections were started by centrifugation of plates (1000×g, 22 °C, 5 minutes) prior to incubation at 37 °C. 200 µl of supernatant was removed in triplicate from each well four hours after infection, centrifuged (1000×g, 22 °C, 5 minutes), and 100 µl  
10 of the supernatant transferred to a fresh 96well plate for assays. To quantitate cell lysis, the amount of lactate dehydrogenase (LDH) released into the culture medium was measured using the LDH cytotoxicity detection kit (Takara) according to the manufacturer's protocol.

**MAM7-inhibition experiments.** For inhibition experiments, tissue culture cells were pre-incubated with either *E. coli* BL21 expressing *V. parahaemolyticus* MAM7 or  
15 recombinant, bead-immobilized MAM7 for 30 minutes as described previously. Generation of BL21-MAM7, cloning of expression constructs for GST and GST-MAM7 fusion protein and protein purifications have been described elsewhere. Purified proteins were immobilized on 1 µm fluorescent orange latex beads (Sigma) as described by El Shazly *et al.* (2007). For inhibition experiments, a total of 7.5 µg protein/10<sup>6</sup> beads/well in PBS was used. After  
20 removing excess bacteria or beads by three PBS washes, cultured cells were infected with clinical isolates for four hours and cytotoxicity measured as described above for LDH release assays.

**Fluorescence microscopy.** Cells were seeded onto cover slips at 150,000 cells/ml and subjected to infection experiments the next day. Following infection experiments, cells were  
25 washed with PBS and fixed with 3.2 % paraformaldehyde in PBS for 15 minutes. Fixed cells were permeabilized with 0.1 % Triton X-100 in PBS for 5 minutes and treated with Hoechst (Sigma) and Alexa Fluor 488-phalloidin (Molecular Probes) for 10 minutes to stain for DNA and F-actin, respectively. Cover slips were mounted onto 10 % (w/v) glycerol and 0.7 % (w/v) propyl gallate in PBS, sealed with nail polish and viewed using a Zeiss LSM510  
30 META Laser Scanning Confocal Microscope. Images were processed using ImageJ and Photoshop software.

## Results

**Isolation and PFGE typing of bacterial pathogens associated with patient wounds.** Bacterial pathogens were cultured after isolation from infected patients treated at the Military Medical Center at San Antonio, Texas, following their evacuation from Afghanistan and Iraq. The characterized a total of twenty different isolates obtained either by superficial or deep wound culture, blood culture (two cases) or urine culture (one case), each of which belonged to one of the four most predominant species of Gram-negative bacteria leading to wound infections, *Acinetobacter baumannii-calcoaceticus* complex, *Pseudomonas aeruginosa* and ESBL-producing *Klebsiella* ssp. and *Escherichia coli* (each represented by five isolates, respectively). Pulsed field gel electrophoresis was used to determine the genotype of all isolates and revealed a high degree of genotypic diversity within each of the four groups. Each of the five isolates within each group represented a distinct genotype (Table 2).

**Clinical isolates from patient wounds vary in their ability to form biofilms, attach to host cells and cause cytotoxicity.** Next, the inventors studied the bacterial isolates for their ability to form biofilms (Table 2) and attach to cultured HeLa epithelial cells and 3T3 fibroblasts using serial dilution plating assays (FIGS. 20A-D). *P. aeruginosa* and *Klebsiella* ssp. isolates showed the greatest tendency to form biofilms (four out of five isolates tested positive for biofilm formation), followed by *A. baumannii* (two out of five) and *E. coli* (one out of five isolates), (Table 2). In contrast, *A. baumannii* isolates had the greatest ability to attach to host cells, followed by *Klebsiella* ssp., *P. aeruginosa* and *E. coli*. However, the inventors observed a large variability in attachment properties within each group, with *E. coli* isolates showing the most variability (attachment ranged from 15% for isolate #2 to 73% for isolate #5). In addition, the inventors tested all twenty isolates for their ability to cause cytotoxicity in host cells within a four hour infection experiment using lactate dehydrogenase (LDH) release assays (FIGS. 20E-H). *A. baumannii* isolates caused the highest overall cytotoxicity, followed by *P. aeruginosa*, *Klebsiella* ssp. and *E. coli*. The variability was largest amongst *Klebsiella* ssp., with cell lysis ranging from 24% for isolate #2 to 69% for isolate #3. Moreover, the inventors noted two interesting features when comparing attachment and cytotoxicity profiles of the tested isolates. First, in most cases the strains showed a slightly higher attachment and cytotoxic effect on 3T3 fibroblasts. Second, in the case of all groups of pathogens except *P. aeruginosa* the inventors observed positive correlation between host cell attachment and pathogen-induced cytotoxicity (FIGS. 20I-L).

**Isolates display a high degree of resistance against a wide range of commonly used antimicrobials.** One of the greatest problems faced in treatment of Gram-negative wound infections is the increasing number of multidrug resistant (MDR) bacteria found in patient isolates. The inventors performed antimicrobial susceptibility testing (AST) by determining the minimal inhibiting concentration (MIC) of a panel of antimicrobials on all twenty bacterial isolates using the BD Phoenix Automated Microbiology System (Table 3). Antimicrobial panels included several drugs from each of the four most commonly used classes of antimicrobials, aminoglycosides,  $\beta$ -lactams, carbapenems and fluoroquinolones. The panels also included antimicrobials from other classes, such as trimethoprim/sulfamethoxazole, but resistance to these was not considered in the classification of isolates as multidrug-resistant. According to the definition of multidrug resistance according to the Center for Disease Control and Prevention (where MDR is defined as resistant to all drugs tested in all or all but one antimicrobial drug classes commonly prescribed to treat Gram-negative infections), (Hospenthal *et al.*, 2011) all tested *A. baumannii* and *P. aeruginosa* strains were found to be MDR. All isolates of ESBL-producing *Klebsiella* ssp. and *E. coli*, even though not conforming to the definition of MDR, displayed a high degree of resistance, being non-susceptible to most aminoglycosides,  $\beta$ -lactams and fluoroquinolones tested (Table 3). All *Klebsiella* ssp. and *E. coli* isolates were susceptible to the tested carbapenems (imipenem, meropenem and ertapenem).

**Anti-adhesion treatment decreases pathogen-mediated cytotoxicity for a large number of clinical isolates.** The inventors have previously demonstrated that a widely conserved adhesin found in Gram-negative bacteria, termed Multivalent Adhesion Molecule (MAM) 7 can be used to block bacterial attachment sites on host cells, thus diminishing infection by gastrointestinal pathogens such as *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Yersinia pseudotuberculosis* and EPEC in tissue culture models of infection as described above. Herein, the inventors investigated if such treatment could be successfully extended to include relevant wound-associated pathogens and tested if MAM7-based anti-adhesion treatment would affect the cytotoxicity mediated by the clinical isolates on HeLa or 3T3 cells using LDH release assays. The inventors used MAM7 either expressed on the surface of non-pathogenic *E. coli* (BL21-MAM7) or recombinant, purified MAM7 protein immobilized on 1  $\mu$ m latex beads to mimic surface display on bacteria (bead-MAM7). Cultured cells were pre-incubated with MAM7-inhibitors at a multiplicity of infection (MOI) of 100 for 30 minutes. Excess inhibitor was subsequently removed by washing and cells were infected with each of the twenty isolates at an MOI of 10. Cytotoxicity towards HeLa and 3T3 cells, either without

pre-incubation or following anti-adhesion treatment with BL21-MAM7 or MAM7-beads, was assessed following four hours of infection (FIGS. 21A-D and FIGS. 22A-D, respectively). Overall, the inventors observed a large decrease in cytotoxicity when cells were pre-incubated with MAM7-based inhibitors, with the extent of inhibition mediated by either BL21-MAM7 or MAM7-beads being very similar. The highest protective effect towards HeLa cells was observed with treatment against *A. baumannii* (mean inhibition of  $76 \pm 8\%$  with BL21-MAM7 and  $68 \pm 6\%$  with MAM7-beads) and *Klebsiella* ssp. isolates (mean inhibition of  $85 \pm 4\%$  and  $71 \pm 18\%$  with BL21 and beads, respectively), (FIGS. 21A, 21C). In contrast, the inhibitory effect against *P. aeruginosa* ( $48 \pm 30\%$  and  $43 \pm 24\%$  for bacteria and beads, respectively) and *E. coli* ( $54 \pm 19\%$ / $51 \pm 20\%$ ) was less pronounced for most strains, but the variability of treatment responses between different isolates within these groups was generally high (FIGS. 21B, 21D). For example in the case of *P. aeruginosa*, cytotoxicity inhibition ranged from  $> 91\%$  for isolate #1 to  $12\%$  for isolate #3. The inhibitory profiles were very similar between HeLa cells and 3T3 cells (FIG. 22A-D).

**Visualization of MAM7 inhibitory potential on bacterial infections using confocal microscopy.** To visualize the detrimental effects of individual pathogens as well as the protective potential of MAM7 inhibitors, the inventors chose representative strains from each of the four groups (marked with asterisks in FIGS. 21A-D and FIGS. 22A-D) and analyzed infections of both HeLa and 3T3 cells as well as inhibition experiments using confocal microscopy (FIGS. 23A-24R). This allowed us to further test and correlate results found in LDH release assays with cellular phenotypes. For this purpose, cells were infected with either *A. baumannii* #1, *P. aeruginosa* #1, *Klebsiella* ssp. #1 or *E. coli* #5 (all of which showed good responses to anti-adhesion treatment with MAM7). In addition, the inventors analyzed infections with *P. aeruginosa* #3, which showed the lowest response to inhibition. Comparative microscopic analyses of HeLa cells infected with either *P. aeruginosa* isolate #1 or #3 demonstrated a large difference between cellular phenotypes following infection with the two isolates, which is in agreement with the inventors' findings from LDH release experiments as well as PFGE analyses. While infection with *P. aeruginosa* #1 caused only limited cell rounding and lysis, even after several hours of infection, and was predominated by an actin phenotype characterized by induction of filopodia and microspikes, isolate #3 causes rapid cell rounding and cell lysis with seemingly no intermediate phenotype (FIGS. 23C-D). *A. baumannii* #1 and *E. coli* #5 both caused rapid cell rounding (FIGS. 23B, 23F), while *Klebsiella* ssp. #1 caused slower and limited rounding and lead to formation of actin protrusions which were distinct from those observed with *P. aeruginosa* isolate #1 (FIG.

23E). With 3T3 cells, it was harder to discern distinct phenotypes of infection, since upon infection with most bacterial isolates the 3T3 cells underwent rapid deterioration characterized by formation of actin stress fibers and microspikes, followed by cell lysis (FIGS. 245A-R). However, with both HeLa and 3T3 cells, pre-incubation with BL21-MAM7 and MAM7-beads markedly slowed down the progress of infection, with only limited cell rounding and lysis visible after infection with either *A. baumannii* #1, *P. aeruginosa* #1, *Klebsiella* ssp. #1 or *E. coli* #5. In all these cases, the remaining cellular phenotypes were limited to changes in actin phenotype, such as formation of stress fibers, filopodia or microspikes (FIGS. 23A-F and FIGS. 24H-R). In contrast, cellular phenotypes following infection with *P. aeruginosa* isolate #3 did not change upon pre-treatment with either BL21-MAM7 or MAM7-beads on either cell type (FIGS. 23A-F and FIGS. 24J, 24P), which is also in agreement with the inventors' results from LDH assays.

## Discussion

Bacterial colonization and infection of wounds is a common cause of complication of treatment in military personnel treated in military medical facilities following evacuation from combat sites. Wound-associated infections with Gram-negative bacteria are predominantly caused by *A. baumannii*, *P. aeruginosa* and ESBL-producing *E. coli* and *Klebsiella* ssp. (Hospenthal *et al.*, 2011). The increasing number of multidrug-resistant pathogens isolated from patients poses a serious concern and underpins the necessity for alternative measures in infection prophylaxis and treatment. Anti-adhesion therapy has been considered and tested as an alternative to small molecule antimicrobials. In most cases, this is based on administering molecular mimics of host cell receptor structures, such as sugars or sugar mimics (Salminen *et al.*, 2007; Hansen *et al.*, 1997; Pieters, 2006). Following the inventors' previous studies exploring the use of MAM7-based inhibitors in anti-adhesion treatment of bacterial infections with gastrointestinal pathogens, the inventors set out to explore the potential of these inhibitory molecules against infection with wound-associated Gram-negative pathogens.

The inventors utilized five representative patient isolates from each of the five above-mentioned Gram-negative bacterial species. PFGE typing showed that all isolates was genotypically distinct. A major problem in the treatment of wound-associated infections is the increasing number of multidrug-resistant organisms encountered. Admission-associated screening over the period of 2003-2009 has revealed that a wide range of patient isolates are

resistant against most antimicrobials commonly used in the clinic and their ability to rapidly acquire additional resistance, such as described in the case of *Acinetobacter baumannii* isolates, which developed colistin resistance during the period of testing (Hospenthal *et al.*, 2011, Jason *et al.*, 2008). This tendency was also found with the isolates tested in the present study – all *Acinetobacter baumannii* isolates and most *Pseudomonas aeruginosa* isolates were multidrug-resistant. While all tested ESBL-producing *Klebsiella* spp. and *E. coli* isolates are not multidrug-resistant according to the CDC's definition (Hospenthal *et al.*, 2011), most of them displayed a very broad resistance profile, excluding most aminoglycosides,  $\beta$ -lactams and fluoroquinolones as treatment options.

The inventors further analyzed a range of parameters described to be important contributing factors for virulence, using *in vitro* and tissue culture assays. These included biofilm formation, attachment to host cells and cytotoxicity in tissue culture models of infection (Dallo and Weitao, 2010; Schierle *et al.*, 2009; Davis *et al.*, 2008; Vance *et al.*, 2005). The majority of strains (64%) were found to form biofilms *in vitro* and all strains displayed an ability to attach to host cells and elucidate host cell killing. However, the degree of attachment and cytotoxicity varied widely across all tested isolates as well as between isolates within the same species, which is in agreement with the genotypic variance found during PFGE profiling of the isolates.

