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(54) Title: BIOFILM MATRIX-BOOSTED VACCINE

(57) Abstract: Some aspects of this disclosure provide engineered exopolysaccharide-associated proteins, engineered bacteria expressing such proteins, and engineered biofilms comprising such proteins. Some aspects of this disclosure provide methods for engineering exopolysaccharide-associated proteins, and for the generation of engineered bacteria and biofilms expressing or comprising such proteins. Some aspects of this disclosure provide compositions and methods useful for the generation of vaccines and the vaccination of subjects, for delivering molecules of interest to a target site, for example, a surface, for purification of molecules of interest, for example, from bioreactors comprising engineered bacteria as provided herein, and for bioremediation applications, such as the cleanup of environmental pollutants.

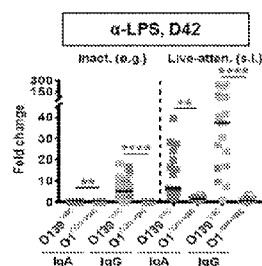


FIGURE 15B

BIOFILM MATRIX-BOOSTED VACCINE

RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/351,895, filed June 17, 2016, which is incorporated by reference herein in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with U.S. Government support under grant NIH AI 50032 awarded by the National Institutes of Health. The U.S. Government has certain rights in this invention.

BACKGROUND

[0003] Bacterial biofilm formation is the process by which bacteria adhere to surfaces to form single or multilayer structures. These biofilm structures are found on biotic surfaces such as the epithelia of animals and on abiotic surfaces such as those of mineral deposits, soil, walls of bioreactors, and air-water interfaces. The bacterial biofilm matrix is comprised of exopolysaccharide, proteins, and DNA.

SUMMARY

[0004] Bacterial biofilms, which are often described as “slime,” have been vilified in medicine and industry. While much research has been performed to develop methods and materials that avoid the formation of biofilms on surfaces, *e.g.*, on surfaces of medical devices or bioreactors, some aspects of this disclosure are based on the recognition that biofilms can be engineered to be useful in a number of biomedical and biotechnological applications.

[0005] In contrast to the conventional paradigm that biofilm exopolysaccharides function as the adhesive material that cements cell-surface and intercellular interactions, some aspects of this disclosure provide that biofilm exopolysaccharides actually serve as a scaffold for cellular proteins which mediate these adhesive interactions. Some aspects of this disclosure are based on the recognition that the cellular proteins mediating cell adhesion in biofilms are abundant in biofilms, and that these cellular proteins can be used to deliver heterologous molecules, *e.g.*, enzymes, antigens, binding agents, detection agents, or small molecules, to biofilms in order to engineer novel biofilm functionalities.

[0006] Some aspects of this disclosure relate to the identification of several secreted proteins that are retained in the bacterial biofilm matrix by their association with the biofilm exopolysaccharide scaffold. As described in more detail elsewhere herein, these exopolysaccharide-associated proteins show different spatial distribution patterns within the biofilm. Some aspects of this disclosure are based on the recognition that such exopolysaccharide-associated proteins can be used to engineer biofilms for various applications, *e.g.*, to serve as reservoirs for surface-active secreted proteins of biomedical, bioengineering, or biotechnological importance. Accordingly, some aspects of this disclosure provide that the biofilm matrix can be exploited, among other uses, as a vehicle for concentration of molecules, *e.g.*, enzymes or antigens, on the surfaces of cells and as a delivery system targeting abiotic surfaces. Because of their affinity for surfaces, biofilms engineered according to aspects of this disclosure are also ideal vehicles for presentation of vaccine antigens and for delivery of enzymes of therapeutic or bioremediative importance to surfaces.

[0007] The technology described herein is broadly applicable, with envisioned applications ranging from vaccine development, treatment of digestive dysfunction, biotechnology (*e.g.*, concentration, isolation, or purification of recombinant proteins from bioreactors), bioremediation (*e.g.*, oil spill clean-up), molecular biology, and others.

[0008] Some aspects of this disclosure provide compositions comprising: (i) a bacterium associated with an exopolysaccharide; (ii) a RbmA protein, and (iii) a heterologous molecule conjugated to the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof.

[0009] In some embodiments, the heterologous molecule is a heterologous protein. In some embodiments, the heterologous protein is an enzyme, or an antigen. In some embodiments, the heterologous protein is fused to the exopolysaccharide-associated protein, or to the exopolysaccharide-binding fragment thereof, thus forming a fusion protein. In some embodiments, the fusion protein is encoded by a recombinant nucleic acid comprised in the bacterium.

[0010] In some embodiments, the RbmA protein comprises an amino acid sequence of SEQ ID NO: 2. In some embodiments, the RbmA protein is a RbmA variant comprising one or more substitution mutations in a surface groove of the RbmA protein. In some embodiments, the one or more substitution mutations are in positions R234, R219, or R116 in SEQ ID NO: 2. In some embodiments, the one or more mutations are R234A, R219A, or

R116A in SEQ ID NO: 2. In some embodiments, the RbmA variant comprises an amino acid sequence of any one of SEQ ID NOs: 38-43.

[0011] In some embodiments, the heterologous molecule comprises an antigen. In some embodiments, the antigen comprises an antigen of a pathogen. In some embodiments, the antigen comprises an antigen of a bacterial toxin. In some embodiments, the bacterial toxin is a cholera toxin. In some embodiments, the cholera toxin is the B subunit of cholera toxin (*e.g.*, MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIYTLNDKIFSATESLAGKRE MAITFKNGAIFQVEVPGSQHIDSQKKAIERMKDTRLRIAYLTEAKVEKLCVWNNKTP HAIAAISMAN (SEQ ID NO: 5)). In some embodiments, the antigen comprises a colonization factor antigen. In some embodiments, the colonization factor antigen is from Enterotoxigenic Escherichia coli (ETEC). In some embodiments, the colonization factor antigen is EtpA. In some embodiments, the EtpA comprise the amino acid sequence of SEQ ID NO: 44. In some embodiments, the antigen comprises a bacterial virulence factor antigen. In some embodiments, the bacterial virulence factor antigen is from Shigella. In some embodiments, the bacterial virulence factor antigen is VirG. In some embodiments, the VirG comprises an amino acid sequence of SEQ ID NO: 45.

[0012] In some embodiments, the heterologous molecule comprises an enzyme. In some embodiments, the enzyme is a therapeutic enzyme. In some embodiments, the enzyme is selected from the group consisting of lactase, a pancreatic enzyme, an oil-degrading enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase.

[0013] In some embodiments, the heterologous molecule comprises a binding agent. In some embodiments, the binding agent is selected from the group comprising an antibody, an antigen-binding antibody fragment, a nanobody, an ScFv, an adnectin, a lectin, a ligand, or an affinity tag. In some embodiments, the heterologous molecule comprises a detection agent.

[0014] In some embodiments, the compositions disclosed herein further comprises a signal peptide fused to the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, or to the heterologous molecule, wherein the signal peptide targets the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, conjugated to the heterologous molecule for secretion.

[0015] In some embodiments, the bacterium is a gram-negative bacterium. In some embodiments, the bacterium is a gram-positive bacterium. In some embodiments, the bacterium is a non-pathogenic bacterium. In some embodiments, the bacterium is a *Vibrio sp.*

bacterium. In some embodiments, the bacterium is a *Vibrio cholerae* bacterium. In some embodiments, the bacterium is an *E. coli* bacterium.

