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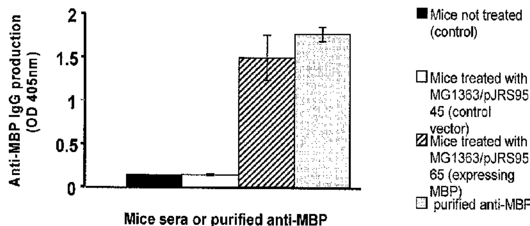

FIG. 19

(57) Abstract: Provided herein are methods and compositions for the display of polypeptides of interest on the tip of pili of Gram-positive bacteria. According to the present invention, the polypeptide of interest is amino terminal to a Gram-positive bacterial pilus tip protein or an active variant or fragment thereof, wherein the active variant or fragment comprises a cleaved cell wall sorting signal (CWSS) motif. The Gram-positive bacterium displaying a polypeptide of interest on the tip of pili that are disclosed herein are useful, for example, in methods for immunizing a subject with an antigen and methods for removing contaminants from a composition.

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METHODS AND COMPOSITIONS FOR THE DISPLAY OF POLYPEPTIDES ON THE PILI OF GRAM-POSITIVE BACTERIA

FIELD OF THE INVENTION

The present invention relates to the field of microbial polypeptide display.

5

BACKGROUND OF THE INVENTION

Heterologous surface display of proteins on recombinant microorganisms involves the targeting and anchoring of heterologous proteins to the outer surface of host-cells such as yeast, fungi, mammalian and plant cells. Display of heterologous proteins at these cells' surfaces can take many forms, varying from the expression of reactive groups such as
10 antigenic determinants, heterologous enzymes, (single-chain) antibodies, polyhistidyl tags, peptides, and other compounds. Heterologous surface display has been applied as a tool for research in microbiology, molecular biology, vaccinology, and biotechnology.

BRIEF SUMMARY OF THE INVENTION

15 The present invention provides methods and compositions for the display of at least one polypeptide of interest on the tip of pili of Gram-positive bacteria. Methods comprise introducing into a Gram-positive bacterium a polynucleotide that encodes a chimeric polypeptide to produce a transformed Gram-positive bacterium expressing the chimeric polypeptide. The chimeric polypeptide comprises the polypeptide of interest and
20 a Gram-positive bacterial pilus tip protein or an active variant or fragment thereof. The pilus tip protein, active variant or fragment thereof comprises a cell wall sorting signal (CWSS) and is carboxyl to the polypeptide of interest. The Gram-positive bacterium expressing the chimeric polypeptide also expresses a tip sortase and a pilus shaft polypeptide. The transformed Gram-positive bacterium is then grown under conditions
25 wherein the pili are formed. The pili produced by the transformed bacteria display the polypeptide of interest.

Compositions comprise Gram-positive bacterium comprising a polypeptide of interest covalently attached to the tip of a pilus, wherein the polypeptide of interest is amino terminal to a Gram-positive bacterial pilus tip protein or an active variant or fragment thereof, wherein the active variant or fragment comprises a cleaved cell wall
5 sorting signal (CWSS) motif. The Gram-positive bacterium displaying a polypeptide of interest on the tip of pili that are disclosed herein are useful in methods for immunizing a subject with an antigen, methods for removing contaminants from a composition (e.g., soil, water), and methods for improving food products.

10 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the FCT region of serotype M3 Group A Streptococcus strain M3 and derived constructs. The positions of HA tags and mutations introduced by site-specific mutagenesis are indicated by arrowheads. Vectors pCR2.1 and pCR-XL are *E. coli* cloning vectors (Invitrogen), and pRegP23 (Barnett *et al.* (2007) *J Bacteriol* 189:1866-1873), is a
15 Gram-positive-*E. coli* shuttle vector. Zähner and Scott (2008) refers to Zähner and Scott (2008) *J Bacteriol* 190:527-535, which is herein incorporated by reference in its entirety.

Figures 2A-2C show the identification of the Cpa(HA)-T3 dimer in *E. coli*. Cell lysates of *E.coli* TOP10 containing plasmid pCR2.1 (lane 1), pJRS1325 (lane 2), and pJRS1326 (lane 3) analyzed:
20 Figure 2A presents a Western blot reacted of whole cell lysates with anti-HA antiserum (reproduced from Zähner and Scott (2008) *J Bacteriol* 190:527-535);

Figure 2B shows the results of immunoprecipitation of a crude extract with "EZview Red Anti-HA Affinity Gel", followed by boiling in SDS and SDS PAGE stained with SYPRORuby. Monomeric Cpa(HA) (Cpa), the putative Cpa-(HA)-T3 dimer (Cpa-
25 T3), and the light chain (lc) and heavy chain (hc) of IgG are indicated on the right; and

Figure 2C presents the protein sequence of T3 (SEQ ID NO: 7) with regions covered by tryptic peptides identified by mass spectrometry indicated in bold. The N-terminal signal peptide (SP) and the C-terminal cell wall sorting signal (CWSS) are underlined.
30 Figures 3A-3D demonstrate the effect of mutations in the CWSS motif of Cpa(HA) (Figures 3A and 3B) and of T3 (Figures 3C and 3D) on formation of the Cpa(HA)-T3 heterodimer in *E. coli*:

Figures 3A and 3B show a Western immunoblot analysis of hot SDS-treated cell lysates of *E. coli* TOP10 containing plasmids pJRS1325 (lane 1), pEU7646 (lane 2), and

pEU7904 (lane 3) reacted with (Figure 3A) monoclonal anti-HA antibody or (Figure 3B) polyclonal anti-T3 antiserum. The anti-T3 antiserum also reacts weakly with Cpa(HA). The locations of the Cpa(HA) monomer (Cpa), the Cpa(HA)-T3 heterodimer (Cpa-T3), and the T3 monomer (T3) and dimer (T3-T3) are indicated on the right; and

5 Figures 3C and 3D show a Western immunoblot analysis of cell lysates of *E. coli* TOP10 containing plasmids pJRS1325 (lane 1), pEU7646 (lane 2), and pEU7905 (lane 3) reacted with (Figure 3C) anti-T3 antiserum or (Figure 3D) anti-HA antibody. The locations of the Cpa(HA) monomer (Cpa), the Cpa(HA)-T3 heterodimer (Cpa-T3), and the T3 monomer (T3) and dimer (T3-T3) are indicated. The sizes of molecular mass standards

10 (in kilodaltons) are indicated to the left.

Figures 4A and 4B shows that the mutation of the Cpa CWSS motif from VPPTG to VP prevents the formation of the Cpa(HA)-T3 heterodimer and incorporation of Cpa into HMW pilus polymers in GAS.

Figure 4A shows a Western immunoblot analysis of cell wall extracts (lanes 1-4)

15 and 10-fold concentrated supernatants (lanes 5-8) from GAS strains JRS4/pJRS9545 (lanes 1 and 5), JRS4/pJRS9550 (lanes 2 and 6), JRS4/pJRS9554 (lanes 3 and 7), and JRS4/pJRS9597 (lanes 4 and 8) analyzed with a monoclonal anti-HA antibody.

Figure 4B shows a Western immunoblot analysis of the same cell wall extracts from JRS4/pJRS9545 (lane 1), JRS4/pJRS9550 (lane 2), JRS4/pJRS9554 (lane 3), and

20 JRS4/pJRS9597 (lane 4) analyzed with polyclonal anti-T3 antiserum. Molecular masses are indicated to the left of the figures. The locations of the Cpa monomer (CPa(HA)), the Cpa-T3 heterodimer (Cpa(HA)-T3), Cpa linked to a T3 homodimer (Cpa(HA)-$(T3)^2$), Cpa linked to a T3 homotrimer (Cpa(HA)-$(T3)^3$), and the T3 monomer (T3) are indicated on the right of Figures 4A and 4B. pJRS9545 is derived from pJRS9508, and consists of the

25 pReg696 backbone and the P23 promoter. (wt): wild type; (vc): vector control

Figure 5 shows a sequence alignment of the amino acid sequences of Group A *Streptococcus* major pilin proteins. Strains shown represent the different serotypes containing: FCT-2 (M1), FCT4 (M12, M28), or FCT-3 (all others) regions. The M type is indicated followed by the strain name. Invariant lysine residues are indicated in red (K43,

30 K81, K100, K106, K173, and K191). Residues predicted to be involved in intramolecular bond formation are indicated in blue (aspartic acid; N180 and N307) and brown (glutamic acid; E129 and E264). The positions indicated for K, N, E are deduced from homology to the T3 sequence. The amino acid sequence of the major pilin protein for M3_MGAS315 strain is set forth in SEQ ID NO: 36; M1_SF370 is SEQ ID NO: 37; M28_MGAS6180 is

SEQ ID NO: 38; emmstD33_D633 is SEQ ID NO: 39; M49_591 is SEQ ID NO: 40; M18_MGAS8232 is SEQ ID NO: 41; emm33_29487 is SEQ ID NO: 42; M5_Manfredo is SEQ ID NO: 43; and M12_A735 is SEQ ID NO: 44.

Figures 6A-C show the effect of replacement of lysine with alanine or arginine in T3 on T3 polymerization in *E. coli*. Western immunoblots reacted with polyclonal anti-T3 antiserum.

Figure 6A shows cell lysates of *E. coli* TOP10 strains containing plasmid: pEU7655 (lane 1), pEU7657 (lane 2), pEU7678 (lane 3), pEU7679 (lane 4), pEU7680 (lane 5), pEU7681 (lane 6), pEU7682 (lane 7);

Figure 6B shows *E.coli* TOP10 strains containing plasmid: pEU7655 (lane 1), pEU7657 (lane 2), pEU7678 (lane 3), pEU7682 (lane 4), and pEU7909 (lane 5); and

Figure 6C shows *E.coli* TOP10 strains containing plasmid: pEU7655 (lane 1), pEU7657 (lane 2), pEU7907 (lane 3), pEU7692 (lane 4), and pEU7908 (lane 5). The position of the T3 monomer (T3), dimer (T3-T3), and trimer $(T3)^3$ are indicated on the right. The sizes of molecular mass standards (in kilodaltons) are indicated on the left.

Figure 7 shows the effect of replacement of lysine with alanine or arginine in T3 on T3 polymerization in GAS. A Western immunoblot analysis of cell wall extracts (lanes 1-4) and supernatants (lanes 5-8) of JRS4/pJRS9536 (lane 1, lane 5), pJRS9541 (lane 2, lane 6), pJRS9543 (lane 3, lane 7), pJRS9538 (lane 4, lane 8) reacted with monoclonal anti-HA antibody is shown. The position of the T3 monomer (T3) is indicated on the right. The sizes of molecular mass standards (in kilodaltons) are indicated on the left.

Figure 8 shows the effect of replacement of lysine with alanine in T3 on Cpa(HA)-T3 heterodimer formation in *E. coli*. Western immunoblots are shown that were reacted with monoclonal anti-HA antibody. Figure 8A shows cell lysates of BL21(DE3)CodonPlus-RIL with pJRS1325 (lane 1), pEU7646 (lane 2), pEU7652 (lane 3), pEU7651 (lane 4), pEU7653 (lane 5), pEU7654 (lane 6), pEU7661 (lane 7). Figure 8B shows *E.coli* TOP10 containing plasmid pEU7646 (lane 1), pEU7687 (lane 2), pEU7688 (lane 3). The Cpa(HA) monomer is labeled Cpa, and the Cpa(HA)-T3 heterodimer is labeled Cpa-T3. The sizes of molecular mass standards (in kilodaltons) are indicated on the left. wt, wild type.

Figures 9A and 9B show the effect of replacement of lysine with alanine in T3 on incorporation of Cpa(HA) and T3 polymerization in GAS. Western immunoblots are shown with cell wall extracts (lanes 1-5) and supernatants (lanes 6-9) of JRS4/pJRS9545 (lane 1), JRS4/pJRS9554 (lanes 2 and 6), JRS4/pJRS9550 (lanes 3 and 7),

JRS4/pJRS9557 (lanes 4 and 8), and JRS4/pJRS9558 (lanes 5 and 9) reacted with monoclonal anti-HA antibody (Figure 9A) or polyclonal anti-T3 antiserum (Figure 9B). (vc)=vector control.

Figure 10 provides photographs of whole-bacteria, negative-stain transmission electron microscopic images of JRS4/pJRS9545 (vector control; panels A and D) or JRS4/pJRS9550 (Cpa(HA), SipA2, T3, and SrtC2; panels B, C, E, and F). In panels A-C, the bacteria were incubated with anti-T3 antiserum, followed by an anti-rabbit secondary antibody conjugated to 12-nm diameter gold particles. In panels D-F, the bacteria were labeled with the anti-T3 antiserum as above, together with anti-HA antibody and an anti-mouse secondary antibody conjugated to 18-nm diameter gold particles. The larger gold particles, specific for Cpa(HA), could be seen at the tips of the pilus fibers (arrows in panels E and F). Scale bars = 500 nm (panels A-D) or 100 nm (panels E and F).

Figure 11 illustrates alternative models of Cpa incorporation into the T3 pilus structure. In the first model (A), the minor pilin, Cpa, is attached by the VPPTG motif in its CWSS to the α-amino group at the N-terminus of the major pilin, T3. In the second model (B), the VPPTG motif (SEQ ID NO: 10) in the CWSS of Cpa is attached to K173 of T3 in place of a T3 subunit. An unknown K residue of Cpa is then used to bond to the QVPTG motif (SEQ ID NO: 9) of the CWSS of T3. This leads to a structure with Cpa interspersed among T3 subunits. In the third model (C), Cpa is linked to a K in T3 other than K173. In the fourth model (D), Cpa can only attach to K173 of T3, and therefore Cpa constitutes the tip of the pilus.

Figure 12 shows the expression of MalE/Cpa fusion constructs in *E. coli* strain XL10. Constructs contain maltose binding protein (MBP)/Cpa through SrtC2 (pJRS9555) or MBP/Cpa through T3 (pJRS9556). The Western immunoblot in panel A was probed with an anti-MBP antibody, while the blot in panel B was probed with an anti-T3 antibody. Lanes 3,6,7 were confirmed by PCR to have the desired insert, lanes 2,4,5 lack this insert and lanes 8-10 lack *srtC2*. Lanes:(1) molecular mass standard, (2) pJRS9555.3, (3) pJRS9555.4, (4) pJRS9555.5, (5) pJRS9555.6, (6) pJRS9555.7, (7) pJRS9555.8, (8) pJRS9556.1, (9) pJRS9556.2, (10) pJRS9556.3.

Figure 13 provides a depiction of the regions encoded by the pJRS9550 and pJRS9565 plasmids.

Figures 14A and 14B show the MBP/Cpa fusion protein (MBP*) is surface exposed in *L. lactis*. Whole cell dot blots of *L. lactis* MG1363 containing the plasmid

pJRS9565 (lanes 1-4, rows E, F, (+)) or the pJRS9566 plasmid (lanes 1-4, row G, (-SrtC2)) were analyzed with a monoclonal anti-MBP antibody (Figure 14A) or a polyclonal anti-T3 antibody (Figure 14B).

Figures 15A and 15B shows that the MBP/Cpa fusion protein (MBP*) is incorporated into T3 pili in *L. lactis*. Western blot analyses are provided of *L. lactis* cell wall extracts of MG1363/pJRS9566 (lane3, (-SrtC2)) and MG1363/pJRS9545 (lane 4, (-)) analyzed with a monoclonal anti-MBP antibody (Figure 15A) or a polyclonal anti-T3 antibody (Figure 15B). Molecular masses are indicated to the left of the figure. The locations of the MBP/Cpa fusion protein (MBP*), the MBP-Cpa-T3 heterotetramer (MBP*-$(T3)^3$) are shown to the right of the figure. HMW = high molecular mass species

Figures 16A-16C provide photographs of immunogold electron microscopy (EM) of MBP*-T3 pili in *L. lactis* MG1363/pJRS9565. Whole-bacteria, negative-stain transmission EM of MG1363/pJRS9565 (Figures 16A and 16B) and MG1363/pJRS9545 (Figure 16C, vector control) incubated with anti-T3 antiserum, followed by an anti-rabbit gold conjugate secondary antibody.

Figures 17A and 17B show that the MBP/Cpa fusion protein (MBP*) is synthesized in an active form in *L. lactis*. Lysates of MG1363/pJRS9545 (lane 1, (vc)), MG1363/pJRS9565 (lane 2, (MBP*)) and MG1363/pJRS9566 (lane 3, (-SrtC2)) were purified with amylose resin and analyzed with a monoclonal anti-MBP antibody (Figure 17A) or polyclonal anti-T3 antiserum (Figure 17B). Molecular masses are indicated to the left of the figure. The locations of the MBP/Cpa fusion protein (MBP*), and the MBP-Cpa-T3 heterodimer (MBP*-T3) are shown to the right of the figure.

Figure 18 shows that T3 pili containing the MBP/Cpa fusion protein (MBP*), but not wild type T3 pili bind to amylose resin. A Western blot analysis is provided of lysates of MG1363/pJRS9565 and MG1363/pJRS9550 that were purified using amylose resin. Samples corresponding to the eluate fraction of MG1363/pJRS9565 (lanes 1 and 6, (E)), and the eluate (lanes 2 and 7, (E)), flow through (lanes 3 and 8, (F)) and crude extract (lanes 4 and 9, (C)) fractions of MG1363/pJRS9550 were analyzed using monoclonal anti-MBP (lanes 1-4) or monoclonal anti-HA (lanes 6-9) antibodies. The locations of the MBP/Cpa fusion protein (MBP*), the MBP-Cpa-T3 heterodimer (MBP*-T3), the Cpa(HA) monomer (Cpa), and the Cpa(HA)-T3 heterodimer (Cpa-T3) are shown. Molecular masses are indicated to the left of the figure. HMW = high molecular mass species. MM = molecular mass standard.

Figure 19 provides a graph depicting the levels of anti-MBP IgG in sera of mice that were intranasally vaccinated with *L. lactis* MG1363/pJRS9545 (control vector) or with MG1363/pJRS9565 (expressing MBP). The bars represent an average of ten mice for the experimental groups and two mice for the control group.

5 Figure 20 provides a graph depicting the levels of anti-MBP IgA in lung lavage fluid of mice subjected that were intranasally vaccinated with *L. lactis* MG1363/pJRS9545 (control vector) or with MG1363/pJRS9565 (expressing MBP). The bars represent an average of ten mice for the experimental groups and two mice for the control group.

10 DETAILED DESCRIPTION OF THE INVENTION

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments
15 are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings.
20 Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended embodiments. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

25 It is to be noted that the term “a” or “an” entity refers to one or more of that entity; for example, “a bacterium” is understood to represent one or more bacteria. As such, the terms “a” (or “an”), “one or more,” and “at least one” can be used interchangeably herein.

Throughout this specification and the embodiments, the words “comprise,” “comprises,” and “comprising” are used in a non-exclusive sense, except where the
30 context requires otherwise.

As used herein, the term “about,” when referring to a value is meant to encompass variations of, in some embodiments ± 50%, in some embodiments ± 40%, in some embodiments ± 30%, in some embodiments ± 20%, in some embodiments ± 10%, in some

embodiments ± 5%, in some embodiments ± 1%, in some embodiments ± 0.5%, and in some embodiments ± 0.1% from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

The presently disclosed subject matter provides methods for producing Gram-positive bacteria having at least one polypeptide of interest attached to the tip of at least one pilus, wherein the method comprises introducing into a Gram-positive bacterium a polynucleotide comprising a nucleotide sequence that encodes a chimeric polypeptide. The chimeric polypeptide comprises the polypeptide of interest and a Gram-positive bacterial pilus tip protein, or an active variant or fragment thereof, wherein the pilus tip protein or active variant or fragment thereof comprises a cell wall sorting signal (CWSS). The chimeric polypeptide is expressed such that the pilus tip protein, variant, or fragment thereof is carboxyl to the heterologous polypeptide. The transformed Gram-positive bacterium additionally expresses a tip sortase and a pilus shaft polypeptide. The transformed Gram-positive bacterium is then grown under conditions that allow formation of the pilus.

Also disclosed herein are compositions comprising Gram-positive bacteria having at least one polypeptide of interest attached to the tip of at least one pilus. These Gram-positive bacteria find use in methods for inducing an immunological response in a subject through the administration of a Gram-positive bacterium where the polypeptide of interest comprises an antigen. The transformed Gram-positive bacteria of the invention also can be used in bioremediation methods, wherein a contaminant is removed from a composition (e.g., soil, water) via the introduction of a Gram-positive bacterium displaying a biosorbent that is capable of adsorbing the contaminant or an enzyme that is capable of degrading the contaminant. Other uses involve biocatalysis, screening for polypeptide expression, the production of biofuels, diagnostics, and use in probiotics.

Without being bound by any theory or mechanism of action, it is believed that the presence of the polypeptide of interest on the tip of the pili will remove the polypeptide further away from the bacterial capsule, enhancing the odds that the polypeptide will fold and function properly. Further, in those embodiments wherein the polypeptide of interest comprises an antigen, it is believed that displaying the polypeptide on the tip of the pili will maximize the exposure of the polypeptide to the cells of the immune system, enhancing the immunological response generated against the antigen.

There are two predominant types of bacteria that are categorized based on the composition and structure of the bacterial cell wall. Whether a given species of bacteria

has one or the other type of cell wall can generally be determined by the cell's reaction to certain dyes. Perhaps the most widely-used dye for staining bacteria is the Gram stain. When stained with this crystal violet and iodine stain, bacteria which retain the stain are called Gram-positive, and those that do not are called Gram negative.

5 As used herein, by "Gram-positive bacteria" is meant a strain, type, species, or genera of bacteria that, when exposed to Gram stain, retains the dye and is, thus, stained blue-purple. The Gram-positive bacterial cell wall contains a relatively thick coat of peptidoglycan.

By contrast, a "Gram-negative bacteria" is meant a strain, type, species, or genera 10 of bacteria that, when exposed to Gram stain does not retain the dye and thus, is not stained blue-purple.

Gram-positive bacteria useful for the presently disclosed methods and compositions include, but are not limited to, bacteria in the following genera: *Actinomyces*, *Bacillus*, *Bifidobacterium*, *Cellulomonas*, *Clostridium*, *Corynebacterium*, 15 *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Micrococcus*, *Mycobactenum*, *Nocardia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. In some embodiments, the Gram-positive bacterium is selected from the group consisting of *Streptococcus pyogenes*, *Streptococcus gordinii*, *Lactococcus lactis*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*. In particular embodiments, the Gram-positive bacterium comprises 20 *Lactococcus lactis*.

The present invention takes advantage of the pili present on the surface of many different species of Gram positive bacteria, including Group A *Streptococcus* (GAS), such as *Streptococcus pyogenes* (Mora, M., G. *et al.* (2005) *Proc. Natl. Acad. Sci.* 102:15641-6.). As used herein, a “pilus” is a hair-like appendage found on the surface of a bacterium.

25 GAS pili have been shown to mediate attachment to primary human keratinocytes and to human tonsillar tissue, as well as to several tissue culture cell lines (Abbot, E. L. *et al.* (2007) *Cell Microbiol.* 9:1822-1833; Manetti, A. G. *et al.* (2007). *Mol. Microbiol.* 64:968-83). They have also been implicated in the formation of biofilms, which may be important for disease development (Manetti, A. G. *et al.* (2007) *Mol. Microbiol.* 64:968-30 83).

Pili on Gram-positive bacteria are composed of multiple subunits of a major backbone protein (referred to herein as the pilus shaft polypeptide) and may also have one or two minor pilin proteins attached thereto (Mandlik, A. *et al.* (2008) *Trends Microbiol.* 16:33-40; Scott, J. R. *et al.* (2006) *Mol. Microbiol.* 62:320-30; Telford, J. L. *et al.* (2006)

Nat. Rev. Microbiol. 4:509-19). Gram-positive pilin subunits are covalently attached to each other and the polymerized pilus is covalently attached to the peptidoglycan of the cell wall (Swaminathan, A. *et al.* (2007) *Mol. Microbiol.* 66:961-974). The minor pilin proteins are not required for assembly of the pilus, although their presence may be important for physiological function and specificity of the pili. Prior to the present disclosure, the location of the minor pilins in the pilus structure was unknown and the method by which they are attached to the shaft was not yet understood.

Pilin proteins have the features typical of Gram-positive surface proteins, including an N-terminal signal sequence and a C-terminal cell wall sorting signal (CWSS), which is composed of a hydrophobic domain, beginning with LPXTG (SEQ ID NO: 1) or a similar motif, followed by a charged tail (Schneewind, O. *et al.* (1993). *Embo J.* 12:4803-11).

Proteins linked covalently to the Gram-positive cell wall are translocated across the cytoplasmic membrane in a Sec-dependent process, which is accompanied by cleavage of the N-terminal signal peptide. In the next step, a membrane-associated transpeptidase, referred to as the “housekeeping” sortase, cleaves the CWSS between the threonine (T) and glycine (G) residues of the LPXTG motif, producing an acyl-enzyme intermediate in which the carboxyl group of the threonine of the CWSS is linked to a cysteine residue of the transpeptidase. Subsequently, the threonine is transferred to an amino group of a constituent of the growing cell wall (the peptide crossbridge or diaminopimelic acid), thereby incorporating the protein into the cell wall (for reviews, see, for example, Marraffini *et al.* (2006) *Microbiol Mol Biol Rev* 70:192-221 and Scott and Barnett (2006) *Annu Rev Microbiol* 60:397-423), each of which are herein incorporated by reference in its entirety.

The genetic locus in which GAS pili are encoded varies between strains and has been named the FCT region for the proteins it encodes (Fibronectin-binding, Collagen-binding, T antigen (Bessen, D. E. *et al.* (2002) *Infect. Immun.* 70:1159-67). The FCT loci of the GAS strains whose sequence is currently available have been grouped into 6 classes (FCT1-6) based on gene content and gene order (Kratovac, Z. *et al.* (2007) *J. Bacteriol.* 189:1299-310, which is herein incorporated by reference in its entirety). A given strain of GAS encodes only a single FCT locus, and therefore produces only a single type of pilus. GAS strains of the serotypes most common in the western world, M1, M3, M5, M18, and M49, contain either an FCT-2 region (M1) or an FCT-3 region (the others). The genes in these two FCT regions are highly homologous and they occur in the same order in each strain. The presently disclosed methods and compositions can utilize polypeptides (e.g.,

pilus tip proteins, pilus shaft polypeptides, tip sortases) from any strain of *S. pyogenes* bacteria. For example, the polypeptides used in the present invention can be a polypeptide encoded by a gene present on a FCT1, FCT2, FCT3, FCT4, FCT5, or FCT6 chromosomal region of a *S. pyogenes* bacterium. One or more of the polypeptides used in the present
5 invention can be a polypeptide encoded by a strain of the serotype M1, M3, M5, M18, or M49 of *S. pyogenes* bacteria. In particular embodiments, one or more of the polypeptides are polypeptides encoded by a strain of serotype M3 of *S. pyogenes* bacteria. In certain embodiments, one or more of the polypeptides used in the invention (e.g., pilus shaft polypeptide, tip sortase, pilus tip polypeptide, pilin chaperone polypeptide) are encoded by
10 the genes found in the FCT-3 region of the AM3 strain of *S. pyogenes*.