It is evident that for many pathogens, close contact has to be maintained with host cells in order to establish a successful infection. Many virulence factors are either soluble, secreted toxins, which directly bind and translocate across the host cell plasma membrane or form pores in the plasma membrane. Both these processes are concentration dependent and therefore require close contact with host cells to avoid loss by diffusion (Matsuda *et al.*, 2010; Kim *et al.*, 2008; Zrimi *et al.*, 2011). Other important virulence factors are injected directly into the host cell's cytoplasm, either by type III, type IV or type VI secretion machinery, which also requires the bacterium to attach to host cells (Cambronne *et al.*, 2006; Filloux *et al.*, 2008; Winnen *et al.*, 2008). For this reason, the inventors analyzed the correlation between host cell attachment and cytotoxicity across each group of pathogen. The inventors generally found a positive correlation between attachment and cytotoxicity for *A. baumannii*, *P. aeruginosa* and *E. coli* isolates. Interestingly, *P. aeruginosa* isolates showed no significant correlation between these two factors. One explanation for this would be a higher potency of virulence mechanisms in some *P. aeruginosa* strains compared to other pathogens or other strains within the same species (and thus only limited need for attachment to elucidate a high degree of cytotoxicity). In this light, it would be interesting to explore if

some of the isolates are hypervirulent compared to others. Another explanation for the atypical behavior of some *P. aeruginosa* isolates would be that they use additional virulence mechanisms which do not strictly depend on direct contact between bacteria and host cells, such as bacterial outer membrane vesicles (OMVs) (Bomberger *et al.*, 2009). OMVs are shedded by a wide variety of bacterial species and can be enriched in certain bacterial proteins (Haurat *et al.*, 2011; Choi *et al.*, 2011; Kulp *et al.*, 2010). In some cases, this may be employed as a mechanism of translocating virulence factors across the host membrane, my means of vesicle/plasma membrane fusion and endocytosis of bacterial components (Jin and Lee, 2011; Parker *et al.*, 2010).

Previously, the inventors have shown that pre-treatment of host cells with MAM7, either presented on the surface of non-pathogenic bacteria or immobilized on polymer beads, markedly decreased the effects of infection with gastro-intestinal pathogens, such as *V. cholera*, *V. parahaemolyticus*, *Y. pseudotuberculosis* and EPEC (see Example 1, above). The inventors further showed that the mechanism of MAM7-based attachment inhibition is likely to be competition for a limited number of host cell receptors (sites rich in both phosphatidic acid and fibronectin), rather than steric hindrance, since the number of attached bacteria or beads plateaus above a certain multiplicity of infection and the MAM7 dose required to achieve inhibition can be kept relatively low (see Example 2, above). Thus, the inventors hypothesized that other pathogens utilizing a MAM7 homolog for host cell attachment might also be responsive to the same mode of treatment and explored the potential of MAM7-based inhibitors in anti-adhesion treatment of infections with wound-associated Gram-negative pathogens. In general, the inventors observed a significant decrease of cytotoxic effects on cultured cells following pre-treatment with MAM7 across all five tested species. A few individual isolates of *P. aeruginosa* and one isolate of ESBL-producing *E. coli*, however, showed very limited responses to MAM7-treatment. Most prominently, the highly cytotoxic isolate *P. aeruginosa* #3 showed almost no response to MAM7 inhibition. In general, the inventors noticed that isolates with weak or no correlation between attachment and cytotoxicity were less responsive to treatment. Given that MAM7 inhibits the attachment of bacteria to host cells, it is conclusive that strains which display a high degree of cytotoxicity even at comparably low levels of attachment, such as *P. aeruginosa* #3, would be less susceptible to anti-adhesion therapy.

The inventors conclude that anti-adhesion prophylaxis or treatment with MAM7-based inhibitors shows promise in the fight against a number of important wound-associated pathogens which might be hard to treat with conventional small molecule antimicrobials.



Thus, experiments presented here, investigating the efficacy of MAM7-based inhibition using tissue culture models of infection, should be extended to include relevant animal models of infection in the future, even though this is beyond the scope of the present study. Most importantly, the inventors demonstrate that the correlation between two important hallmarks  
5 of virulence which can easily be tested for in tissue culture, attachment and cytotoxicity, could serve as a useful predictor for the success of MAM7-based inhibition against bacterial infections, and potentially other molecules used in anti-adhesion therapy.

**Table 2 - Isolation and genotypic characterization of bacterial isolates**

Species	Isolate No.	Year of isolation	Source	PFGE type	Biofilm formation
<i>Acinetobacter baumannii</i>					
	1	2006	Blood	ABC PFT 4	N
	2	2006	Blood	ABC PFT 3	Y
	3	2007	Wound culture superficial	ABC PFT 1	Y
	4	2007	Wound culture deep	ABC PFT 2	N
	5	2008	Wound culture deep	ABC PFT 5	N
<i>Pseudomonas aeruginosa</i>					
	1	2008	Wound culture deep	PA PFT 7	N
	2	2007	Wound culture deep	PA PFT 2	Y
	3	2009	Wound culture deep	PA PFT 1	Y
	4	2007	Wound culture deep	PA PFT 4	Y
	5	2008	Wound culture superficial	PA PFT 5	Y
<i>Klebsiella ssp.</i>					
	1	2007	Wound culture deep	KP PFT 1	Y
	2	2008	Wound culture deep	KP PFT 2	Y
	3	2009	Wound culture deep	KP PFT 7	Y
	4	2007	Wound culture deep	KP PFT 3	N
	5	2008	Wound culture deep	KP PFT 9	Y
<i>Escherichia coli</i>					
	1	2007	Wound culture deep	EC PFT 3	N
	2	2007	Wound culture superficial	EC PFT 4	N
	3	2009	Wound culture deep	EC PFT 1	N
	4	2010	Wound culture deep	EC PFT 7	N
	5	2008	Urine culture	EC PFT 2	Y

Table 3 - Strain susceptibility against commonly screened antibiotics

Bacterial species	Isolate#	Amik.	Amox C.	Amp.	Az.	Cef o.	Ceft.	Cef u.	Ceph.	Cipr o.	Gati.	Gent.	Im.	Lev.	Mer o.	Nf.	Pip.	Tet.	Tob.	Tms m.	
<i>Acinetobacter baumannii</i>																					
	1	>32 R	>16/8 R	>16 R	>1/6 R	>32 R	>16 R	>16 R	>16 R	>2 R	>4 R	>8 R	2 S	>4 R	2 S	>64 R	>64 R	>8 R	8 I	>2/38 R	
	2	>32 R	>16/8 R	>16 R	>1/6 R	>32 R	>16 R	>16 R	>16 R	>2 R	>4 R	>8 R	>8 R	>4 R	>8 R	>64 R	>64 R	4 S	>8 R	>2/38 R	
	3	32 I	>16/8 R	>16 R	>1/6 R	>32 R	8 S	>16 R	>16 R	>2 R	4 I	>8 R	>8 R	4 I	>8 R	>64 R	>64 R	>8 R	>8 R	>2/38 R	
	4	>32 R	>16/8 R	>16 R	>1/6 R	>32 R	>16 R	>16 R	>16 R	>2 R	>4 R	>8 R	2 S	>4 R	≤1 S	>64 R	>64 R	>8 R	>8 R	>2/38 R	
	5	>32 R	>16/8 R	>16 R	>1/6 R	>32 R	>16 R	>16 R	>16 R	>2 R	>4 R	>8 R	>8 R	>4 R	>8 R	>64 R	>64 R	8 I	4 S	>2/38 R	
<i>Pseudomonas aeruginosa</i>		Amik.	Amp.	Amp S.	Az.	Cef a.	Cef e.	Cx.	Cd.	Co.	Cef u.	Cipr o.	Gen t.	Im.	Lev.	Mer o.	Nf.	Pip T.	Tet.	Tob.	Tms m
	1	≤8 S	>16 R	>16/8 R	>1/6 R	>16 R	16 I	>16 R	>16 R	>32 R	>16 R	>2 R	4 S	>8 R	>4 R	4 S	>64 R	>64/4 R	>8 R	≤2 S	>2/38 R
	2	16 S	>16 R	>16/8 R	>1/6 R	>16 R	>16 R	>16 R	8 S	>32 R	>16 R	>2 R	4 S	>8 R	>4 R	>8 R	>64 R	>64/4 R	>8 R	>2/38 R	
	3	16 S	>16 R	>16/8 R	>1/6 R	>16 R	>16 R	>16 R	>16 R	>32 R	>16 R	>2 R	8 I	>8 R	>4 R	>8 R	>64 R	>64/4 R	>8 R	≤2 S	>2/38 R
	4	32 I	>16/8 R	>16 R	>1/6 R	>16 R	>16 R	>16 R	>16 R	>32 R	>16 R	>2 R	>8 R	>8 R	>4 R	>8 R	>64 R	32/4 S	>8 R	>2/38 R	
	5	32	>16	>16/8	>1/6	>16	>16	>16	>16	>32	>16	>2	>8	2	>4	4	>64	>64/4	>8	>2/38	

	I	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	TmS m
<i>Klebsiella</i> spp.	Ani k.	Amox C.	Amp.	Az .	Cef a.	Cef e.	Cef o.	Cx.	Cd.	Co.	Cipr o.	Ert.	Gen t.	Im.	Lev.	Mer o.	Nf.	Pip T.	Tet.	Tob.	TmS m		
1	≤8 S	16/8 R	>16 R	>1 6 R	NA	>16 R	>32 R	NA	>16 R	NA	>2 R	NA	>8 R	≤1 S	>4 R	≤1 S	>64 R	NA	>8 R	8 I	>2/38 R		
2	≤8 S	16/8 R	>16 R	>1 6 R	>16 R	>16 R	>32 R	≤4 S	>2 R*	>32 R	>2 R	≤0.5 S	>8 R	≤1 S	≤1 S	≤1 S	>64 R	32/4 I	>8 R	>8 R	≤0.5/9, 5 S		
3	≤8 S	8/4 R	>16 R	>1 6 R	>16 R	2 R*	≤4 S	>16 R	>2 R*	16 R	1 S	≤0.5 S	8 I	≤1 S	≤1 S	≤1 S	>64 R	16/4 S	>8 R	8 I	≤0.5/9, 5 S		
4	≤8 S	>16/8 R	>16 R	>1 6 R	>16 R	>16 R	>32 R	8 S	>2 R*	>32 R	≤0.5 S	≤0.5 S	>8 R	≤1 S	≤1 S	≤1 S	>64 R	>64/ 4 R	>8 R	8 I	≤0.5/9, 5 S		
5	>32 R	>16/8 R	>16 R	>1 6 R	NA	2 R*	>32 R	NA	>16 R	NA	≤0.5 S	NA	>8 R	≤1 S	≤1 S	≤1 S	>64 R	NA	>8 R	>8 R	>2/38 R		
<i>Escherichia coli</i>	Ani k.	Amp. S.	Amp. S.	Az .	Cef a.	Cef e.	Cipr o.	Cd.	Co.	Cef u.	Cipr o.	Gen t.	Im.	Lev.	Mer o.	Nf.	Pip T.	Tet.	Tob.	TmS m			
1	≤8 S	>16 R	>16/8 R	>1 6 R	>16 R	>16 R	8 S	>16 R	>32 R	>16 R	>2 R	>8 R	≤1 S	>4 R	≤1 S	32 S	32/4 I	>8 R	>8 R	>2/38 R			
2	≤8 S	>16 R	>16/8 R	>1 6 R	>16 R	>16 R	≤4 S	>16 R	>32 R	>16 R	>2 R	>8 R	≤1 S	>4 R	≤1 S	≤16 S	≤2/4 S	≤2 S	>8 R	>2/38 R			
3	≤8 S	>16 R	>16/8 R	>1 6 R	>16 R	>16 R	8 S	>16 R	>32 R	>16 R	>2 R	>8 R	≤1 S	>4 R	≤1 S	≤16 S	32/4 I	>8 R	>8 R	>2/38 R			
4	≤8 S	>16 R	>16/8 R	>1 6 R	>16 R	>16 R	>16 R	>16 R	>32 R	>16 R	>2 R	≤2 S	≤1 S	>4 R	≤1 S	≤16 S	16/4 I	>8 R	≤2 S	>2/38 R			
5	≤8 S	>16 R	>16/8 R	8 R*	>16 R	8 R*	8 S	4 R*	>32 R	>16 R	>2 R	≤2 S	≤1 S	>4 R	≤1 S	≤16 S	32/4 I	>8 R	>8 R	>2/38 R			

Table 3 Abbreviations: Amik, Amikacin; AmoxC., Amoxillin-Calvulanate; Amp., Ampicillin; AmpS., Ampicillin-Sulbactam; Az., Aztreonam; Cd., Ceftazidime; Cefa., Cefazolin; Cefe., Cefepime; Cefo., Cefotaxime; Ceft., Ceftazidime; Cefu., Cefuroxime; Ceph., Cephalotin, Co., Ceftriaxone; Cx., Cefoxitin; Cipro., Ciprofloxacin; Ert., Ertapenem; Gati., Gatifloxacin; Gent., Gentamicin; Im., Imipenem; Lev., Levofloxacin; Mero., Meropenem; Nf., Nitrofurantoin; Pip., Piperacillin; PipT., Piperacillin-Tazobactam; Tet., Tetracycline; Tob., Tobramycin; TmSm., Trimethoprim-Sulfamethoxazole; NA, data not available; Values given are minimal inhibiting concentrations in µg/ml and interpretation of the result in terms of R, resistant; I, intermediate; S, susceptible; \*As a rule, when ESBL is detected, an interpretation of susceptible or intermediate for penicillins, cephalosporins and aztreonam is reported as resistant.