[0016] In some embodiments, the bacteria toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC). In some embodiments, the non-toxic STa variant comprises a A14H mutation (STa^{A14H}). In some embodiments, the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}. In some embodiments, wherein the fusion protein is encoded by an nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.

[0017] Other aspects of this disclosure provide compositions comprising: (i) a RbmA protein, and (ii) a heterologous molecule conjugated to the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof.

[0018] In some embodiments, the heterologous molecule comprises a heterologous protein. In some embodiments, the heterologous protein is an enzyme, or an antigen. In some embodiments, the heterologous protein is fused to the exopolysaccharide-associated protein, or to the exopolysaccharide-binding fragment thereof, thus forming a fusion protein.

[0019] In some embodiments, the RbmA protein comprises an amino acid sequence of SEQ ID NO: 2. In some embodiments, the RbmA protein is a RbmA variant comprising one or more substitution mutations in a surface groove of the RbmA protein. In some embodiments, the one or more substitution mutations are in positions R234, R219, or R116 in SEQ ID NO: 2. In some embodiments, the one or more mutations are R234A, R219A, or R116A in SEQ ID NO: 2. In some embodiments, the RbmA variant comprises an amino acid sequence of any one of SEQ ID NOs: 38-43.

[0020] In some embodiments, the heterologous molecule comprises an antigen. In some embodiments, the antigen comprises an antigen of a pathogen. In some embodiments, the antigen comprises an antigen of a bacterial toxin. In some embodiments, the bacterial toxin is a cholera toxin. In some embodiments, the cholera toxin is the B subunit of cholera toxin. In some embodiments, the B subunit of cholera toxin comprises the sequence of (SEQ ID NO: 5). In some embodiments, antigen comprises a colonization factor antigen. In some embodiments, the colonization factor antigen is from Enterotoxigenic Escherichia coli (ETEC). In some embodiments, the colonization factor antigen is EtpA. In some embodiments, the EtpA comprise the amino acid sequence of SEQ ID NO: 44. In some embodiments, the antigen comprises a bacterial virulence factor antigen. In some embodiments, the bacterial virulence factor antigen is from Shigella. In some embodiments,

the bacterial virulence factor antigen is VirG. In some embodiments, the VirG comprises an amino acid sequence of SEQ ID NO: 45.

[0021] In some embodiments, the heterologous molecule comprises an enzyme. In some embodiments, the enzyme is a therapeutic enzyme. In some embodiments, the enzyme is selected from the group consisting of lactase, a pancreatic enzyme, an oil-degrading enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase.

[0022] In some embodiments, the heterologous molecule comprises a binding agent. In some embodiments, the binding agent is selected from the group comprising an antibody, an antigen-binding antibody fragment, a nanobody, an ScFv, an adnectin, a lectin, a ligand, or an affinity tag. In some embodiments, the heterologous molecule comprises a detection agent.

[0023] In some embodiments, the compositions disclosed herein further comprise a signal peptide fused to the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, or to the heterologous molecule, wherein the signal peptide targets the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, conjugated to the heterologous molecule for secretion.

[0024] In some embodiments, the bacteria toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC). In some embodiments, the non-toxic STa variant comprises a A14H mutation (STa^{A14H}). In some embodiments, the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}. In some embodiments, wherein the fusion protein is encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.

[0025] Further provided herein are vaccines comprising the composition described herein. In some embodiments, the fusion protein is expressed by the bacterium. In some embodiments, the exopolysaccharide is an exopolysaccharide secreted by the bacterium. In some embodiments, the exopolysaccharide is bound by the bacterium. In some embodiments, the fusion protein is bound by the exopolysaccharide. In some embodiments, the antigen is an antigen of a pathogen.

[0026] In some embodiments, the vaccine further comprises an adjuvant. In some embodiments, the adjuvant is a Cholera toxin subunit A (CtxA) variant. In some embodiments, the CtxA variant comprises mutations corresponding to N189D, A190S, P191S, R192G, S193T, S194I, M195T, S196G, N197I, N197D, or L211A in SEQ ID NO: 46. In some embodiments, the CtxA variant has reduced activity. In some embodiments, the CtxA variant activity is reduced by at least 10-100 fold. In some embodiments, the CtxA

variant is expressed by the bacterium. In some embodiments, the CtxA variant is constitutively expressed.

[0027] In some embodiments, the adjuvant is a multiple-mutated Cholera toxin (mmCT). In some embodiments, the mmCT is encoded by a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 47.

[0028] In some embodiments, the vaccine is a killed whole cell vaccine. In some embodiments, the vaccine is a live attenuated vaccine.

[0029] In some embodiments, the bacterium is a pathogenic bacterium. In some embodiments, the bacterium is *V. Cholerae*. In some embodiments, the bacterium is *E. Coli*. In some embodiments, the *V. cholerae* is inactivated in ctxA. In some embodiments, the ctxA is deleted from the *C. cholerae* genome.

[0030] Other aspects of this disclosure provide methods of vaccinating a subject against a pathogen, the methods comprising administering to the subject an effective amount of the vaccine described herein.

[0031] In some embodiments, the vaccine is administered in an amount sufficient to elicit an immune response against the bacterium and/or against the antigen. In some embodiments, the vaccine is administered in an amount sufficient to immunize the subject against the bacterium and/or against the antigen. In some embodiments, the vaccine is administered sublingually. In some embodiments, the vaccine is administered orally.

[0032] In some embodiments, the subject is 0-13 years of age.

[0033] In some embodiments, the vaccine stimulates systemic immune response to the antigen. In some embodiments, the vaccine stimulates mucosal immune response to the antigen. In some embodiments, the vaccine elicits antigen-specific antibodies in the subject. In some embodiments, the antigen-specific antibody is IgG. In some embodiments, the antigen-specific antibody is IgA.

[0034] Further provided herein are methods for delivering a molecule to a target site, the method comprising delivering to the target site a bacterium associated with an exopolysaccharide that binds a RbmA protein, wherein the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, binds the molecule. In some embodiments, the target site is a surface. In some embodiments, the target site is an air/water interface. In some embodiments, the heterologous molecule comprises a polypeptide. In some embodiments, the polypeptide is fused to the exopolysaccharide-associated protein or the exopolysaccharide binding protein fragment.

[0035] In some embodiments, the bacterium comprises a recombinant nucleic acid encoding the polypeptide fused to the exopolysaccharide-associated protein or the exopolysaccharide binding protein fragment. In some embodiments, the molecule comprises a therapeutic protein. In some embodiments, the method comprises administering an effective amount of the bacterium to a subject. In some embodiments, the bacterium is a non-pathogenic bacterium. In some embodiments, the molecule is an enzyme and wherein the target site is a digestive organ of the subject. In some embodiments, the target site is the intestinal tract of the subject.

[0036] In some embodiments, the molecule is lactase, a pancreatic enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase. In some embodiments, the bacterium can colonize the intestinal tract of the subject. In some embodiments, the bacterium is administered orally. In some embodiments, the molecule is a mucus-digesting enzyme, and wherein the target site is the lung of the subject. In some embodiments, the mucus-digesting enzyme is mucinase or alginase.

[0037] In some embodiments, the bacterium can colonize the lung of the subject. In some embodiments, the bacterium is administered via an aerosol. In some embodiments, the molecule is an enzyme that can digest a contaminant. In some embodiments, the contaminant is oil. In some embodiments, the molecule is an oil-digesting enzyme. In some embodiments, the target site is an environmental water-air interface.

[0038] Also provided herein are biofilms comprising the bacterium described herein.