The protein encoded by the first gene in the FCT-3 operon (Figure 1), *cpa,* is a minor pilin protein that has been shown to bind collagen (Podbielski, A. *et al.* (1999) *Mol. Microbiol.* 31:1051-64). For the M3 strain used in studies presented herein (the AM3 strain), the second gene (*sipA2*) is essential for pilus polymerization and probably acts as a
15 chaperone (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35, which is herein incorporated by reference in its entirety). This gene is followed by *tee3*, which encodes the shaft protein T3, and by *srtC2*, encoding the pilin polymerase (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75, which is herein incorporated by reference in its entirety). Recently Mora *et al.* elegantly demonstrated that the shaft protein of GAS pili corresponds
20 to the trypsin-resistant (T) antigen long used for serological typing in GAS (Mora, M. *et al.* (2005) *Proc. Natl. Acad. Sci.* 102:15641-6). The last gene in the cluster, referred to as *orfB*, also encodes a minor pilin whose homologue was found by immunogold electron microscopy to be associated with the pilus structure of a serotype M1 strain (Mora, M. *et al.* (2005) *Proc. Natl. Acad. Sci.* 102:15641-6). OrfB and Cpa can each be added to the
25 pilus structure in the absence of the other, but, prior to the present disclosure, the residues linking these minor pilins to the major pilin were not defined.

The pilin proteins in the FCT-2, FCT-3, and FCT-4 regions of GAS strains contain CWSSs with motifs that differ from the canonical LPXTG (SEQ ID NO: 1) CWSS motif (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). This may indicate that their
30 polymerization requires a transpeptidase different from the housekeeping sortase. This has been demonstrated for the T3 protein, whose anchoring to the cell wall requires SrtC2, encoded in the FCT region, and not the housekeeping SrtA (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). Previous results indicate that the noncanonical CWSS motif is

needed for polymerization of the T3 protein, as the replacement of this noncanonical CWSS motif with the canonical LPSTG (SEQ ID NO: 2) motif prevents formation of T3 polymers (Zähner and Scott (2008) *J Bacteriol* 190:527-535).

The present invention provides Gram-positive bacterium having a polypeptide of interest covalently attached to the tip of a pilus through the introduction of a polynucleotide encoding a chimeric polypeptide into the bacterium. The chimeric polypeptide includes the polypeptide of interest linked in the proper reading frame to a pilus tip protein such that the polypeptide of interest is expressed as part of the pili on the transformed bacteria.

The terms "nucleic acid," "polynucleotide," or "oligonucleotide" generally are used herein in their art-accepted manners to refer to a polymer of nucleotides. As used herein, an oligonucleotide is typically less than 100 nucleotides in length. Polynucleotides can be single-stranded (with or without a secondary structure, e.g., hairpin) or double-stranded. Naturally occurring nucleic acids include deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The polynucleotide or oligonucleotide may include natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), or synthetic nucleosides, such as, nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, C5-propynylcytidine, C5-propynyluridine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine), and/or nucleosides comprising chemically or biologically modified bases, such as those ribonucleosides that are substituted at the 2' position, for example, with an alkyl or alkyloxy group (e.g., methylated bases, such as those that are 2'-O-methylated, and 2'-O-methoxyethylated) or a fluoro group, intercalated bases, and/or modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose). The phosphate groups in a polynucleotide or oligonucleotide are typically considered to form the internucleoside backbone of the polymer. In naturally occurring nucleic acids (e.g., DNA or RNA), the backbone linkage is via a 3' to 5' phosphodiester bond. Polynucleotides and oligonucletides containing modified backbones or non-naturally occurring internucleoside linkages, however, also can be used in the presently disclosed subject matter. Such modified backbones include backbones that have a phosphorus atom in the backbone and others that do not have a phosphorus atom in the backbone. Examples of modified linkages include, but are not limited to, phosphorothioate and 5'-N-phosphoramidite

linkages. Polynucleotides and oligonucleotides need not be uniformly modified along the entire length of the molecule. For example, different nucleotide modifications, different backbone structures, and the like, may exist at various positions in the polynucleotide or oligonucleotide. Any of the polynucleotides described herein may utilize these
5 modifications.

According to the presently disclosed methods, a polynucleotide comprising a nucleotide sequence that encodes a chimeric polypeptide is introduced into a Gram-positive bacterium. As used herein, the terms “polypeptide” or “peptide” or “protein” can be used interchangeably throughout, and refer to any monomeric or multimeric protein or
10 peptide comprised of a polymer of amino acid residues. The term applies to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

As used herein, the terms “encoding” or “encoded” when used in the context of a
15 specified nucleic acid mean that the nucleic acid comprises the requisite information to direct translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (*e.g.*, introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (*e.g.*, as in cDNA).
20 A “coding sequence” refers to a nucleotide sequence (e.g., DNA) that encodes a specific RNA or polypeptide.

The term “expression” has its meaning as understood in the art and refers to the process of converting genetic information encoded in a DNA sequence (coding sequence) into RNA (e.g., mRNA, rRNA, tRNA, or snRNA) through “transcription” of a
25 polynucleotide (e.g., via the enzymatic action of an RNA polymerase), and for polypeptide-encoding polynucleotides, into a polypeptide through “translation” of mRNA. Thus, an “expression product” is, in general, an RNA transcribed from the coding sequence (e.g., either pre- or post-processing) or a polypeptide encoded by an RNA transcribed from the DNA coding sequence (e.g., either pre- or post-modification).
30 The use of fragments and variants of the disclosed polynucleotides and polypeptides are also encompassed by the present invention. By "fragment" is intended a portion of the polynucleotide or polypeptide and include active fragments that retain the biological activity of the polypeptide or the ability to encode an active polypeptide fragment. Alternatively, fragments of a polynucleotide that are useful as hybridization

probes or PCR primers need not retain this biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, about 500 nucleotides, about 1000 nucleotides, and up to the full-length polynucleotide.

5 Thus, a fragment of the polynucleotide may encode a polypeptide that is biologically active or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A polynucleotide that encodes an active polypeptide can be prepared by isolating a portion of the polynucleotide (e.g., by recombinant expression *in vitro*) or chemically synthesizing the polynucleotide and 10 assessing the activity of the encoded polypeptide. Polynucleotides that encode active fragments of the polypeptides of the invention have a nucleotide sequence comprising at least 10, 20, 30, 50, 100, 200, 500, or 1000 contiguous nucleotides of the sequences of the invention, or up to the number of nucleotides present in a polynucleotide that encodes a full-length polypeptide.

15 "Variants" is intended to mean substantially similar sequences. A variant comprises a polynucleotide having deletions (i.e., truncations) at the 5' and/or 3' end; deletion and/or addition of one or more nucleotides at one or more internal sites in the native polynucleotide; and/or substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide comprises a 20 naturally occurring nucleotide sequence. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis (but which still retain the 25 activity of the polynucleotides of the invention). Generally, variants of a particular polynucleotide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters as described elsewhere herein.

30 "Variant" polypeptide is intended to mean a polypeptide derived from the native polylpeptide by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the native polypeptide; deletion and/or addition of one or more amino acids at one or more internal sites in the native polypeptide; or substitution of one or more amino acids at one or more sites in the native polypeptide. Variant

polypeptides encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native polypeptide. Such variants may result from, for example, genetic polymorphism or from human manipulation. In general, biologically active variants of a native polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native polypeptide as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a polypeptide of the invention may differ from that polypeptide by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The polynucleotides that encode the polypeptides useful in this invention can be used to isolate variants of the polynucleotide sequences from any organism. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire polynucleotides sequences set forth herein or to variants and fragments thereof are useful for the present invention. In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding polynucleotide sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known polynucleotide is used as a probe that selectively hybridizes to other corresponding polynucleotides present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Methods for preparation of probes for hybridization and for construction of cDNA and genomic

libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire T3 polynucleotide disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding pilus shaft-encoding polynucleotides and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among pilus shaft-encoding polynucleotide sequences and are optimally at least about 10 nucleotides in length, and most optimally at least about 20 nucleotides in length. Such probes may be used to amplify corresponding pilus shaft-encoding polynucleotides from a chosen bacterium by PCR. This technique may be used to isolate additional coding sequences from a desired bacterium. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions, wherein a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. Stringency conditions can be adjusted to allow the identification of 100% complementary sequences or sequences with lower degrees of similarity. Generally, a probe is less than about 1000 nucleotides in length, optimally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash

buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is optimal to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

The percent sequence identity between two sequences can be determined using alignment methods that are well known in the art, such as mathematical algorithms. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 872264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity, including, but not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra.* A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra.* BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra.* When utilizing BLAST, Gapped

BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10, which uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. The default gap creation and extension penalty values can be used for sequence alignments.

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions,

dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

The polynucleotides that are introduced into a Gram-positive bacterium can further comprise one or more regulatory sequences that are operably linked to the polynucleotide encoding the chimeric polypeptide that facilitate expression of the polynucleotide. “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. *See*, for example, Goeddel (1990) in *Gene Expression Technology: Methods in Enzymology* 185 (Academic Press, San Diego, California). Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences. A polynucleotide comprising regulatory sequences operably linked to coding sequences can be referred to as expression cassettes.

Regulatory sequences are operably linked with a coding sequence to allow for expression of the polypeptide encoded by the coding sequence. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide that encodes a polypeptide and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polypeptide. Operably linked elements may be contiguous or non-contiguous. Polynucleotides may be operably linked to regulatory sequences in sense or antisense orientation. When used to refer to the joining of two protein coding regions, by operably linked is intended that the coding regions are in the same reading frame.

The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the coding polynucleotides may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the coding polynucleotides may be heterologous to the host cell or to each other. As used herein, “heterologous” in reference to a sequence or a polypeptide is a sequence or polypeptide that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide

was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

In particular embodiments wherein the polynucleotide encoding the chimeric polypeptide comprises regulatory sequences, the polynucleotide can further comprise additional coding sequences. In some of these embodiments, the regulatory sequences can be operably linked to more than one coding sequence. For example, a single promoter can be operably linked to more than one coding sequence, wherein the coding sequences are co-transcribed from the single promoter into a single polycistronic transcript, which is separately translated into more than one polypeptide.

It will be appreciated by those skilled in the art that the design of the expression cassette can depend on such factors as the choice of the host cell to be transformed, the level of expression of the presently disclosed polynucleotides, and the like. Such expression cassettes typically include one or more appropriately positioned sites for restriction enzymes, to facilitate introduction of the nucleic acid into a vector.

It will further be appreciated that appropriate promoter and/or regulatory elements can readily be selected to allow expression of the presently disclosed polynucleotides in the cell of interest.

"Promoter" refers to a polynucleotide capable of controlling the expression of a polynucleotide. In general, the polynucleotide to be transcribed is located 3' to a promoter sequence. The promoter sequence may comprise proximal and more distal upstream elements; the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a polynucleotide, which can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, polynucleotide fragments of different lengths may have identical promoter activity.

The promoters used in accordance with the present invention may be constitutive promoters or regulated promoters. Common examples of useful regulated promoters include those of the family derived from the nisin promoter (see, for example, U.S. Patent No. 5,914,248 and Kleerebezem *et al.* (1997) *Appl Environ Microbiol* 63:4581-4584, each of which are herein incorporated by reference in its entirety); and the tetracycline-

inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156, which are herein incorporated by reference). Regulated promoters utilize promoter regulatory proteins in order to control transcription of the gene of which the promoter is a part. Where a regulated promoter is used herein, corresponding promoter regulatory protein(s) will also be part of an expression system according to the present invention. Examples of promoter regulatory proteins include the NisR and NisK proteins for use with the *nisA* promoter. Many regulated-promoter/promoter-regulatory protein pairs are known in the art.

Promoter regulatory proteins interact with or are activated or repressed by an effector compound, i.e. a compound that reversibly or irreversibly associates with or activates or represses the regulatory protein so as to enable the protein to either release or bind to at least one DNA transcription regulatory region of the gene that is under the control of the promoter, thereby permitting or blocking the action of a transcriptase enzyme in initiating transcription of the gene. A non-limiting example of an effector compound is tetracycline or nisin for use with tetracycline-regulated promoter systems or nisin-regulated systems, respectively. Effector compounds are classified as either inducers or co-repressors, and these compounds include native effector compounds and gratuitous inducer compounds. Many regulated-promoter/promoter-regulatory-protein/effector-compound systems are known in the art. Although an effector compound can be used throughout the cell culture or fermentation, in some embodiments in which a regulated promoter is used, after growth of a desired quantity or density of host cell biomass, an appropriate effector compound is added to the culture to directly or indirectly result in expression of the desired gene(s) encoding the protein or polypeptide of interest.

Other non-limiting examples of useful promoters for expression in Gram positive cells are the P_{ami}, P_{spac}, P_{veg}, and P23 promoters (see, for example Biswas *et al.* (2008) *Microbiology* 154:2275-2282, which is herein incorporated by reference in its entirety).

Other regulatory elements may be included in an expression cassette, including but not limited to, transcriptional enhancer sequences, translational enhancer sequenes, other promoters, activators, translational start and stop signals, transcription terminators, cistronic regulators, polycistronic regulators, signal sequences (e.g., Sec dependent signal sequences), or tag sequences, such as nucleotide sequence "tags" and "tag" polypeptide coding sequences, which facilitates identification of the polypeptide or cell expressing the polypeptide. A non-limiting example of a tag polypeptide is the hemagglutinin (HA) peptide.

Regulatory sequences found within expression cassettes can include a 3' non-coding region. The "3' non-coding region" or "terminator region" refers to DNA or RNA sequences located downstream of a coding sequence and may include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

Proteins linked covalently to the Gram-positive cell wall are translocated across the membrane in a Sec-dependent process accompanied by cleavage of the signal peptide by a signal peptidase. Thus, in some embodiments, the expression cassette comprises a nucleotide sequence that encodes for an appropriate signal peptide that is inserted into the expression cassette in such a manner that it encodes a polypeptide of interest with the signal peptide fused to the amino terminal end of the polypeptide of interest. Sec-dependent signal sequences are known in the art and generally consist of a short (about 30 amino acids), mainly hydrophobic sequence comprising the following three domains: (i) a positively charged n-region with at least one arginine or lysine residue, (ii) a hydrophobic h-region and (iii) an uncharged but polar c-region. The cleavage site for the signal peptidase is located in the c-region. However, the degree of signal sequence conservation and length, as well as the cleavage site position, can vary between different proteins. The signal sequence aids protein export and is cleaved off by a periplasmic signal peptidase when the exported protein reaches the periplasm. In some embodiments, the signal peptide encoded by the expression cassette comprises the signal peptide derived from the *S. pyogenes* pilus tip polypeptide that is fused to the polypeptide of interest to be displayed on the pili tip (such as the signal peptide set forth in SEQ ID NO: 100 from the Cpa protein). In other embodiments, the signal peptide is derived from the polypeptide of interest. The signal peptide can also be heterologous to both the polypeptide of interest and the *S. pyogenes* pilus tip polypeptide (such as a consensus Sec-dependen signal sequence).

For suitable expression systems for prokaryotic cells, *see* Chapters 16 and 17 of Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York), which are herein incorporated by reference. See also Goeddel (1990) in *Gene Expression Technology: Methods in Enzymology* 185 (Academic Press, San Diego, California), which is herein incorporated by reference in its entirety.

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase
5 (HPT). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su et *al.* (2004) *Biotechnol Bioeng 85:*610-9 and Fetter *et al.* (2004) *Plant Cell 16:*215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science 117*:943-54 and Kato *et al.* (2002) *Plant Physiol 129*:913-42), and yellow florescent protein (for example, PhiYFP™
10 from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science 117*:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612;
15 Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.*
20 (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents*
25 *Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); and Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting as any selectable marker gene can be used in the present invention.

30 In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide

for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

Such expression cassettes can be contained in a vector which allow for the
5 introduction of the expression cassette into a cell. In specific embodiments, the vector allows for autonomous replication of the expression cassette in a cell or may be integrated into the genome of a cell. Such vectors are replicated along with the host genome. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of
10 expression vectors, such as viral vectors.

According to the present invention, polynucleotides encoding the chimeric polypeptides are introduced into a cell. "Introducing" is intended to mean presenting to the cell the polynucleotide in such a manner that the sequence gains access to the interior of the cell. The methods of the invention do not depend on a particular method for
15 introducing a sequence into a cell, only that the polynucleotide gains access to the interior of the cell. Methods for introducing polynucleotides into cells are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

"Stable transformation" is intended to mean that the polynucleotide introduced into
20 a cell integrates into the genome of the cell and is capable of being inherited by the progeny thereof. "Transient transformation" is intended to mean that a polynucleotide is introduced into the cell and does not integrate into the genome of the cell.

Exemplary art-recognized techniques for introducing foreign polynucleotides into a host cell include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-
25 mediated transfection, lipofection, particle gun, or electroporation and viral vectors. Suitable methods for transforming or transfecting host cells can be found in U.S. Pat. No. 5,049,386, U.S. Pat. No. 4,946,787; and U.S. Pat. No. 4,897,355, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) and other standard molecular biology laboratory manuals. One of
30 skill will recognize that depending on the method by which a polynucleotide is introduced into a cell, the polynucleotide can be stably incorporated into the genome of the cell, replicated on an autonomous vector or plasmid, or present transiently in the cell. In some embodiments, transient expression may be desired. In those cases, standard transient

transformation techniques may be used. Such methods include, but are not limited to viral transformation methods, and microinjection of DNA or RNA, as well other methods well known in the art.

Host organisms containing the introduced polynucleotide are referred to as "transgenic" or "transformed" organisms. By "host cell" is meant a cell that contains an introduced polynucleotide construct and supports the replication and/or expression of the construct. The host cells of the present invention are Gram-positive bacteria.

The skilled artisan will recognize that different independent transformation events will result in different levels and patterns of expression (Jones *et al.* (1985) *EMBO J. 4*:2411-2418; De Almeida *et al.* (1989) *Mol. Gen. Genetics 218*:78-86), and thus that multiple events may have to be screened in order to obtain cells displaying the desired expression level and pattern. Such screening may be accomplished by PCR or Southern analysis of DNA to determine if the introduced polynucleotide is present in complete form, and then northern analysis or RT-PCR to determine if the expected RNA is indeed expressed.

According to the present invention, a polypeptide of interest is displayed on the surface of a bacterium as a chimeric polypeptide, wherein the polypeptide of interest is covalently attached directly or indirectly to a pilus tip protein. As used herein, a "chimeric polypeptide" or "fusion polypeptide" refers to a polypeptide comprising two polypeptides operably linked to one another, wherein the two polypeptides are not covalently bound to one another through peptide bonds in nature (without any human intervention). As described elsewhere herein, "operably linked" is intended to mean a functional linkage between two or more elements. For example, two polypeptides within a chimeric polypeptide are operably linked if the two polypeptides are fused to each other directly or indirectly through a peptide bond so that both polypeptides fulfill the proposed function attributed to each polypeptide. The chimeric polypeptides of the invention are created through the joining of the coding sequences for each polypeptide, wherein the two coding sequences are operably linked within the same reading frame to allow for the expression of the chimeric polypeptide. The polypeptide of interest could be fused indirectly to the pilus tip protein or active fragment or variant thereof, wherein additional amino acid residues can serve as a linker between the two polypeptides. The use of a linker sequence can increase the likelihood that the two polypeptides (polypeptide of interest and pilus tip protein) fold properly. The linker sequence can consist of 1 amino acid to about 100 amino acid residues or more.

The chimeric polypeptides of the invention comprise a heterologous polypeptide and a Gram-positive bacterial pilus tip polypeptide (or an active variant or fragment thereof). As used herein, a "pilus tip polypeptide" or "pilus tip protein" is a polypeptide that is present at the end of a bacterial pilus that extends out from the surface of the
5 bacteria. The pilus tip polypeptide is distinct from the major pilin polypeptide that forms the shaft of the pilus (the pilus shaft polypeptide). While pilus shaft polypeptides can be localized at the tip of some pili, they are not considered pilus tip polypeptides, as they also comprise the major proteins found within the pilus shaft. In general, the pilus shaft polypeptide is the major pilin polypeptide and the pilus tip polypeptide is a minor pilin
10 polypeptide within a given Gram-positive bacterium. In general, the major pilin protein is the T antigen that is often used for serological typing of *S. pyogenes* (Mora *et al.* (2005) *Proc Natl Acad Sci USA* 102:15641-15646; Schneewind *et al.* (1990) *J Bacteriol* 172:3310-3317).

In some embodiments, the pilus tip polypeptide comprises a pilus tip polypeptide
15 from a *Streptococcus* bacterium. In some of these embodiments, the pilus tip polypeptide comprises a *Streptococcus pyogenes* pilus tip polypeptide. Data presented elsewhere herein demonstrate that the minor pilin protein Cpa from the M3 strain of *Streptococcus pyogenes* is present on the tip of pili. Cpa is a putative adhesin protein that is capable of binding to collagen. Thus, in some embodiments, the pilus tip polypeptide is an adhesin.
20 An "adhesin" is a polypeptide that binds to an extracellular matrix protein, host cell surface protein, or other host cell-associated protein that facilitates bacterial-host cell interactions. An additional, non-limiting example of a *Streptococcus pyogenes* adhesin protein is the fibronectin-binding protein F1.

In general, the pilus tip protein of any given *Streptococcus pyogenes* strain is the
25 protein encoded by the first non-regulatory gene present in the FTC region of the bacterial chromosome. In some embodiments, the *S. pyogenes* pilus tip polypeptide can be selected from the group consisting of Cpa, protein F1, OrfB, Spy0128, Spy0130, FctA, FctX, and FctB. In particular embodiments, the pilus tip polypeptide comprises a Cpa polypeptide (also known as Cpa49). In some of these embodiments, the pilus tip polypeptide
30 comprises the Cpa polypeptide from the AM3 strain of *S. pyogenes* (sequence set forth in SEQ ID NO: 3), which is encoded by the nucleotide sequence set forth in SEQ ID NO: 4.

To determine if a given polypeptide functions as a pilus tip protein, one can use assays that are known in the art to localize a polypeptide to the tip of a pilus, including but not limited to assays presented elsewhere herein (see Experimental Example 1). Assays

used to determine if a polypeptide is polymerized into a pilus structure, in general, involve extracting the cell wall fraction of bacteria (with mutanolysin with or without lysozyme), boiling the extract in SDS and separating the proteins using SDS-PAGE. Pilus proteins appear as high molecular weight ladders in immunoblots. The *E. coli* expression system and the mutational analysis of the pilus shaft polypeptide and pilus tip polypeptide used elsewhere herein can be used to determine if the polypeptide is indeed localized to the pilus tip. Other methods known in the art can be used to localize the pilus tip protein to the leading edge of pili, including but not limited to, visualization by fluorescence microscopy or negative staining (e.g., immunogold electron microscopy).

The chimeric polypeptide displayed on the tip of the Gram-positive pili can comprise an active variant or fragment of a pilus tip polypeptide. An active variant or fragment of a pilus tip polypeptide is a polypeptide that retains the ability to be localized to the tip of a bacterial pilus. In some embodiments, the active variant or fragment of the pilus tip polypeptide comprises the cell wall sorting signal (CWSS). In particular embodiments, the active fragment of the pilus tip polypeptide comprises at least one amino acid residue amino terminal to (i.e., preceding) the CWSS and the CWSS itself. In certain embodiments, the polypeptide fragment of the *S. pyogenes* pilus tip polypeptide comprises at least 2, at least 3, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1000 amino acid residues amino terminal to the CWSS up to the full length pilus tip polypeptide sequence. In some embodiments, an active fragment comprises amino acids 594-744 of SEQ ID NO: 3 (this region is set forth in SEQ ID NO: 6). In some embodiments, the polypeptide of interest is fused (directly or indirectly) to an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher sequence identity to the amino acid sequence set forth in SEQ ID NO: 6.

In some embodiments, active variants of the pilus tip polypeptide have an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher sequence identity to the amino acid sequence set forth in SEQ ID NO: 3. In certain embodiments, active variants of the pilus tip polypeptide are encoded by a nucleotide sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher sequence identity to the nucleotide sequence set forth in SEQ ID NO: 4.

The cell wall sorting signal is present in all Gram-positive bacterial surface displayed proteins and is comprised of the cell wall sorting signal (CWSS) motif, which is generally a five amino acid residue motif, a hydrophobic domain carboxyl to the CWSS motif, and a charged tail region carboxyl to the substantially hydrophobic domain. The CWSS motif is recognized and cleaved by the "housekeeping" sortase A, which is a membrane-associated transpeptidase. Canonical CWSS motifs generally comprise a LPXTG (SEQ ID NO: 1) amino acid sequence. The motif is generally cleaved at the threonine by the sortase to form an acyl-enzyme intermediate, wherein the carboxyl group of the threonine (T) of the CWSS is linked to a cysteine (C) residue of the transpeptidase. Subsequently, the threonine is transferred to an amino group of the peptidoglycan molecule within the peptide crossbridge of the growing cell wall, thereby incorporating the protein into the cell wall (for reviews see Marraffini, L. A. *et al.* (2006) *Microbiol. Mol. Biol. Rev*. 70:192-221; Scott, J. R. *et al.* (2006) *Annu. Rev. Microbiol*. 60:397-423). As used herein, a "cleaved CWSS motif" comprises the remains of a CWSS motif sequence following the cleavage of the motif by a sortase transpeptidase enzyme. A cleaved canonical CWSS motif, thus, has the sequence of LPXT.

The *S. pyogenes* pilus tip protein or variant or fragment thereof that is carboxy terminal to a polypeptide of interest displayed on the tip of a pilus on a Gram-positive bacterium comprises a cleaved cell wall sorting signal (CWSS) motif. In some embodiments, variants of the pilus tip protein have an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher sequence identity to the amino acid sequence set forth in SEQ ID NO: 101. In other embodiments, the variant of the pilus tip protein comprising a cleaved CWSS motif has an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher sequence identity to the amino acid sequence set forth in SEQ ID NO: 103. In certain embodiments, the pilus tip protein comprising a cleaved cell wall sorting signal motif has the sequence set forth in SEQ ID NO: 101. In other embodiments, the pilus tip protein comprising a cleaved cell wall sorting signal motif has the sequence set forth in SEQ ID NO: 103.

Polymerization of pilin proteins in Gram-positive bacteria requires a sortase family transpeptidase (pilin polymerase) and therefore is generally assumed to proceed by a process similar to that demonstrated for the *Staphylococcus aureus* housekeeping sortase (Ton-That, H. *et al.* (1999) *Proc. Natl. Acad. Sci.* 96:12424-9; Ton-That, H. (2004)*Trends*

Microbiol. 12:228-34; for a review of sortases, see Marrafifini *et al.* (2006) *Microbiol Mol Biol Rev* 70:192-221, both of which are herein incorporated in their entireties). It is believed that the pilin polymerase catalyzes formation of a peptide bond between the threonine in the CWSS motif of one subunit and an ε-amino group of a lysine in the next subunit of the growing pilus chain.