\* \* \* \* \*

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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## **WHAT IS CLAIMED IS:**

1. A use of a composition comprising a non-pathogenic bacterium expressing a MAM polypeptide comprising at least 6 mce repeat regions for preventing or inhibiting a pathogenic bacterial infection in a subject caused by a pathogenic bacterium expressing a MAM polypeptide.
2. The use of claim 1, wherein said subject is a mammal.
3. The use of claim 2, wherein said subject is a human.
4. The use of claim 1, wherein said pathogenic bacterium is Gram-negative.
5. The use of claim 4, wherein said pathogenic bacterium is selected from the group consisting of *Vibrio parahaemolyticus*, *Vibrio cholera*, *Yersinia pseudotuberculosis*, and enteropathogenic *Escherichia coli*.
6. The use of claim 1, wherein said non-pathogenic bacterium expresses a native MAM polypeptide.
7. The use of claim 1, wherein said non-pathogenic bacterium expresses a heterologous MAM polypeptide.
8. The use of claim 7, wherein said non-pathogenic bacterium is *Escherichia coli* strain BL21 or *Escherichia coli* strain HS.
9. The use of claim 7, wherein said non-pathogenic bacterium comprises a gene encoding a MAM polypeptide in an extrachromosomal self-replicating vector.
10. The use of claim 7, wherein said non-pathogenic bacterium comprises a gene encoding a MAM polypeptide integrated into a chromosome of said non-pathogenic bacterium.
11. The use of claim 1, wherein said composition further comprises a probiotic.
12. The use of claim 11, wherein said composition is formulated for oral administration.

13. The use of claim 11, wherein said composition is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, pet food, a tablet, a liquid bacterial suspension, dried oral supplement and wet oral supplement.
14. The use of claim 1, wherein the MAM polypeptide of said non-pathogenic bacterium comprises 6 or 7 mce repeat regions.
15. The use of claim 1, wherein the MAM polypeptide of said non-pathogenic bacterium comprises more than 7 mce repeat regions.
16. The use of claim 1, wherein the MAM polypeptide comprises 6 mce repeat regions.
17. The use of claim 1, wherein the MAM polypeptide comprises 7 mce repeat regions.
18. The use of claim 1, wherein said composition is formulated for administration more than once.
19. The use of claim 1, wherein said composition is formulated for topical administration.
20. The use of claim 19, wherein said subject has suffered a burn injury.
21. The use of claim 19, wherein said composition is formulated as a cream, a gel, a salve, an ointment or a powder.
22. The use of claim 19, wherein said composition is comprised in or disposed on a wound dressing.
23. The use of claim 1, wherein said composition is comprised in or disposed on a surgical mesh or implantable device.
24. A use of a composition comprising a MAM peptide or protein comprising at least 6 mce repeat regions coupled to a particle or bead for preventing or inhibiting a pathogenic bacterial infection in a subject caused by a pathogenic bacterium expressing a MAM polypeptide.

25. The use of claim 24, wherein said subject is a mammal.
26. The use of claim 25, wherein said subject is a human.
27. The use of claim 24, wherein said pathogenic bacterium is Gram-negative.
28. The use of claim 27, wherein said pathogenic bacterium is selected from the group consisting of *Vibrio parahaemolyticus*, *Vibrio cholera*, *Yersinia pseudotuberculosis*, and enteropathogenic *Escherichia coli*.
29. The use of claim 24, wherein said composition is formulated for oral delivery.
30. The use of claim 24, wherein the MAM peptide or protein comprises 6 or 7 mce repeat regions.
31. The use of claim 24, wherein the MAM peptide or protein of said composition comprises more than 7 mce repeat regions.
32. The use of claim 24, wherein the MAM peptide or protein comprises 6 mce repeat regions.
33. The use of claim 24, wherein the MAM peptide or protein comprises 7 mce repeat regions.
34. The use of claim 24, wherein said composition is formulated for administration more than once.
35. The use of claim 24, wherein said particle or bead is comprised of a polymer, a metal, or a lipid.
36. The use of claim 24, wherein said particle or bead is made of polystyrene, latex, a metal oxide, or polylactic-coglycolic acid (PLGA).
37. The use of claim 24, wherein said particle or bead is a microsphere, a liposome, a nanoparticle, or a quantum dot.
38. The use of claim 24, wherein said composition is formulated for topical administration.
39. The use of claim 38, wherein said subject has suffered a burn injury.

40. The use of claim 38, wherein said composition is formulated as a cream, a gel, a salve, an ointment or a powder.
41. The use of claim 24, wherein said composition is comprised in or disposed on a wound dressing, surgical mesh or implantable device.

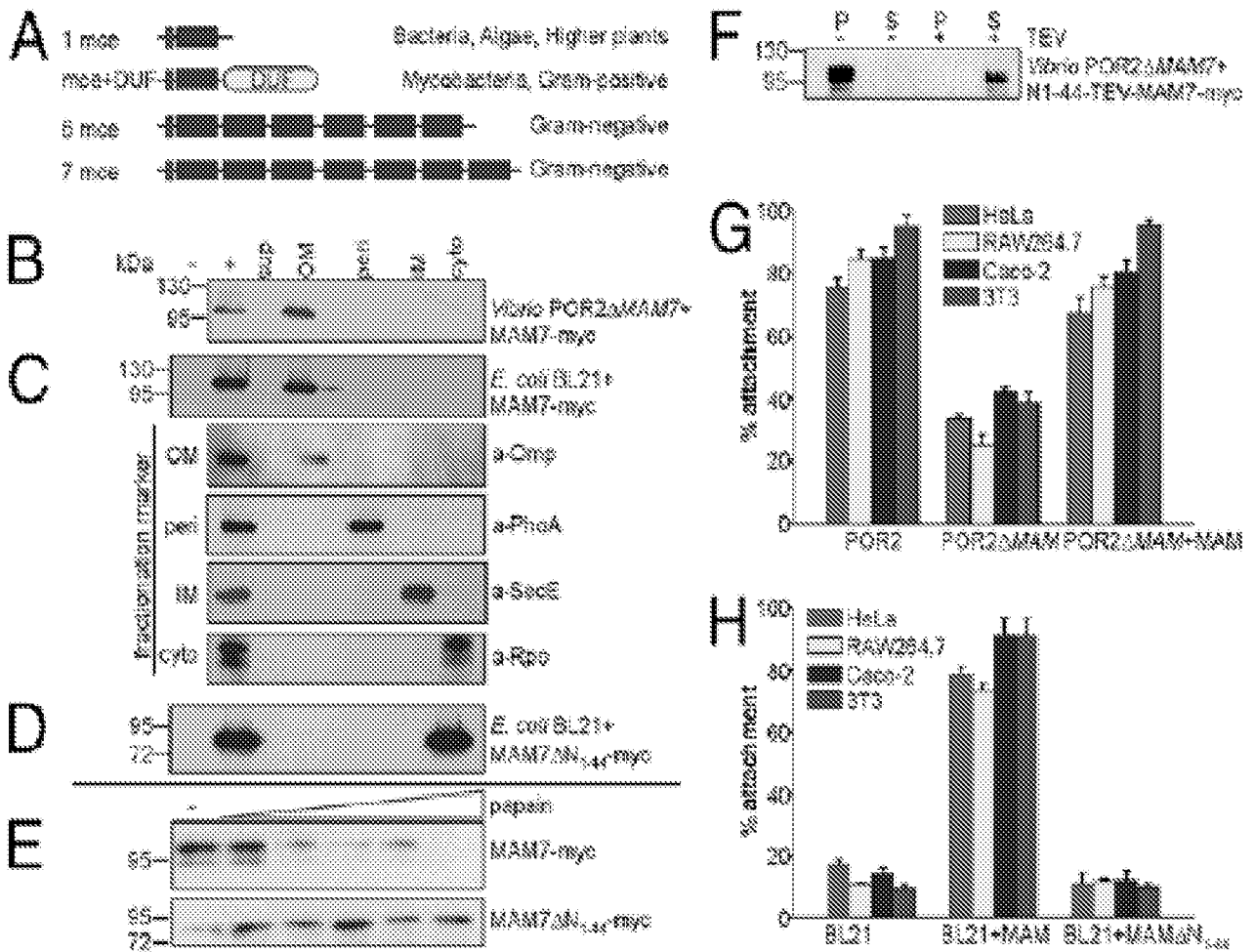


FIG. 1A-H

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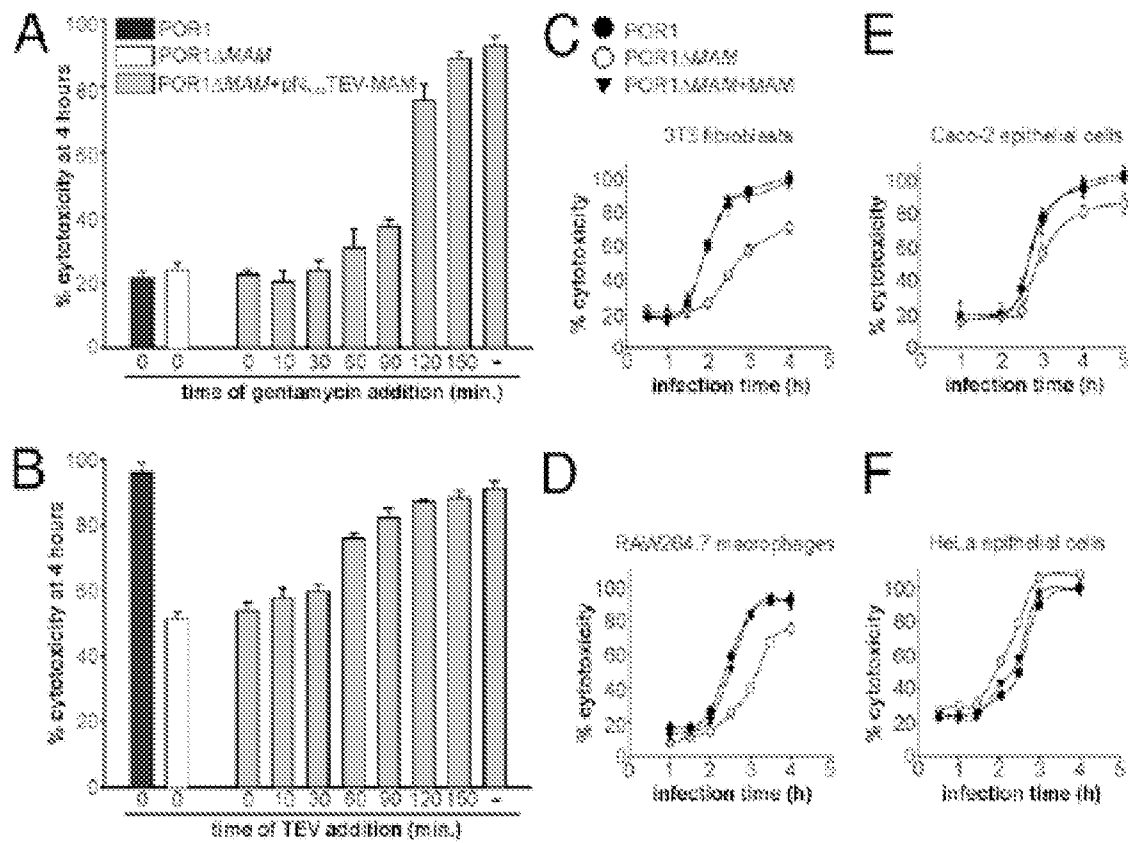


FIG. 2A-F



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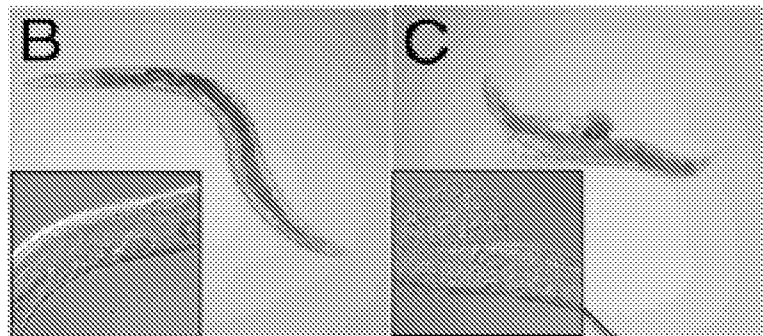
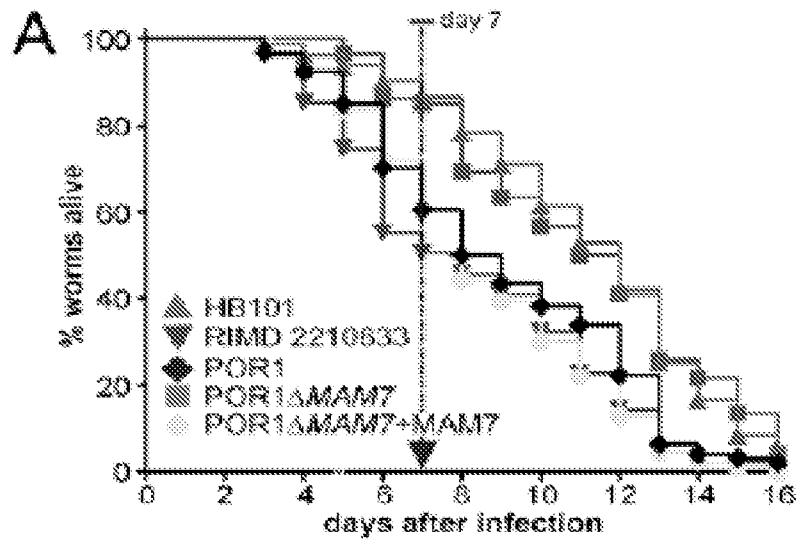