[0039] Yet other aspects of this disclosure provide methods for modifying the surface of a bacterium, the method comprising contacting a bacterium associated with an exopolysaccharide with a composition comprising a heterologous molecule conjugated to a RbmA protein, wherein the RbmA protein binds to the exopolysaccharide. In some embodiments, the composition is the composition disclosed herein.

[0040] Yet another aspect of the present disclosure provides methods of targeting a molecule to a biofilm, the method comprising contacting a biofilm that comprises an exopolysaccharide with a RbmA protein, conjugated to the molecule. In some embodiments, the biofilm comprises a pathogen and the molecule comprises a polypeptide toxic to the pathogen.

[0041] Other aspects of the present disclosure provide vaccines comprising: (i) a *Vibrio cholerae* bacterium associated with an exopolysaccharide; (ii) a RbmA protein; and (iii) an antigen conjugated to the RbmA protein, or an exopolysaccharide-binding fragment

thereof. In some embodiments, the RbmA protein comprises the amino acid sequence of SEQ ID NO: 2.

[0042] In some embodiments, the antigen is a bacterial toxin. In some embodiments, the bacterial toxin is a cholera toxin. In some embodiments, the cholera toxin is the B subunit of cholera toxin (CtxB). In some embodiments, the CtxB comprises the amino acid sequence of (SEQ ID NO: 5).

[0043] In some embodiments, the bacterial toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC). In some embodiments, the bacterial toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC).

[0044] In some embodiments, the non-toxic STa variant comprises a A14H mutation (STa^{A14H}). In some embodiments, the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}. In some embodiments, the fusion protein is encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.

[0045] In some embodiments, the antigen is bacterial lipopolysaccharide (LPS). In some embodiments, the LPS is from Vibrio cholerae.

[0046] In some embodiments, the vaccine further comprises an adjuvant. In some embodiments, the adjuvant is a Cholera toxin subunit A (CtxA) variant. In some embodiments, the CtxA variant comprises mutations corresponding to N189D, A190S, P191S, R192G, S193T, S194I, M195T, S196G, N197I, N197D, or L211A in SEQ ID NO: 46. In some embodiments, the CtxA variant has reduced activity. In some embodiments, the adjuvant is a multiple-mutated Cholera toxin (mmCT). In some embodiments, the mmCT is encoded by a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 47. In some embodiments, the adjuvant is expressed by the Vibrio cholera bacterium. In some embodiments, the adjuvant is constitutively expressed. In some embodiments, the adjuvant is under the control of a LacZ promoter. In some embodiments, the RbmA protein and the antigen conjugated to the RbmA protein are under the control of a native RbmA promoter.

[0047] In some embodiments, the Vibrio cholera bacterium lacks endogenous CtxA. In some embodiments, the vaccine is a live attenuated vaccine.

[0048] Further provided herein are methods of vaccinating a subject against a pathogen, the method comprising administering to the subject an effective amount of the vaccine described herein. In some embodiments, the vaccine is administered in an amount sufficient to elicit an immune response against the bacterium and/or against the antigen. In

some embodiments, the vaccine is administered sublingually. In some embodiments, the vaccine is administered orally.

[0049] In some embodiments, the subject is 0-13 years of age.

[0050] In some embodiments, the vaccine stimulates systemic immune response to the antigen. In some embodiments, the vaccine stimulates mucosal immune response to the antigen. In some embodiments, the vaccine elicits antigen-specific antibodies in the subject. In some embodiments, the antigen-specific antibody is IgG. In some embodiments, the antigen-specific antibody is IgA.

[0051] The summary above is meant to illustrate, in a non-limiting manner, some of the embodiments, advantages, features, and uses of the technology disclosed herein. Other embodiments, advantages, features, and uses of the technology disclosed herein will be apparent from the Detailed Description, the Drawings, the Examples, and the Claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0053] **Figures 1A to 1B.** An RbmA-CtxB fusion protein is retained in the biofilm matrix. **Figure 1A.** Immunofluorescent imaging of the distribution of RbmA-FLAG (RbmA), ChiA-2-FLAG, RbmA-ChiA-2-FLAG, or RbmA-CtxB fusion protein (RbmA-CtxB) in a biofilm formed by wild-type *V. cholerae* carrying a plasmid encoding this protein. RbmA-FLAG and the fusion protein were visualized with anti-FLAG and anti-CtxB antibodies, respectively. Bacterial DNA was stained with DAPI. FLAG-tagged ChiA does not stay associated with the cells, while the RbmA-ChiA12 and RbmA-CTB fusion proteins remained cell-associated. **Figure 1B.** An expanded view of the distributions of RbmA-FLAG and RbmA-CtxB in the biofilm. RbmA-CTB fusion protein co-localizes with the cells.

[0054] **Figure 2.** An enzymatically active RbmA-ChiA-2-FLAG fusion protein is retained in the biofilm matrix. **(A)** Immunofluorescent imaging of the distribution of ChiA-2-FLAG or RbmA-ChiA-2-FLAG in a biofilm formed by wild-type *V. cholerae* carrying a plasmid encoding each of these proteins. The proteins were visualized with an anti-FLAG antibody. Bacterial DNA was stained with DAPI. As a control, a biofilm formed by wildtype *V. cholerae* carrying an empty vector was developed with an anti-FLAG antibody (CTL). **(B)** An expanded view of the distribution of RbmA-ChiA-2-FLAG in the biofilm. Chitinase activity measured in the biofilms **(C)** and supernatants **(D)** of wild-type *V. cholerae* carrying

an empty vector (CTL) or a plasmid encoding RbmA-CtxB (RbmACtxB), ChiA-2-FLAG (ChiA-2), or RbmA-ChiA-2-FLAG (RbmA-ChiA-2). The chitinase activity in the biofilm of the strain expressing RbmA-ChiA-2-FLAG was approximately 40 times higher. This difference was statistically significantly different from that in all other biofilms ($p \leq 0.0003$). Similarly, chitinase activity in the supernatants of strains expressing either RbmA-ChiA-2-FLAG or ChiA-2-FLAG was significantly different from that of strains carrying the control vector ($p = 0.007$ and $p = 0.0215$, respectively) or the RbmA-CtxB fusion ($p = 0.0025$ or $p = 0.0149$, respectively). The difference in chitinase activity between the supernatants of the strains expressing RbmA-ChiA-2-FLAG and ChiA-2-FLAG was not significantly different ($p = 0.07$). Using a commercial kit for chitinase activity, chitinase activity was found in the supernatant (**F**) of both a strain expressing ChiA, a normally secreted protein, and a strain expressing RbmA-ChiA, indicating that there are some RbmA-ChiA fusion proteins in the supernatant that either did not stick onto the cell or become dissociated from the cell surface. Chitinase activity was only detected in the cell pellet of a strain expressing RbmA-ChiA fusion protein on a plasmid, but not in the pellets of strains only expressing unconjugated ChiA (**E**).