Along with the five amino acid residue CWSS motif, the CWSS also comprises a carboxyl terminal substantially hydrophobic domain and a charged tail region. By "substantially hydrophobic" is intended a region of a polypeptide, wherein at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more of the amino acid residues making up the region are hydrophobic. In some embodiments, the hydrophobic region is at least about 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, or at least 60 amino acid residues in length. In particular embodiments, the hydrophobic region is at least about 25 amino acid residues in length. In certain embodiments, the hydrophobic region comprises the sequence set forth in amino acids 714-738 of SEQ ID NO: 3.

The hydrophobic region of the CWSS is followed by a charged tail region. At least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more of the amino acid residues making up the charged tail region have a positive or negative charge at physiological pH. In some embodiments, the charged tail region comprises about 5 to about 20 amino acid residues, including, but not limited to, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20. In certain embodiments, the charged tail region comprises the sequence RKGTKK (SEQ ID NO: 5), which corresponds to the extreme carboxy terminus of Cpa (set forth in SEQ ID NO: 3).

It was demonstrated elsewhere herein that the sortase C2 polypeptide is capable of polymerizing the major pilin T3 polypeptide and is required for the covalent attachment of the minor pilin Cpa to T3 polypeptides at the tip of pili. Thus, according to the presently disclosed methods and compositions, the Gram-positive bacteria useful for the display of polypeptides of interest express a tip sortase as well as a pilus shaft polypeptide. As used herein, a "tip sortase" is a sortase enzyme capable of covalently attaching a pilus tip polypeptide to the pilus shaft. The tip sortase can be from any organism. In some embodiments, a tip sortase comprises a sortase C enzyme. In particular embodiments, the

tip sortase comprises a sortase C1 or sortase C2 enzyme. In some embodiments, the sortase C enzyme comprises a SrtC1 polypeptide, which is found in the M1 strains of *S. pyogenes* (see Barnett *et al.* (2004) *J Bacteriol* 186:5865-5875).

In other embodiments, the tip sortase comprises a sortase C2 enzyme, such as the sortase C2 enzyme encoded within the FCT-3 and FCT-4 chromosomal regions of *S. pyogenes* bacteria (including, but not limited to the FCT-3 or FCT-4 regions from M3, M5, M12, M18, and M49 strains of *S. pyogenes*; see Barnett *et al.* (2004) *J Bacteriol* 186:5865-5875).

In particular embodiments, the sortase C2 enzyme comprises the srtC2 from the AM3 strain of *S. pyogenes* (with the amino acid sequence set forth in SEQ ID NO: 7). In some embodiments, the tip sortase has an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 96%, at least 97%, at least 98%, at least 99%, or more sequence identity to the sequence set forth in SEQ ID NO: 7. The AM3 sortase C2 polypeptide is encoded by the nucleotide sequence set forth in SEQ ID NO: 8. In some embodiments, the tip sortase is encoded by a nucleotide sequence having at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 96%, at least 97%, at least 98%, at least 99%, or more sequence identity to the sequence set forth in SEQ ID NO: 8. Active variants of SEQ ID NO: 7 retain the ability to attach a pilus tip polypeptide to a pilus shaft. Suitable assays for determining if a given polypeptide exhibits this activity include any method known in the art or described elsewhere herein (see Experimental Example 1).

Previous results have demonstrated that the sortase C2 enzyme is capable of attaching the T3 shaft polypeptide of the pilus from the M3 strain of *S. pyogenes* to the cell wall through a non-canonical CWSS motif. The CWSS motif of the T3 polypeptide (also referred to as Orf100) comprises a QVPTG (set forth in SEQ ID NO: 9) amino acid sequence. Results presented elsewhere herein demonstrate this non-canonical CWSS motif is also utilized by SrtC2 to attach the T3 polypeptides to one another. Further presented herein are data that show the SrtC2 enzyme also catalyzes the covalent attachment of the Cpa minor pilin to the T3 protein. Similar to the T3 polypeptide, Cpa also comprises a non-canonical CWSS motif with the amino acid sequence of VPPTG (SEQ ID NO: 10). These data suggest sortase C2 polypeptides recognize and cleave non-canonical CWSS motifs. Thus, in some embodiments, both the major shaft polypeptide expressed by the Gram-positive bacterium and the pilus tip polypeptide (or active variant or fragment thereof) fused to the displayed heterologous polypeptide comprise a non-

canonical CWSS motif. In particular embodiments, a tip sortase is one that is capable of covalently attaching a pilus tip polypeptide with a non-canonical CWSS motif to a growing pilin chain or polymerizing a pilus shaft polypeptide having a non-canonical CWSS motif. As used herein, a "non-canonical CWSS motif' is one wherein the sequence
5 does not follow the consensus canonical CWSS motif of LPXTG (SEQ ID NO: 1), wherein X is any amino acid. In some embodiments, the non-canonical CWSS motif comprises a XXPTG (SEQ ID NO: 11) motif. In some of these embodiments, the first amino acid comprises a glutamine or a valine. In other embodiments, the second amino acid comprises a valine or a proline. In yet other embodiments, the first amino acid
10 comprises a glutamine or a valine and the second amino acid comprises a valine or a proline. In certain embodiments, the non-canonical motif is one comprising a XXPTG motif (SEQ ID NO: 11), wherein the first amino acid is not a leucine residue. In other embodiments, the second amino acid residue is not a proline.

The Gram-positive bacteria of the invention comprise a major pilin that functions
15 as the pilus shaft polypeptide. A "pilus shaft polypeptide" is a polypeptide that comprises the shaft of the pilus. In some embodiments, the pilus shaft polypeptide comprises at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, 96%, 97%, 98%, 99%, or higher of the polypeptides comprising a pilus. In some embodiments, the pilus shaft polypeptide comprises the major pilin protein. In general, the major pilin
20 protein is the T antigen that is often used for serological typing of *S. pyogenes* (Mora *et al.* (2005) *Proc Natl Acad Sci USA* 102:15641-15646; Schneewind *et al.* (1990) *J Bacteriol* 172:3310-3317). In some embodiments, the pilus shaft polypeptide comprises a non-canonical CWSS motif within its cell wall sorting signal. In particular embodiments, the pilus shaft polypeptide comprises the T3 polypeptide. In some of these embodiments, the
25 T3 polypeptide is from a M3 strain of *S. pyogenes*. In some of these embodiments, the T3 polypeptide comprises the T3 polypeptide from the AM3 strain of *S. pyogenes*, the amino acid sequence of which is set forth in SEQ ID NO: 12. In particular embodiments, the pilus shaft polypeptide has an amino acid sequence having at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at
30 least 98%, at least 99% or high sequence identity to the sequence set forth in SEQ ID NO: 12. The AM3 T3 polypeptide is encoded by the nucleotide sequence set forth in SEQ ID NO: 13. In some embodiments, the pilus shaft polypeptide is encoded by a nucleotide sequence having at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or higher sequence

identity to the nucleotide sequence set forth in SEQ ID NO: 13. Pilus shaft polylpeptides and active variants thereof retain the ability to polymerize into a pilus and to be covalently attached to a peptidoglycan molecule in the bacterial cell wall. Assays for detecting this activity include any method known in the art (see, for example, Barnett *et al.* (2004) *J Bacteriol* 186:5865-5875) and methods disclosed elsewhere herein (see Experimental Example 1). As disclosed elsewhere herein, the lysine residue at position 173 within the T3 protein is required for covalent attachment of T3 to Cpa. Specifically, the K173 residue is covalently attached to the threonine residue within the CWSS motif of Cpa. The lysine corresponding to amino acid residue 173 of T3 is conserved throughout the major pilin proteins found in at least the FCT-2, FCT-3, and FCT-4 chromosomal regions (see Figure 5). Thus, in some embodiments, the pilus shaft polypeptide comprises a lysine residue within the major pilin protein in a similar region of the polypeptide as the K173 in T3 protein, which can be determined through alignment of the sequences using methods described elsewhere herein.

In some embodiments of the present invention, the tip sortase functions as a pilin polymerase, facilitating the polymerization of the pilin shaft polypeptides, in addition to its role in attaching the pilus tip polypeptide to the pilin shaft polypeptide. Additionally, in certain embodiments, the tip sortase has the ability to attach the pilus to peptidoglycans within the cell wall. In other embodiments, the tip sortase that attaches the pilus tip polypeptide to the pilus shaft polypeptide is distinct from the pilin polymerase that facilitates attachment of the pilus shaft polypeptides to one another or is distinct from the sortase enzyme that attaches the pilus to the cell wall (e.g., the housekeeping sortase A). In these embodiments, the transformed Gram-positive bacteria further express or comprise a pilin polymerase that can polymerize the pilin shaft polypeptide, a sortase that attaches the pilus to the cell wall (e.g., the housekeeping sortase A), or both.

Attachment of the T3 polypeptide and the Cpa polypeptide to the cell wall of M3 strains of *S. pyogenes* requires the SipA pilin chaperone polypeptide. As used herein, a pilin chaperone polypeptide is a polypeptide that is required for the stabilization of pilin proteins and that facilitates the polymerization and cell wall attachment of a pilus. Thus, in some embodiments, the Gram-positive bacteria further express a pilin chaperone polypeptide. In certain embodiments, the pilin chaperone polypeptide comprises a SipA polypeptide from an M3 strain of *S. pyogenes* (Zähner and Scott (2008) *J Bacteriol* 190:527-535). In some embodiments, the SipA polypeptide is from the AM3 strain of *S. pyogenes*, the amino acid sequence of which is set forth in SEQ ID NO: 14. In some of

these embodiments, the pilin chaperone polypeptide has an amino acid sequence having at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or higher sequence identity to the sequence set forth in SEQ ID NO: 14. The AM3 SipA polypeptide is encoded by the nucleotide sequence set forth in SEQ ID NO: 15. In some embodiments, the pilin chaperone polypeptide is encoded by a nucleotide sequence having at least about 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or higher sequence identity to the sequence set forth in SEQ ID NO: 15. A given polypeptide sequence (or a polypeptide encoded by any nucleotide sequence) can be assessed for its ability to function as a pilin chaperone using any method known in the art (see Zähner and Scott (2008) *J Bacteriol* 190:527-535), including those methods disclosed elsewhere herein (see Experimental Example 1).

In particular embodiments wherein Cpa or an active variant or fragment thereof serves as the pilus tip protein that is fused to the polypeptide of interest, the Gram positive bacterium further expresses or comprises the tip polymerase SrtC2, the T3 pilus shaft polypeptide, and the SipA2 chaperone protein. In some of these embodiments, the SrtC2 tip polymerase has the sequence set forth in SEQ ID NO: 7, the T3 pilus shaft polypeptide has the sequence set forth in SEQ ID NO: 12, and the SipA2 chaperone protein has the sequence set forth in SEQ ID NO: 14.

In some embodiments, at least one of the pilus shaft polypeptide, sortase C polypeptide, and pilin chaperone polypeptide are heterologous to the Gram-positive bacterium that is displaying a polypeptide of interest. In some embodiments, one or all of the polypeptides can be expressed within the bacteria through the introduction of an expression cassette that comprises a polynucleotide that comprises a nucleotide sequence that encodes for at least one of the three polypeptides. In some embodiments, the expression cassette that comprises the polynucleotide that encodes the chimeric polypeptide also comprises a coding sequence for at least one of a SrtC, pilus shaft, and SipA polypeptides. In other embodiments, the expression cassette that comprises a polynucleotide sequence that encodes at least one of the SrtC, pilus shaft, and SipA polypeptides is different from the expression cassette that comprises the polynucleotide that encodes the chimeric polypeptide. In yet other embodiments, each of the polypeptides is encoded by a coding sequence present on a distinct expression cassette.

The transformed Gram-positive bacterium displaying the polypeptide of interest can display more than one polypeptide of interest. In some of these embodiments, the

Gram-positive bacterium comprises at least two groups of pili, wherein each group expresses a distinct polypeptide of interest. This can be due to the introduction of at least two distinct polynucleotides, each encoding for a distinct chimeric polypeptide. Alternatively, one polynucleotide can be introduced into the bacterium, wherein the
5 polynucleotide comprises coding sequences for each of the polypeptides that are to be displayed on the surface of the bacterium. In these embodiments, the coding sequence for each chimeric polypeptide (the polypeptide of interest fused to a Gram positive bacterial pilus tip protein or an active variant or fragment thereof) can be operably linked to the same regulatory sequences (monocistronic) or to separate regulatory sequences
10 (polycistronic).

According to the methods of the invention, following the introduction of the polynucleotide comprising an expression cassette encoding the chimeric polypeptide, the Gram-positive bacterium is grown under conditions that allow for the generation of the pilus. The growth conditions used for this step of the presently disclosed methods can be
15 any growth condition known in the art for growth of the Gram-positive bacterium that is displaying the polypeptide of interest. In general, the bacteria can be grown in liquid or solid culture medium. Growth in liquid culture often is facilitated through aeration of the culture medium (e.g., through shaking of the container comprising the medium). In some embodiments, particularly those embodiments wherein the Gram-positive bacterium is a *S.*
20 *pyogenes* bacterium, the bacterium is grown in Todd-Hewitt medium (such as the Todd-Hewitt medium that is commercially available from BD, Sparks, MD). In some of these embodiments, growth supplements are added to the medium. A non-limiting example of a growth supplement is yeast extract. In some embodiments, Todd-Hewitt medium is supplemented with yeast extract at a 0.2% concentration. Another non-limiting example
25 of a growth media for Gram positive bacteria, including *L. lactis*, is M17 media (such as the M17 media available from Oxoid Limited, Hampshire, UK). The M17 media can be supplemented with glucose (for example, at a concentration of 0.5%).

As used herein, a “polypeptide of interest” refers to any full-length, variant, or fragment of any naturally-occurring polypeptide from any organism (prokaryotic or
30 eukaryotic) or a synthetically derived polypeptide that would find use in the display on the surface of a bacterium. In some embodiments, the polypeptide of interest that is displayed on the surface of the bacterium retains the activity (e.g., enzymatic activity) of the naturally occurring polypeptide or the same polypeptide that has not been fused to the pilus tip polypeptide. As non-limiting examples, the polypeptide can comprise an enzyme,

an antigen, or a biosorbent. The polypeptide of interest may be native to the Gram-positive bacterium that is displaying the polypeptide of interest or the polypeptide of interest may be heterologous to the bacterium.

The compositions and methods of the invention can be used for any use known in the art for surface displayed polypeptides (see, for example, Hansson *et al.* (2001) *Combinatorial Chemistry & High Throughput Screening* 4:171-184; Wu *et al.* (2008) *Trends in Microbiology* 16:181-188; Wernerus and Stahl (2004) *Biotechnol. Appl. Biochem.* 40:209-228; Chen and Georgiou (2002) *Biotechnol Bioeng* 79:496-503; Lee *et al.* (2003) *Trends in Biotechnology* 21:45-52; Wernerus *et al.* (2002) *Journal of Biotechnology* 96:67-78, each of which are herein incorporated by reference). For example, the compositions and methods of the invention are useful in methods for inducing an immunological response in a subject, methods for screening for expression of a heterologous polypeptide, methods for removing a contaminant from a composition (e.g., soil, water), methods for producing ethanol, and methods for improving food and nutritional additives.

As used herein, an "antigen" comprises any polypeptide that can mount an immune response in a subject and is, thus, immunologically active. The present invention provides immunological compositions or vaccines comprising a Gram-positive bacterium displaying an antigen on the tip of a pilus, wherein the antigen is amino terminal to a *Streptococcus pyogenes* pilus tip protein or an active variant or fragment thereof, wherein said pilus tip protein or active variant or fragment thereof comprises a cleaved cell wall sorting signal (CWSS) motif.

The immunological compositions comprising the Gram-positive bacteria displaying an antigen can be used to prevent the development of a particular disease or unwanted condition through the administration of the compositions to a subject. As used herein, the term "prevent" refers to obtaining a desired pharmacologic and/or physiologic effect. Administration of the immunological composition might lead to complete or partial prevention of a particular infection or disease or sign or symptom thereof.

Methods for inducing an immunological response in a subject comprise administering to a subject a composition comprising a Gram-positive bacterium displaying an antigen on the tip of a pilus, wherein the antigen is amino terminal to a *Streptococcus pyogenes* pilus tip protein or an active variant or fragment thereof, wherein said active variant or fragment comprises a cleaved cell wall sorting signal (CWSS) motif.

When referring to the Gram-positive bacteria of the invention or compositions comprising the same, the term "administering," and derivations thereof, comprises any method that allows for the Gram-positive bacteria or compositions comprising the same to contact a cell within the subject to which the composition was administered.

5 By "subject" is intended an animal, including a mammal, such as a human, and including, but by no means limited to, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., avian species, such as 10 chickens, turkeys, songbirds, etc., *i.e.*, for veterinary medical use. In particular embodiments, the subject is a human.

A non-limiting example of an antigen that can be displayed on the tip of a Gram-positive bacterial pili includes domain 1' (residues 168-258) of the protective antigen of the anthrax toxin of *Bacillus anthracis* (the nucleotide and amino acid sequence of which 15 is set forth in SEQ ID NO 91 and 92, respectively), which is the domain that remains at the N-terminus of the toxin following its proteolytic cleavage by proteases ubiquitously present in host tissue. This domain, called the "LEF domain", is involved in binding to the other subunits of the anthrax toxin, LF (lethal factor), and EF (edematous factor). This antigen is useful for methods involving the administration of the Gram-positive bacterium 20 displaying such an antigen for the purposes of providing protection against an infection of *Bacillus anthracis*. Another domain of the anthrax toxin that can be used as an antigen for the purpose of stimulating an immunological response to a *Bacillus anthracis* bacteria is domain 4 (residues 596-735 of SEQ ID NO: 92), called "RBD", which is responsible for binding of the toxin to host cell receptors. The RBD and LEF domains are antigenic as 25 DNA vaccines, have been inserted into the influenza virus fused within the hemagglutinin protein, and have been shown to provide passive protection against the toxin (Li *et al* (2005) *J Virol* 79:10003-10012).

Other examples of antigens that can be displayed on the pili tip of a Gram-positive bacterium according to the presently disclosed methods include a mutant nontoxic form of 30 the heat labile toxin LT A or LT B proteins, and CooD, an ETEC adhesin, which are useful in providing protection against enterotoxigenic *Escherichia coli* (ETEC). In some embodiments, the mutant LT A protein comprises a triple LT mutant (R7K, S63K, V53E) in which three residues required for toxin activity have been changed in ways that don't alter the protein structure (Pizza *et al* (1994) *J Exp Med* 180:2147-2153; the nucleotide

and amino acid sequences of the LT A protein are set forth in SEQ ID NO: 95 and 96, respectively and the nucleotide and amino acid sequences of the LT B protein are set are set forth in SEQ ID NO: 93 and 94, respectively). In those embodiments, wheren the antigen comprises CooD, the *cooD* gene from a CS1 ETEC strain (the nucleotide and amino acid sequence of which is set forth in SEQ ID NO: 89 and 90, respectively) can be used and its chaperone gene *cooB* will also be introduced into the Gram-positive bacteria displaying the antigen (Voegele, Sakellaris & Scott (1997) *Proc Natl Acad Sci USA* 94:13257-13261).

Vaccine delivery or immunization via attenuated bacterial vector strains expressing distinct antigenic determinants against a wide variety of diseases is now commonly being developed. Recently, mucosal (for example nasal or oral) vaccination using such vectors has received a great deal of attention. For example, both systemic and mucosal antibody responses against an antigenic determinant of the hornet venom were detected in mice orally colonized with a genetically engineered human oral commensal *Streptococcus gordonii* expressing the antigenic determinant on its surface (Medaglini *et al.* (1995) *Proc Natl Acad Sci USA* 2:6868-6872). Also, a protective immune response was elicited by oral delivery of a recombinant bacterial vaccine wherein tetanus toxin fragment C was expressed constitutively in *Lactococcus lactis* (Robinson *et al.* (1997) *Nature Biotechnology* 15:653-657). Mucosal immunization as a means of inducing IgG and secretory IgA antibodies directed against specific pathogens of mucosal surfaces is considered an especially effective route of vaccination. In addition, the existence of a common mucosal immune system permits immunization on one specific mucosal surface to induce secretion of antigen-specific IgA and other specific immune responses at distant mucosal sites. Thus, in some of these embodiments, the Gram-positive bacteria that display an antigen comprise attenuated pathogenic bacteria.

An alternative approach avoids the use of attenuated bacterial strains that may become pathogenic themselves by using recombinant commensal bacteria as vaccine carriers, such as *Streptococcus spp*. and *Lactococcus spp* (see, for example, Buccato *et al.* (2006) *Journal of Infectious Diseases* 194:331-340). In some embodiments, the Gram-positive bacteria that display an antigen comprise live, non-pathogenic bacteria. Non-limiting examples of non-pathogenic Gram-positive bacteria useful for the development of vaccines include *Streptococcus gordinii*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*. Non-pathogenic bacteria can include, but are not limited to food-grade bacteria. A non-limiting example of a food-grade bacterium is *Lactococcus lactis*. *Lactococcus*

lactis is currently used as a probiotic and has been reported to have adjuvant properties. Although it can colonize the intestines temporarily, it is not normally found in the human microflora. Further, it is likely that a continuous cold chain would not be required for delivery of an *L. lactis* vaccine and it would be inexpensive to produce. See Raha *et al.* 5 (2005) *Appl Microbiol Biotechnol* 68:75-81, which is herein incorporated in its entirety, for a review on the use of *L. lactis* as a vaccine vector)

The presently disclosed immunological compositions can be formulated for delivery, i.e., administering to the subject, by any available route including, but not limited, to parenteral (e.g., intravenous), intradermal, subcutaneous, oral, nasal, bronchial, 10 opthalmic, transdermal (topical), transmucosal, rectal, and vaginal routes. In some embodiments, the route of delivery is intravenous, parenteral, transmucosal, nasal, bronchial, vaginal, or oral.

The presently disclosed compositions also can include a Gram-positive bacterium with a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically 15 acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds also can be incorporated into the compositions.

As one of ordinary skill in the art would appreciate, a presently disclosed 20 pharmaceutical composition is formulated to be compatible with its intended route of administration. Solutions or suspensions used for parenteral (e.g., intravenous), intramuscular, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial 25 agents, such as benzyl alcohol or methyl parabens; antioxidants, such as ascorbic acid or sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid; buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, 30 disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use typically include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water,

Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). The composition should be sterile and should be fluid to the extent that easy syringability exists. In some embodiments, the pharmaceutical compositions are stable under the conditions of manufacture and storage and should be preserved against the contaminating
5 action of microorganisms, such as bacteria and fungi. In general, the relevant carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures 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 in the case of
10 dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In some embodiments, isotonic agents, for example, sugars, polyalcohols, such as manitol or sorbitol, or sodium chloride are included in the formulation. Prolonged absorption of the injectable
15 formulation can be brought about by including in the formulation an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., polynucleotide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered
20 sterilization. In certain embodiments, solutions for injection are free of endotoxin. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In those embodiments in which sterile powders are used for the preparation of sterile injectable solutions, the solutions can be prepared by vacuum drying
25 and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral
30 compositions also can be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches, and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient, such as starch or

lactose, a disintegrating agent, such as alginic acid, Primogel, or corn starch; a lubricant, such as magnesium stearate or Sterotes; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring. Compositions for oral delivery can advantageously incorporate agents to improve stability within the gastrointestinal tract and/or to enhance absorption.

For administration by inhalation, the presently disclosed compositions can be delivered in the form of an aerosol spray from a pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Liquid aerosols, dry powders, and the like, also can be used.

Systemic administration of the presently disclosed compositions also can be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds also can be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical or cosmetic carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of individuals. Guidance regarding dosing is provided elsewhere herein.

Depending on the route of administration, the agent may be coated in a material to protect it from the action of enzymes, acids and other natural conditions which may inactivate the agent. For example, solutions or suspensions used for parenteral,

intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite;
5 chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

10 To administer an agent by other than parenteral administration, it may be necessary to coat the agent with, or co-administer the agent with, a material to prevent its inactivation. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol.

In some embodiments, the polypeptide of interest dispayed on the tip of Gram
15 positive bacterial pili comprises an enzyme. As used herein, an enzyme is any polypeptide that can catalyze a chemical reaction. Thus, the presently disclosed Gram-positive bacteria that display enzymes can be used in methods requiring whole cell biocatalysts. Enzymes useful for the presently disclosed methods and compositions include those enzymes that are capable of degrading organic matter, those that are involved in the
20 production of biofuels, or those that find use in improving the nutritive quality of food products, such as probiotics.

For the production of ethanol, Gram positive bacterium expressing an enzyme at the pilus tip that catalyzes a step in the degradation of plant materials such as starch, cellulosic, lignocellulosic materials or the like can be added to the plant materials. Non-
25 limiting examples of enzymes useful for this purpose include starch degrading enzymes such as α-amylases (EC 3.2.1.1), glucuronidases (E.C. 3.2.1.131); exo-1,4-α-D glucanases such as amyloglucosidases and glucoamylase (EC 3.2.1.3), β-amylases (EC 3.2.1.2), α-glucosidases (EC 3.2.1.20), and other exo-amylases; and starch debranching enzymes, such as a) isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and the like; b) cellulases
30 such as exo-1,4-3-cellobiohydrolase (EC 3.2.1.91), exo-1,3-β-D-glucanase (EC 3.2.1.39), β-glucosidase (EC 3.2.1.21), endo-1,4-β-glucanase (EC 3.2.1.4) and the like; c) endoglucanases such as endo-1,3-β-glucanase (EC 3.2.1.6); d) L-arabinases, such as endo-1,5-α-L-arabinase (EC 3.2.1.99), α-arabinosidases (EC 3.2.1.55) and the like; e)

galactanases such as endo-1,4-β-D-galactanase (EC 3.2.1.89), endo-1,3-β-D-galactanase (EC 3.2.1.90), α-galactosidase (EC 3.2.1.22), β-galactosidase (EC 3.2.1.23) and the like; f) mannanases, such as endo-1,4-β-D-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-mannosidase (EC 3.2.1.24) and the like; g) xylanases, such as endo-1,4-β-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), 1,3-β-D-xylanase, and the like; h) other enzymes such as α-L-fucosidase (EC 3.2.1.51), α-L-rhamnosidase (EC 3.2.1.40), levanase (EC 3.2.1.65), inulanase (EC 3.2.1.7) and the like, enzymes capable of degrading maltose maltotriose and α-dextrins obtained from the first degradation of starch, include maltases, α-dexitrinase, α-1,6-glucosidases, glucoamylases (α-1,4-glucan glucohydrolases), and the like, and enzymes capable of modifying monosaccharides, such as glucose isomerase, invertase, and the like.