FIG. 3A-C

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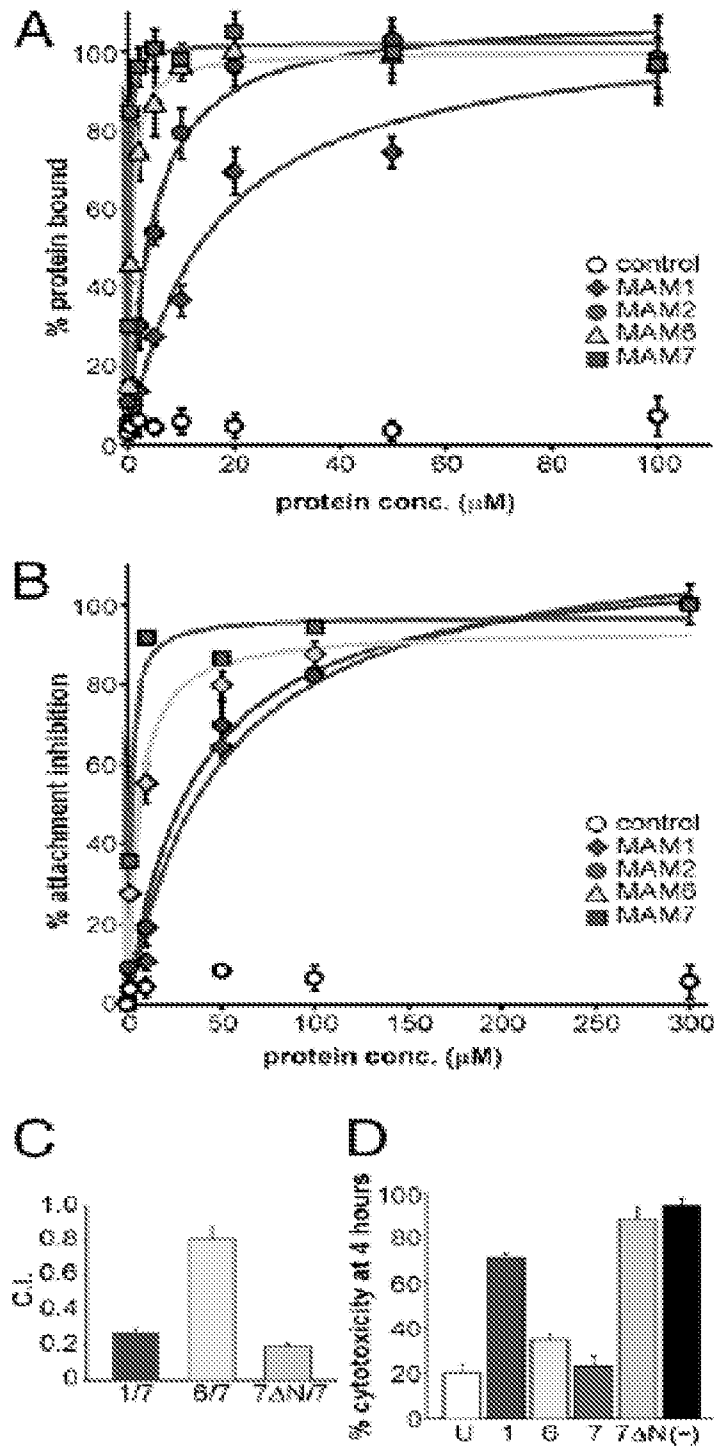


FIG. 4A-D

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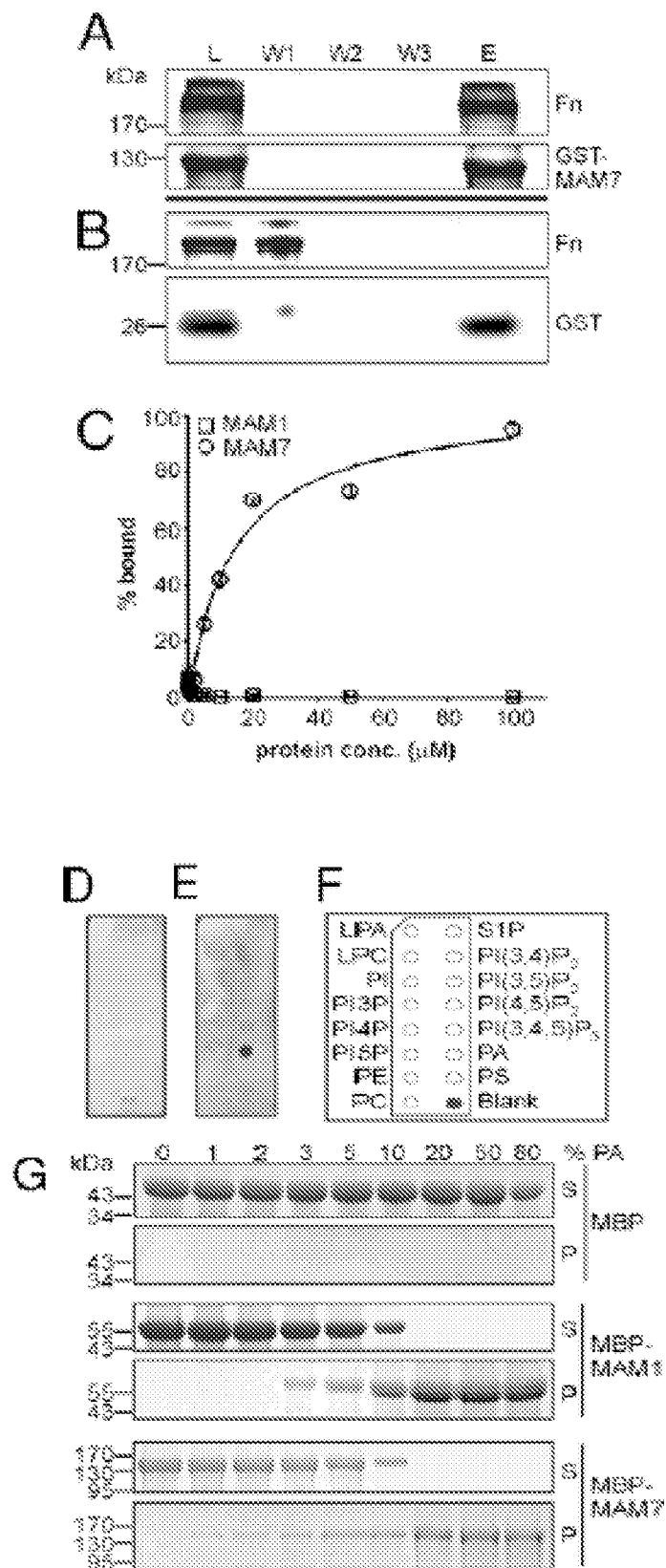


FIG. 5A-G

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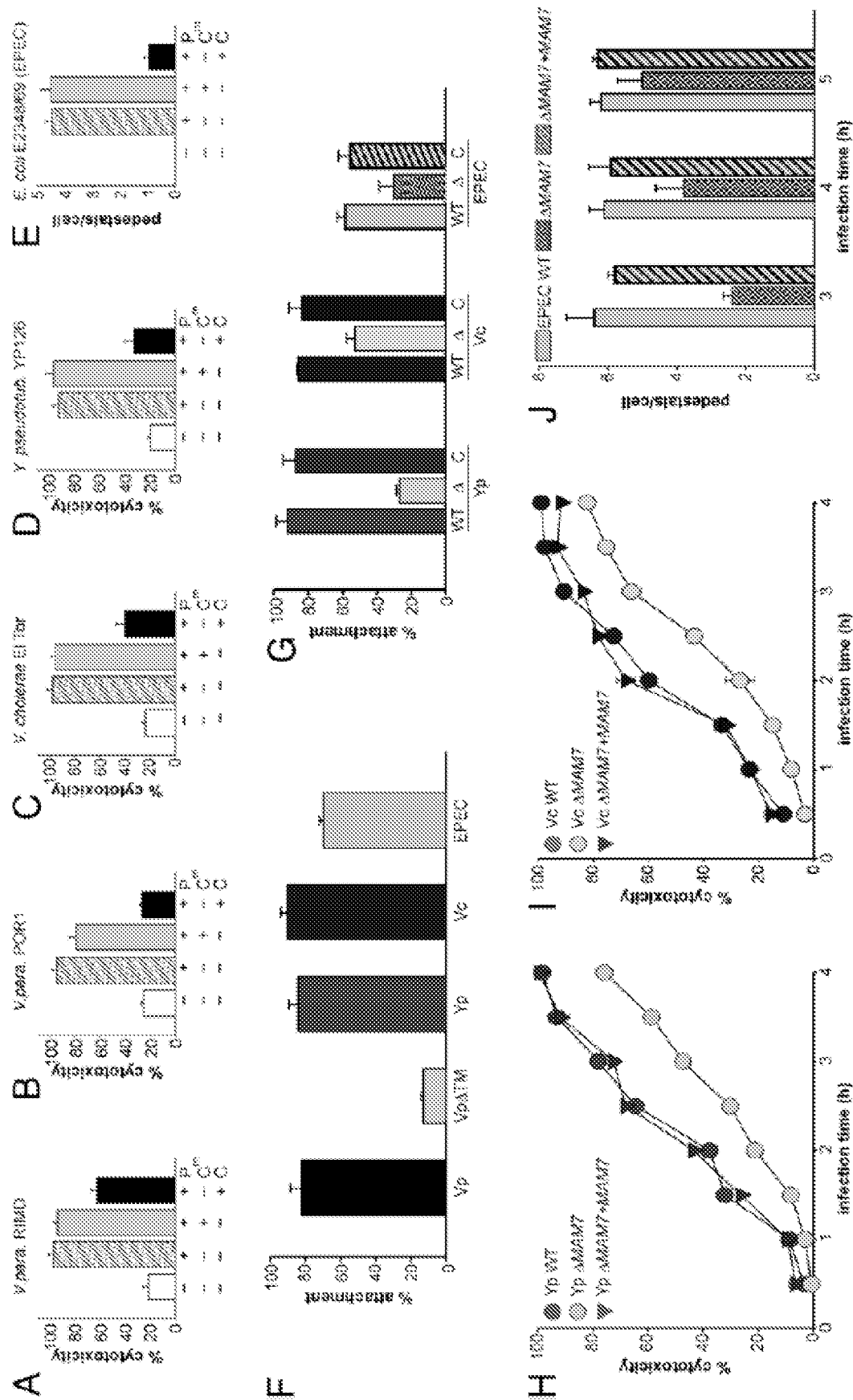


FIG. 6A-J

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**A**

	10	20	30	40	50	60
mce1 (aa.046-138)	AGQRVQIYFSDAAGLVAGRTTIRYQGLEVGMRDINLSE----	DLGSIYVDADIYPEATK				
mce2 (aa.161-251)	QGLNIKLSRLDGGVSIQ-SQIVYEKIPICAVYSYQLDE----	DAKSITIQANIQEQRH				
mce3 (aa.281-373)	EGIAIKIALPDDNKVSSEGAPIMYRGIEIGQVTLDSLSE----	GREVILASAAIQPAFSD				
mce4 (aa.396-481)	-SIAIRKLISDINSFGLDSG-ANYLYKGIIVVGSIIINVGLVDEKKQTKHEVFMDVLIDHEYKH					
mce5 (aa.523-611)	GSKTLMLEFASELPPIISKQ-SPLLYRNLEPVGNVSDFHL-----	VDGGVLIKATIENRFAY				
mce6 (aa.641-732)	EGRVISLETDTGTQEVLENG-MPIEYQGVKVGCVTLVVPNF----	RRNLVEVTARILPEYVE				
mce7 (aa.750-840)	EGVMFTLQSEQPGSVQVG-TFVLYRQMEVGQVTEVPLGE----	FADRVVSTIKIKPEYAY				
	.	.	:	.	:	*
	70	80	90			
mce1 (aa.046-138)	-LLNDKTRFWLVKPTASLTGVSGLDALVSGNYISIQFG					
mce2 (aa.161-251)	-IINDRSRFFWNVSGIGASIGFEGVDVRLE-SMSALLGG					
mce3 (aa.281-373)	-MLTTGTRFVLEEAKVSLSGVENIANLVKGNFLTIVPG					
mce4 (aa.396-481)	-LIKSNRFRFYVTGSASAELETESGLSVTVF-----					
mce5 (aa.523-611)	-LVTFQTVFWMRSGIEIDASLSGVSVKHAH-PLKSLIEG					
mce6 (aa.641-732)	NEAVEGTHFWLTEPEIGLGGVKNLGALVS-KSISVEPG					
mce7 (aa.750-840)	-LVRQNSVFWNVSGVDVSGITSGANIKAG-TIDSLVRG					
	:	.	*	.		