[0055] **Figure 3.** A *V. cholerae* $\Delta bap1 \Delta rbmA \Delta rbmC$ triple mutant does not make a biofilm but can recruit the chitinase activity of RbmA-ChiA-2-FLAG to the cell surface. **(A)** Quantification of biofilms formed by wild-type *V. cholerae* (WT), an exopolysaccharide mutant ($\Delta vpsL$), and a $\Delta bap1 \Delta rbmA \Delta rbmC$ mutant (triple) carrying either an empty vector (pCTL) or a plasmid encoding RbmA (pRbmA), and **(B)** the pellicle formed by a $\Delta bap1 \Delta rbmA \Delta rbmC$ mutant carrying either an empty vector (CTL) or plasmids encoding RbmA-FLAG (RbmA), RbmA-CtxB (RbmA-CtxB), ChiA-2-FLAG (ChiA-2), or RbmA-ChiA-2-FLAG (RbmA-ChiA-2). Chitinase activity in the cellular fraction (**C**) and supernatants (**D**) of *V. cholerae* $\Delta bap1 \Delta rbmA \Delta rbmC$ mutant carrying an empty vector or a plasmid encoding RbmA-CtxB, ChiA-2-FLAG, or RbmA-ChiA-2-FLAG. Chitinase activity in the cellular fraction of the mutant expressing RbmA-ChiA-2-FLAG was significantly different from strains expressing all other recombinant proteins ($p < 0.0001$). The chitinase activity in the supernatants of mutants expressing ChiA-2-FLAG and RbmA-ChiA-2-FLAG was significantly different from those of the mutant carrying an empty vector ($p = 0.034$ and $p = 0.0172$) and the mutant expressing RbmA-CtxB ($p = 0.039$ and $p = 0.0215$). The difference in chitinase activity between the supernatants of the strains expressing RbmA-ChiA-2-FLAG and ChiA-2-FLAG was not significantly different ($p = 0.535$).

[0056] **Figure 4.** Structures of β -prism domain proteins: **(A)** In Bap1 and RbmC, the β -prism domain is surrounded by FG-GAP domains that likely form a β -propeller. HlyA consists of spatially separated cytolysin, β -trefoil, and β -prism domains. **(B)** Crystal structure of HlyA.

[0057] **Figure 5.** Bap1 and RbmC are required for anchoring of the biofilm to a surface, while RbmA stabilizes intercellular attachments. Wild-type *V. cholerae* as well as Δ vpsL, Δ rbmA, Δ bap1 Δ rbmC, and rescued Δ bap1 Δ rbmC mutant biofilms undisturbed (U), after shaking (S), or after vortexing (V). A Δ rbmA biofilm is dispersed by vortexing, while the Δ bap1 Δ rbmC mutant falls to the bottom of the tube when shaken gently.

[0058] **Figure 6.** Distinct distributions of the proteins RbmA and Bap1 in the biofilm matrix: Immunofluorescent analysis of biofilms formed by strains containing a chromosomally encoded affinity tag at the C-terminus of RbmA **(A)** or Bap1 **(B)**. Immunofluorescence shows that RbmA is distributed throughout the biofilm, while Bap1 is concentrated at the surface. **(C)** Fluorescent intensity plotted as a function of biofilm height (Z-axis) for each of these biofilms.

[0059] **Figure 7.** RbmA mediates cell-cell interactions in the biofilm. RbmA contains two Fn III domains and functions as a homodimer (bottom panel). It is secreted and attaches to the outside of cells to mediate cell-cell interactions. There are two grooves in an RbmA dimer: the wide groove and the narrow groove. The wide groove, which has a net negative charge, is important in recruiting VPS (planktonic) cells into biofilm. The narrow groove, which has a net positive charge, is important in cell-cell adhesion within the biofilm, and key arginine residues within the groove have been found to be key contributors to this charge and cell adhesion property. Knocking out RbmA impairs biofilm formation. When the wild-type biofilm is vortexed, it breaks up into small aggregates of cells; RbmA mutant biofilms are easily dispersed by vortexing (top panel). Adding purified protein exogenously to the RbmA mutant during biofilm formation results in the vortex-resistant aggregate phenotype seen in WT biofilm (middle panel). RbmA mediates cell-cell interactions.

[0060] **Figure 8.** Confirmation that RbmA-CTB fusion protein co-localizes with cells. A prototype formalin-killed whole cell vaccine that carries RbmA-CTB on the surface of the cell was developed. The Western blot shows that cholera toxin B (CTB) is expressed by itself from a plasmid is only detected in the supernatant; however, when it is linked to RbmA, the fusion protein is found in the cellular fraction. Furthermore, in a simple whole cell vaccine, the RbmA-CTB fusion protein is found at levels equivalent to the amount of purified recombinant CTB added to the Dukoral® vaccine, showing that the CTB protein

antigen is effectively incorporated into the whole cell vaccine. The RbmA protein presentation platform may be an efficient way to achieve antigen presentation.

[0061] **Figure 9.** Schematic and results of the initial vaccine trial with prototype vaccine. BALB/c mice were administered formalin-killed whole cell vaccines by oral gavage, followed by a regimen of one initial inoculation and two boosters spaced two weeks apart. For comparison, formalin-killed V cholera whole cells, similar to Shanchol™, and formalin-killed whole cells given with purified CTB, similar to Dukoral®, were also studied. The primary outcome was the presence of CTB-specific antibodies in serum and stool. As shown in the graphs, four weeks after the final booster, only the test group similar to Dukoral®, which contains purified CTB, shows a CTB-specific response in serum. No CTB-specific antibodies were detected in stool in any of the groups. * $p < 0.02$

[0062] **Figure 10.** Schematic and results of a test of a live-attenuated version of the whole cell vaccine. Live-attenuated vaccines have several advantages over an inactivated vaccine, including having documented better responses in young children, a single dose of live-attenuated vaccine can be sufficient to induce an adequate and sustained immune response. The vaccine was delivered sublingually to avoid the use of bicarbonate and to bypass the stomach completely. The strain used, *ΔctxA/prbmA-ctxB*, does not express ctxA, the effector subunit of the cholera toxin. The study included 10 mice, and serum and stool were collected every two weeks to measure the presence of CTB-specific antibodies. The results, lower panel, show that CTB-specific IgG was not detected in the serum, while the serum levels of CTB-specific IgA showed a slightly increasing trend by four weeks after administration of the final booster. In contrast to the inactivated vaccine, there was a significant rise in the secretory IgA specific to CTB, which waned by four weeks after the final booster. While the level of CTB-specific secretory IgA is low, it is statistically significant compared with levels of non-specific antibody detection before vaccination. Furthermore, levels of total IgA can be seen to have remained constant while the CTB-specific IgA levels increased, therefore, the live-attenuated vaccine may show more promise than the inactivated vaccine.

[0063] **Figure 11.** Both the live-attenuated and the killed whole-cell vaccine generated vibriocidal antibodies. Serum levels of vibriocidal antibodies have been recognized as an indication of protection, both in cases of cholera infection and vaccination. To determine if the vaccines were properly administered, the levels of vibriocidal antibodies in the serum were examined. They were present in both the formalin-killed and live-attenuated vaccines. In particular, the increase in vibriocidal antibody titer for the live-

attenuated vaccine corresponded well with a decrease in the number of live vibrio shedding in the stool post-vaccination.

[0064] Figure 12. Schematic of the treatment protocol to test the immunogenicity of a whole cell vaccine expressing RbmA-CTB. BALB/c mice were vaccinated with either an oral killed whole cell vaccine (similar to the current cholera vaccine Shanchol™), or with a live-attenuated strain (*ΔctxA*) that expresses RbmA-CTB but not ctxA (the effector subunit of cholera toxin) via the sublingual route. Each protocol included an initial vaccination followed by two boosters, each two weeks apart. Immune responses were then examined four weeks after the final booster for the oral killed vaccine group, and every two weeks during vaccination for the live-attenuated vaccine group.