Methods for improving the nutritive quality of food products (e.g., probiotics) include, but are not limited to, the addition of food-grade Gram-positive bacterium displaying an enzyme that assists in digestion of certain food products (e.g., carbohydrates) to food products for animal consumption. Such supplemented food products are particularly useful for subjects that exhibit enzymatic deficiencies and are less able to digest particular food products. In specific embodiments of these methods, the Gram-positive bacteria comprise lactic acid bacteria, which are bacteria that are capable of converting sugars, including lactose and other carbohydrates, into lactic acid. Non-limiting examples of lactic acid bacteria include bacteria from the genera *Lactobacillus* or *Bifidobacterium*. Non-limiting examples of *Lactobacillus* species useful as probiotics include *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. acidophilus*, *L. bulgaricus*, *L. plantarum*, *L. salivarius*, *L. johnsonii*, and *L. helveticus*. Non-limiting examples of *Bifidobacterium* include *B. lactis*, *B. infantis*, *B. longum*, *B. animalis*, and *B. bifidum*. The addition of lactic acid bacteria to food products is particularly useful for the administration of the food products to people with lactose intolerance. Bacterial strains useful for probiotics are known in the art (see, for example, Sanders (2007) *Functional foods & nutraceuticals*; June 2007:pp.36-41, which is herein incorporated by reference in its entirety). Enzymes that are useful in improving the nutritive quality of food products (e.g., for human or other animals) are known in the art and can be expressed on the pili of Gram positive bacteria (e.g,. *Lactobacillus, Bifidobacterium*) using the methods described herein. Non-limiting examples of such enzymes include lactase, hemi-cellulase, and phytase.

In other embodiments, the polypeptide of interest that is displayed on the Gram positive pili comprises a biosorbent. As used herein, a biosorbent comprises a polypeptide that specifically binds with a substantially high affinity to a particular molecule. Non-limiting examples of biosorbents include polypeptides with a cellulose-binding domain, or a metal-binding domain, such as a metallothionein or a phytochelatin.

Gram-positive bacteria displaying a biosorbent find use in bioremediation methods. Specifically, the presently disclosed subject matter provides for methods for removing a contaminant from a composition (e.g., soil, water), wherein the method comprises introducing to the composition a Gram-positive bacterium with a polypeptide of interest displayed on the tip of the pilus, wherein the polypeptide of interest comprises a biosorbent capable of specifically binding to the contaminant or an enzyme capable of degrading the contaminant.

As used herein, the term "contaminant" refers to any inorganic or organic molecule that is not desirable in a particular composition (e.g., soil, water). Non-limiting examples of contaminants include environmental chemicals, radioactive elements, bacteria or organisms, the byproduct of the growth of bacteria or organisms, decomposing material, or waste. In some embodiments, the composition comprising the contaminant is soil or water. In some of these embodiments, the contaminant comprises a heavy metal. In these embodiments, the polypeptide of interest comprises a biosorbent, wherein the biosorbent comprises a metal binding polypeptide that specifically binds to heavy metals. In some of these embodiments, the metal binding polypeptide comprises a metallothionein or a phytochelatin.

In other embodiments, the contaminant comprises an organic contaminant. In these embodiments, the heterologous polypeptide comprises an enzyme capable of degrading the organic contaminant. In some embodiments, the organic contaminant comprises an organophosphate. In some of these embodiments, the heterologous polypeptide comprises organophosphorous hydrolase (OPH).

As used herein, the term "removing" when referring to a contaminant means there is less than 99%, less than 98%, less than 97%, less than 96%, less than 95%, less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 5%, less than 1% or less of the contaminant remaining in the composition after the introduction of the bacterium displaying the biosorbent or degrading enzyme relative to the same composition prior to its introduction.

The presently disclosed Gram-positive bacteria also find use in diagnostic methods, wherein the Gram-positive bacteria display a detection reagent, which is a peptide (including, but not limited to, an antibody or a fragment thereof) capable of specifically detecting a disease-associated protein or ligand. In some of these
5 embodiments, the displayed peptide further comprises a detectable label (e.g., a radiolabel, a fluorescent label). The Gram-positive bacteria displaying the detection reagent can be administered to a subject, followed by detection of the bacteria through the detectable label attached thereto. In some of these embodiments, the Gram-positive bacteria that is displaying the detection reagent comprise attenuated pathogenic bacteria or non-
10 pathogenic commensal bacteria.

The following examples are offered by way of illustration and not by way of limitation.

15 EXPERIMENTAL

Example 1. Cpa pilins are present on the tip of Group A *Streptococcus* pili

To study the covalent linkage of major and minor pilin subunits catalyzed by the pilin polymerase SrtC2, an expression system in *Escherichia coli* has been established
20 (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35). In this system, the only GAS genes present are those encoding SrtC2, the pilins T3 and Cpa, and the chaperone, SipA2. Because complete pili are not synthesized in *E. coli*, a large fraction of the total pilin protein consists of the low molecular weight pilin polymers. This genetic approach has allowed the definition of the pilin residues required for covalent linkage of these subunits.
25 In this work, the linkage of the minor pilin protein Cpa to the backbone protein of T3 pili of GAS was investigated. The results indicate that the noncanonical CWSS motif in Cpa is required for its attachment to the T3 protein by SrtC2. Evidence is also provided that addition of Cpa to T3 requires the same lysine residue in T3 that is needed for polymerization of T3 subunits. This implies that addition of Cpa to a T3 subunit leaves
30 only the C-terminus of this T3 subunit available for addition of another subunit. Therefore, the results strongly suggest that Cpa is located exclusively at the tip of the T3 pilus, and, based on this, a model for biogenesis of these pili has been suggested.

Cpa is not linked to the N-terminus of T3

In previous analyses of T3 pilus polymerization in *E. coli*, a presumptive T3-Cpa heterodimer using an HA-tagged derivative of Cpa was identified (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35). This protein was encoded on pJRS1326, which also encodes
5 SipA2, T3 and SrtC2, all derived from the M3 GAS strain AM3 (see Figure 1). To identify the Cpa(HA) monomer, pJRS1325, a plasmid derived from pJRS1326 by deletion of *srtC2* was used. Monomeric Cpa(HA) appeared as a band of approximately 75 kDa on western blots of whole cell lysates of TOP10/pJRS1325 boiled in SDS (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35 and Figure 2A lane 2). In the presence of SrtC2, a second
10 strong band that reacted with both anti-T3 and anti-HA antisera was visible (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35 and Figure 2A and B lanes 3). The molecular mass difference between this approximately 105 kDa band and the monomeric Cpa(HA) band is approximately that of the mature T3 protein (about 32 kDa). To confirm that the 105 kDa band is a heterodimer of Cpa(HA) covalently bound to T3, a whole cell lysate of *E. coli*
15 TOP10/pJRS1326 was subjected to immunoprecipitation using an anti HA-antibody. The immunoprecipitate was boiled in SDS to dissociate noncovalent protein interactions and separated by SDS-PAGE. The 105 kDa band (Figure 2B, lane 3) was recovered from a SyproRuby-stained SDS gel, digested with trypsin and subjected to mass spectrometry (MS) for peptide identification. Both T3 and Cpa(HA) peptides were present in the
20 immunoprecipitated sample (see Table 1).

Table 1. T3/Cpa peptides identified by mass-spectrometry [a]

CpaHA					
Start[b]	End[b]	Before[c]	Sequence	SEQ ID NO:	After[c]
46	54	G[d]	AEEQSVPNK	47	Q
72	80	K	GYPDYSPLK	48	T
87	93	K	VNLDGSK	49	E
120	130	K	KLEGTNENFIK	50	L
121	130	K	LEGTNENFIK	51	L
137	148	R	IEDGQLQQNILR	52	I
149	158	R	ILYNGYPNDR	53	N
164	176	K	GIDPLNAILVTQN	54	A
193	202	K	AFQQEETDLK	55	L
236	245	Y	QLSIFQSSDK	56	T
271	282	K	**YPYDVPDYA**TEK[e]	57	T
289	297	R	KYAEGDYSK	58	L
290	297	K	YAEGDYSK	59	L
298	305	K	LLEGATLK	60	L

306	317	K	LAQIEGSGFQEK	61	I
318	323	K	IFDSNK	62	S
347	355	Y	GVATPITFK	63	V
365	376	K	NKEGQFVENQNK	64	E
367	376	K	EGQFVENQNK	65	E
450	460	K	YTHVSGYDLYK	66	Y
467	475	R	DKDADFFLK	67	H
469	475	K	DADFFLK	68	H
494	501	K	TLTEAQFR	69	A
528	534	K	GYHGFDK	70	L
594	603	K	QAPIIPITHK	71	L
609	618	K	TVTGTIADKK	72	K
T3					
Start	End	Before	Sequence	SEQ ID NO:	After
29	38	A[d]	ETAGVSENAK	73	L
76	86	K	DGLEIKPGIVN	74	G
107	114	K	STEFDFSK	75	V
115	124	K	VVFPGIGVYR	76	Y
125	130	R	YTVSEK	77	Q
131	142	K	QGDVEGITYDTK	78	K
144	153	W	TVDVYVGNK	79	E
154	161	K	EGGGFEPK	80	F
191/296	202/308	K/K	KNVSGNTGELQK/ TDESADEIVVTNK[f]	81/82	E/R
218	226	K	KDQIVSLQK	83	G
219	226	K	DQIVSLQK	84	G
243	253	K	LKNGESIQLDK	85	L
245	253	K	NGESIQLDK	86	L
254	261	K	LPVGITYK	87	V
262	273	K	VNEMEANKDGYK	88	T

[a] Mass spectrometry performed on trypsin-digested band isolated from SDS-PAGE (105 kDa)
[b] numbering refers to the position in the sequence of the preprotein
[c] residue before/after the peptide cleavage site
[d] residue preceding the predicted signal peptide cleavage site in the preprotein
[e] HA-tag sequence indicated in bold letters
[f] peptides linked by intramolecular isopeptide bond; residues predicted to form bond are underlined.

Since the sample was boiled prior to separation by SDS-PAGE, it is concluded that the 105 kDa band corresponds to a covalently linked T3-Cpa heterodimer produced in *E. coli* in the presence of SrtC2.

Because a sortase forms an amide bond between the carboxyl group at the C terminus of one protein and an amino group of a second protein, it seemed possible that the Cpa protein was attached at the α-amino group of the distal T3 subunit in the pilus. T3 is synthesized as a preprotein that is predicted to be cleaved by the signal peptidase between alanine 28 and glutamate 29 (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35). Consistent with this, the peptide representing the N-terminus of the mature T3 protein (E29-K38) was recovered by MS, while the first 28 residues of the preprotein of T3 were not among the peptides seen (Figure 2C). Since trypsin is not expected to cleave between A28 and E29, recovery of peptide E29-K38 indicates that cleavage occurred in *E. coli*, and not during MS sample preparation. Therefore, recovery of the N-terminal peptide of T3 from the Cpa-T3 heterodimer demonstrates that the α-amino group at the T3 N-terminus is not bound to Cpa(HA).

The VPPTG motif in the CWSS of Cpa is required for linkage of Cpa to T3

Because covalent linkage of Cpa to T3 requires the sortase family enzyme SrtC2, the motif at the start of the CWSS of Cpa was expected to be required for this reaction. However, this motif, VPPTG, differs from the canonical LPXTG motif found in substrates of the housekeeping sortase SrtA, like the M protein. It also differs from the CWSS motif in T3 (QVPTG), which is required for its polymerization by SrtC2 (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). Therefore, studies were initiated to establish whether the VPPTG motif in the CWSS of Cpa is essential for linkage of Cpa to T3. LPSTG was substituted for the VPPTG motif of Cpa to test the importance of this motif in formation of the Cpa-T3 heterodimer.

The HA-tagged derivative of Cpa was used in the assay that had been established in *E. coli* (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35) to investigate this reaction. The desired motif replacement was constructed by site-directed mutagenesis of pEU7646 (Figure 1). DNA sequencing was used to confirm the presence of the mutation in the resulting plasmid, pEU7904. The formation of the T3-Cpa(HA) heterodimer was examined, as well as the T3-T3 homodimer, by analysis of extracts of *E. coli* strains that had been boiled in SDS to disrupt non-covalent bonds. TOP10/pJRS1325, which lacks *srtC2*, was used to facilitate identification of the monomeric form of Cpa (Figure 3A lane 1) and T3 (Figure 3B lane 1). As expected, in extracts of strain TOP10/pEU7646, which encodes Cpa(HA) with the wild type CWSS, western blot analysis using an anti-HA antibody showed bands corresponding to the sizes for both the Cpa(HA) monomer (75

kDa) and the Cpa(HA)-T3 heterodimer (105kDa) (Figure 3A lane 2). However, no Cpa(HA)-T3 heterodimer was detected in extracts of the strain in which LPSTG replaced the VPPTG motif of the Cpa CWSS (TOP10/pEU7904; Figure 3A lane 3). Instead, only the Cpa(HA) monomer and its characteristic degradation product, migrating at about 32 kDa, were present. To verify that the absence of the Cpa(HA)-T3 dimer was due to the introduction of the mutation in the Cpa(HA) CWSS motif, and not to an undetected second mutation that might affect the function of SipA2 or SrtC2, the same cell lysates were analyzed by western blot using anti-T3 antiserum (Figure 3B). In both TOP10/pEU7646 and TOP10/pEU7904 (Figure 3B lanes 2 and 3), T3 dimers were present, while they were absent from the strain lacking SrtC2 (Figure 3B lane 1). This indicates that in both plasmids, the genes required for pilus formation functioned normally. The presence of polymerized forms of T3, combined with the absence of the T3-Cpa(HA) dimer in strain TOP10/pEU7904, demonstrate that the VPPTG motif in the CWSS of Cpa is required for linkage of Cpa to T3.

In addition to replacing the VPPTG motif of Cpa with the LPSTG sequence, the VPPTG sequence of the pJRS9550 plasmid was mutated to delete the "PTG" residues of the motif, leaving just "VP" (pJRS9597; Figure 1). This plasmid was transformed into the heterologous serotype M6 GAS strain JRS4, which lacks the FCT-3 region containing the genes required for T3 pilus production and thus does not express T3 pili (see Figure 10). A vector control, pJRS9545 (derived from pJRS9508), which consists of the pReg696 backbone and the P23 promoter, was transformed into JRS4 as a negative control. Cell wall fractions of this strain and strains JRS4/pJRS9550 (wt), JRS4/pJRS9554 (-SrtC2), and JRS4/pJRS9597 (VP) that had been boiled in SDS to dissociate noncovalent bonds were examined for formation of the Cpa(HA)-T3 heterodimer and incorporation of Cpa(HA) into HMW T3 polymers using an anti-HA antibody (Figure 4A, lanes 1-4). Although the high molecular weight (HMW) banding pattern characteristic of pili on Gram-positive bateria (Mora *et al.* (2005) *Proc Natl Acad Sci U S A* 102:15641-15646; Zähner and Scott (2008) *J Bacteriol* 190:527-535) was detected in cell wall extracts of JRS4/pJRS9550, the positive control, no pilus bands were visible in extracts of JRS4/pJRS9597 (the VP mutant) or of the SrtC2- control JRS4/pJRS9554. To be sure that SipA2 and SrtC2 remained functional in the mutant, the production of pili in cell wall fractions was also examined using anti-T3 (Figure 4 B). The presence of high molecular weight (HMW) forms of T3 in the mutant extract indicated that lack of incorporation of Cpa into pili in JRS4/pJRS9597 is not due to a defect in polymerization of T3. As

expected, HMW forms of T3 were present in cell wall extracts of JRS4/pJRS9550 (positive control) but not in those of its SrtC2- derivitive.

The absence of HMW polymers containing Cpa(HA) in cell wall extracts of the mutant might result either from lack of polymerization or from lack of covalent attachment of the pili to the cell wall. If the latter were correct, pilus polymers should be present in the culture supernatant. Therefore, concentrated supernatants were analyzed for the presence of HMW forms containing Cpa(HA) and polymerized T3 (Figure 4 A, lanes 5-8 and data not shown). As expected, pili containing T3 were present in the concentrated supernatant and Cpa-containing pili were present in the supernatant from the positive control strain. However, Cpa(HA) was not present in HMW pilus forms in the supernatant of the VP mutant. The absence of incorporation of Cpa into the pili when the VPPTG motif is partially deleted indicated that the VPPTG motif at the start of the CWSS of Cpa is required for addition of Cpa to T3 pili.

The QVPTG motif in the T3 CWSS is not necessary for formation of the T3-Cpa heterodimer

Previously, it was shown that the QVPTG motif (SEQ ID NO: 9) in the CWSS of T3 is required for polymerization of T3 by SrtC2 (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). To determine whether this motif is also needed for the formation of the Cpa(HA)-T3 heterodimer, it was replaced in pEU7646 with the canonical LPSTG motif (SEQ ID NO: 2; Figure 1: pEU7905). Whole cell lysates of *E. coli* TOP10/pEU7905 were examined by western blots using anti-T3 antiserum and anti-HA antibody (Figure 3 C and D). As described above for the Cpa CWSS motif replacement experiments, TOP10/pJRS1325, which lacks *srtC2*, was used to identify the monomeric forms of T3 (Figure 3C lane 1) and Cpa (Figure 3D lane 1). Whole cell lysates of the control strain TOP10/pEU7646, which encodes T3 with the wild type CWSS, showed bands corresponding to the T3 monomer, the T3 dimer, and the T3-Cpa(HA) heterodimer (Figure 3C lane 2) as well as the Cpa(HA) monomer and the Cpa(HA)-T3 heterodimer (Figure 3D, lane 2). As previously found (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75), replacement of the native CWSS motif in T3 by LPSTG (in TOP10/pEU7905) prevented formation of multimers of T3, although the T3 monomer was still present (Figure 3C lane 3). Most importantly, the T3-Cpa(HA) heterodimer was also visible in lysates of this strain (Figures 3C and D lanes 3). Thus, although the T3 CWSS motif is necessary for the polymerization of T3, it is not required for the formation of the T3-Cpa(HA) heterodimer.

Lysine residues forming putative intramolecular bonds in T3 are not required for its polymerization

To identify lysine residues in the T3 protein that might be involved in pilus formation, available sequences of the predicted pilus backbone proteins of the FCT-2, FCT-3 and FCT-4 regions of different GAS strains were compared (Figure 5). Sequence alignment revealed 6 fully conserved lysine residues that correspond to K43, K81, K100, K106, K173, and K191 in the T3 protein. Using pEU7657 (Figure 1), which expresses T3, SrtC2 and the chaperone-like protein SipA2 (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35) as a template, each of these lysines were replaced with alanine and analyzed polymerization of T3 for each mutant in extracts of *E. coli*.

T3 dimers, and usually trimers, were visible on western blots of the cell extracts of the mutants with K to A corresponding to K43, K81, K100, K106, and K191 developed with anti-T3 antiserum (Figure 6A). Surprisingly, the monomeric and polymeric forms of T3 mutant proteins K43A and K191A migrated with an increased apparent molecular mass relative to the wild type T3 protein (Figure 6A, lanes 3 and 7). The homologs of K43 and K191 in T1, the homolog of T3 in the FCT-2 region, (Figure 5), have been shown to be involved in intramolecular bonds (Kang *et al.* (2007) *Science* 318:1625-1628). Therefore, these two lysine residues are predicted to participate in intramolecular isopeptide bonds in T3. In support of this, the trypsin fragments of the Cpa(HA)-T3 heterodimer identified by MS analysis included one containing two T3 peptides linked by an isopeptide bond between K191 and N307 (Table 1).

The formation of these intramolecular bonds would be prevented by substituting alanine for the glutamate residue catalyzing formation of this bond (Kang *et al.* (2007) *Science* 318:1625-1628) or the lysine residue participating in the bond. Thus, it seems likely that the altered running behavior of the mutant proteins is a result of lack of formation of the intramolecular bonds. In agreement with this, the double mutant K43A,K191A protein migrates even more slowly than either single mutant protein (Figure 6B). However, for both single mutants and for the double mutant, dimers of T3 protein were present and trimers were also visible on some gels (Figure 6A and 6B and data not shown). This indicates that if these lysine residues are essential for intramolecular bond formation, they are not a requirement for polymerization of T3.

Lysine residue 173 of T3 is required for T3 polymerization

Multimeric forms of T3 were present for all mutants except one: K173A (Figure 6C). In the K173A mutant (Figure 6C, lane 3), the presence of monomeric T3, which has an apparent molecular mass of 32 kDa when analyzed by SDS PAGE, indicates that the mutant protein is expressed and is stable in this strain. Because it seemed possible that the charge on the lysine was the reason it was required for T3 polymerization, a second mutant in which K173 was replaced with arginine (R) was constructed (Figure 1 : pEU7692). Extracts of the *E. coli* strain containing this mutation also showed only T3 monomers (Figure 6C, lane 4), indicating the importance of the lysine group for polymerization of the T3 pilin. As a control, the adjacent lysine, K174, was also replaced with A (Figure 1:pEU7908). As expected, this had no visible effect on T3 polymerization (Figure 6C, lane 5). This demonstration that K173 is essential for T3 polymerization is consistent with the recent X-ray crystalographic and MS analysis of the M1 major pilin (Kang *et al.* (2007) *Science* 318:1625-1628; see Figure 5).

The role of K173 in T3 pilus formation was also examined in GAS. Previous studies (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35) of T3 polymerization in GAS utilized pJRS9536 (Figure 1), which contains the same DNA fragment from the GAS serotype M3 strain AM3 as used above in *E. coli* (plasmid pEU7657), except that an HA tag was added to the T3 protein (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). This plasmid and its derivatives were analyzed in the heterologous serotype M6 GAS strain JRS4, which lacks the FCT-3 region containing the genes required for T3 pilus production. The high molecular weight (HMW) banding pattern, which is characteristic of pili on Gram-positive bacteria (Mora, M. *et al.* (2005) *Proc. Natl. Acad. Sci.* 102:15641-6; Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35) was detected with anti-HA antibody in western blots of cell wall extracts that had been boiled in SDS to dissociate noncovalent bonds. Plasmid pJRS9536 was used as a template for site-directed mutagenesis to introduce the mutations K173A and K173R in T3(HA), resulting in plasmids pJRS9541 and pJRS9543, respectively (Figure 1). As a control, a K81A mutation was introduced into T3(HA) in plasmid pJRS9536 because this residue did not affect pilus formation in *E. coli* (Figure 6A). This mutation had no effect on T3 polymerization in GAS as expected, although the total amount of all forms of T3 in GAS was reduced in this mutant relative to its wild type parent (Figure 7, lane 4 vs 1). In GAS, replacing K173 with either A or R resulted in a loss of HMW T3(HA) polymers, while the monomer remained plentiful (Figure 7, lanes 2 and 3 vs. lane 1), which is in agreement with the results in *E. coli*. The

weak protein band that migrated with an apparent mass of about 65 kDa, consistent with the expected molecular weight of a T3(HA) dimer, does not appear to be a precursor to pilus formation since no higher molecular weight bands were visible. All four cell wall extracts also contained degradation products of approximately 20 kDa (Figure 7, lanes 1-
5 4).

The absence of HMW T3 polymers in cell wall extracts of the K173A mutant might result either from lack of polymerization or from lack of covalent attachment to the cell wall. If the latter were correct, pilus polymers should be present in the culture supernatant. To determine whether the T3(HA) monomer and/or its polymers are released
10 into the culture medium, concentrated supernatants were analyzed for the presence of T3(HA). The supernatant from GAS strain JRS4/pJRS9536, which expresses the HA-tagged T3 protein along with the rest of the genes needed for pilus synthesis, showed a HMW banding pattern similar to that seen with the cell wall extract from this strain (Figure 7, lanes 1 and 5). The culture supernatant of the K81A mutant (JRS4/pJRS9538)
15 showed a weak, but discernable, HMW pattern, indicating that polymerization of the T3 protein occurred (Figure 7, lane 8), although there was less total T3(HA) relative to the amount released from the wild type parent (pJRS9536). In contrast, no HMW bands were detected in culture supernatants of either the K173A or K173R mutants of T3 (JRS4/pJRS9541 or JRS4/pJRS9543, respectively) (Figure 7, lane 6 and 7). The culture
20 supernatants of all four strains contained a similar pattern of degradation products. The additional band of approximately 65 kDa seen in cell extracts (see above) was also present in supernatants from both the K173A and K173R mutants. From the absence of HMW bands of T3 in both the cell wall and supernatant it is concluded that K173 is essential for polymerization of T3(HA).

25

Lysine residue 173 of T3 is also required for attachment of Cpa(HA) to T3

To identify the lysine in T3 required for the formation of a covalent bond to Cpa(HA), the conserved lysines (Figure 5) were replaced with alanine using plasmid pEU7646, which encodes Cpa(HA), SipA2, T3 and SrtC2 (Figure 1). Following
30 confirmation of the induced mutation by sequencing, plasmids were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL, and the formation of T3-T3 homodimer and T3-Cpa heterodimer was analyzed by western blot of whole cell lysates. The monomeric form of Cpa(HA) was identified in an extract from a strain lacking SrtC2 (Figure 8A lane 1), as

before. The Cpa(HA)-T3 heterodimer was present in lysates from T3 mutants K43A, K81A, K100A, K106A, and K191A (Figure 8A, lanes 3-7), although extracts from mutants K81A and K106A appeared to contain less heterodimer. However, the lysate from mutant K173A showed no heterodimer, while K174A, used as a further control, produced the Cpa(HA)-T3 complex (Figure 8B). This demonstrates that lysine 173 of T3 is not only required for polymerization of T3, but is also needed for attachment of Cpa(HA) to the T3 shaft protein in *E. coli*.

To demonstrate the role of K173 of T3 in attachment of Cpa to the growing pilus in GAS, the M3 pilus cluster regions from pJRS1325, pEU7646, pEU7687 and pEU7688 (Figure 1) were cloned into the pReg696-derivative pJRS9508 (Barnett *et al.* (2007) *J Bacteriol* 189:1866-73), resulting in plasmids pJRS9554, pJRS9550, pJRS9557 and pJRS9558 respectively. Production of T3 pili by JRS4/pJRS9550 was confirmed by electron microscopy (Figure 10). A vector control derived from pJRS9508, consisting of the pReg696 backbone and the P23 promoter, was also constructed (pJRS9545). To identify monomeric Cpa(HA) and show its incorporation into the high molecular mass pilus ladder, cell wall extracts of JRS4 with each of these plasmids were prepared, boiled in SDS, and analyzed by western blot using an anti-HA antibody. Since these constructs differ from those used above (Figure 7) by encoding Cpa(HA) and an untagged version of T3, the same cell wall extracts were also analyzed using an anti-T3 antibody to confirm that in this genetic context K173 is essential for polymerization of T3 and for formation of the T3-Cpa heterodimer in GAS (Figure 9B). In addition, supernatants of each culture were concentrated 10-fold by TCA precipitation and analyzed by western blot as described above. Strain JRS4/pJRS9554, which lacks *srtC2* was used as a control to identify the band corresponding to monomeric T3 (Figure 9B lanes 2 and 6) and monomeric Cpa(HA) (Figure 9A lanes 2 and 6). Cell wall extracts and supernatants from strain JRS4/pJRS9550, encoding Cpa(HA), SipA2, T3 and SrtC2, show the HMW forms of the pilus ladder, indicating that Cpa is present in these species (Figure 9A lanes 3 and 7). Bands corresponding to sizes expected for monomeric Cpa(HA), the Cpa(HA)-T3 heterodimer, the Cpa(HA)-(T3)^2 hetero-trimer and the Cpa(HA)-(T3)^3 hetero-tetramer are also visible (Figure 9A lanes 3 and 7). While the Cpa(HA) species mentioned above were also visible in cell wall extracts and supernatants of JRS4/pJRS9558 (T3 K174A) (Figure 9A, lanes 5 and 9), the cell wall extract and supernatant from strain JRS4/pJRS9557 (T3 K173A) resembled that of the strain lacking SrtC2 (JRS4/pJRS9554): it lacked the

prominent bands of the Cpa-T3 heterodimer and higher order polymers observed in the wild type and K174A mutant (compare Figure 9A lanes 2 and 6 with lanes 4 and 8). Thus, K173 is required for attachment of Cpa to T3 in GAS as well as in *E. coli*.