FIG. 7A

	10	20	30	40	50	60
V. para	MSDQNN	SQTSYV	PDVKR	PSKGIS	PLWLLP	ILTMVL
V. cholerae	MSQENIT	QTSYTF	ENKRRG	ISPLWLL	PIVIMF	LAGWLV
Y. pseudot	-MQQETP	STPTEA	HVKHKK	RISPFW	LLPFIA	LLITG
EPEC	-MSQETP	PASTTEA	QIKNKK	RISPFW	LLPFIA	LLMIAG
	.....	..	.....	: ****:****:.....:****:	.	: : * : * * .: *
	70	80	90	100	110	120
V. para	VAGRTT	IRYQG	LEVGM	VRDINL	SEIDL	SGSIYV
V. cholerae	IAGETT	IRYQG	LEVGM	VEDIKL	SEGLDS	SIYVEA
Y. pseudot	VAGETT	IRYQG	VLVGL	VQSIRL	DDNLR	NIKVTAS
EPEC	VPGRTE	VPYRQ	GVVGV	TVQDIS	LDLSE	DLKIEV
	..***:****:..*	*.*	..**	..* * ..*	:	. * : ****:*.****:*
	130	140	150	160	170	180
V. para	VSGLDAL	VSGNYI	SIQPG	--DGQ	EFETTE	HHALLS
V. cholerae	VSGLDAL	VSGNYI	AIQPG	STHQED	YPTQY	QALDS
Y. pseudot	VSGLDAL	VGGNYI	IGMPG	---EGK	PQSHET	ALDTQ
EPEC	VSGLDAL	VGGNYI	IGMPG	---KGK	EQDHEV	ALDTQ
	*****	*****	: **	.	: ***: *	* * * .: ****:.. *
	190	200	210	220	230	240
V. para	QIVKIP	ICAVS	IQLEA	SITIAN	QEQ	PHILNDR
V. cholerae	QIVKIP	ICAVS	IQLEA	SITIAN	QEQ	PHILNDR
Y. pseudot	LVYKIP	ICAVS	IQLEA	SITIAN	QEQ	PHILNDR
EPEC	LVYKIP	ICAVS	IQLEA	SITIAN	QEQ	PHILNDR
	*	: ****:*	*.*	: :	: ****:*	*****

FIG. 7B

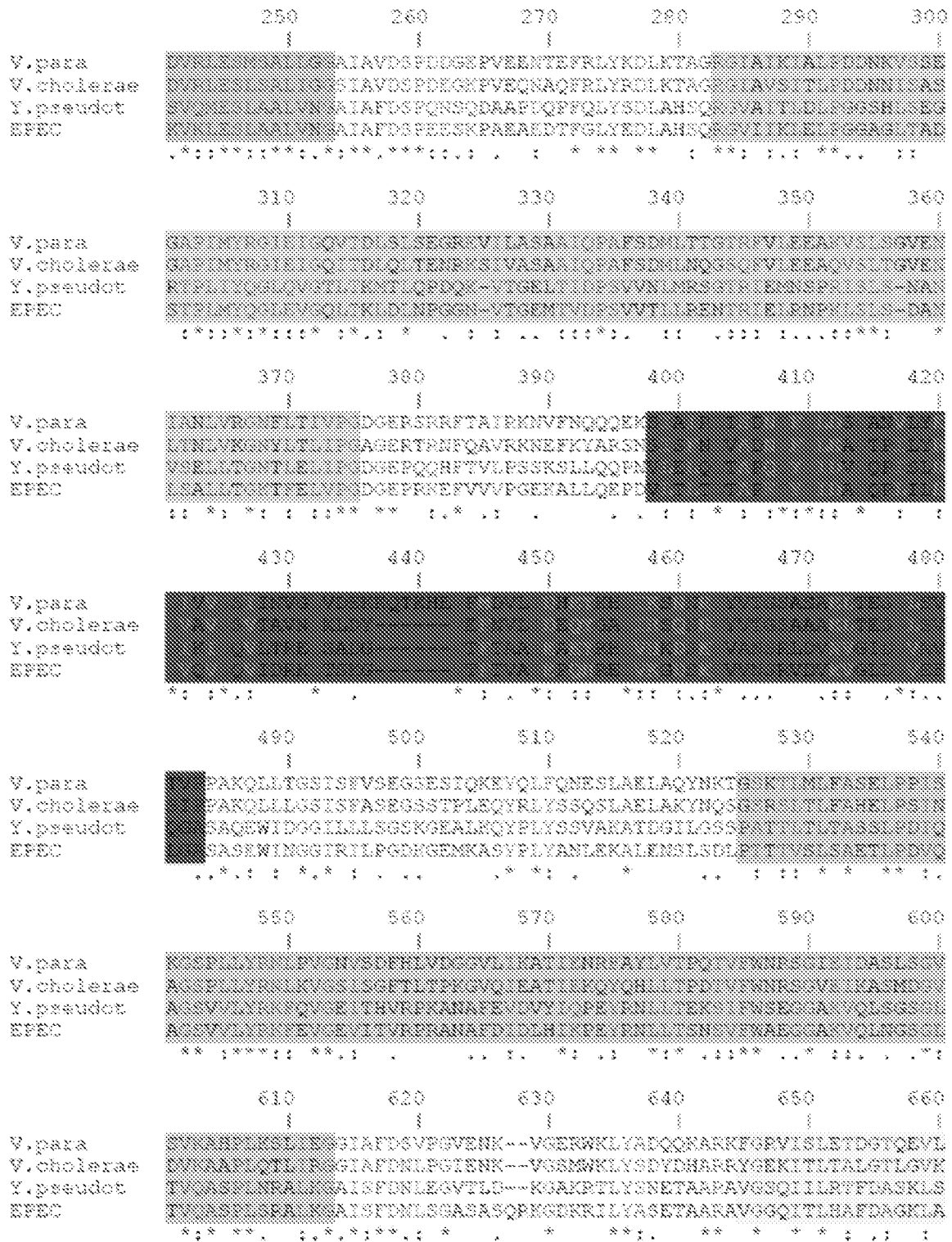


FIG. 7B(cont.)

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	670	680	690	700	710	720
V. para	KGMETIEYQGVKVGCVTLVVPNFRNLVEVTARILPEYVENIAVEGTHFWLTPETIIGLGGV					
V. cholerae	VGTPVQYQGVQIGEVFEIIPDFESDFVKLAARIEPQYAPKIAKQNSQFWLSQAKIGLSGI					
Y. pseudot	AGMPIRYLGIDIGQVESLKLAPERNEVLAKAVLYPEYVQNFTRAGTFHSIVSPEISAAGV					
EPEC	VGMPIRYLGIDIGQIQTLDLITARNEVQAKAVLYPEYVQTFARGGTFHSVVTTPQISAAGV					
	* * : , * * : : * : : : : : : : * * : * : * : : : : * : : : * : : : * : : *					
	730	740	750	760	770	780
V. para	KNLGALVSKSISVEPGNGKAKFDFQLEKGFDR---VEGVMTLSEERGVQVITPVL					
V. cholerae	ENVQNLGQSIIEVQPGNGESRFEFELHKEARHG---GAGNTYTLSEERGVQVITPVL					
Y. pseudot	NNLETLFQPYINVEPGKGGPLRNFEQTATITDSRYLDLSITLSEERGVQVITPVL					
EPEC	ENHLDTLQPYINVEPGRCNPRDFELQEATITDSRYLDLSITLSEERGVQVITPVL					
	::: : : * : * : * : * : : : * : : : : : : : * : : : : * : : * : * : *					
	790	800	810	820	830	840
V. para	EQGVVQVITPVLSEERGVQVITPVLSEERGVQVITPVLSEERGVQVITPVL					
V. cholerae	EDIEVQVITPVLSEERGVQVITPVLSEERGVQVITPVLSEERGVQVITPVL					
Y. pseudot	EGIEVQVITPVLSEERGVQVITPVLSEERGVQVITPVLSEERGVQVITPVL					
EPEC	EGIEVQVITPVLSEERGVQVITPVLSEERGVQVITPVLSEERGVQVITPVL					
	* : * * * * : : * * : * * * : : * * : : : * : * : * * * * * * : * : * : * : *					
	850	860	870	880	890	
V. para	TIQSLPAGIAFSTPEQSQIPFAAKRGHSFYLYPRADESWVQWRTPIPKP					
V. cholerae	TIQSLPAGIETATPEQKQLTPAAPEGHTFYLYPQAQEWTWRTPIPKP					
Y. pseudot	TIQSLPAGIAFATPPTIPLAPRANVNQHFLLAPEEPKDWPKWGTALPRS					
EPEC	TIQSLPAGIAFATPPGTPLAPKAEQGHFLQSEPKEWPEWGTALPK-					
	* : : : : * * * : * * : : * * : : * * : : * * : * : * : *					

FIG. 7B(cont.)



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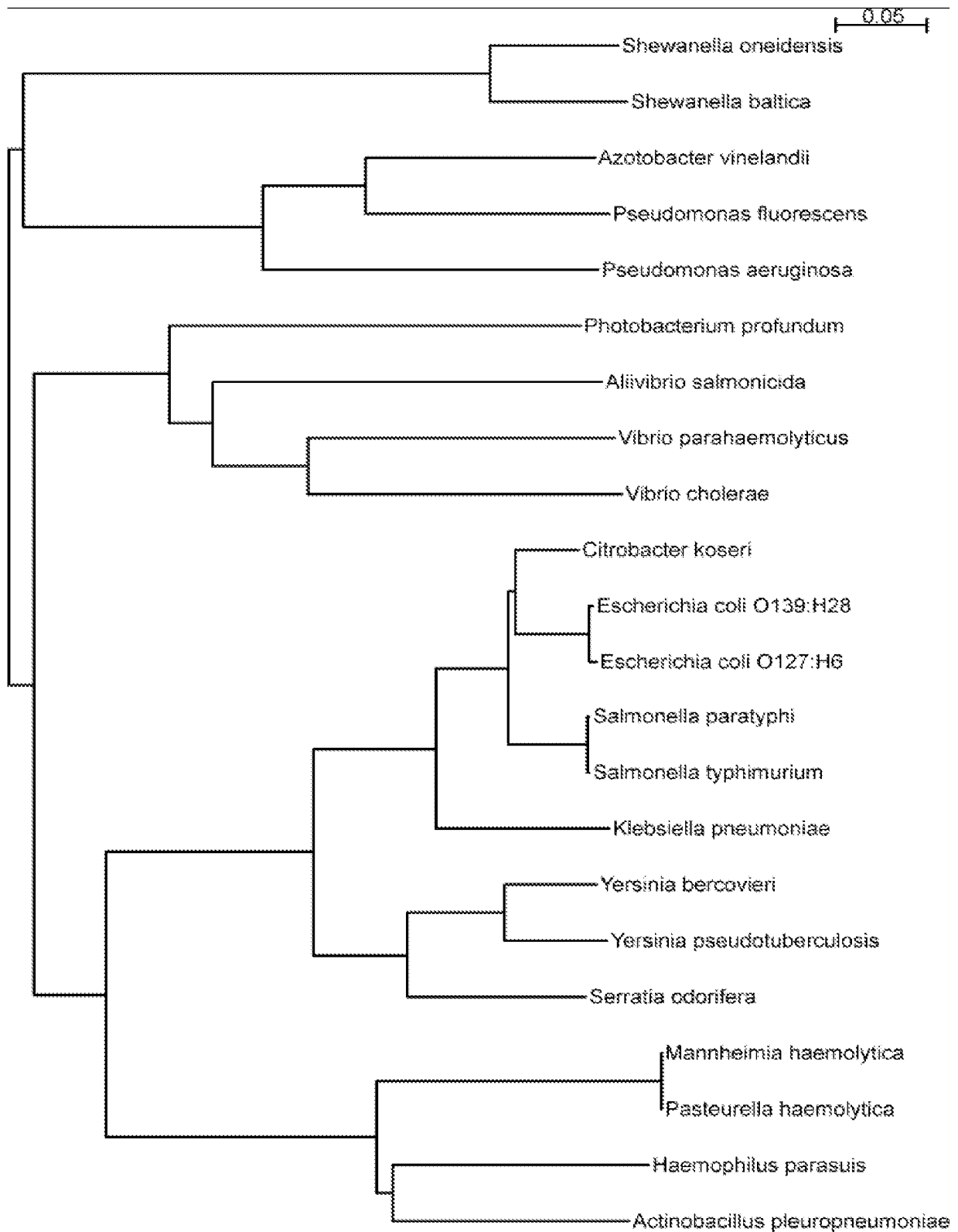


FIG. 8

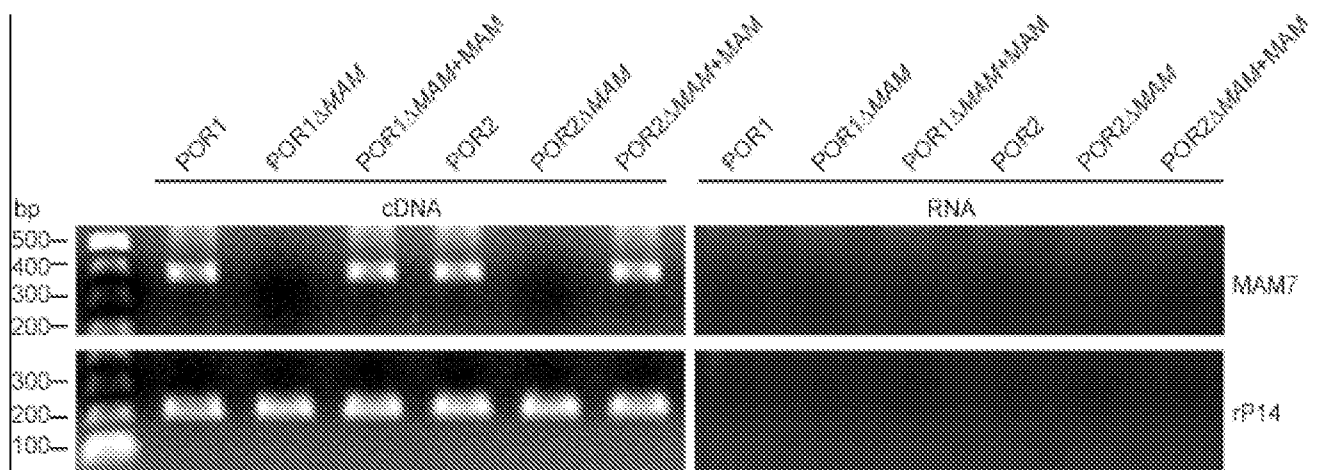


FIG. 9

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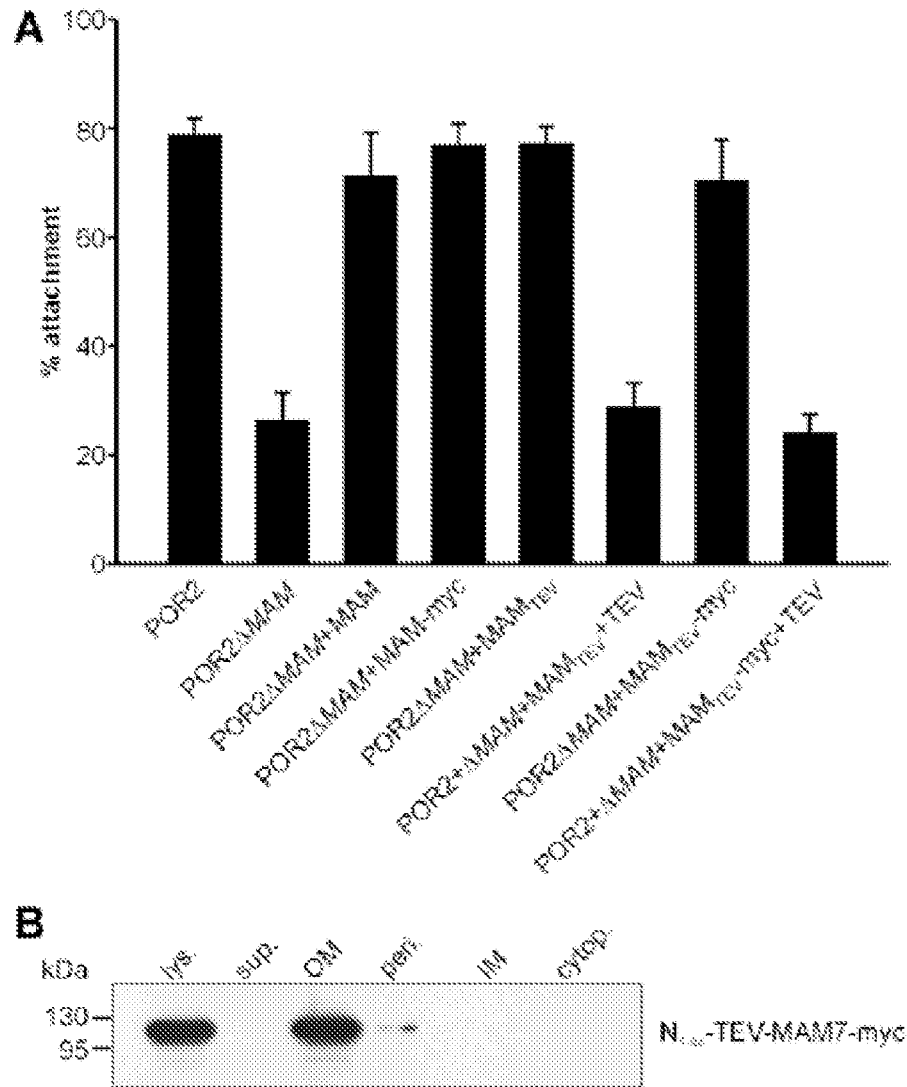