[0065] Figures 13A to 13C. Antigen-specific antibody responses. No CTB-specific immune response was seen in the control group given the oral killed whole cell vaccine, but the live attenuated vaccine, administered sublingually, induced an antigen-specific response in the serum, dominated by IgA (Figure 13A). The live-attenuated vaccine also induced the production of antigen-specific secretory IgA at the intestinal mucosa (Figure 13C). The rise in antigen-specific secretory IgA is seen by the first booster, suggesting that a single dose of the sublingual vaccine may be sufficient to induce this response.

[0066] Figures 14A-14D. Generation of a prototype biofilm matrix protein vaccine. RbmA contains two fibronectin III (Fn III) domains and functions as a homodimer (Figure 14A). Schematic of how cholera toxin B subunit (CTB), a secreted protein, can be anchored to the surface of the cell when linked to the C terminus of RbmA (Figure 14B). The RbmA-CTB fusion protein (R-CTB) is detected by Western blot in the cellular fraction using antibodies against CTB, whereas unfused CTB is detected only in the supernatant (Figure 14C). The amount of R-CTB in a Vc(pR-CTB) prototype whole cell vaccine is comparable to the amount of purified CTB included in the Dukoral® (WC-rBS) vaccine (Figure 14D).

[0067] Figures 15A-15N. The prototype vaccine elicits LPS-specific antibodies, protects against cholera challenge, and generates antigen-specific mucosal antibodies. Vaccination study scheme. Open triangles indicate vaccination. Arrows indicate blood and stool collection (Figure 15A). Fold change in serum antibodies against O139 or O1 LPS compared against PBS immunization sera (inactivated) or pre-immune sera (live-attenuated) (Figure 15B). Serum vibriocidal antibody titer at day 42 against wild-type MO10 (O139) (Figure 15C). Colonization of the small and large intestines in suckling mice challenged with wild-type MO10. Pups were born to unvaccinated dams (Ctrl) or to dams that received the live-attenuated vaccine (Vacc) (Figure 15D). Weight of the small and large intestines of pups

after challenge (Figure 15E). Fluid accumulation in the large intestine and cecum (triangle) is visible in the control pups and absent in protected pups (Figure 15F). Reduced skin turgor present in control pups and absent in protected pups. Scale bars = 10 cm (Figure 15G). Fold change of CTB-specific antibodies after immunization with a control strain or vaccine strain expressing R-CTB (Figures 15H-15J). Components of the detoxified cholera toxin in the supernatant after overnight culture in LB broth (Figure 15K). LPS-specific antibodies in vaccine strains with and without mmCT. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. $p \geq 0.05$ using unpaired, two-tailed Mann-Whitney rank-sum test. Horizontal bars mark the median (Figures 15L-15N). Each vaccination group included ten mice. Vaccinated and unvaccinated litters included 16 and 11 pups, respectively. Fold change calculated against pre-immune background levels unless otherwise stated.

[0068] Figures 16A-16I. A live-attenuated vaccine delivering ETEC antigens and a detoxified cholera toxin elicits antigen-specific antibodies and enhances LPS-specific antibody response. Schematic of the vaccine strain, based on an O139 strain where CTX phage elements, including the recombination sites, have been deleted. The ETEC antigens LTb and STa was cloned to the 3' end of *rbmA* on the chromosome to create a chimeric protein expressed from the native *rbmA* promoter. Additionally, the vaccine strain harbors a deletion of the major colonization factor, *tcpA*, and expresses mmCT (Figure 16A). The RbmA-LTb-STa protein (R-LT/ST) detected by anti-STa Western blot in the cell pellet, and not the supernatant, after overnight growth (Figure 16B). Vaccination scheme for the ETEC antigen-boosted whole cell vaccine. Open triangles indicate vaccination. Arrows indicate blood and stool collection (Figure 16C). Fold change of LTb/CTB-specific antibodies after immunization with a control strain expressing only mmCT or vaccine strain co-expressing R-LT/ST and mmCT (Figures 16D-16F). Fold change of STa-specific antibodies after immunization with the control strain or the R-LT/ST vaccine strain. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. $p \geq 0.05$ using unpaired, two-tailed Mann-Whitney rank-sum test. Horizontal bars mark the median (Figures 16G-16I). Each vaccination group included ten mice. Fold change calculated against pre-immune background levels unless otherwise stated.

[0069] Figure 17. Western blot analysis of cell-associated R-CTB after formalin treatment. Formalin treatment of the prototype vaccine expressing pR-CTB resulted in protein crosslinking. R-CTB (arrow), but not native CTB, is detected in the cellular fraction after formalin treatment.

[0070] Figures 18A-18C. Inactivated prototype vaccine given orogastrically did not elicit antigen-specific antibody responses. a-c, Fold change of CTB-specific IgA and IgG in the serum and CTB-specific IgA in the stool. Antibody levels were measured four weeks after the second vaccine booster. Fold change calculated against background levels in PBS immunized sera. Each vaccination group included ten mice. Horizontal bars mark the median. * $p \leq 0.05$, **** $p \leq 0.0001$, n.s. not significant using unpaired, two-tailed Mann-Whitney test.

[0071] Figure 19. Dissemination of live bacteria after sublingual immunization occurs at low levels. Live *V. cholerae* recovered from stool pellets after sublingual immunization. Bacterial shedding ceased after 24 hours. The limit of detection was estimated to be 440 CFU/g and is denoted by the dotted line. Groups that received vaccine strains with *tcpA* and vaccine strains without *tcpA* included 20 and 40 mice, respectively. Horizontal bars mark the median.

[0072] Figures 20A-20C. Expression of mmCT by the prototype vaccine significantly enhances production of antibodies against the O-antigen. Fold change of IgA and IgG specific to O-antigen in the serum and IgA against the O-antigen in the stool. Fold change calculated against pre-immune sera. Each vaccination group included ten mice. Horizontal bars mark the median. * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. $p \geq 0.05$ using unpaired, two-tailed Mann-Whitney test.

[0073] Figures 21A-21C. Inclusion of the adjuvant, mmCT, in the prototype R-CTB vaccine abrogated the immune response to CTB. Fold change of CTB-specific antibodies after immunization with a vaccine strain expressing R-CTB and mmCT. *** $p \leq 0.001$, n.s. $p \geq 0.05$ using unpaired, two-tailed Mann-Whitney test. Horizontal bars mark the median. Each vaccination group included ten mice. Fold change calculated against pre-immune background levels.

[0074] Figure 22. The mmCT adjuvant does not contribute significantly to the amount of CTB delivered in a vaccine dose. Quantification of the amount of CTB delivered as R-CTB and as part of mmCT. Error bars denote standard deviation.

[0075] Figure 23. Each dose of the R-LT/STa^{A14H} vaccine delivers 30 femtomoles of STa. Quantification of the amount of STa delivered as part of R-LT/STa^{A14H}. Error bar denotes standard error.

DETAILED DESCRIPTION

Introduction

[0076] The occurrence of surface-associated bacterial structures known as biofilms is viewed as an undesirable phenomenon in the context of many biomedical and biotechnological applications. Biofilm formation can lead to failure of biomedical devices, to greatly reduced continuous production times and yields in production-scale bioreactors, and to many other detrimental consequences. Accordingly, biofilms are the target of intense antimicrobial research efforts.

[0077] In contrast to the conventional paradigm that biofilms are undesirable contaminants, some aspect of this disclosure provide that biofilms can be engineered to confer novel structural and/or functional characteristics upon them, making them useful tools in many biomedical and biotechnological applications. For example, as described in more detail herein, engineered biofilms, bacteria, and biofilm-associated proteins provided herein are useful for vaccine development and production, isolation and purification of bioreactor products, delivery of molecules to a target site in vivo, and bioremediation applications, such as cleaning up environmental pollutants.