5 *Cpa(HA) is located at the pilus tip*

Next, the position of Cpa(HA) in the T3 pili expressed by GAS was examined using whole-bacteria, negative-stain transmission electron microscopy (EM) coupled with immunogold localization. As expected, strain JRS4 containing the vector-only control plasmid pJRS9545 lacked any detectable pilus fibers (Figure 10, panel A and D). In
10 contrast, strain JRS4/pJRS9550, encoding Cpa(HA), SipA2, T3, and SrtC2, produced abundant surface fibers (Figure 10, panels B, C, E, and F). The identity of the surface fibers were identified by incubating the bacteria with anti-T3 rabbit polyclonal antiserum, followed by detection with a secondary anti-rabbit antibody conjugated to 12-nm diameter gold particles. The fibers produced by JRS4/pJRS9550 were abundantly labeled by the
15 gold particles (Figure 10, panel B and C), whereas only a few stray gold particles were visible on the EM grid with the vector control strain (Figure 10, panel A). To localize Cpa(HA) in the T3 pili, a dual labeling analysis was performed. For these experiments, the 12-nm gold particles were used to identify T3 pilins, and Cpa(HA) was detected using an anti-HA mouse monoclonal antibody followed by an anti-mouse secondary antibody
20 conjugated to 18-nm diameter gold particles. Dual labeling of the vector control strain again resulted in the presence of only a few, non-specific gold particles (Figure 10, panel D). However, with strain JRS4/pJRS9550, the larger, HA-specific gold particles could clearly be seen at what appeared to be the tips of some of the pilus fibers labeled by the smaller, T3-specific gold particles (Figure 10, panels E and F). The EM data suggests that
25 Cpa(HA) can be located at the pilus tips.

Discussion

Motif in the CWSS

Like other proteins covalently linked to the cell wall of Gram-positive bacteria,
30 pilins contain a CWSS at their C termini. Because the enzyme required for pilin polymerization is a member of the sortase family of transpeptidases, it is expected to behave like sortases, which are responsible for covalent attachment of surface proteins to the cell wall. These enzymes recognize the motif at the beginning of the CWSS, usually LPXTG, cleave the substrate protein between the T and G and attach the T residue to an

amino group of a second substrate. Unlike all the pilins of the three serologically different *Corynebacterium diphtheriae* pili, as well as pili of *S. pneumoniae*, *S. agalactiae*, and *Bacillus cereus*,which contain the canonical LPXTG motif, some pilins of Streptococci contain non-canonical motifs in their CWSSs (Scott, J. R. *et al.* (2006) *Mol. Microbiol.*
5 62:320-30). For the T3 pilus of GAS, not only is the motif of each of the three pilins that form the pilus non-canonical, but it differs for each of these proteins. Nevertheless, SrtC2 catalyzes both polymerization of T3 and association of the minor pilin, Cpa, with the T3 pilus shaft. It had previously been demonstrated that substitution of the canonical LPSTG motif (SEQ ID NO: 2) for the QVPTG motif (SEQ ID NO: 9) found in the shaft protein,
10 T3, prevents its polymerization (Barnett, T. C. *et al.* (2004) *J. Bacteriol.* 186:5865-75). The minor pilin, Cpa, has now been examined and it was found that substituting LPSTG (SEQ ID NO: 2) for VPPTG (SEQ ID NO: 10) in this protein prevents its attachment to the shaft protein. In addition, a deletion within the VPPTG motif (SEQ ID NO: 10) in this protein prevents its attachment to the shaft protein, highlighting the necessity of a specific
15 motif at the start of the CWSS for this minor pilin. Thus, it appears that the two different motifs in T3 pilins (XXPTG; SEQ ID NO: 11) are both recognized by SrtC2.

The Second Partner in the Intermolecular Bond Between T3 subunits

For formation of the peptide bond, the pilin polymerase must recognize a specific
20 motif N-terminal to the CWSS in the second pilin substrate, since the CWSS is cleaved and removed from the pilin. In *C. diphtheriae* SpaA, SpaD and SpaH pilins, Ton-That and Schneewind identified a conserved "pilin motif" WxxxVxVYPK (SEQ ID NO: 97; Ton-That, H. *et al.* (2003) *Mol. Microbiol.* 50:1429-38) that plays this role. By site-specific mutagenesis, they showed that the K at the end of this motif is required for pilin
25 polymerization, and later demonstrated that this motif, together with the CWSS, is sufficient to cause an unrelated *S. aureus* surface protein to be incorporated into SpaA pili (Ton-That, H. *et al.* (2004) *Mol. Microbiol.* 53:251-61.). They suggested, therefore, that the ε- amino group of this K participates in the peptide bond. A similar pilin motif is recognizable in pilins of some other Gram-positive bacteria, but not in all known or
30 putative pilins. It is not present in any of the proteins that constitute GAS pili.

For the GAS T1 major pilin protein, the K that is linked to the T of the CWSS motif of the next T1 subunit was recently identified by structural analysis (Kang, H. J. *et al.* (2007) *Science* 318:1625-8). The corresponding residue in the homologous T3 pilin is K173, as shown by sequence alignment (Figure 4). In this work, it was demonstrated that

substitution of an A or an R for this residue abrogates T3 polymerization, thus providing biological confirmation of the conclusions of Kang *et al.* that this bond is required for pilus polymerization.

The presence of two intra-molecular isopeptide bonds within the T1 shaft protein was discovered recently by Kang *et al.* (Kang, H. J. *et al.* (2007) *Science* 318:1625-8). Each of these bonds is formed between the ε-amino group of a lysine residue and the carboxyl group of an asparagine (N) residue within the same protein. By site-specific mutagenesis, they demonstrated that a glutamate (E) residue located near each of the two intramolecular bonds is required for formation of this link in a reaction that appears to be spontaneous. Since the wild type T1 protein is more resistant to trypsin digestion than is the mutant protein lacking intramolecular bonds, Kang *et al.* suggested that the role of the intramolecular bonds might be similar to that of disulfide bonds commonly found in pilins of Gram-negative bacteria i.e. they might stabilize the folded protein and make it more resistant to forces it might encounter in nature. The importance of these intramolecular peptide bonds is suggested by conservation of the residues (KEN) required for their formation in the T1 protein in other pilus backbone proteins (Figure 5). The MS analysis of the Cpa(HA)-T3 heterodimer (Table 1) identified one of the peptide fragments of T3 formed by an intramolecular isopeptide bond between K191 and N307, as predicted by homology with the T1 protein (Kang *et al.* (2007) *Science* 318:1625-1628). The inability to identify a peptide containing the second predicted intramolecular bond should not be regarded as significant because the MS coverage of the T3 protein was limited. It was found, however, that substitution of an A for either or both of the K residues predicted to participate in intramolecular bonds does not prevent T3-T3 polymerization or addition of Cpa to T3. Thus, if these K residues are essential for formation of these bonds, then the intramolecular peptide bonds are not required for pilus biogenesis, although they may still play a role in the biological function of the pili.

Linkage of Cpa to T3

Attachment of Cpa to T3 is catalyzed by the same pilin polymerase, SrtC2, as that required for linkage of T3 subunits to each other (3, 43). This enzyme catalyzes formation of a bond between a T in the CWSS and the ε-amino group of a lysine in the next pilus subunit. If linkage of Cpa to T3 proceeds by the same enzymatic mechanism, there are four alternative models of integration of a minor pilin into the pilus structure (Figure 11), two of which have been proposed previoiusly (Telford *et al.* (2006) *Nat Rev Microbiol*

4:509-519). It is theoretically possible for Cpa to be located exclusively at the tip of the T3 polymer and for the T in the CWSS motif of Cpa to be linked to the α-amino group of the N-terminal amino acid of T3 (Figure 11, model A). Because the intact N-terminal peptide of the mature T3 protein in the Cpa-T3 heterodimer was identified in our mass spectrometric analysis, this possibility can be ruled out. It is also possible for the T of the CWSS of T3 to be linked to a K of Cpa. There are two variants of this model. In the one shown in Figure 11, Model B, Cpa is interspersed within the T3 polymer (a model proposed by Telford *et al* (2006) *Nat Rev Microbiol* 4:509-519). In the other version of this model (not shown), a minor pilin is anchored directly to the cell wall and forms the base of the pilus, as occurs in GBS and C. diphtheriae (Nobbs *et al.* (2008) *Infect Immun* 76:3550-3560; Mandlik *et al.* (2008) *Trends Microbiol* 16:33-40). If either variant of Model B were correct for Cpa, the Cpa-T3 heterodimer should still be formed when: (i) the VPPTG (SEQ ID NO: 10) of Cpa is changed (top T3 monomer linked to Cpa in Model B), or (ii) K173 of T3 is mutated to another residue. However, the Cpa-T3 heterodimer for either of these mutations was not seen. Therefore, Model B is unlikely to be correct for Cpa. Model C assumes that Cpa is attached to the T3 polymer by a lysine in T3 different from K173, which links the T3 subunits to each other (Figure 11, Model C; branched model proposed by Telford *et al.* (2006) *Nat Rev Microbiol* 4:509-519). In this case, as in Models A and B, K173 of T3 would not be needed to form the Cpa-T3 heterodimer. Since it was found that a K173A mutation of T3 abolished formation of the Cpa-T3 heterodimer, Model C can also be eliminated. The remaining possibility is for the T of the CWSS of Cpa to be linked to T3 by K173 (Figure 11, model D). This model predicts that mutation of K173 of T3 would prevent formation of the Cpa-T3 heterodimer, which is what was found. Additional support for this model is that replacement of the QVPTG motif (SEQ ID NO: 9) of T3 with LPSTG (SEQ ID NO: 2) did not prevent formation of the dimer with Cpa. In summary, it was found that the same K173 residue that links T3 monomers to each other is required for attachment of Cpa to T3 and the VPPTG motif of Cpa is needed for this attachment, while the QVPTG (SEQ ID NO: 9) of T3 is not required. Thus, model 8D appears to be correct and it appears that Cpa can only be located on the T3 pilus tip.

Localization of Cpa in T3 Pili by Immunogold EM

Model D (Figure 11) indicates that the minor pilin, Cpa, must be located at the pilus tip. This location was supported by our immunogold EM analysis of the T3 pili

expressed by intact GAS bacteria. The electron micrographs indicate that the pilus fibers protrude from the bacterial surface and may well extend beyond the capsule surrounding the GAS strain. The T3 pili appeared as long, thin fibers that tended to twist and bundle together. Dual labeling of the bacteria to detect both the major pilin T3 and minor pilin Cpa demonstrated the presence of Cpa at what appeared to be the pilus tips. This localization was consistently observed, although quantitative analysis of Cpa localization was not possible due to the flexible nature of the pili.

Location of Pilus Adhesin

The tip location of the Cpa minor pilin is similar to that found for the adhesin protein of pili on Gram-negative bacteria that are assembled by the chaperone-usher or alternate chaperone-usher pathways (e.g. Pap and CS1 pili respectively). In these cases, distal location of the adhesin is generally considered to facilitate its interaction with the receptor to which the pilus attaches. However, for GAS, the role of Cpa in adherence of T3 pili or in adherence of the homologus T1 pili is not clear. Abbot *et al*. (Abbot, E. L. *et al.* (2007) *Cell Microbiol*. 9**:**1822-1833) have shown that in a strain producing T1 pili, these pili are required for adherence to primary human keratinocytes or human tonsilar epithelial cells, which are likely to represent the cells to which GAS must attach for initiation of infection. However, in this strain, pili are not needed for attachment to A549 or HEp-2 cells. The Cpa protein of a serotype M49 GAS strain, which has an FCT-3 pilus locus similar to that of the T3 pilus, has been found to bind to type 1 collagen, an important extracellular matrix protein in the human host and to mediate adherence to HEp-2 cells (Kreikemeyer, B.*et al.* (2005) *J. Biol. Chem*. 280:33228-39). However, for the M1 GAS strain studied by Kehoe's group, collagen binding does not appear to be important for adherence to primary human keratinocytes or human tonsillar epithelial cells, since preincubation of either type of human cell with collagen did not affect GAS adherence (Abbot, E. L. *et al.* (2007) *Cell Microbiol*. 9**:**1822-1833).

The adhesin for *Streptococcus pneumoniae* (Nelson, A. L. *et al.* (2007) *Mol. Microbiol*. 66:329-40), *S. agalactiae* (Dramsi, S. *et al.* (2006). *Mol. Microbiol*. 60:1401-13; Krishnan, V. *et al.* (2007) *Structure* 15:893-903.) and *C. diphtheriae* (Mandlik, A. *et al.* (2007) *Mol. Microbiol*. 64:111-24) is a minor pilin protein, encoded by the first gene in the pilus locus, and the pilus shaft protein is dispensable for adherence to the cells studied. In contrast, for the T1 pili of the M1 GAS strain, all three pilin proteins are required for adherence: deletion of the genes for any of the three pilin proteins prevented adherence

(Abbot, E. L. *et al.* (2007) *Cell Microbiol.* 9:1822-1833). However, it is still possible that Cpa is a specific adhesin of T1 and T3 pili, since the shaft protein may be required only to present the adhesin so that it is external to the cell capsule. The role and location of the other minor pilin for T1 or T3 pili has not been investigated yet. It may be interspersed along the shaft of the pilus (Model 8C) as occurs for pilins in *S. agalactiae* (Rosini, R. *et al.* (2006) *Mol. Microbiol.* 61:126-41) and *S. pneumoniae* (Barocchi, M. A. *et al.* (2006) *Proc. Natl. Acad. Sci.* 103:2857-62; Hilleringmann, M., *et al.* (2008) *PLoS Pathog.* 4:e1000026), or it may be located exclusively at the tip in place of Cpa on some T3 pili. The latter location would produce pili with different specificities on the same bacterial cell.

Comparison with Pili of Gram-negative Bacteria: Model for Pilus Assembly

Unlike the much larger flagellae in which new subunits are transported through the structure and added at the tip (Macnab, R. M. *et al.* (2003) *Annu. Rev. Microbiol.* 57:77-100), pili on Gram-negative bacteria grow from the base out. In the well-studied Pap pili, the tip protein is added first and serves to nucleate formation of the pilus structure (reviewed by Sauer, F. G. *et al.* (2004) *Biochim. Biophys. Acta* 1694:259-67; Thanassi, D. G. *et al.* (2005) *Mol. Membr. Biol.* 22:63-72). This is accomplished by the strong affinity of a tip-chaperone complex for the usher protein, which forms a pore in the outer membrane of the Gram-negative cell. Interaction with the usher is proposed to alter the configuration of the tip-chaperone complex so that a shaft subunit can now displace the tip protein from the usher to allow addition of further subunits, leading to continued pilus growth.

Although assembly of pili on Gram-positive bacteria requires a specific pilin polymerase, in both Gram-positive and Gram-negative bacteria, the Sec system is used to transport pilins across the membrane of the cell. It is likely that pili on Gram-positive bacteria also grow by adding new subunits from the bottom because, based on the presence of a predicted membrane-spanning domain, the pilin polymerase is expected to be membrane located. However, the minor pilins are not required to nucleate formation of the pilus structure since they are dispensable for formation of pili in *C. diphtheriae, S. agalactiae, S. pneumoniae* and GAS. It was found that the minor pilin, Cpa, is likely to be located exclusively at the T3 pilus tip, therefore it must be added first as the pilus grows. In agreement with this idea, Cpa is found in all the HMW bands of growing pili in GAS. Ordered subunit incorporation might be accomplished by a mechanism involving

differential affinity, similar to that used for Pap pilus assembly. The membrane-located "gating" protein in Pap pili is the usher, while in Gram-positive bacteria it would be the pilin polymerase. This assembly model predicts that for GAS T3 pili, the polymerase SrtC2 will be found to have a greater affinity for Cpa than for T3. The relative abundance of the Cpa-T3 heterodimer vs. the T3 homodimer in Figure 3C lane 2 is in agreement with this prediction. When more Cpa becomes available in the cell, growth of a new pilus should be initiated as long as there is an excess of SrtC2 and all other sites and proteins (e.g. SipA2) required. This would lead to limitation of growth of old pili. Thus, regulation of synthesis of the pilin proteins should be important in determining the length and number of pili on the GAS surface.

In summary, the residues required in Cpa and T3 for SrtC2-catalyzed peptide bond formation have been identified. It was also learned that the K residues that appear to be involved in formation of the recently described intramolecular peptide bonds in the shaft protein of the T3 pilus are not required for pilus polymerization, suggesting that the intramolecular peptide bonds are not needed for this process. Finally, because it was found that K173 of T3 is required for addition of Cpa as well as for T3-T3 polymerization, it is likely that Cpa is located exclusively at the tip of the T3 pilus structure.

Materials and Methods for Experimental Example 1

Bacterial strains and growth conditions

GAS strain JRS4 is a spontaneous streptomycin-resistant derivative of the serotype M6 strain D471 (32). GAS strains were grown in Todd-Hewitt medium supplemented with 0.2% yeast extract (Difco). *E. coli* strains TOP10 (Invitrogen) and BL21-CodonPlus(DE3)-RIL (Stratagene) were grown in Luria broth (LB) (30). Antibiotics were used in the following concentrations: kanamycin 50 μg/ml and spectinomycin 100 μg/ml. IPTG at a final concentration of 1 mM was used for induction.

Site specific mutagenesis

Mutagenesis was performed using the QuikChange II XL mutagenesis Kit (Stratagene) according to the manufacturer's protocol using the primers shown in Table S1. Mutagenized plasmids were transferred into *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene) or TOP10 (Invitrogen). Correct nucleotide replacement was confirmed by DNA sequencing of the mutagenized gene.

Preparation of cell lysates and cell wall extracts

Cell lysates of *E. coli* were obtained from overnight cultures grown with antibiotics and IPTG if appropriate. Samples were prepared from *E. coli* and GAS as described previously (Zähner, D. *et al.* (2008) *J. Bacteriol.* 190:527-35).

5

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblot analysis: Proteins were separated by SDS-PAGE on 4 to 12% gradient gels (NuPAGE, Invitrogen) and transferred to nitrocellulose membrane (BioRad) for immunoblot analysis. The monoclonal anti-HA antibody (clone HA-7, Sigma) was used at a 1:2,000 dilution.
10 The polyclonal anti-T3 antiserum, used at a 1:250 dilution is a T3 typing serum provided by Dr. B. Beall (CDC, Atlanta). T3 typing sera have been demonstrated to cross-react with Cpa and other proteins encoded in the FCT region (15).

Immunoprecipitation of Cpa(HA)-T3. E. coli

15 Top10/pJRS1326 cells were grown to OD_{600nm} of 1.2, and the cell pellet resuspended in 1/50 volume of RIPA buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris; pH 8.0). Cells were disrupted by sonication, and insoluble cell debris was removed by centrifugation at 12,000 x g for 15 min at 4°C. Immunoprecipitation of crude cell extracts was performed
20 using "EZview Red Anti-HA Affinity Gel" (Sigma) according to the manufacturer's protocols. The immunoprecipitated protein was subjected to SDS-PAGE on a 4 to 12% gradient gel (NuPAGE, Invitrogen) followed by visualization with SYPRORuby (Invitrogen) according to the manufacturer's instructions. The band migrating with an apparent molecular weight of 105 kDa, corresponding to the Cpa(HA)-T3 heterodimer
25 was excised, and stored at 4°C until analysis by mass spectrometry.

Peptide preparation and mass spectrometry

The excised protein band was subjected to trypsin digestion and mass spectrometric analysis (MALDI-TOF-MS/MS analysis) (Medzihradszky, K. F. *et al.*
30 (2000) *Anal. Chem.* 72:552-8; Venkataraman, N. *et al.* (2005) *J. Immunol.* 175:7560-7) at the microchemical and proteomics facility at Emory University as described previously (Freeman, W. M. *et al.* (2005) *Pharmacogenomics J.* 5:203-14; Tseung, C. W. *et al.* *Biochem J.* 380:211-8). GPS Explorer 2.0 software (Applied Biosystems) and a MASCOT

(www.matrixscience.com/) search engine were used for identification of peptide fragments. The National Center for Biotechnology Information nonredundant database was used for the searches.

5 *Electron microscopy*

For immunogold-EM, *S. pyogenes* strains JRS4/pJRS9545 (vector control) or JRS4/pJRS9550 (expressing T3 pili with Cpa(HA)) were grown as described above, harvested, washed with PBS, and then adsorbed to polyvinyl formal-carbon-coated grids (E.F. Fullam, Inc.) for 2 minutes and fixed with 1% glutaraldehyde for 1 minute. For 10 single labeling experiments, the grids were washed twice with PBS, blocked with PBS + 1% BSA, and then incubated for 1 hour with a 1:200 dilution (in PBS + 1% BSA) of the rabbit polyclonal anti-T3 antiserum described above. The grids were washed three times with PBS and then incubated for 1 hour with a 1:50 dilution (in PBS + 1% BSA) of anti-rabbit IgG antibody conjugated to 12-nm diameter colloidal gold particles (Sigma-15 Aldrich). The grids were washed three times with PBS and twice with water, and then negatively stained with 0.5% phosphotungstic acid (Ted Pella, Inc.) for 35 seconds. For dual labeling experiments, grids prepared as described above were then incubated for 1 hour with 1:50 dilutions of both the anti-T3 antiserum and the anti-HA mouse monoclonal antibody described above. The grids were washed three times with PBS and then 20 incubated for 1 hour with 1:50 dilutions of both the 12-nm gold anti-rabbit IgG antiserum and an anti-mouse IgG antibody conjugated to 18 nm diameter colloidal gold particles (Sigma-Aldrich). The grids were then washed and stained as described above. The grids containing the negatively stained bacteria were examined on an FEI TECNAI 12 BioTwin G02 microscope (FEI) at 80 kV accelerating voltage. Digital images were acquired with 25 an AMT XR-60 CCD digital camera system (Advanced Microscopy Techniques).

Table 2 presents information on the primers that were used in these studies.

Table 2. Primers used in these experiments[A].

Primer	SEQ ID NO:	Sequence (5'-3')	Plasmids[B]
Orf100K43A_F	16	CCGAAAATGCAAAATTAATAGTAAAAgctACATTTGACTCTTATACAGAC	pEU7646→pEU7652, pEU7657→pEU7678
Orf100K43A_R	17	GTCTGTATAAGAGTCAAATGTagcTTTTACTATTAATTTTGCATTTTCGG	" " " "
Orf100K81A-F	18	CGAAAGACGGTTTAGAGATTGCTCCAGGTATTGTTAATGGTTTAACAG	pEU7646→pEU7651, pEU7657→pEU7679
Orf100K81A-R	19	CTGTTAAACCATTAACAATACCTGGAGCAATCTCTAAACCGTCTTTCG	" " " "
Orf100K100A_F	20	CAGCTATACTAATACTGATgcaCAGATAGTAAAGTTAAAAGTACAGAG	pEU7646→pEU7653, pEU7657→pEU7679
Orf100K100A_R	21	CTCTGTACTTTTAACTTTACTATCTGGtgcATCAGTATTAGTATAGCTG	" " " "
Orf100K106A_F	22	CTGATAAACCAGATAGTAAAGTTgcaAGTACAGAGTTTGATTTTTCAAAAG	pEU7646→pEU7654, pEU7657→pEU7681
Orf100K106A_R	23	CTTTTGAAAAATCAAACTCTGTACTtgcAACTTTACTATCTGGTTTATCAG	" " " "
Orf100K173A_F	24	CTAAGGAACAAGGAACAGACGTCgcAAAACCAGTTAATTTTAACAAC	pEU7646→pEU7687, pEU7657→pEU7907
Orf100K173A_R	25	GTTGTTAAAATTAACTGGTTTTcgGACGTCTGTTCCTTGTTCCTTAG	" " " "
Orf100K173R_F	26	CTAAGGAACAAGGAACAGACGTCcgAACCAGTTAATTTTAACAAC	pEU7657→pEU7692

Orf100K173R_R	27	GTTGTTAAAATTAAC TGGTTTTcgGACGTCT GTTCCTTGTTCCTTA G	" "
Orf100K174A_F	28	CTAAGGAACAAGGA ACAGACGTCAAAgcA CCAGTTAATTTTAAC AAC	pEU7646→pEU7688, pEU7657→pEU7908
Orf100K174A_R	29	GTTGTTAAAATTAAC TGGTgcTTTGACGTCT GTTCCTTGTTCCTTA G	" " " "
Orf100K191A_F	30	GCAACTACTTCGTTA AAAGTTAAGgcaAAT GTATCGGGGAATACT GG	pEU7646→pEU7661, pEU7657→pEU7682, pEU7678→pEU7909
Orf100K191A_R	31	CCAGTATTCCCCGAT ACATTtgcCTTAACTTT TAACGAAGTAGTTGC	“ “ “ “ “ “
Cpa_LPSTG_Sense	32	GAAAACCGAAAAGA TCTTcTCCCAtCAACT GGTTTGACAACAGAT GG	pEU7646→pEU7904
Cpa_LPSTG_Anti	33	CCATCTGTTGTCAAA CCAGTTGaTGGGAgA AGATCTTTTCGGTTT TC	" "
T3_LPSTG_Sense	34	GTCACAAATAAGCGT GACACTCtACCttCAA CTGGTGTTGTAGGCA CCCTTGCTCC	pEU7646→pEU7905
T3_LPSTG_Anti	35	GGAGCAAGGGTGCC TACAACACCAGTTGa AggTaGAGTGTCACGC TTATTTGTGAC	" "
Cpa_VP1_Sense	45	GAAAACCGAAAAGA TCTTGTCCCATTGAC AACAGATGG	pJRS9550→pJRS9597
Cpa_VP1_Anti	46	CCATCTGTTGTCAAT GGGACAAGATCTTTT CGGTTTTC	" "

[A] Uppercase letters represent bases complementary to GAS sequence. Lowercase letters represent bases added or changed to facilitate cloning or mutagenesis.
[B] Templates used in PCR with according primer pairs, and the resulting plasmids.

5

Example 2. Expression of a polypeptide fused to Cpa in *E. coli*

Constructs containing a polynucleotide encoding a fusion protein comprising the maltose binding protein (encoded by the *malE* gene) and amino acid residues 594-744 of Cpa (SEQ ID NO: 6) were transformed into *E. coli* strain XL10. The fusion protein
5 further comprised an amino-terminal Sec-dependent signal sequence. Constructs used in this study were pJRS9555 (comprises the FCT-3 region from M3 GAS strain AM3 from the MBP/Cpa gene through SrtC2) or pJRS9556 (comprises the same FCT-3 region from M3 GAS strain AM3 from the MBP/Cpa through T3), which are derivatives of the pJRS1326 construct (see Figure 1). Cell lysates and cell wall extracts were prepared as
10 described in Zähner and Scott (2008) *J Bacteriol* 190:527-535. The extracts were treated with hot SDS to dissociate molecules that are not covalently linked. Immunoblot analysis with an anti-MBP and anti-T3 antibody was performed as described in Experimental Example 1 and results are shown in Figure 12. Lanes 3, 6, and 7 have the genes needed to link the MBP/Cpa fusion protein to the growing T3 polymer (MBP/Cpa, T3, SipA, and
15 SrtC2). In lanes 3, 6, and 7 MBP/Cpa is covalently linked to T3. Lanes 8, 9, and 10 are negative controls lacking the gene encoding the enzyme that catalyzes this process (SrtC2). Lanes 3, 6, and 7 were confirmed by PCR analysis to have the desired insert; lanes 2, 4, and 5 lack the insert and lanes 8-10 lack srtC2. Therefore, the MBP/Cpa fusion protein can be added to the T3 polymer in *E. coli*.