FIG. 10A-B

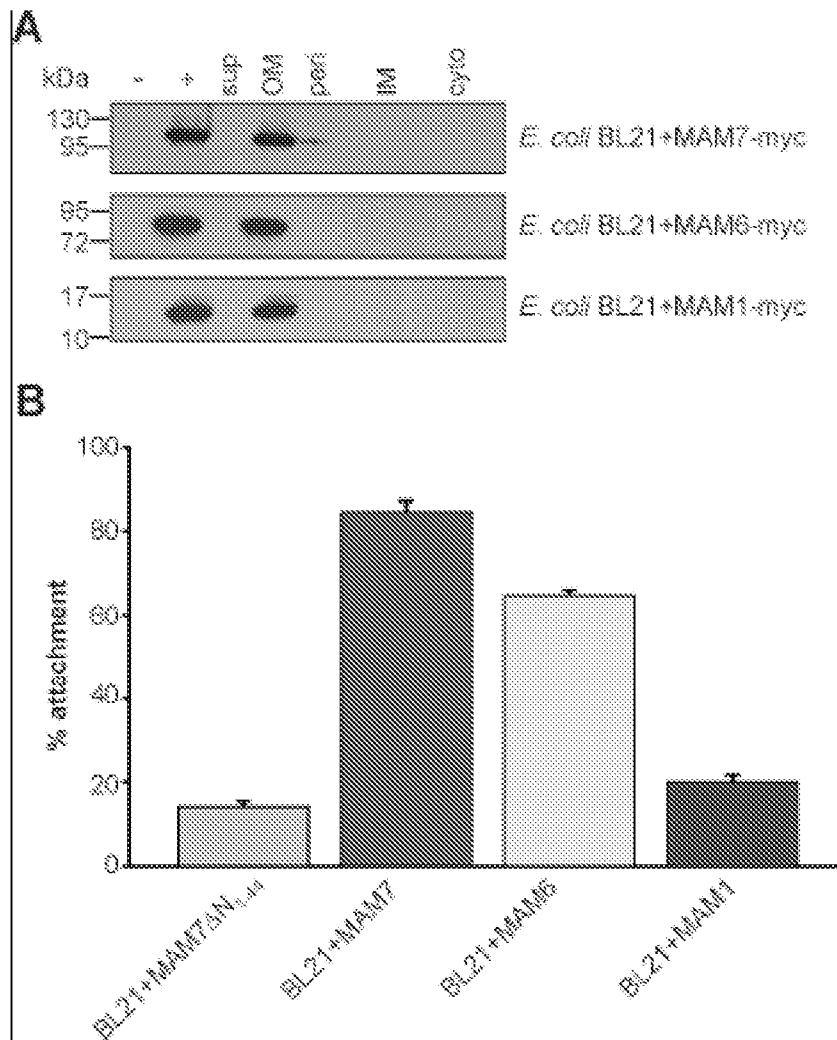


FIG. 11A-B

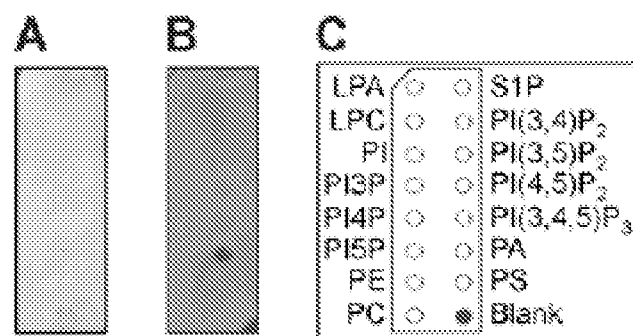


FIG. 12A-C

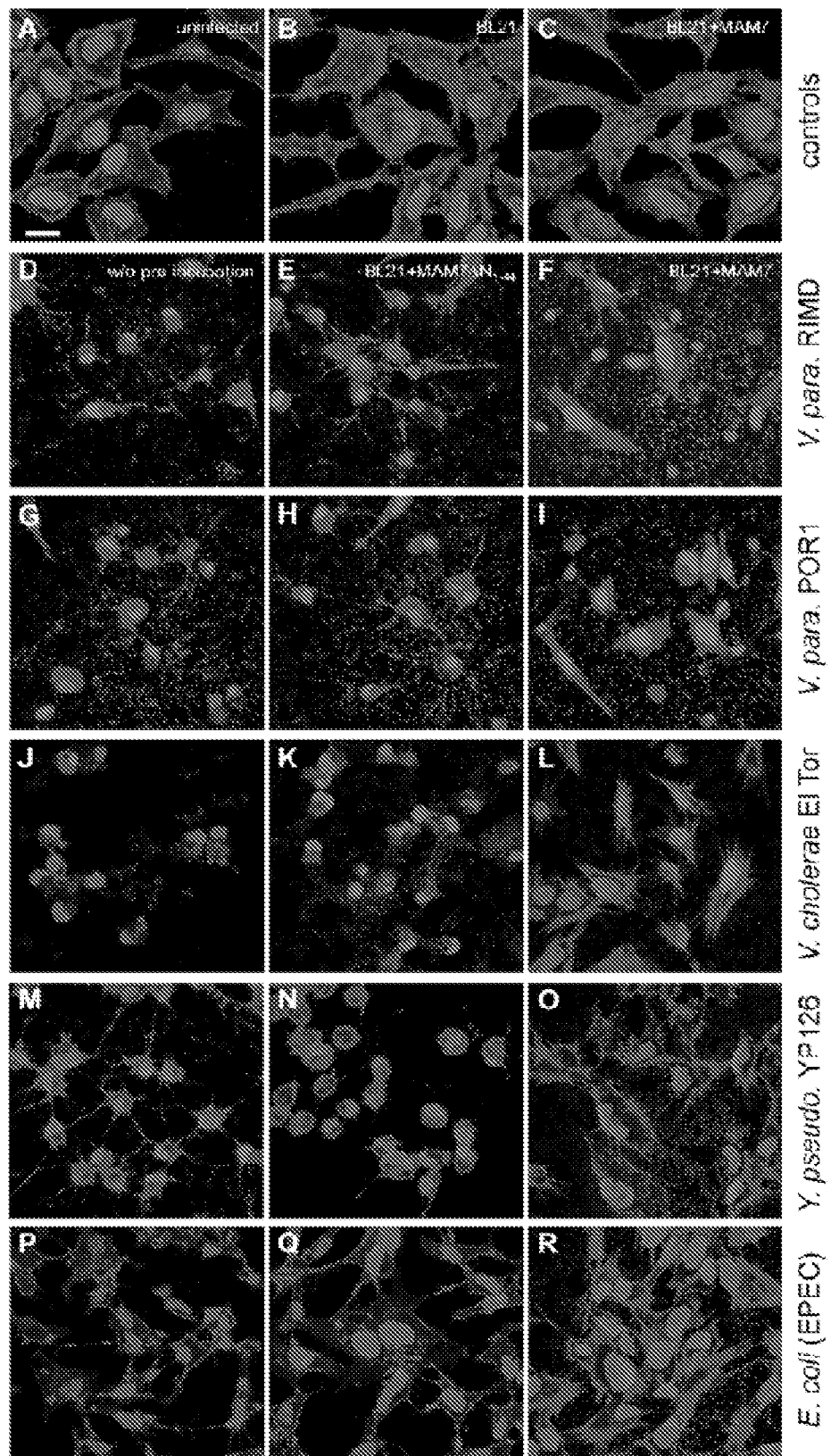


FIG. 13A-R

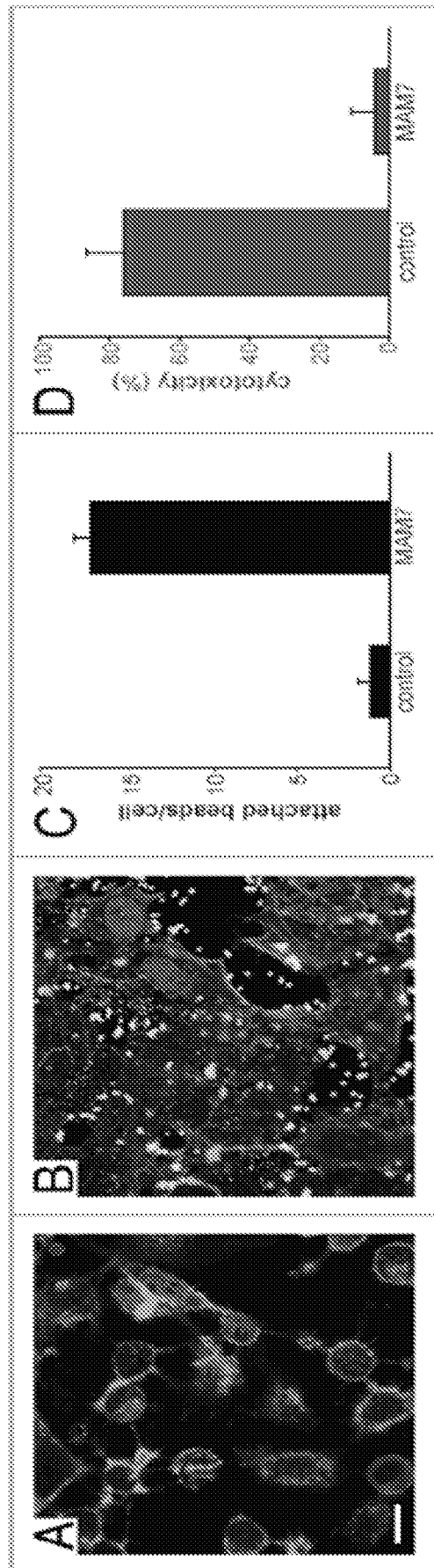


FIG. 14A-D

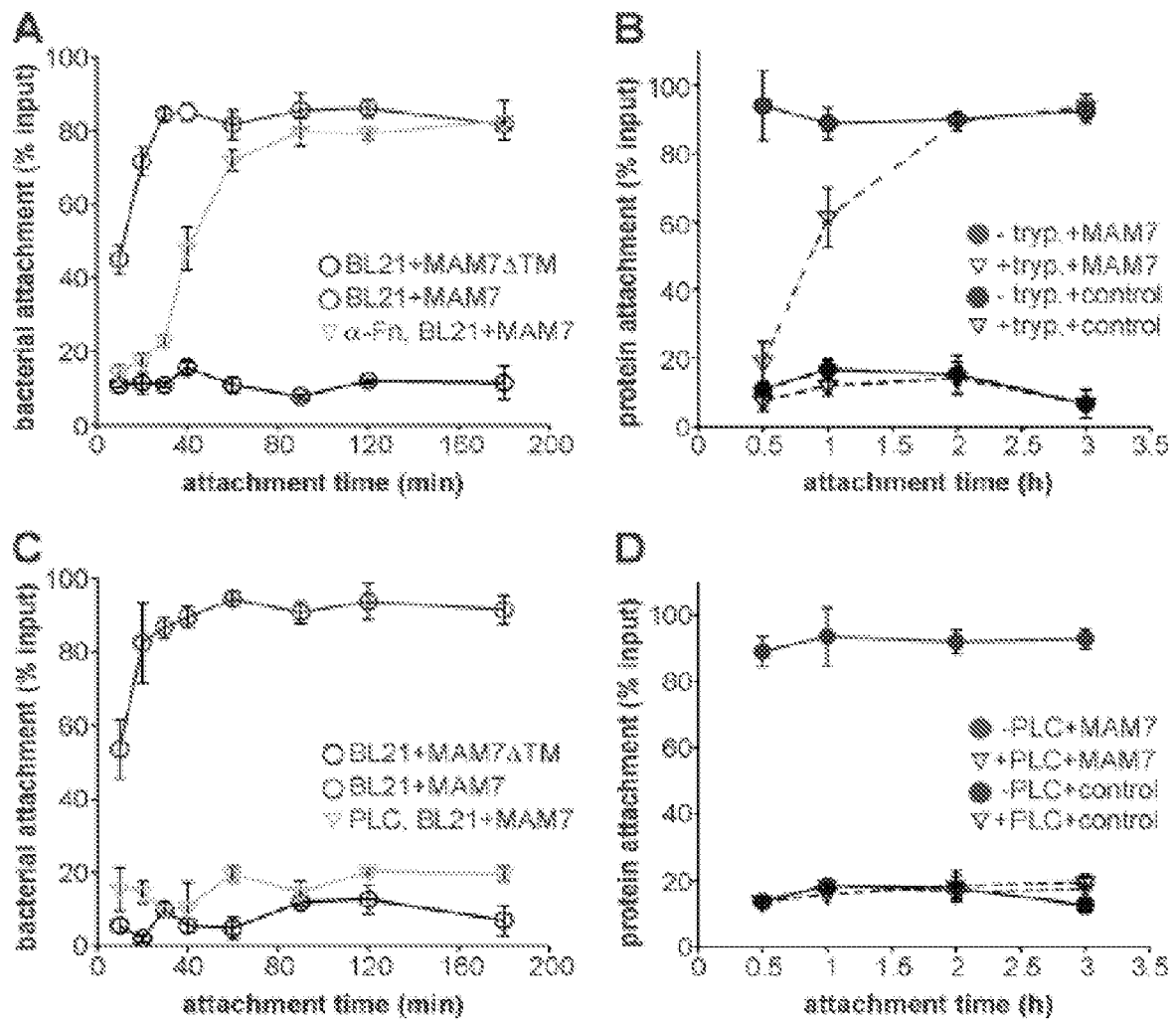