[0078] This paradigm shift away from the view that biofilms are undesirable contaminants and towards the view that biofilms can be engineered to serve highly desirable purposes in a variety of applications is based, in part, on the recognition of how biofilms form and maintain their structure and their adhesion to surfaces. In contrast to the conventional view that exopolysaccharides shed by bacteria in a biofilm function as the “glue” that mediates adhesion of the cells within the biofilm to each other and also adhesion of the biofilm to a surface, some aspects of this disclosure provide that biofilm exopolysaccharides function in a different way. Namely, as described in more detail herein, biofilm exopolysaccharides provide a scaffold to which cellular proteins, secreted by or expressed on the surface of the cells within the biofilm, adhere. These cellular proteins are referred to herein as exopolysaccharide-associated proteins.

[0079] Some aspects of this disclosure are based on the recognition that the cellular proteins mediating cell adhesion in biofilms, exopolysaccharide-associated proteins, are abundant in biofilms, and that different exopolysaccharide-associated proteins exhibit different spatial distributions throughout a given biofilm. Some aspects of this disclosure relate to the identification and characterization of several secreted or extracytoplasmic proteins that are retained in the bacterial biofilm matrix by their association with the biofilm

exopolysaccharide scaffold. Some aspects of this disclosure provide engineered exopolysaccharide-associated proteins, for example conjugated with a heterologous molecule, *e.g.*, a protein, an enzyme, antigen, binding agent, detection agent, or small molecules. Such engineered exopolysaccharide-associated proteins can be used to deliver heterologous molecules to biofilms, for example, in order to engineer novel biofilm functionalities and structures. Depending on the spatial distribution of a given exopolysaccharide-associated protein, a heterologous molecule can be delivered to the surface, the interior, or evenly throughout a given biofilm according to some aspects of this disclosure.

[0080] Some aspects of this disclosure are based on the recognition that engineered exopolysaccharide-associated proteins in bacteria, as provided herein, can be used to engineer biofilms for various applications, *e.g.*, to serve as reservoirs for surface-active secreted proteins of biomedical, bioengineering, or biotechnological importance. Accordingly, some aspects of this disclosure provide that the biofilm matrix can be exploited, among other uses, as a vehicle for concentration of molecules, *e.g.*, enzymes or antigens, on the surfaces of cells and as a delivery system targeting abiotic surfaces. Because of their affinity for surfaces, biofilms engineered according to aspects of this disclosure are also ideal vehicles for presentation of vaccine antigens and for delivery of enzymes of therapeutic or bioremediative importance to surfaces.

[0081] Some aspects of this disclosure provide engineered exopolysaccharide-associated proteins, engineered bacteria expressing such proteins, and engineered biofilms comprising such proteins. Some aspects of this disclosure provide methods for engineering exopolysaccharide-associated proteins, and for the generation of engineered bacteria and biofilms expressing or comprising such proteins. Some aspects of this disclosure provide compositions and methods useful for the generation of vaccines and the vaccination of subjects, for delivering molecules of interest to a target site, for example, a surface, for purification of molecules of interest, for example, from bioreactors comprising engineered bacteria as provided herein, and for bioremediation applications, such as the cleanup of environmental pollutants.

[0082] The technology described herein is broadly applicable to any biofilm comprising an exopolysaccharide scaffold and exopolysaccharide-associated proteins mediating cell-cell adhesion and/or cell-surface adhesion within the biofilm. Accordingly, exemplary envisioned applications range from, without limitation, therapeutic and prophylactic medical uses, *e.g.*, vaccine development and treatment of digestive dysfunction, to biotechnological uses (*e.g.*, concentration, isolation, or purification of recombinant

proteins or other products from bioreactors), to bioremediation (*e.g.*, oil spill clean-up), to molecular biology, and others. For example, the technology described herein represents an economical and versatile new platform for delivery of protein antigens or immune adjuvants in whole cell vaccines. For another example, the technology described herein can be used to deliver functional proteins to surfaces. For instance, a commensal bacterium such as *E. coli* or a commonly used probiotic might be used to deliver a digestive enzyme, for example, lactase or a pancreatic enzyme, to the intestinal brush border of a subject with a deficiency in the digestive enzyme, (*e.g.*, subjects with lactase deficiency or cystic fibrosis). A nonpathogenic bacterium colonizing the lung of a cystic fibrosis patient might be re-engineered according to some aspects of this disclosure to deliver mucinase or alginase, thus helping to clear biofilm-associated *Pseudomonas aeruginosa* from the lung. For another example, the technology described herein can be used to deliver enzymes that are useful in the digestion of an environmental pollutant, *e.g.*, oil, to contaminated surfaces, *e.g.*, polluted water-air surfaces of lakes or oceans. For yet another example, secreted proteins destined for purification, *e.g.*, from a culture in a production-scale bioreactor, could be fused to an exopolysaccharide-associated protein. Cells expressing such a fusion protein would retain the secreted protein and could be used as a “biocolumn.” Such cells could be grown, either in suspension or as a biofilm, and subsequently pelleted to isolate the secreted protein of interest. If the fusion protein comprises a protease cleavage site separating the secreted protein of interest from the exopolysaccharide-associated protein, the protein of interest can be released from the bacterial pellet by protease digestion and subsequent elution.

[0083] Some aspects of this disclosure demonstrate the feasibility of an application of the bacterial biofilm matrix exopolysaccharide as a scaffold for localization and presentation of proteins and for delivery of functional enzymes to surfaces. The “proof of principle” experiments in the diarrheal pathogen *V. cholerae* described in more detail in the Examples section herein can be extended to other bacteria that also form biofilms incorporating structural exopolysaccharides and exopolysaccharide-associated proteins. Such bacteria include, for example, other biofilm-forming diarrheal pathogens [10-13]. However, it will be understood by those of skill in the art that the inventive concepts described herein can be applied to any biofilm matrix exhibiting similar characteristics to the biofilms specifically described. Additional applications of the technology provided herein will be apparent to those of skill in the art based on the instant disclosure. The exemplary embodiments listed above serve to illustrate the versatility of the instantly disclosed technology. The disclosure is not limited in this respect.

[0084] The term *bacterium* refers to a prokaryotic microorganism from the taxon *Bacteria*. Names of bacteria described herein are provided according to international rules for the naming of bacteria and taxonomic categories and for the ranking of them in the International Code of Nomenclature of Bacteria by the International Committee on Systematic Bacteriology (ICSB).

[0085] The term *Gram staining* refers to a method of staining bacteria developed by Hans Christian Gram, which allows differentiating bacterial species into two large groups (Gram-positive and Gram-negative, see, e.g., Gram, HC (1884). *Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten* (German). *Fortschritte der Medizin* 2: 185–189. English translation in: Brock, T.D. (1999). *Milestones in Microbiology 1546-1940* (2 ed.). ASM Press. pp. 215–218. ISBN 1-55581-142-6.); the contents of each of which are incorporated herein in their entirety). Gram staining detects peptidoglycan in Gram positive bacteria via crystal violet staining. Gram-positive bacteria retain crystal violet, resulting in a purple/blue color. In some embodiments, Gram staining also utilizes a counter stain, e.g., fuchsin or safranin, for detecting Gram-negative bacteria, typically resulting in a pink/red color.