20

Example 3. Expression of a polypeptide fused to Cpa in *Lactococcus lactis*

A plasmid, referred to herein as pJRS9565, was constructed which comprises the FCT-3 region from the M3 GAS strain AM3 including the Cpa gene, SipA2, T3, and SrtC2 (see Figure 13), wherein the gene encoding maltose binding protein was inserted
25 within the Cpa gene. The plasmid thus encodes a MBP/Cpa fusion protein which is referred to herein as MBP*. The nucleotide and amino acid sequences of the MBP/Cpa fusion are set forth in SEQ ID NOs: 98 and 99, respectively. The nucleotide sequence comprises 30 nucleotides in the 5' untranslated region of Cpa, followed by the first 56 codons of the coding sequence, the MBP coding sequence, and then the nucleotide
30 sequence encoding amino acid residues 594-744 of Cpa. This construct comprises the coding sequence for the Sec-dependent signal peptide sequence of Cpa (the amino acid sequence is set forth in SEQ ID NO: 100). Once the expressed protein is translocated, the

Sec-dependent signal peptide is cleaved, resulting in a MBP/Cpa fusion protein comprising the sequence set forth in amino acids SEQ ID NO: 102. A control plasmid pJRS9566, which lacks SrtC2, was also constructed and used in the following studies.

Lactococcus lactis strain MG1363 was transformed with the pJRS9565 or the control pJRS9566 plasmid and the exposure of the MBP* antigen and T3 on the surface of intact MG1363/pJRS9565 was examined by whole cell dot blot with a monoclonal anti-MBP antibody and polyclonal anti-T3 antiserum. The MBP* antigen and T3 are both surface exposed in MG1363/pJRS9565 as demonstrated by reaction with the anti-MBP antibody and anti-T3 antiserum (Figures 14A and 14B, lanes 1-4, rows E, F (+)). As expected, MG1363/pJRS9566, which lacks SrtC2, does not react with either of these antibodies (Figures 14A and 14B lanes 1-4, row G (-SrtC)).

To examine whether the MBP* antigen is incorporated into HMW polymers characteristic of pili in Gram-positive bacteria (Scott and Zähner (2006) *Mol Microbiol* 62:320-330; Telford *et al.* (2006) *Nat Rev Microbiol* 4:509-519; Mandlik *et al.* (2008) *Trends Microbiol* 16:33-40), cell wall fractions of strains MG1363/pJRS9565 (MBP*), MG1363/pJRS9566 (-SrtC2), and MG1363/pJRS9545 (vector control) were prepared and analyzed by western blot with anti-MBP and anti-T3. The MBP*-T3 heterodimer (80kDa) and the high molecular mass ladder characteristic of pili are seen in cell wall extracts of MG1363/pJRS9565 analyzed with anti-MBP and anti-T3, indicating that MBP* is incorporated into the pilus structure and that T3 pilus polymerization occurs normally (Figures 15A and 15B, lanes 1 and 2). As expected, analysis of the cell wall fraction from MG1363/pJRS9566 (SrtC2-) with anti-MBP and anti-T3 shows only the monomeric forms of MBP* and T3, with apparent molecular masses of 57 kDa and 32 kDa respectively (Figures 15A and 15B, lane3). No cross reactivity with either the anti-MBP antibody or the anti-T3 antiserum is observed with the cell wall fraction of MG1363/pJRS9545.

Negative-stain transmission electron microscopy of whole bacteria coupled with immunogold localization (performed using similar methods as those described in Experimental Example 1) reveals that abundant surface fibers are expressed by MG1363/pJRS9565. Analysis with polyclonal anti-T3 antiserum followed by detection with a secondary anti-rabbit gold-conjugate antibody indicates that these fibers are composed of the T3 protein (Figures 16A and 16B). As expected, MG1363 containing the vector control does not express pili or react with the T3 antibody (Figure 16C).

To determine whether MBP* is synthesized in MG1363 in an active form, lysates of MG1363/pJRS9565 and MG1363/pJRS9566 were applied to amylose resin, and the

eluate, flow through and crude lysate fractions were analyzed by western blot with the anti-MBP antibody and the anti-T3 antiserum for the presence of HMW pilus polymers. Lysates of MG1363/pJRS9545 treated in the same fashion were used as a negative control. HMW pilus forms are detected by both the anti-MBP antibody and the anti-T3 antiserum
5 in the eluate fraction of MG1363/pJRS9565, indicating that MBP remains active and confers the ability to bind amylose resin to hybrid pili (Figures 17A and 17B, lane 2). As expected, only the monomeric form of the MBP* is seen in the eluate fraction of MG1363/pJRS9566 when analyzed in this fashion (Figures 17A and 17B, lane 3). No cross reactivity with either the anti-MBP antibody or the anti-T3 antiserum is observed
10 with extracts of MG1363/pJRS9545.

To examine the possibility that the binding of MBP* pili to the amylose resin is nonspecific in nature, lysates of MG1363/pJRS9550, which produces wild type (wt) T3 pili, were purified using the amylose resin and analyzed with anti-MPB and anti-T3 as described above. Duplicate samples corresponding to the elution (E) fraction of
15 MG1363/pJRS9565 and the elution (E), flow through (F) and crude lysate fractions of MG1363/pJRS9550 were transferred to nitrocellulose. The membrane was cut down the middle (slide 10 lane 5) and half was analyzed with monoclonal anti-MBP antibody (Figure 18, lanes 1-4) while the other half was analyzed with monoclonal anti-HA antibody (Figure 18, lanes 6-9). As expected, the pili produced by MG1363/pJRS9565
20 (MBP*) bind to the amylose resin and can be eluted with maltose as HMW forms that react with anti-MBP (Figure 18, lane 1). In contrast, the pili of MG1363/pJRS9550 do not bind to the amylose beads, and are detected in the flow through and crude lysate fractions using the anti-HA antibody (Figure 18, lanes 8, 9). These data demonstrate that the binding of hybrid pili to the amylose resin is not a result of interactions between the wild
25 type pilus subunits and the amylose beads, but is rather due to the ability to bind amylose conferred upon hybrid pili by MBP*.

<u>*Materials and Methods for Experimental Example 3*</u>

Strains, plasmids and growth conditions

30 *Lactococcus lactis* MG1363 was cultured without shaking at 30°C in M17 media (OXOID) supplemented with 0.5% glucose (GM17). MG1363 was made competent by the method of Holo and Nes (Holo and Nes (1989) *Appl Environ Microbiol* 55:3119-3123). Spectinomycin was used at a concentration of 100 μg/mL.

Cell wall extraction

Cell wall fractions of MG1363 were obtained using a modification of the procedure of Buccato *et al.* (2006) *J Infect Dis* 194:331-340, as follows. Overnight cultures of MG1363 were centrifuged at 4000 rpm for 10 minutes at 4°C in an Eppendorf 5810R tabletop centrifuge with an A-4-62 swinging bucket rotor. The pellet was resuspended in 1/10 volume of saline (0.9% m/v NaCl), transferred to a 1.5 mL Eppendorf tube, and centrifuged at 13000 rpm for 1 minute at 4°C in a Spectrafuge 16M microcentrifuge. The pellet was resuspended in the same volume of saline solution, and the optical density at 600 nm (OD_{600}) was determined at a dilution of 1:100. The concentration of cells in cell units/mL [CU/mL] was calculated as previously described (Biswas *et al.* (2001) *Infect Immun* 69:7029-7038). Four CU was transferred to a new 1.5 mL tube, and centrifuged as above. Cell wall extraction was performed in 160 μL of lysis buffer (50 mM Tris-HCl 6.8, 30% raffinose, Roche Complete protease inhibitors, 4 mg/mL lysozyme, 400 U/mL mutanolysin) at 37°C for 3 hours with gentle rotation. Samples were centrifuged at 13000 rpm for 1 minute at room temperature, and the supernatant was transferred to a new tube, and recentrifuged at 13000 rpm for 4 minutes at room temperature. Then, 75 μL of the second supernatant was combined with 25 μL of 4X SDS sample buffer (Sambrook *et al.*, 1989) in a new tube and samples were heated to 100°C for 10 min.

Dot blot

Dot blot was performed by a slight modification of the procedure of Biswas *et al.* (2001) *Infect Immun* 69:7029-7038. Briefly 5 μL of an overnight culture of MG1363 that had been washed in saline solution as described above, was spotted onto a nitrocellulose membrane (Bio-Rad) and dried for 3 hours at room temperature. Membranes were blocked at room temperature in blocking solution (3% BSA in TBS 7.6, 0.02% NaN_3) for 30 minutes with gentle orbital rotation, followed by analysis with the appropriate antibody.

Amylose purification of hybrid pili

Overnight cultures of MG1363 were centrifuged at 4000 rpm at 4°C for 10 minutes, and the pellets were resuspended in 1/10 volume of saline solution at 4°C. The OD_{600} was used to calculate the number of cell units/mL [CU/mL] as previously described (Biswas *et al.* (2001) *Infect Immun* 69:7029-7038). Ten CU were transferred to a sterile 1.5 mL Eppendorf tube and centrifuged at 13000 rpm for 1 minute at 4°C. Samples were incubated in lysis buffer (50 mM Tris-HCl 6.8, Roche Complete protease inhibitors, 4 mg/mL lysozyme, 400 U/mL mutanolysin) for 30 min at 37°C. Samples were then incubated at 4°C for 10 minutes followed by sonication at 4°C for 2 X 15 seconds, with 15 second pauses between sonications. Reactions were centrifuged at 13,000 rpm for 5 minutes at 4°C, and the supernatant was transferred to a new tube and recentrifuged at 4°C for 10 min at 13000 rpm. A sample of this supernatant, corresponding to crude lysate, was saved for later analysis. The remainder was transferred to a 200 µL slurry volume of amylose resin (New England Biolabs), which had been washed and pre-equilibrated with column wash buffer (20 mM Tris-HCl 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). Samples were batch purified by incubation at 4°C for 30 minutes with gentle tapping every 5 minutes. Reactions were then centrifuged at 6000 rpm for 1 min at 4°C and a sample corresponding to the flow-through fraction was stored for later analysis. The resin was washed with 3 X 1 mL of column wash buffer at 4°C with a 1 minute centrifugation at 6000 rpm between washes. Bound protein was eluted in 350 µL column wash buffer containing 25 mM maltose. SDS sample buffer (Sambrook *et al.*, 1989) was added and samples were heated to 100°C for 10 minutes.

SDS PAGE and western blot

SDS PAGE was conducted using NuPAGE 4-12% gradient gels (Invitrogen) with MES running buffer as previously described (Zähner and Scott (2008) *J Bacteriol* 190:527-535). Proteins were transferred to nitrocellulose membranes (Bio-Rad) using a Bio-Rad mini Trans-Blot® system with transfer buffer (25 mM Tris 8.3, 192 mM glycine) at a constant voltage of 100V for 1 hour at 4°C. Blocking solution (3% BSA in TBS 7.6, 0.02% NaN_3) was used to block membranes and for incubation of primary and secondary antibodies. The polyclonal anti-T3 antiserum was used at a dilution of 1:250. The mouse monoclonal anti-MBP antibody, a product of New England Biolabs, and the mouse monoclonal anti-HA antibody (HA-7), a product of Sigma-Aldrich, were used at dilutions

of 1:2,000. Goat anti-mouse and goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Sigma-Aldrich) were used at a dilution of 1:3000. Signals were detected using a nitrotetrazolium blue (NBT), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (BCIP) detection system.

5

Example 4. Intranasal vaccination of mice with *Lactococcus lactis* expressing the maltose binding protein on the pilus tip

To determine if the *L. lactis* bacteria comprising the pJRS9565 plasmid could elicit an immune response to the displayed MBP protein in mice, CD1 mice were vaccinated
10 intranasally with the MG1363/pJRS9565 (encodes MBP*) or the MG1363/pJRS9545 (vector control) bacteria. Blood samples and lung lavage fluids were obtained from the mice. Anti-MBP IgG or IgA antibodies in the fluids were measured using an ELISA. As seen in Figures 19 and 20, mice that were vaccinated with the plasmid expressing the MBP-Cpa fusion protein had developed an immune response to the displayed MBP.
15 Therefore, polypeptides displayed on the tips of bacterial pili are effective at mounting an immune response in mice, demonstrating the utility of such methods for the development of vaccines to various antigens.

Materials and Methods for Experimental Example 4

20 *Mouse immunization*

Cells (MG1363/pJRS9545 or MG1363/pJRS9565) grown at 30° C in M17 with glucose containing 100 μg/ml spectinomycin, were washed and resuspended in PBS to give 5×10^7 cfu/ml. Female CD1 mice were vaccinated intranasally by administration of 20 μl of cell suspension (10^9 CFU). The mice were vaccinated every 10 days with a dose
25 of 10^9 CFU for three consecutive days, (i.e., the animals were vaccinated on days 1, 2, 3, 14, 15, 16, and on days 27, 28, and 29). Blood samples were collected every 10 days (on days 1, 14, 27, and 39). The mice were sacrificed on the 39th day, and lung lavage fluids were obtained post mortem by inserting a nylon cannula into the exposed trachea, which was tied in place. A 1.0 ml syringe was used to inject and withdraw 1ml of 0.9% sodium
30 chloride solution three times, the supernatants were then stored at -80°C.

ELISA detection of antigen-specific antibodies in serum and lung lavage

A 96-well EIA/RIA microplate (Costar, Corning Inc.) was coated overnight at 4°C with 100 ng of MBP per well. The coated plate was blocked with 5% soy milk in PBS-Tween to prevent nonspecific binding. Sera (1:50 dilution) or lung fluid was reacted with the coated wells Antibody production was detected by using anti-mouse IgG or anti-mouse IgA secondary antibodies coupled to alkaline phosphatase (Sigma). Absorbance was measured at 405 nm after 45 min following the addition of *p*-nitrophenyl phosphate hexahydrate disodium salt (pNPP) tablets dissolved in diethanolamine buffer solution (KPL). The values were corrected for background by subtracting the reading obtained with sera or lung fluid of non- immunized mice.

Example 5. Development of a live *Lactococcus lactis* vaccine

As model epitopes, two different domains of the protective antigen subunit of the anthrax toxin are used to provide protection against *Bacillus anthracis*. Domain 1' (residues 168-258 of SEQ ID NO: 92), which is the domain that remains at the N-terminus of the toxin following its proteolytic cleavage by proteases ubiquitously present in host tissue is used. This domain, called "LEF domain", is involved in binding to the other subunits of the anthrax toxin, LF (lethal factor), and EF (edematous factor). The second domain used is domain 4 (residues 596-735 of SEQ ID NO: 92), called "RBD", which is responsible for binding of the toxin to host cell receptors. The RBD and LEF domains are antigenic as DNA vaccines, have been inserted into the influenza virus fused within the hemagglutinin protein, and have been shown to provide passive protection against the toxin (Li *et al* (2005) *J Virol* 79:10003-10012).

Two other model antigens are used that are likely to be protective against enterotoxigenic *Escherichia coli* (ETEC): a mutant nontoxic form of the heat labile toxin LT, and CooD, an ETEC adhesin.

The following two antigens are used separately to generate vaccines: 1) a triple LT A mutant (whose nucleotide and amino acid sequences are set forth in SEQ ID NOs: 95 and 96, respectively) is constructed (R7K, S63K, V53E) in which three residues required for toxin activity have been changed in ways that don't alter the protein structure (Pizza *et al* (1994) *J Exp Med* 180:2147-2153); and 2) *cooD* from a CS1 ETEC strain (whose nucleotide and amino acid sequences are set forth in SEQ ID NOs: 89 and 90, respectively), which will be cloned into the plasmid together with its chaperone gene *cooB* (Voegele, Sakellaris & Scott (1997) *Proc Natl Acad Sci USA* 94:13257-13261). Using

standard recombinant DNA technology, a fusion protein is engineered that has the entire antigenic protein (either LT or CooD) fused to the C terminus of the pilus tip protein (Cpa) in a plasmid that contains all the genes needed to make T3 pili from *S. pyogenes*. Following DNA sequence confirmation of each plasmid construction, western blots are used to show that, in *Escherichia coli*, the guest protein is polymerized with T3. Each plasmid is transformed into *L. lactis* and a western blot is used to identify the presence of the guest antigen in polymerized pili. Whole cell dot immunoblots is used to confirm surface localization in *L. lactis* of the guest antigen.

Each of the two *L. lactis* strains are introduced intraperitoneally and intranasally into mice. Serum is collected and tested in the ELISA assay for IgG and IgA reactivity with LT or CooD. Mice are sacrificed and IgA assayed in nasal lavage, bronchio-alveolar lavage, and gut lavage fluid.

Upon detection of antibody, the ability of the anti-LT to neutralize toxicity of whole LT-producing ETEC bacteria or anti-CooD to prevent adherence of CS1 ETEC bacteria is determined.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended embodiments.

THAT WHICH IS CLAIMED:

1. A method for producing a Gram-positive bacterium having at least one polypeptide of interest covalently attached to the tip of at least one pilus, wherein said method comprises:

a) introducing into said Gram-positive bacterium a polynucleotide comprising a nucleotide sequence that encodes a chimeric polypeptide, said chimeric polypeptide comprising said polypeptide of interest and a *Streptococcus pyogenes* pilus tip protein, active variant or active fragment thereof, wherein said pilus tip protein or active variant or fragment thereof comprises a cell wall sorting signal (CWSS), and said pilus tip protein or fragment thereof is carboxyl to said polypeptide of interest, and wherein said Gram-positive bacterium expresses a tip sortase and a pilus shaft polypeptide; and

b) growing said Gram-positive bacterium under conditions wherein said chimeric polypeptide is expressed and said pilus is formed.

2. The method of claim 1, wherein said *Streptococcus pyogenes* pilus tip protein comprises a polypeptide encoded by the first gene in the FCT region of the chromosome of a *Streptococcus pyogenes* bacterium.

3. The method of claim 1, wherein said *Streptococcus pyogenes* pilus tip protein comprises an adhesin protein.

4. The method of claim 1, wherein said *Streptococcus pyogenes* pilus tip protein is selected from the group consisting of Cpa, protein F1, Spy0130, FctX, and FctB.

5. The method of claim 1, wherein said active variant of said *Streptococcus pyogenes* pilus tip protein has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 3.

6. The method of claim 1, wherein said *Streptococcus pyogenes* pilus tip protein has the amino acid sequence set forth in SEQ ID NO: 3.

7. The method of claim 1, wherein said active fragment of said *Streptococcus pyogenes* pilus tip protein has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 6.

5 8. The method of claim 7, wherein said active fragment of said *Streptococcus pyogenes* pilus tip protein has the amino acid sequence set forth in SEQ ID NO: 6.

9. The method of claim 1, wherein said active fragment of said *Streptococcus pyogenes* pilus tip protein comprises at least one amino acid preceding said CWSS.

10

10. The method of claim 1, wherein said CWSS comprises a non-canonical CWSS motif.

11. The method of claim 1, wherein said CWSS comprises:

15 a) a CWSS motif, wherein said CWSS motif has an amino acid sequence of X_1X_2PTG, wherein X_1 and X_2 is any amino acid;

b) a substantially hydrophobic domain carboxyl to said CWSS motif; and

c) a charged tail region carboxyl to said substantially hydrophobic 20 domain.

12. The method of claim 11, wherein said X_1 is glutamine or valine.

13. The method of claim 11 or claim 12, wherein said X_2 is valine or proline.

25

14. The method of any one of claims 11-13, wherein said substantially hydrophobic domain comprises at least 25 amino acid residues.

15. The method of any one of claims 11-14, wherein said charged tail region 30 comprises at least 6 amino acid residues.

16. The method of claim 1, wherein said polynucleotide further comprises a promoter that is active in said Gram-positive bacterium, wherein said promoter is operably linked to said polynucleotide.

17. The method of claim 1, wherein said tip sortase comprises a *S. pyogenes* sortase C enzyme.

5 18. The method of claim 1, wherein said tip sortase has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 7.

19. The method of claim 18, wherein said tip sortase has the amino acid sequence set forth in SEQ ID NO: 7.

10

20. The method of claim 1, wherein said tip sortase, said pilus shaft polypeptide, or both polypeptides are heterologous to said Gram-positive bacterium.

21. The method of claim 1 or 16, wherein said polynucleotide further
15 comprises at least one of a nucleotide sequence encoding said tip polymerase and a nucleotide sequence encoding said pilus shaft polypeptide.

22. The method of claim 1, wherein said pilus shaft polypeptide comprises a *Streptococcus pyogenes* pilus shaft polypeptide.

20

23. The method of claim 1, wherein said pilus shaft polypeptide has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 12.

24. The method of claim 23, wherein said pilus shaft polypeptide has the amino
25 acid sequence set forth in SEQ ID NO: 12.

25. The method of claim 1, wherein said Gram-positive bacterium further expresses a pilin chaperone polypeptide.

30 26. The method of claim 25, wherein said pilin chaperone polypeptide is heterologous to said Gram-positive bacterium.

27. The method of claim 1, 16 or 21, wherein said polynucleotide further comprises a nucleotide sequence encoding said pilin chaperone polypeptide.

28. The method of claim 25, wherein said pilin chaperone polypeptide comprises a *Streptococcus pyogenes* SipA polypeptide.

5 29. The method of claim 25, wherein said pilin chaperone polypeptide has an amino sequence having at least 80% sequence identity to SEQ ID NO: 14.

30. The method of claim 29, wherein said pilin chaperone polypeptide has the amino acid sequence set forth in SEQ ID NO: 14.

10

31. The method of claim 1, wherein said nucleotide sequence encoding said chimeric polypeptide further comprises a nucleotide sequence encoding an amino terminal signal sequence.

15 32. The method of claim 1, wherein said Gram-positive bacterium belongs to a genus selected from the group consisting of *Actinomyces*, *Bacillus*, *Bifidobacterium*, *Cellulomonas*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Mycobactenum*, *Nocardia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*.

20

33. The method of claim 32, wherein said Gram-positive bacterium comprises *Lactococcus lactis*.

34. The method of claim 1, wherein said polypeptide of interest is heterologous
25 to said Gram-positive bacterium.

35. The method of claim 1, wherein said polypeptide of interest is selected from the group consisting of an antigen, an enzyme, a biosorbent, or an antibody or fragment thereof.

30

36. A Gram-positive bacterium produced by any one of the methods of claims 1-35.

37. A Gram-positive bacterium comprising at least one polypeptide of interest covalently attached to the tip of at least one pilus, wherein said polypeptide of interest is amino terminal to a *Streptococcus pyogenes* pilus tip protein or an active variant or fragment thereof, wherein said pilus tip protein or active variant or active fragment thereof
5 comprises a cleaved cell wall sorting signal (CWSS) motif.

38. The Gram-positive bacterium of claim 37, wherein said Gram-positive bacterium belongs to a genus selected from the group consisting of *Actinomyces*, *Bacillus*, *Bifidobacterium*, *Cellulomonas*, *Clostridium*, *Corynebacterium*, *Enterococcus*,
10 *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Mycobactenum*, *Nocardia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*.

39. The Gram-positive bacterium of claim 38, wherein said Gram-positive bacterium comprises *Lactococcus lactis*.

15

40. The Gram-positive bacterium of claim 37, wherein said *Streptococcus pyogenes* pilus tip protein comprises a polypeptide encoded by the first gene in the FCT region of the chromosome of a *Streptococcus pyogenes* bacterium.

20 41. The Gram-positive bacterium of claim 37, wherein said *Streptococcus pyogenes* pilus tip protein comprises an adhesin protein.

42. The Gram-positive bacterium of claim 37, wherein said *Streptococcus pyogenes* pilus tip protein is selected from the group consisting of Cpa, protein F1,
25 Spy0130, FctX, and FctB.

43. The Gram-positive bacterium of claim 37, wherein said active variant of said *Streptococcus pyogenes* pilus tip protein has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 103.

30

44. The Gram-positive bacterium of claim 37, wherein said *Streptococcus pyogenes* pilus tip protein has the amino acid sequence set forth in SEQ ID NO: 103

45. The Gram-positive bacterium of claim 37, wherein said active fragment of said *Streptococcus pyogenes* pilus tip protein has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 101.

5 46. The Gram-positive bacterium of claim 45, wherein said active fragment of said *Streptococcus pyogenes* pilus tip protein has the amino acid sequence set forth in SEQ ID NO: 101.

47. The Gram-positive bacterium of claim 37, wherein said active fragment of 10 said *Streptococcus pyogenes* pilus tip protein comprises at least one amino acid preceding said cleaved CWSS motif.

48. The Gram-positive bacterium of claim 37, wherein said cleaved CWSS motif had a non-canonical CWSS motif prior to being cleaved.

15

49. The Gram-positive bacterium of claim 37, wherein said cleaved CWSS motif has an amino acid sequence of X_1X_2PT, wherein said X_1 is any amino acid except leucine, and wherein X_2 is any amino acid except proline.

20 50. The Gram-positive bacterium of claim 49, wherein said X_1 is glutamine or valine.

51. The Gram-positive bacterium of claim 49 or claim 50, wherein said X_2 is valine or proline.

25

52. The Gram-positive bacterium of claim 37, wherein said pilus comprises a pilus shaft polypeptide, and wherein said pilus shaft polypeptide comprises a *S. pyogenes* pilus shaft polypeptide.

30 53. The method of claim 37, wherein said pilus comprises a pilus shaft polypeptide, wherein said pilus shaft polypeptide has an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 12.

54. The method of claim 53, wherein said pilus shaft polypeptide has the amino acid sequence set forth in SEQ ID NO: 12.

55. The Gram-positive bacterium of claim 37, wherein said polypeptide of interest is heterologous to said Gram-positive bacterium.

56. The Gram-positive bacterium of claim 37, wherein said polypeptide of interest is selected from the group consisting of an antigen, an enzyme, a biosorbent, or an antibody or fragment thereof.

57. A method for inducing an immunological response comprising administering to a subject a composition comprising a Gram-positive bacterium of any one of claims 36-56, wherein said polypeptide of interest comprises an antigen.

58. The method of claim 57, wherein said Gram-positive bacterium comprises an attenuated pathogenic bacterium or a non-pathogenic bacterium.

59. The method of claim 57, wherein said Gram-positive bacterium is selected from the group consisting of *Streptococcus gordinii*, *Lactococcus lactis*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*.