FIG. 15A-D



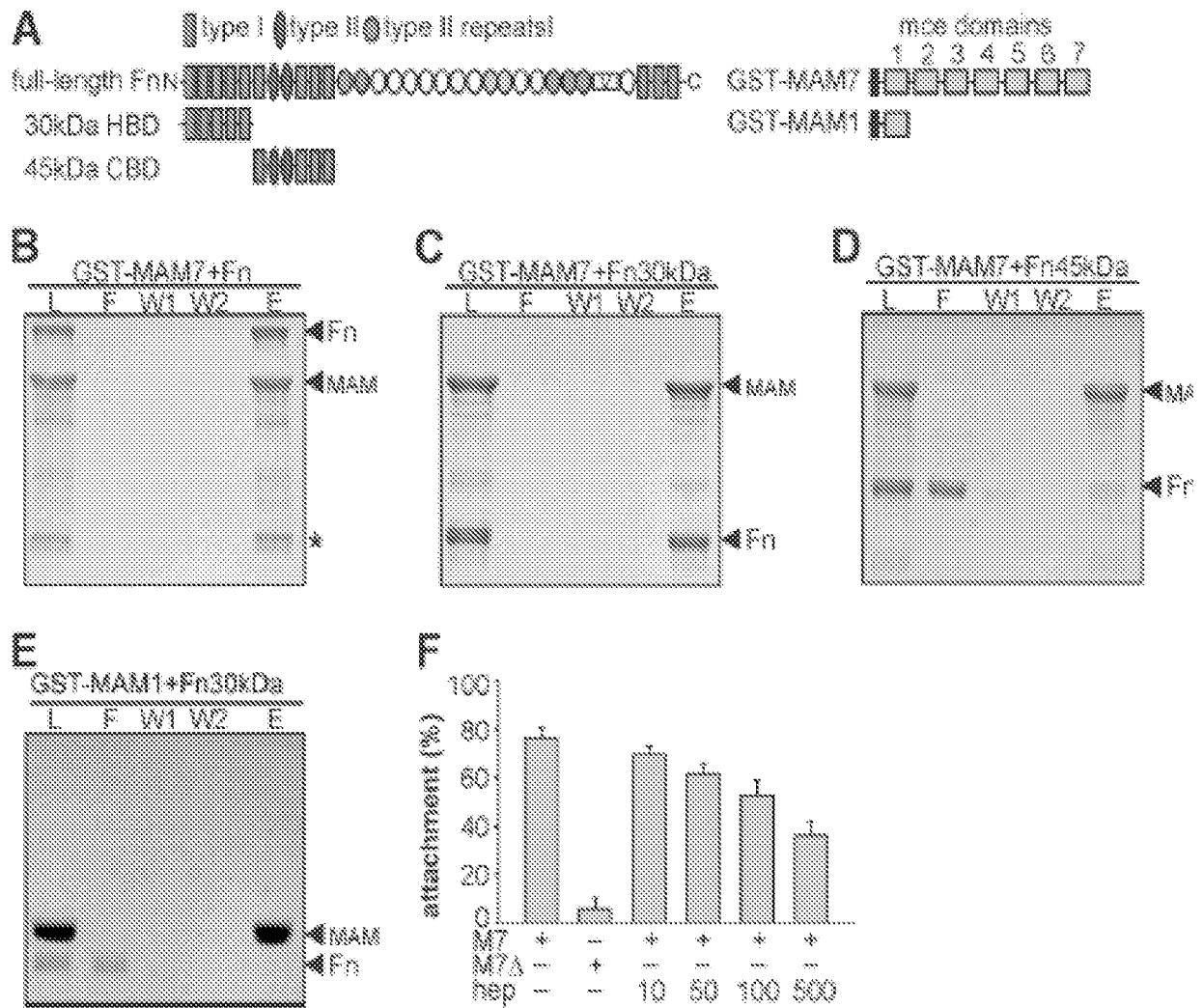


FIG. 16A-F

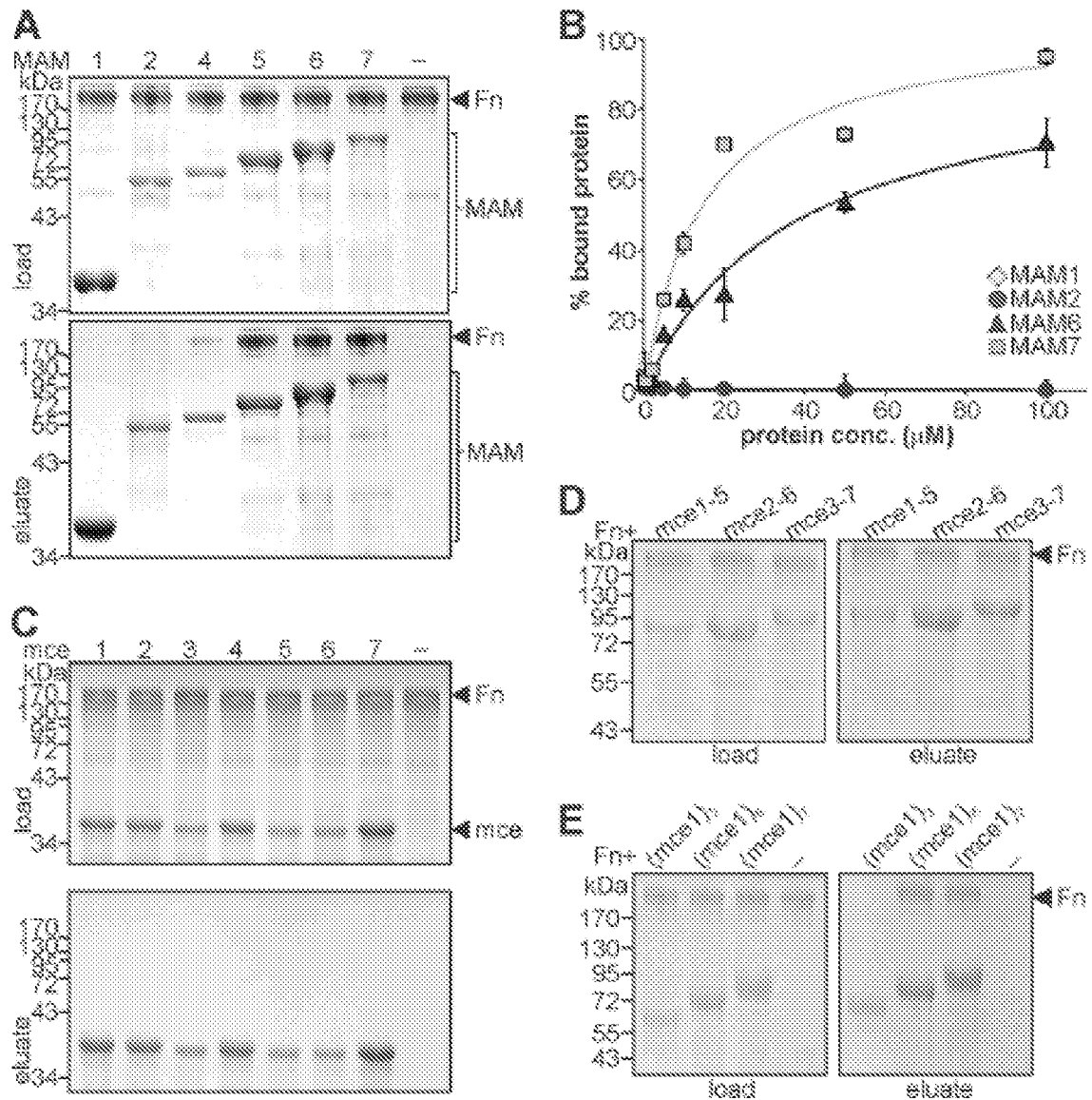


FIG. 17A-E

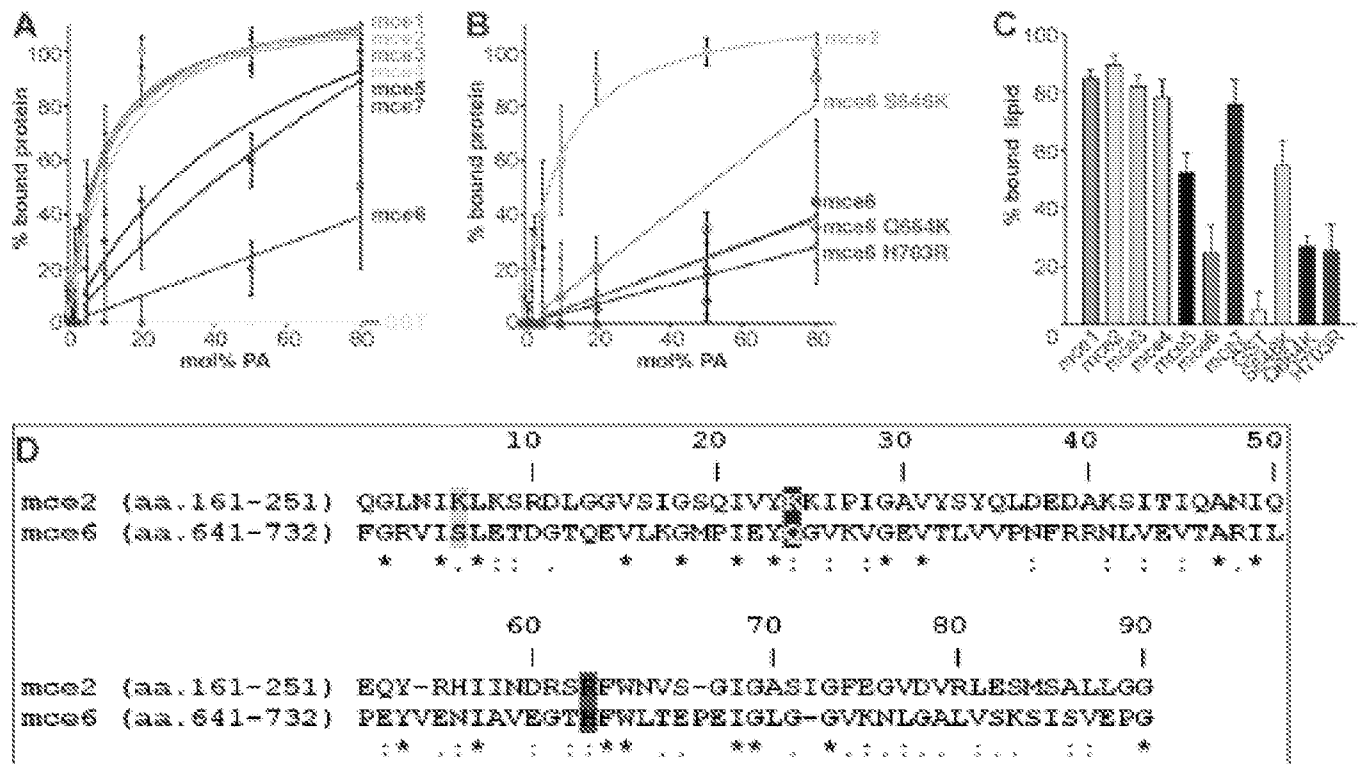


FIG. 18A-D

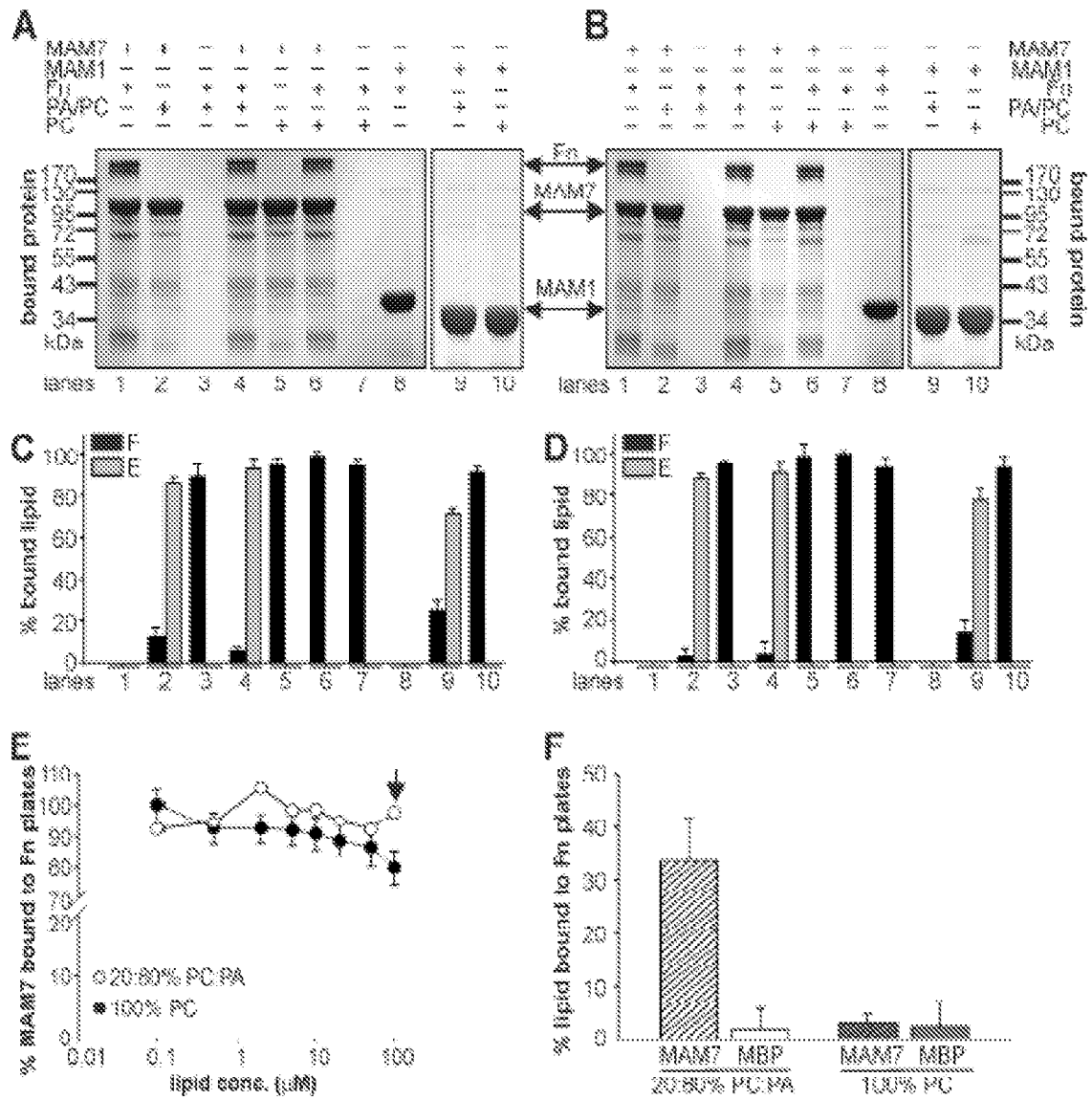


FIG. 19A-F