[0086] The term *gram-negative*, in the context of bacteria, refers to bacteria that are not stained dark blue or violet by Gram staining, because they cannot retain the crystal-violet stain used in Gram staining. In some embodiments of Gram staining, a counterstain is used (e.g., safranin or fuchsin) that is retained by Gram-negative bacteria, staining them red or pink. Some Gram-negative bacteria are pathogens in humans, for example, some species of *Escherichia* sp., *Enterobacter* sp. (e.g., *Enterobacter cloacae*), *Salmonella* sp. (e.g., *Salmonella enteritidis*, *Salmonella typhi*), *Shigella* sp., *Pseudomonas* sp. (e.g., *Pseudomonas aeruginosa*), *Moraxella* sp. (e.g., *Moraxella catarrhalis*), *Neisseria* sp. (e.g., *Neisseria gonorrhoeae*, *Neisseria meningitidis*), *Helicobacter* sp., (e.g., *Helicobacter pylori*) *Stenotrophomonas* sp., *Vibrio* sp. (e.g., *Vibrio cholerae*), *Legionella* sp. (*Legionella pneumophila*), *Hemophilus* sp. (e.g., *Hemophilus influenzae*), *Klebsiella* sp. (e.g., *Klebsiella pneumoniae*), *Proteus* sp. (e.g., *Proteus mirabilis*), *Serratia* sp. (*Serratia marcescens*).

[0087] The term *Gram-positive*, in the context of bacteria, refers to bacteria that are stained dark blue or violet by Gram staining. Gram-positive bacteria retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. The cell walls of Gram-positive bacteria typically lack the outer membrane found in Gram-negative bacteria. Some Gram-positive bacteria are pathogens in humans, for example, some species of *Streptococcus* sp., *Staphylococcus* sp., *Corynebacterium* sp., *Listeria* sp., and *Clostridium* sp..

[0088] The term *non-pathogenic* refers to a microorganism, *e.g.*, a bacterium, that does not typically cause a disease in a subject exposed to it. In some embodiments, whether or not a bacterium is pathogenic depends on the type of exposure or administration of the bacterium to the subject. For example, a bacterium may be non-pathogenic or even beneficial if administered into the gastrointestinal tract, but may be pathogenic, *e.g.*, causing inflammation or other disease or disorder, upon exposure of an open wound, administration into the bloodstream, or inhalation. In some embodiments, a non-pathogenic bacterium is a bacterium that does not cause a disease or disorder in a subject, *e.g.*, a human subject, when administered orally, parenterally, subcutaneously, intravenously, intramuscularly, into the lung, into the blood, topically, or into the respiratory tract.

[0089] The term *pathogen* refers to an agent or organism that causes a disease or disorder in a subject, *e.g.*, a human subject. In some embodiments, the pathogen is a bacterium. Bacterial pathogens are well known to those of skill in the art. Bacterial genera comprising bacterial pathogens and exemplary bacterial pathogens include, without limitation, *Bacillus sp.* (*e.g.*, *Bacillus anthracis*) *Bordetella sp.* (*e.g.*, *Bordetella pertussis*); *Borrelia sp.* (*e.g.*, *Borrelia burgdorferi*); *Brucella sp.* (*e.g.*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*); *Campylobacter sp.* (*e.g.*, *Campylobacter jejuni*); *Chlamydia sp. and Chlamydophila sp.* (*e.g.*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila psittaci*); *Clostridium sp.* (*e.g.*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*); *Corynebacterium sp.* (*e.g.*, *Corynebacterium diphtheriae*); *Enterococcus sp.* (*e.g.*, *Enterococcus faecalis*, *Enterococcus faecium*); *Escherichia sp.* (*e.g.*, *Escherichia coli*, *Enterotoxigenic E. coli*, *enteropathogenic E. coli*; *E. coli* O157:H7); *Francisella sp.* (*e.g.*, *Francisella tularensis*); *Haemophilus sp.* (*e.g.*, *Haemophilus influenzae*); *Helicobacter sp.* (*e.g.*, *Helicobacter pylori*); *Legionella sp.* (*e.g.*, *Legionella pneumophila*); *Leptospira sp.* (*e.g.*, *Leptospira interrogans*); *Listeria sp.* (*e.g.*, *Listeria monocytogenes*); *Mycobacterium sp.* (*e.g.*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*); *Mycoplasma sp.* (*e.g.*, *Mycoplasma pneumoniae*); *Neisseria sp.* (*e.g.*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*); *Pseudomonas sp.* (*e.g.*, *Pseudomonas aeruginosa*); *Rickettsia sp.* (*e.g.*, *Rickettsia rickettsii*); *Salmonella sp.* (*e.g.*, *Salmonella typhi*, *Salmonella typhimurium*); *Shigella sp.* (*e.g.*, *Shigella sonnei*); *Staphylococcus sp.* (*e.g.*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*); *Streptococcus sp.* (*e.g.*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*); *Treponema sp.* (*e.g.*, *Treponema pallidum*); *Vibrio sp.* (*e.g.*, *Vibrio cholerae*); *Yersinia sp.* (*e.g.*, *Yersinia pestis*).

[0090] The term *biofilm* refers to an aggregate of microorganisms, *e.g.*, bacteria, on a surface. In some embodiments, a biofilm comprises bacterial cells that are embedded within an extracellular matrix comprising extracellular polysaccharides, also sometimes referred to as EPS. Extracellular polysaccharides are also referred to herein as exopolysaccharides. In some embodiments, extracellular matrix components, *e.g.*, exopolysaccharides, are produced and secreted by the cells within the biofilm. Bacterial cells within a biofilm adhere to the extracellular matrix of the biofilm by molecules, *e.g.*, proteins, expressed on the surface of the cells that bind to the biofilm EPS.

[0091] The term *exopolysaccharide* refers to a high-molecular-weight polysaccharide that is secreted by a microorganism. Typically, exopolysaccharides comprise a polymer of monosaccharides. Some exopolysaccharides, however, also comprise non-carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate). Exemplary exopolysaccharides include, without limitation acetan (*Acetobacter xylinum*), alginate (*Azotobacter vinelandii*), cellulose (*Acetobacter xylinum*), chitosan (*Mucorales sp.*), curdlan (*Alcaligenes faecalis var. myxogenes*), cyclophorans (*Agrobacterium sp.*, *Rhizobium sp.* and *Xanthomonas sp.*), dextran (*Leuconostoc mesenteroides*, *Leuconostoc dextranicum* and *Lactobacillus hilgardii*), emulsan (*Acinetobacter calcoaceticus*), galactoglucopolysaccharides (*Achromobacter sp.*, *Agrobacterium radiobacter*, *Pseudomonas marginalis*, *Rhizobium sp.* and *Zooglea sp.*), gellan (*Aureomonas elodea* and *Sphingomonas paucimobilis*), glucuronan (*Sinorhizobium meliloti*), N-acetyl-glucosamine (*Staphylococcus epidermidis*), N-acetyl-heparosan (*Escherichia coli*), hyaluronic acid (*Streptococcus equi*), indican (*Beijerinckia indica*), kefiran (*Lactobacillus hilgardii*), lentinan (*Lentinus elodes*), levan (*Alcaligenes viscosus*, *Zymomonas mobilis*, *Bacillus subtilis*), pullulan (*Aureobasidium pullulans*), scleroglucan (*Sclerotium rolfii*, *Sclerotium delfinii* and *Sclerotium glucanicum*), schizophyllan (*Schizophyllum commune*), stewartan (*Pantoea stewartii subsp. stewartii*), succinoglycan (*Alcaligenes faecalis var myxogenes*, *Sinorhizobium meliloti*), xanthan (*Xanthomonas campestris*), and welan (*Alcaligenes sp.*).