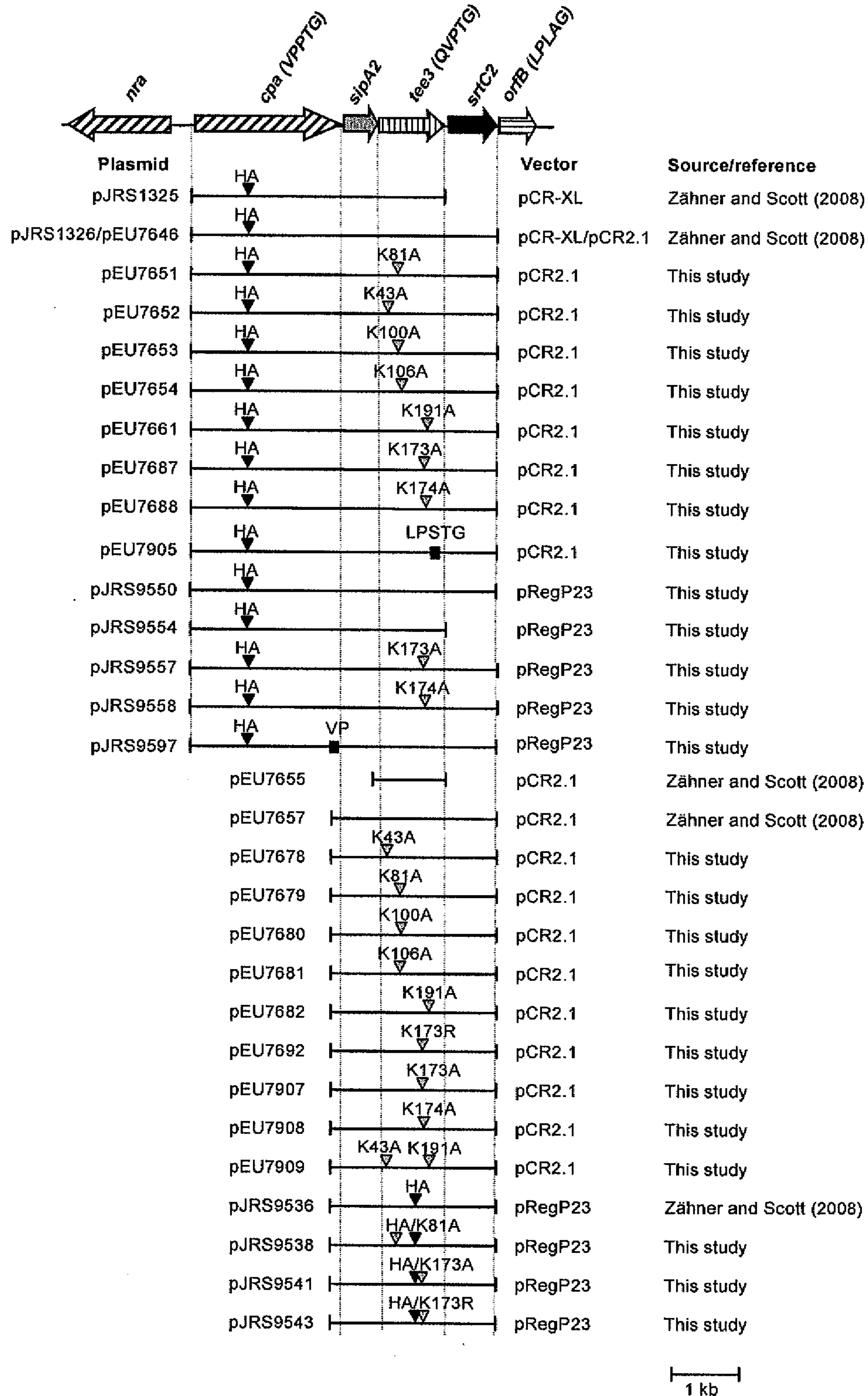

FIG. 1

Cpa(HA) - + +
T3 - + +
SipA - + +
SrtC2 - - +

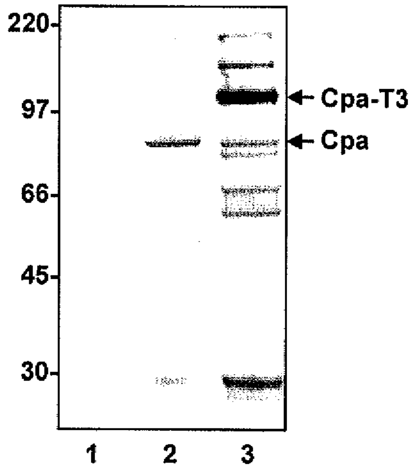

FIG. 2A

Cpa(HA) - + +
T3 - + +
SipA- + +
SrtC2 - - +

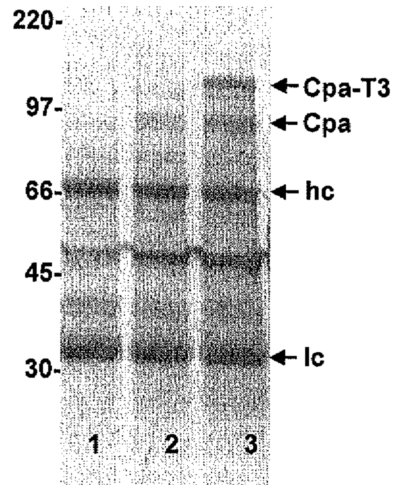

FIG. 2B

```
                          SP                                  K43
  1 MKKNKLLLATAILATALGTASLNQNVKAETAGVSENAKLIVKKTFDSYTD

 51 NEVLMPKADYTFKVEADSTASGKTKDGLEIKPGIVNGLTEQIISYTNTDK

101 PDSKVKSTEFDFSKVVFPGIGVYRYTVSEKQGDVEGITYDTKKWTVDVYV
                              K173                K191
151 GNKEGGGFEPKFIVSKEQGTDVKKPVNFNNSFATTSLKVKKNVSGNTGEL

201 QKEFDFTLTLNESTNFKKDQIVSLQKGNEKFEVKIGTPYKFKLKNGESIQ

251 LDKLPVGITYKVNEMEANKDGYKTTASLKEGDGQSKMYQLDMEQKTDESA

301 DEIVVTNKRDTQVPTGVVGTLAPFAVLSIVAIGGVIYITKRKKA
                          CWSS
```

FIG. 2C

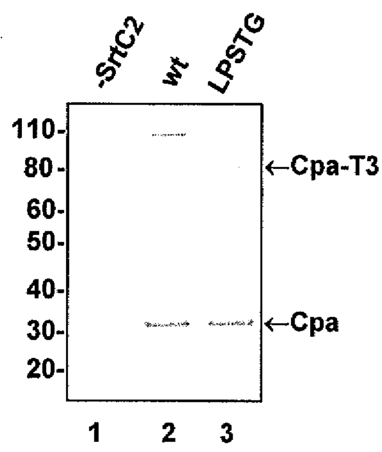

FIG. 3A

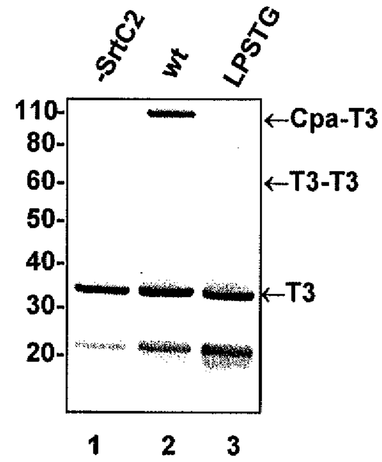

FIG. 3B

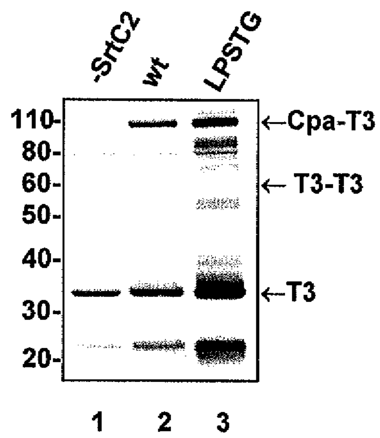

FIG. 3C

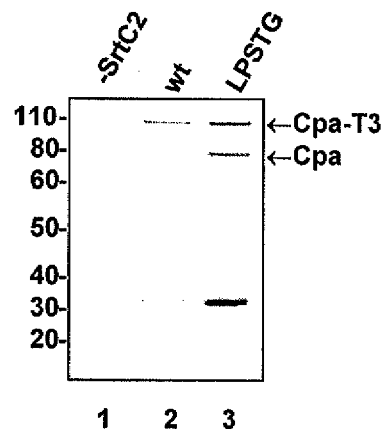

FIG. 3D

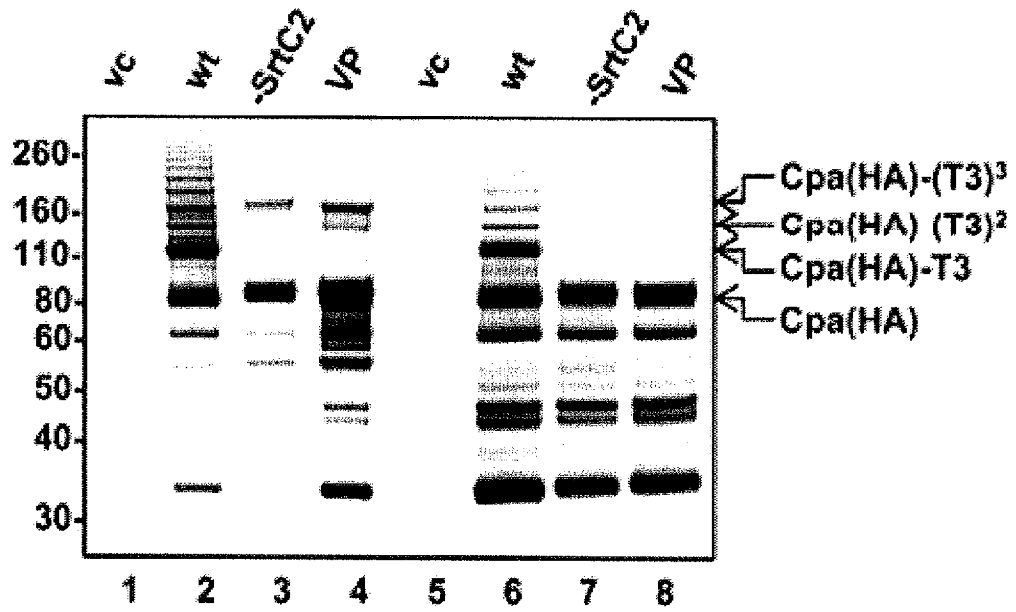

FIG. 4A

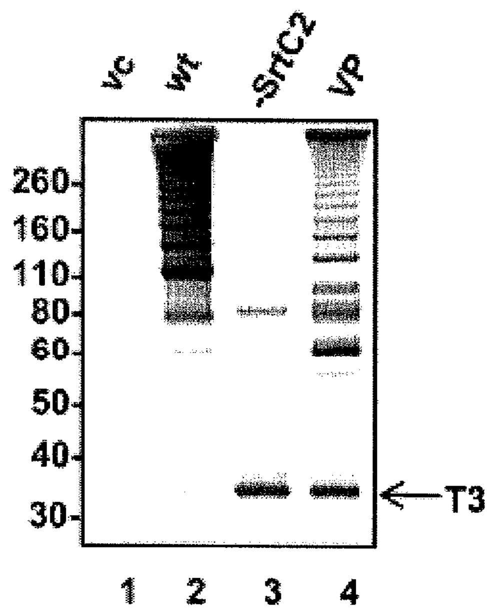

FIG. 4B

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                          K43                                          K81
M3_MGAS315      29 ETAGVSENAKLIVKKTFDSYTDNEVLMPKADYTFKVEADSTAS--GKTKDGLEIKPGIVN 86
M1_SF370        22 HGETVVNGAKLTVTKNLDLVNSN-ALIPNTDFTFKIEPDTTVN-----EDGNKFKG--VA 72
M28_MGAS6180    29 ETAGVSENAKLIVKKTFDSYTDNEVLMPKADYTFKVEADSSAT--DKTKDGLEIKPGVTE 86
emmstD33_D633   29 ETAGVTNGTQLTIKKTIANYNDSEVLMPKATFTFEVKPDNSVTGVEKTVDGLTIKAGIAE 88
M49_591         29 ETAGVIDGSTLVVKKTFPSYTDDNVLMPKADYSFKVEADDNAK--GKTKDGLDIKPGVID 86
M18_MGAS8232    29 ETAGVIDGSTLVVKKTFPSYTDDKVLMPKADYTFKVEADDNAK--GKTKDGLDIKPGVID 86
emm33_29487     29 ETAGVVTGKTLPITKSMI-YTDNEILMPKTTFTFTIEPDTTAS--GKTKDGLEIKSGETT 85
M5_Manfredo     29 ETAGVVTGKSLQVTKTMT-YDDEEVLMPETAFTFTIEPDMTAS--GK-EGSLDIKNGIVE 84
M12_A735        29 ETAGVVSSGQLTIKKSITNFNDDTLLMPKTDYTFSVNPDSAAT---GTESNLPIKPGIAV 85

                   K100 K106                    E129
M3_MGAS315      87 GLT-EQIISYTNTDKPDSKVKSTEFDFSKVVFPGIGVYRYTVSEK-QGDVEGITYDTKKW 144
M1_SF370        73 LNTPMTKVTYTNSDKGGSNTKTAEFDFSEVTFEKPGVYYYKVTEEKIDKVPGVSYDTTSY 133
M28_MGAS6180    87 GLTTEQTIAYDNSVKPSDKSKTATFDFSTVKFPEVGVYRYTVSEI-DSKVSGIKYDTKTW 145
emmstD33_D633   89 GLVKTGNVEYSNTDKVENKDKTTTFDFSTVKFPEVGVYRYTVSET-DSKVSGIKYDTKTW 147
M49_591         87 GLENTKTIRYSNSDKITAKEKSVNFEFANVKFPGVGVYRYTVAEV-NGNKAGITYDSQQW 145
M18_MGAS8232    87 GLENTKTIHYGNSDKTTAKEKSVNFDFANVKFPGVGVYRYTVSEV-NGNKAGIAYDSQQW 145
emm33_29487     86 GLTTKAIVSYDNTDKESAKNKTSNFNFETVTFSGIGIYRYTVSEQ-NDGIEGIQYDGKKW 144
M5_Manfredo     85 GLDKQVTVKYKNTDKTSQKTKIAQFDFSKVKFPAIGVYRYMVSEK-NDKKDGITYDDKKW 143
M12_A735        86 NNQ-DIKVSYSNTDKTSGKEKQVVVDFMKVTFPSVGIYRYVVTEN-KGTAEGVTYDDTKW 143

                                            K173   N180      K191
M3_MGAS315     145 TVDVYVGNKEGG--GFEPKFIVSKEQGTDVKKPVNFNNSFATTSLKVKKNVSGNTGELQK 202
M1_SF370       134 TVQVHVLWNEEQQ-KPVATYIVGYKEGS--KVPIQFKNSLDSTTLTVKKKVSGTGGDRSK 190
M28_MGAS6180   146 IVDVYVVNDGNG--GFKARYIVSKEKGQNDKKPVVFENSFKTTSLKVEKQVTGNTGELKK 203
emmstD33_D633  148 IVDVYVVNDGNG--GFKAQYIVSKEKGQNDKKPVVFENSFKTTSLKVEKQVTGNTGELKK 205
M49_591        146 TVDVYVVNKEGG--GFEVKYIVSTEVGQSEKKPVLFKNSFDTTSLKIEKQVTGNTGEHQR 203
M18_MGAS8232   146 TVDVYVVNREDG--GFEAKYIVSTEGGQSDKKPVLFKNFFDTTSLKVTKKVTGNTGEHQR 203
emm33_29487    145 TVDVYVGNKEGG--GFEPKYVVSKEVNSDVKKPIRFENSFKTTSLKIEKQVTGNTGELQK 202
M5_Manfredo    144 TVDVYVGNKANNEEGFEVLYIVSKEGTSSTKKPIEFTNSIKTTSLKIEKQITGNAGDRKK 203
M12_A735       144 LVDVYVGNNEKG--GLEPKYIVSKKGDSATKEPIQFNNSFETTSLKIEKEVTGNTGDHKK 201

M3_MGAS315     203 EFDFTLTLNESTNFKKDQIVSLQKGNE------KFEVKIGTPYKFKLKNGESIQLDKLPV 256
M1_SF370       191 DFNFGLTLKANQYYKASEKVMIEKTTKGGQAPVQTEASIDQLYHFTLKDGESIKVTNLPV 250
M28_MGAS6180   204 DFNFTLTINPNDNFVAGQVIKLEKGGI------KADVKIGEPYKFALKNGEKVTLSKLPV 257
emmstD33_D633  206 DFNFTLTINPNDNFVAGQVIKLEKGGI------KADVKIGEPYKFALKNGEKVTLSKLPV 259
M49_591        204 LFSFTLLLTPNECFEKGQVVNILQGGE------TKKVVIGEEYSFTLKDKESVTLSQLPV 257
M18_MGAS8232   204 SFSFTLLLTPNECFEKGQVVNILQGGE------TKKVVIGEEYSFTLKDKESVTLSQLPV 257
emm33_29487    203 DFNFTLILEASALYEKGQVVKIIQDGQ------TKDVVIGQEYKFTLHDHQSIMLAKLPI 256
M5_Manfredo    204 SFNFTLTLQPSEYYKTGSVVKIEQDGS------KKDVTIGTPYKFTLGHGKSVMLSKLPI 257
M12_A735       202 AFTFTLTLQPNEYYEASSVVKIEENGQ------TKDVKIGEAYKFTLNDSQSVILSKLPV 255

                       E264                                      N307
M3_MGAS315     257 GITYKVNEMEANKDGYKTTASLKE-GDGQSKMYQLDM-EQKTDESADEIVVTNKRDTQVP 314
M1_SF370       251 GVDYVVTEDDYKSEKYTTNVEVSPQDGAVKNIAGNSTEQETSTDKDMTITFTNKKDFEVP 310
M28_MGAS6180   258 GVTYSIIEDEADKDGYTTNAKITD-GTAAPVEYKLGN-QQLADESADEIVVTNNRDTQVP 315
emmstD33_D633  260 GITYSIIEDDAGKDGYKTTAILKD-GEQS-STYELGK-NQKTDESADEIVVTNKRDTQVP 316
M49_591        258 GIEYKLTEEDVTKDGYKTSATLKD-GEQS-STYELGK-DHKTDKSADEIVVTNKRDTQVP 314
M18_MGAS8232   258 GIEYKVTEEDVTKDGYKTSATLKD-GDVT-DGYNLGD-SKTTDKSTDEIVVTNKRDTQVP 314
emm33_29487    257 GISYKLTEDKA--DGYTTTATLKE-GEIDAKEYVLGN-LQKTDESADEIVVTNKRDTQVP 312
M5_Manfredo    258 GINYYLSEDEANKDGYTTTATLKEQGKEKSSDFTLSTQNQKTDESADEIVVTNKRDTQVP 317
M12_A735       256 GINYKVEEAEANQGGYTTTATLKD--GEKLSTYNLGQ-EHKTDKTADEIVVTNNRDTQVP 312