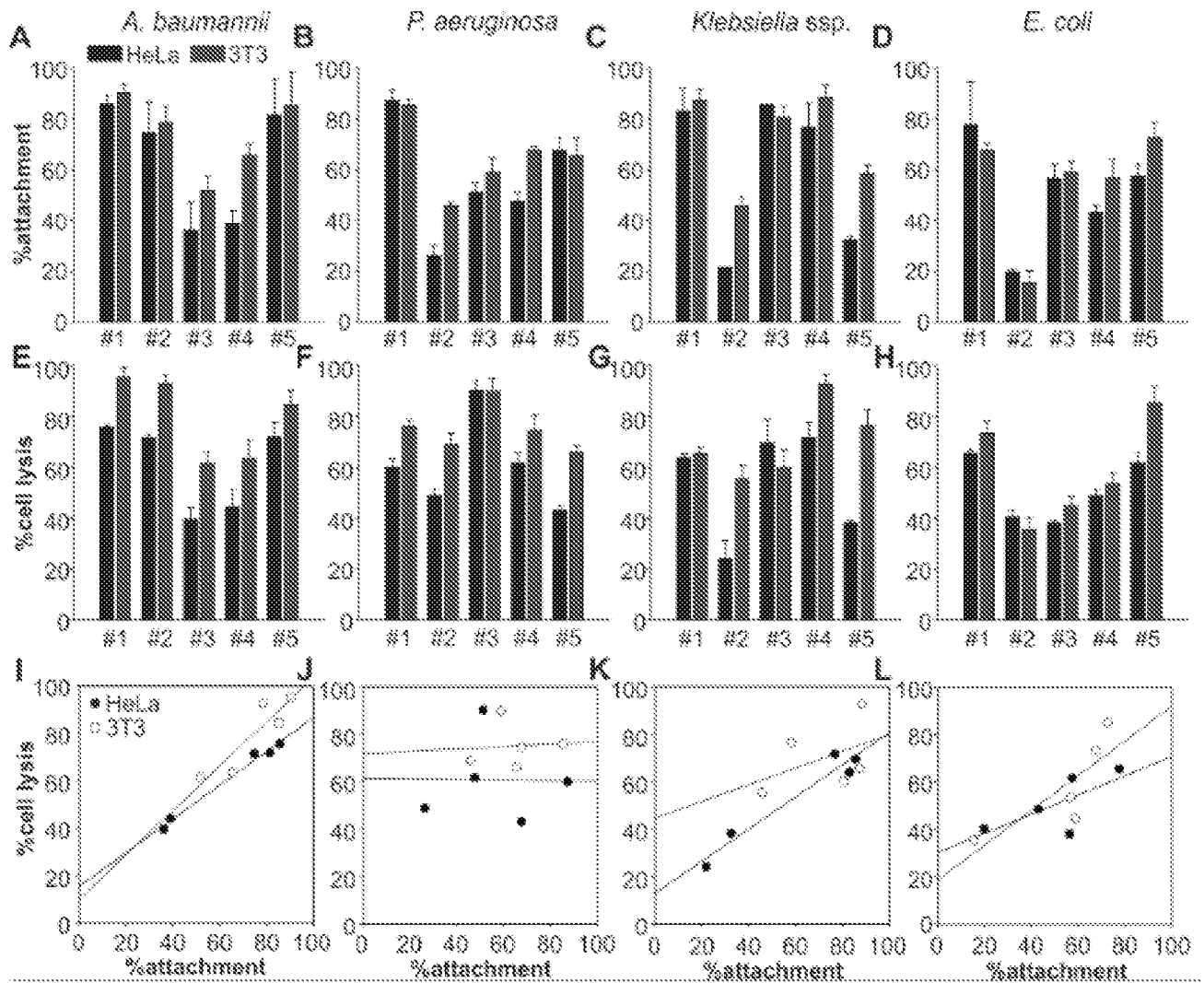


FIG. 20A-L

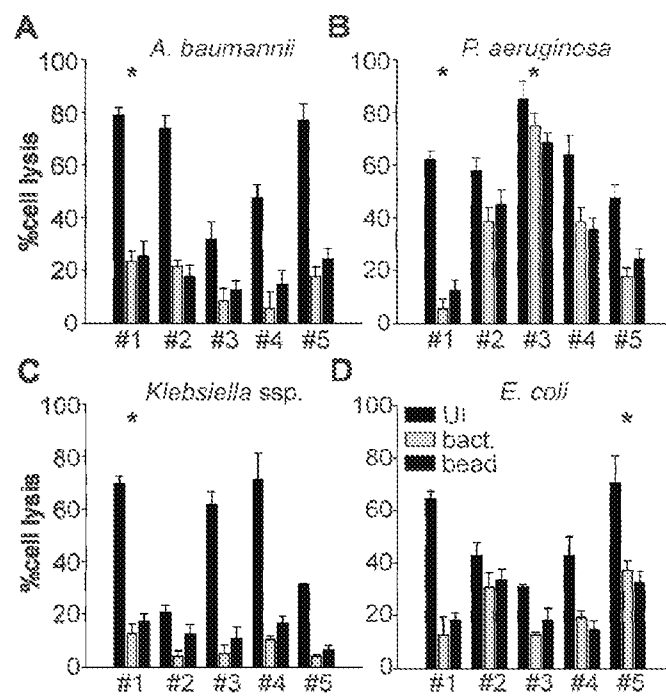


FIG. 21A-D

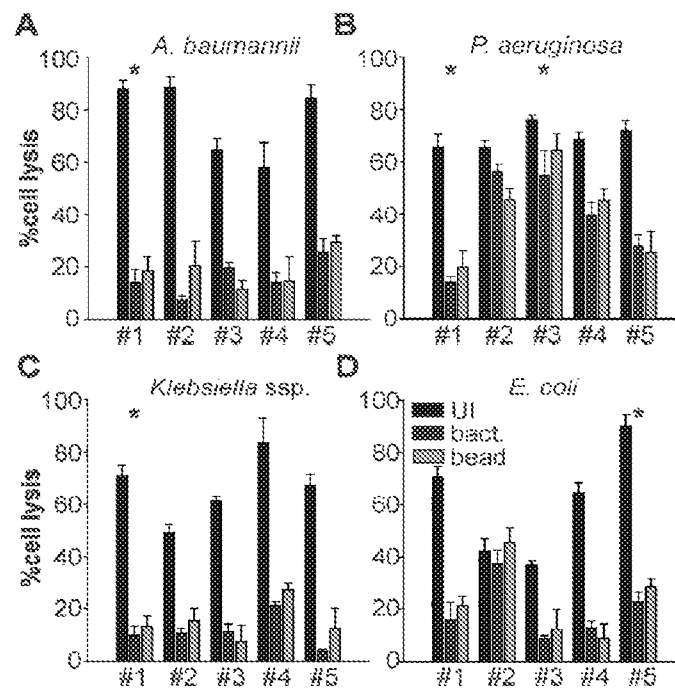


FIG. 22A-D

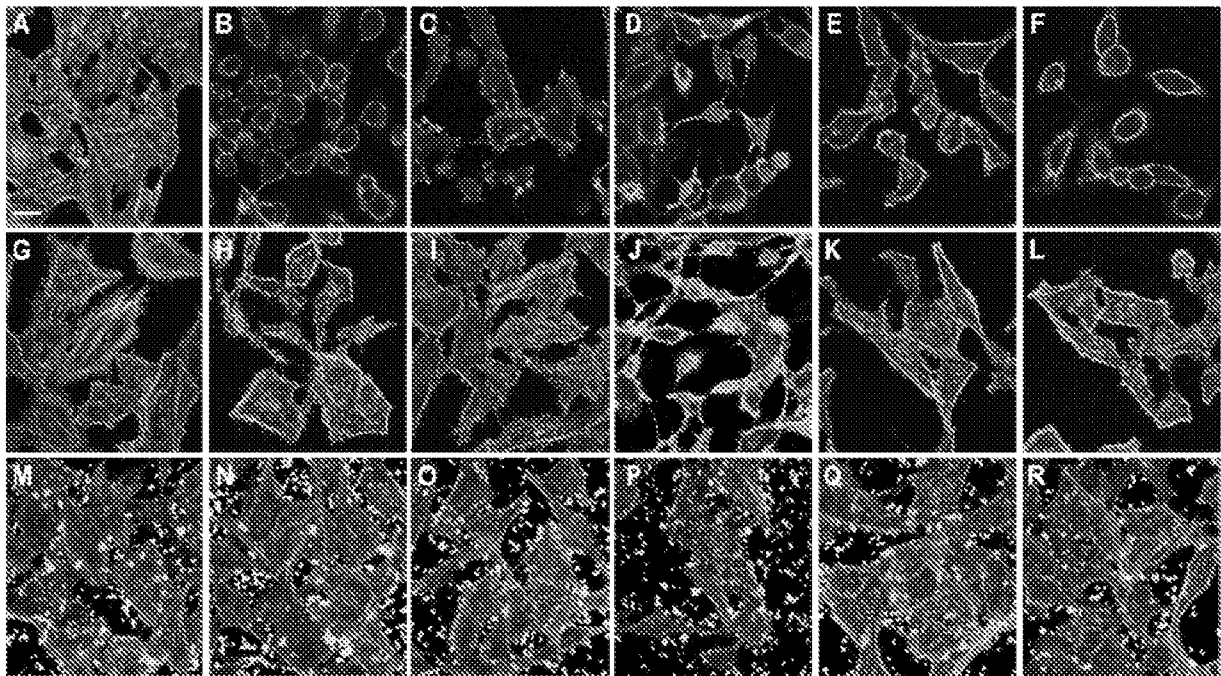


FIG. 23A-R

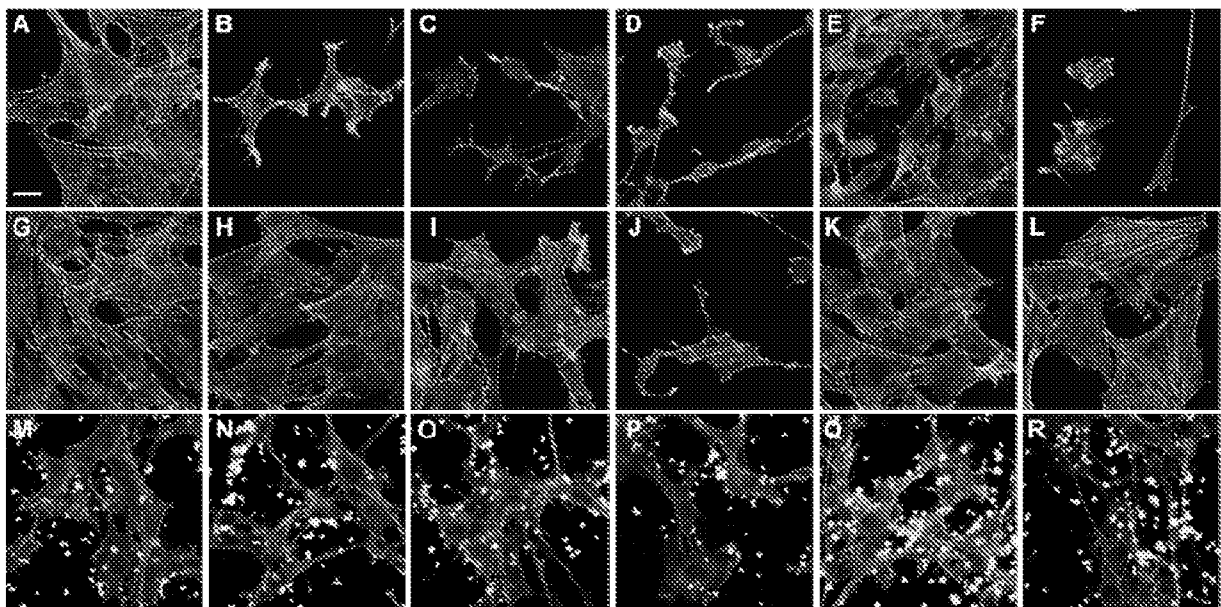


FIG. 24A-R