M3_MGAS315     315 TG 316
M1_SF370       311 TG 312
M28_MGAS6180   316 TG 317
emmstD33_D633  317 TG 318
M49_591        315 TG 316
M18_MGAS8232   315 TG 316
emm33_29487    312 TG 313
M5_Manfredo    318 TG 319
M12_A735       313 TG 314
```

FIG. 5

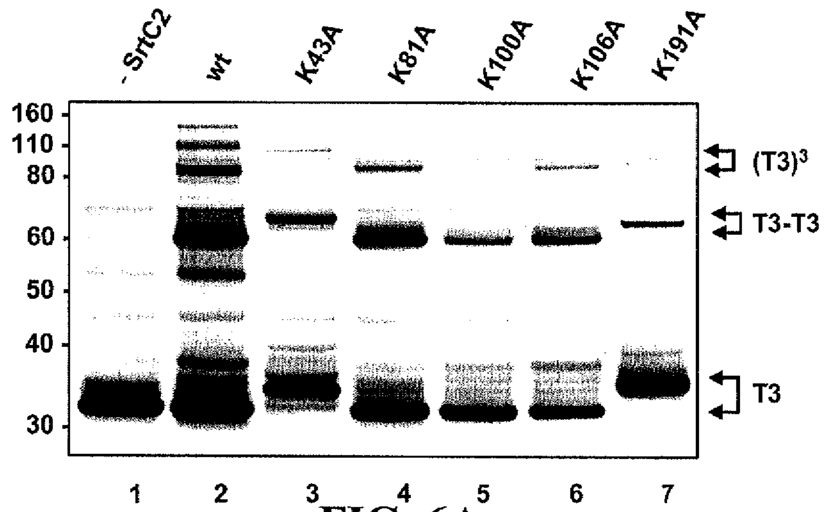


FIG. 6A

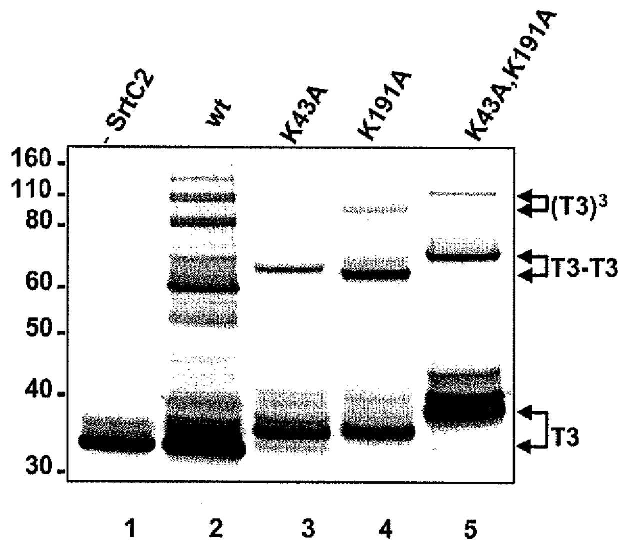


FIG. 6B

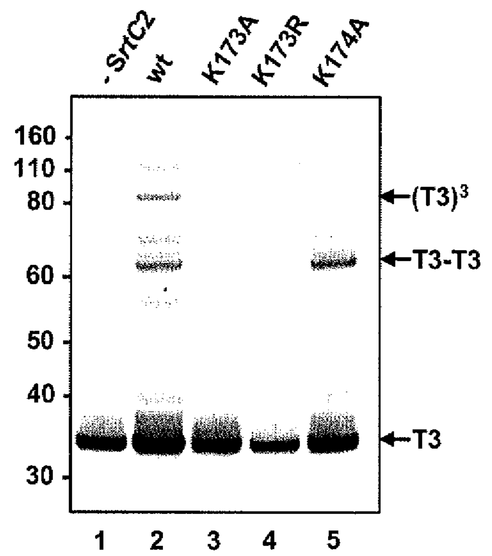


FIG. 6C

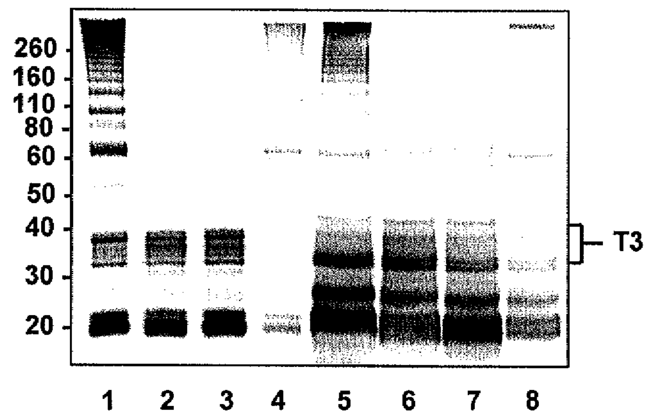


FIG. 7

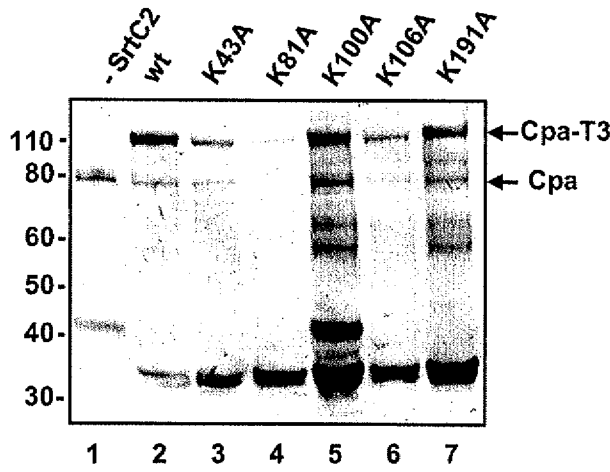


FIG. 8A

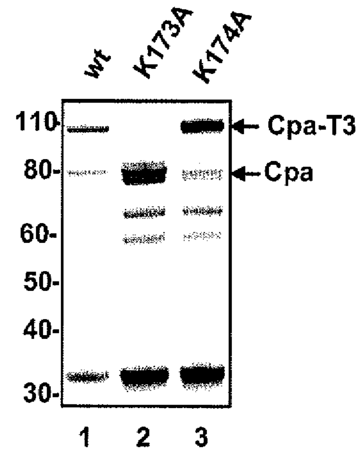


FIG. 8B

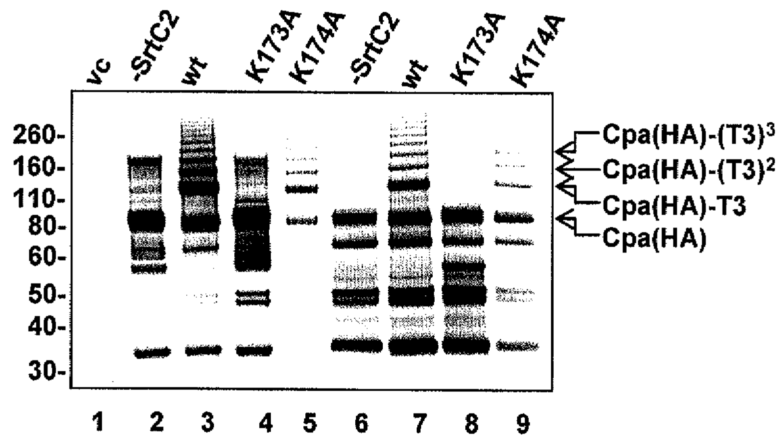


FIG. 9A

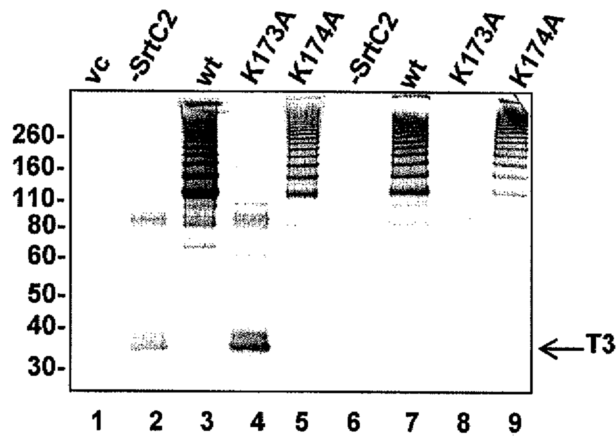


FIG. 9B

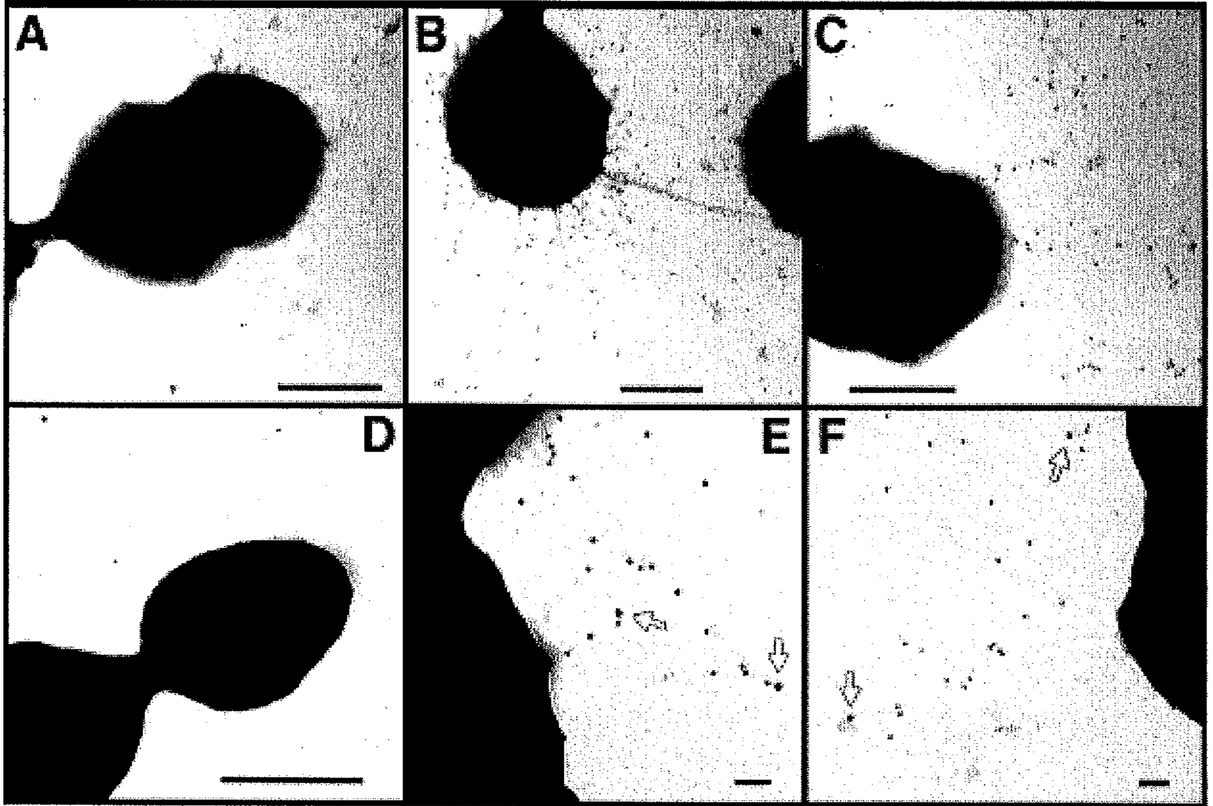


FIG. 10

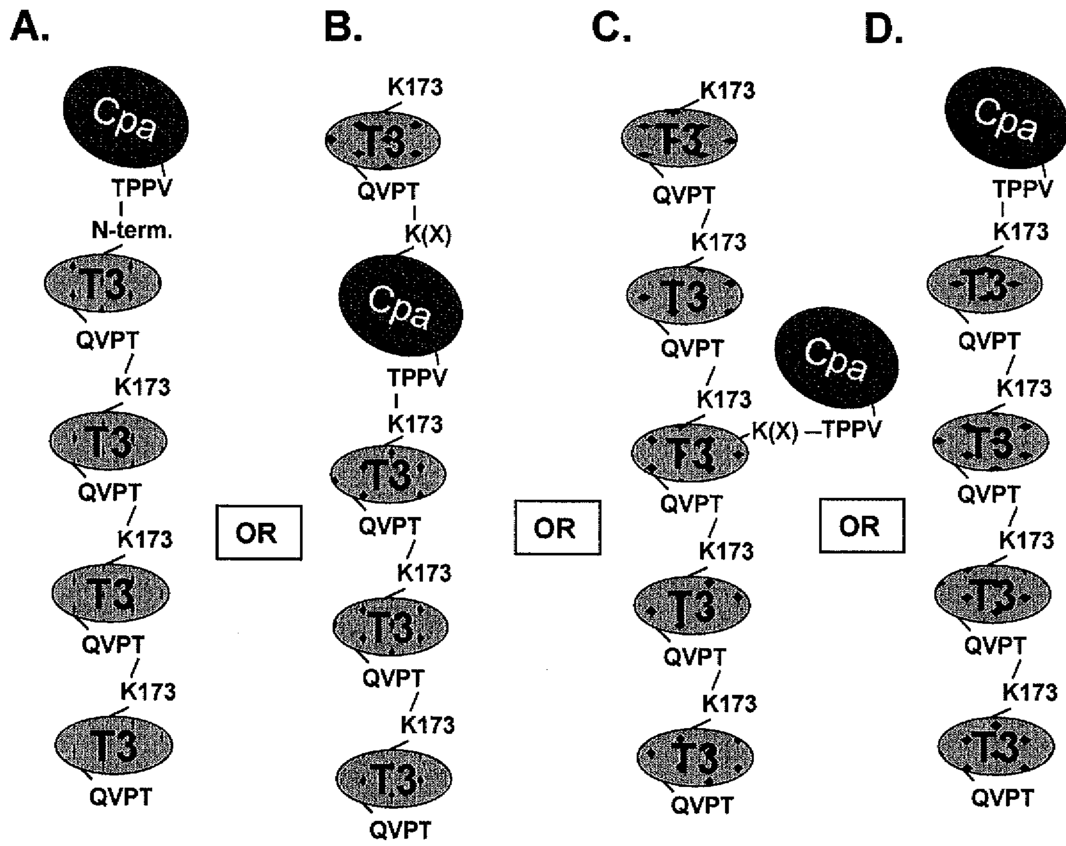
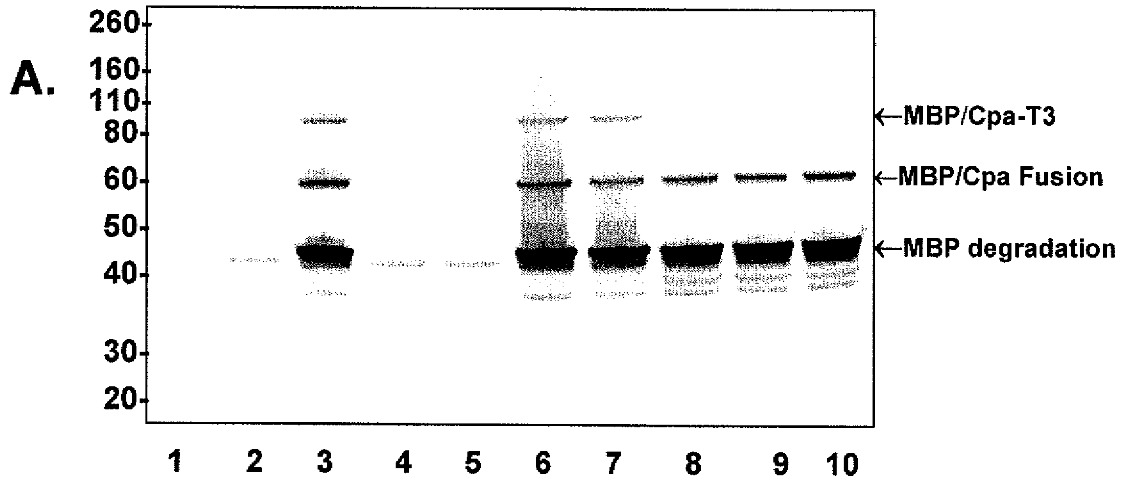


FIG. 11



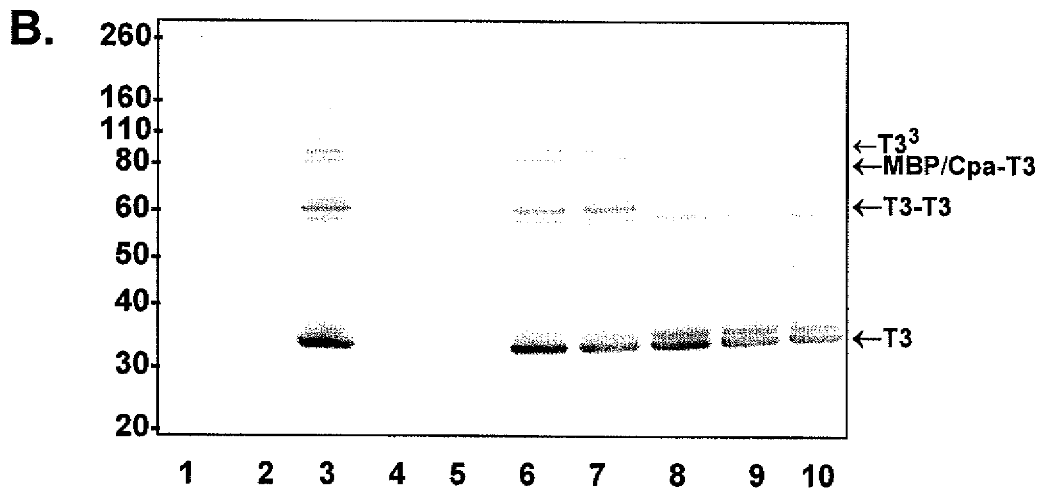


FIG. 12

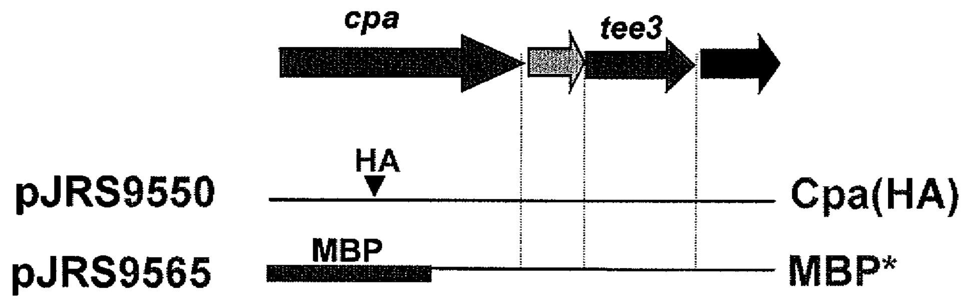


FIG. 13

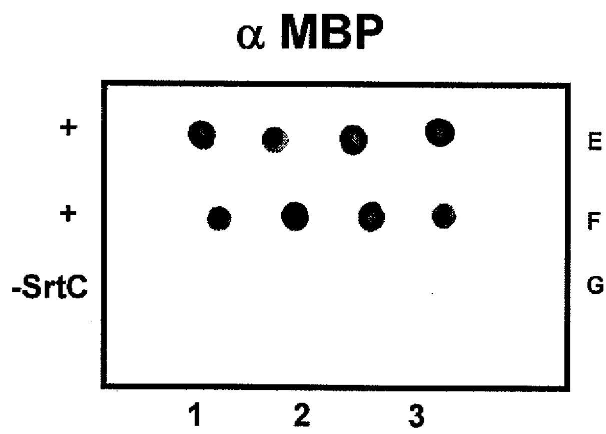


FIG. 14A

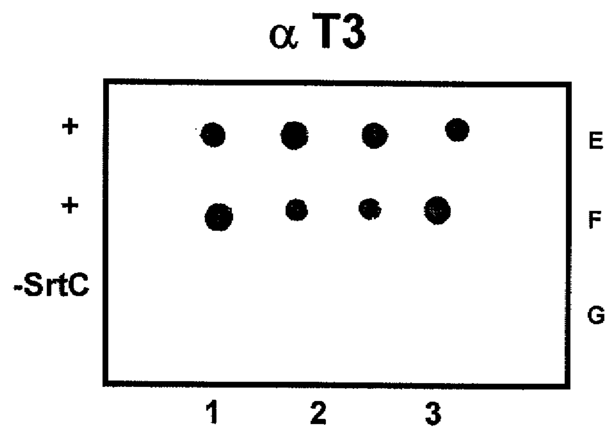


FIG. 14B

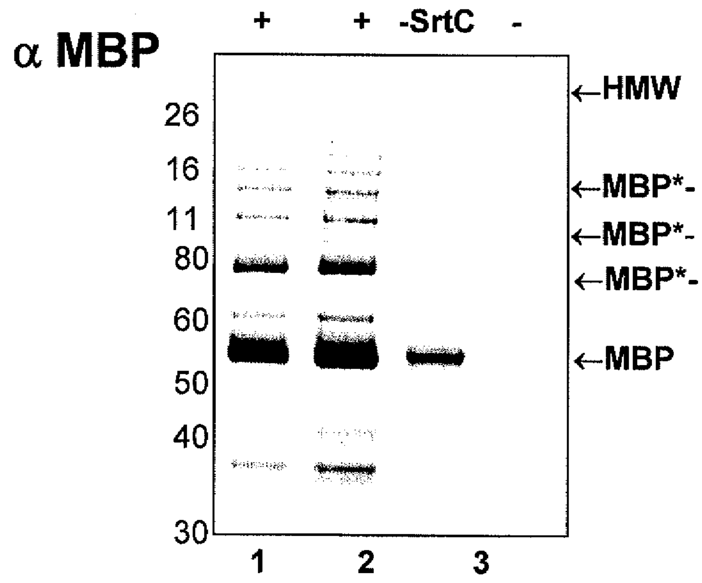


FIG. 15A

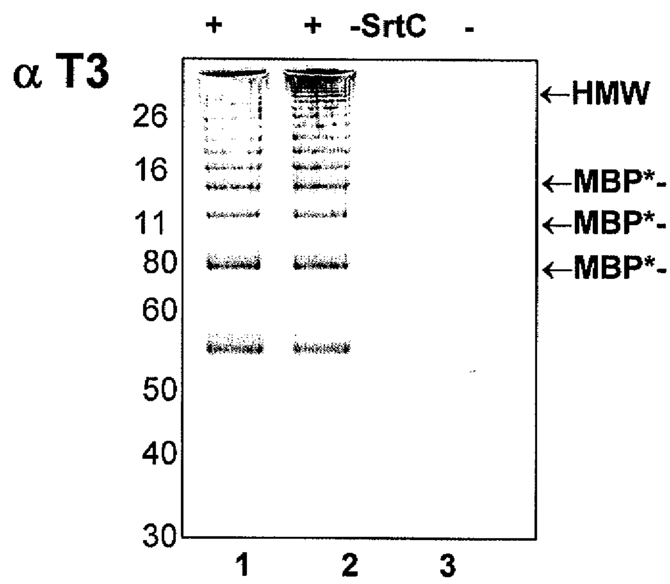


FIG. 15B

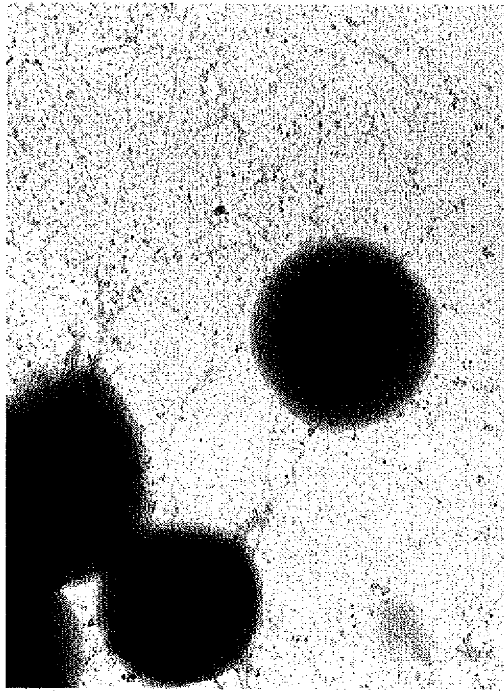


FIG. 16A

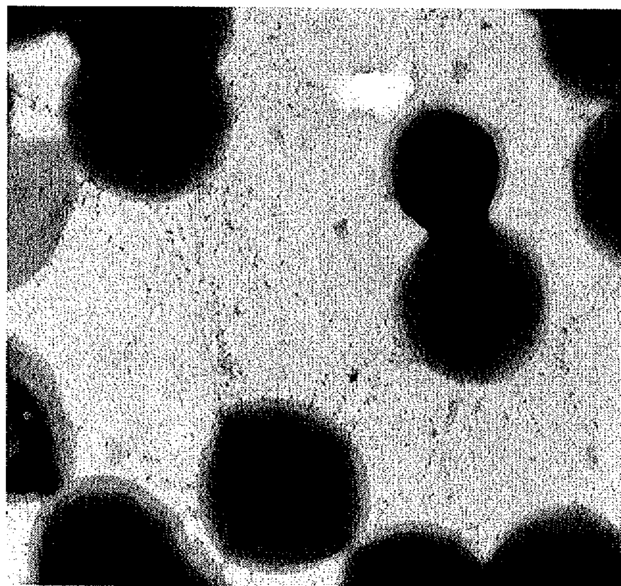


FIG. 16B

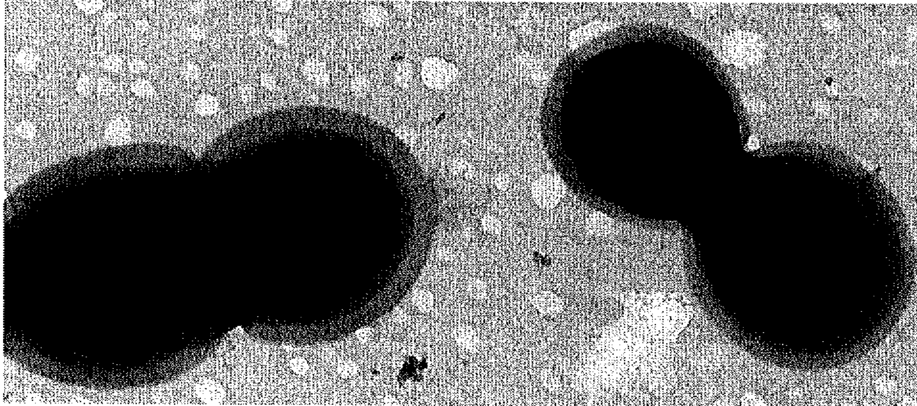


FIG. 16C

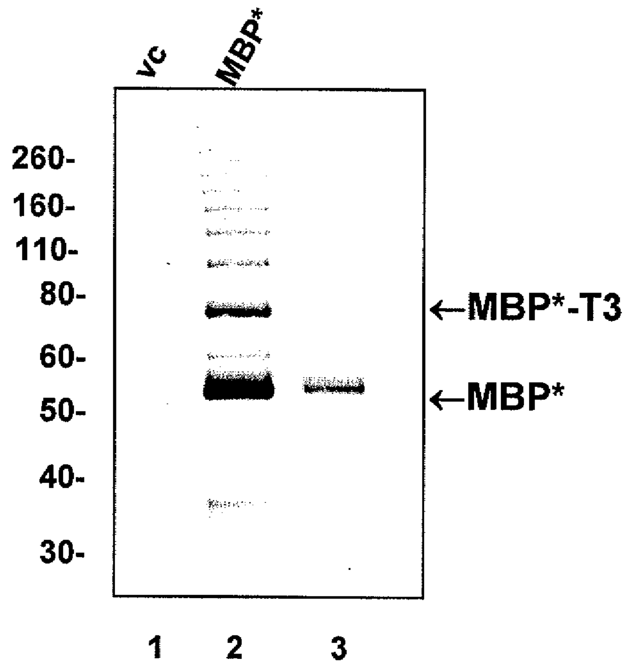


FIG. 17A

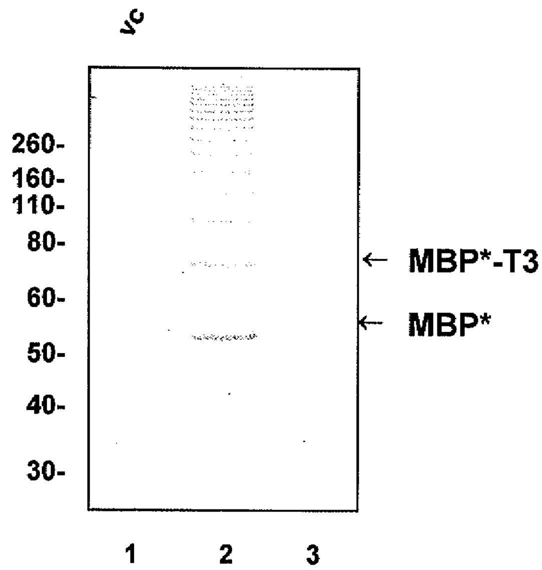


FIG. 17B

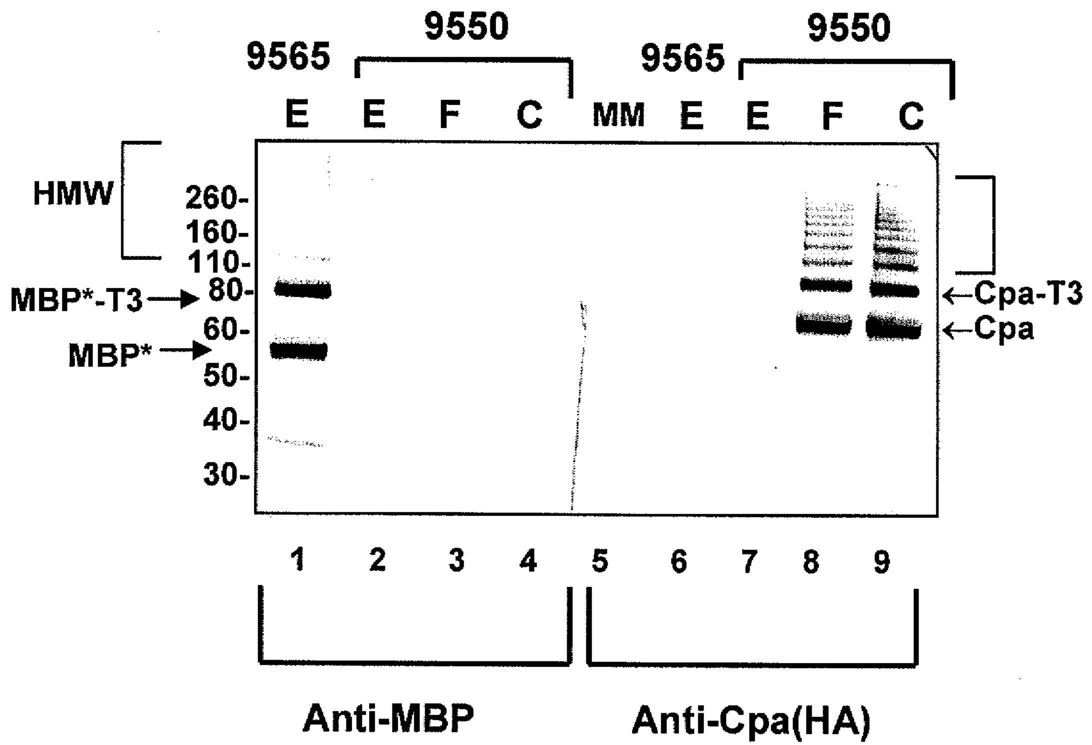


FIG. 18

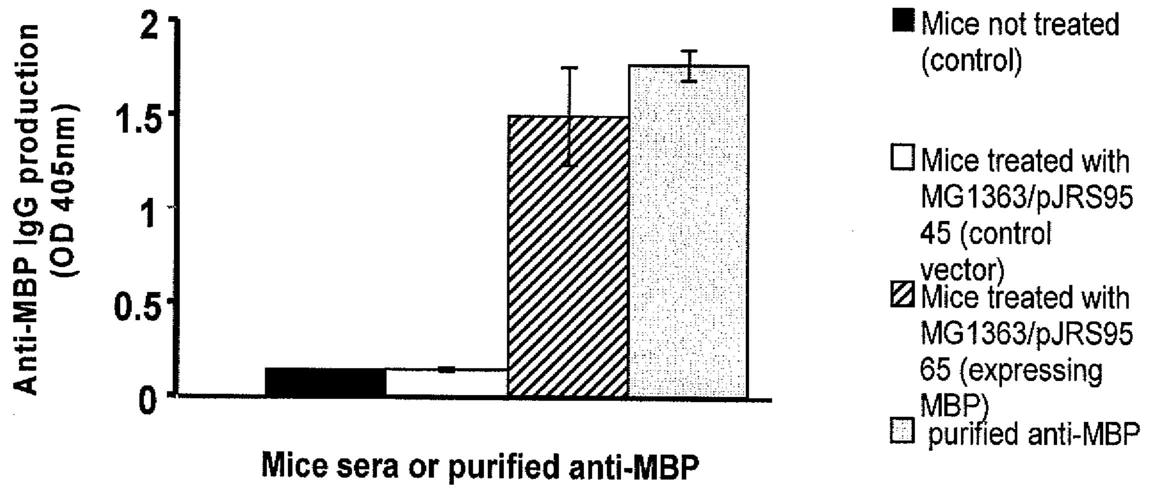


FIG. 19

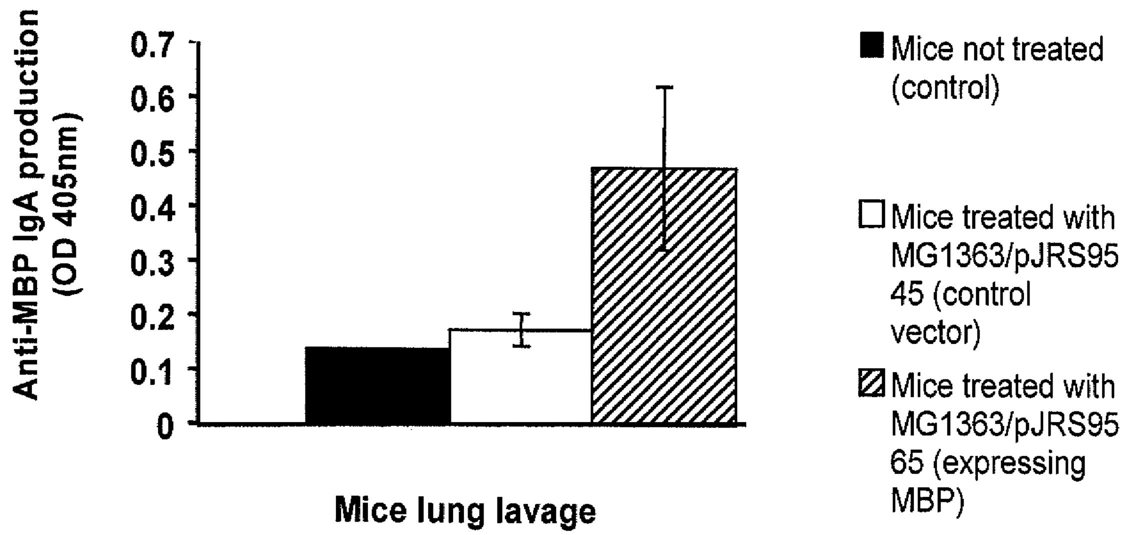


FIG. 20