[0092] The term *exopolysaccharide-associated protein* refers to a protein that binds to an exopolysaccharide via non-covalent interactions with a K_D of $<10^{-5}$ M, $<10^{-6}$ M, $<10^{-7}$ M, $<10^{-8}$ M, $<10^{-9}$ M, $<10^{-10}$ M, $<10^{-11}$ M, or $<10^{-12}$ M. The term *exopolysaccharide-binding fragment*, in the context of exopolysaccharide-associated proteins, refers to a fragment of an exopolysaccharide-associated protein, wherein the fragment retains the exopolysaccharide binding characteristics of the parent protein, or binds the exopolysaccharide with a K_D of $<10^{-5}$ M, $<10^{-6}$ M, $<10^{-7}$ M, $<10^{-8}$ M, $<10^{-9}$ M, $<10^{-10}$ M, $<10^{-11}$ M, or $<10^{-12}$ M.

Exopolysaccharide-associated proteins are known to those of skill in the art. Some exemplary exopolysaccharide-associated proteins and exopolysaccharide-binding fragments thereof are described herein, including, but not limited to Bap1, RbmA, RbmC, and HlyA. Below are exemplary, representative sequences of Bap1, RbmA, RbmC, and HlyA from *Vibrio cholerae*. It is to be understood that these sequences are for illustration purposes only and are not meant to limit the scope of this disclosure. Those of skill in the art will know or will be able to ascertain additional sequences of Bap1, RbmA, RbmC, and HlyA, and of additional exopolysaccharide-binding proteins, both from *Vibrio* sp. and from other bacteria based on this disclosure and knowledge in the art. The disclosure is not limited in this respect.

[0093] Bap1

>vch:VC1888 hemolysin-like protein (A)

MKQTKTLTAISVLALSHLMTQSTAFASSSSDIQTKLKWSWSTSVFHPESNQVMAAPI
 VVQLNDDNGDGKIDEKDVADIIVVTFEGNKYANGGYIRALSGVDGSELWSYSNGGV
 IADARYAPAAADLDGDGLIEIVSTSALTPYINILDHQGNIKKQLLKSASGWRSVGDIA
 LADINGDGNIEILAADGVYSYESGLLFSHDWAPSSIAFDSNGDGQREVFANGTLYQN
 NGAYLWQYQANDTVWFSSVANLDGDDKPELVVSVPASLSTPENSEIAVLEHDGSVK
 WRVNNLSNPGGSVQAVSSFLGKPSSSATTVDAQSAVYGYTDWAHQQRVLAENHQL
 AIRSGAVVDAIGANSQNMIGGSGGSLSTIDTSKVRAIDVTYGKNKYTWKYGVLEMSF
 TLDNGAKVTVGSKDSAFTYLGLEWKTKTVPYLGVWRTKTVSYWFFGWHTKQVA
 YLAPVWKEKTIPYAVPVTLSKSTTVRYDIPQGSQLLGMNVWSKEKHLFKHKQQVNA
 VQFLVGKVTADQSHMGIVYAGYYAVDMYDAQGNKVWSVANDDLNSGKIGVSAYD
 FTGDGIDEVLVQDRLRMRILDGQTGRVMGIIANSSGTLWEYPVVADLEGNNNASLIM
 VANDYDRESQVNHGVFVYESANPSKPWRNATRIWNQYAFNFSDINANGTIPTNAQP
 SWLTHNSFRSATIRVPLK (SEQ ID NO: 1)

EF Hand domain: residues 63-78

VCBS domain: residues 77-135

PQQ enzyme repeat: residues 90-113

FG-GAP repeat: residues 119-13

VCBS domain: residues 125-184

FG-GAP repeat: residues 170-185

FG-GAP repeat: residues 246-267

FG-GAP repeat: residues 555-571

[0094] RbmA

>vch:VC0928 hypothetical protein (A)

MSNFKGSIMNKRHYYLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
 ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
 TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
 QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
 DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 2)

Signal peptide: residues 1-30

[0095] RbmC

>vch:VC0930 hemolysin-like protein (A)

MTSHYIALAVGLLSLSSNVVQATTNEAEGCIISRLNGEKYCLKVGERSGYSLPSWIYA
 HPVDVQAPSGVSVMLSDWDNLSYNRLAVFDRTYGNEDLKNVKAYNLAYLDFSKPR
 SMRVLASETYPEACIVSRQTGERFCLKEGERSGYSLPAYIYGHEVDVEAPLGLGVML
 SDWDNLSYNRLAVFGGNTQNEQMRAVKAYNGETLDFSKPRSMRVVPYDGDSSALN
 MKLKWSWQGSFAFPNSNQVMVTPIVAQLNDDNGDGKIDEKDVADLIVVTFEGNKY
 ANGGLVRALSGVDGSELWSYANGGVIADARYSPA VGDLDGDGIVEIVTTNNRDQFI
 TILDNQGNIKKQIPTTESGWRIVGDITLADLDHDGSVEILAADGVYNYHSGLVFNHP
 WAPSSINVDVDGDQQQEVFSGGTLFQNNGAINWQYQANDAVWFSSLVNLDNDAEP
 EIVASVPATFATGDNARFAVLEHDGTIKWEINNTANPGGGVQAVSNFLGKAQAVETS
 EFSKVYGYQPNNNPASIALAVDGKISVRSGFAIDAIGASASTLVGGTGGNLNAAVNV
 KDIKAIDLTWGKYYWGGYHLLALDFRMSNGSVISMGSKNYAYSQKQTERFTVPAGSR
 IKGIKAWTAGWLLDGVQFELATQNGTNDLDVKGIVYAGYAAVDMYNSKGERVWS
 VANDDTGSGKIGVSA YDFDNDGIDEVLVQDHARVRVLDGKTGKERASLAHSTATL
 WEYPIVVDLEGDNNAELIVAANDFDRQYSINHGVIYVYQSADSSKPWKNA TRIWNQH
 AFHLTNINQDGTLPFVEPSWLSHNTYRSSTLRAAVGGESPIFGYSNTQQSQRVVTAD
 NLMYLRSGFAIDAIGTTVNNLVGGPVQGTNGGVLRAPIALDQLQSVEVTSGLYNWG
 GYHIVAIFKFTMKDGSSVLLGSTHYASNKKVETYTPVQPKRIKQINVWTGGWLVEGF
 QFVY (SEQ ID NO: 3)

Signal peptide: residues 1-20

FG-GAP repeat: residues 311-328

FG-GAP repeat: residues 684-709prism

Jacalin type lectin (beta prism) domain: residues 529-639

Jacalin type lectin (beta prism) domain: residues 832-957

[0096] HlyA

>vch:VCA0219 haemolysin; K10948 hemolysin (A)

MPKLNRCIAIIFTILSAISSPTLLANINEPSGEAADIISQVADSHAIKYNAADWQAED
NALPSLAELRDLVINQQKRVLVDFSQISDAEGQAEMQAQFRKAYGVGFANQFIVITE
HKGELLFTFPDQAEVDPQLLEAPRTARLLARSGFASPAPANSETNTLPHVAFYISVN
RAISDEECTFNNSWLWKNEKGSRPFCCKDANISLIYRVNLERSLQYGIVGSATPDAKIV
RISLDDDDSTGAGIHLNDQLGYRQFGASYTTLDAYFREWSTDAIAQDYRFVFNASNNK
AQILKTFPVDNINEKFERKEVSGFELGVTGGVEVSGDGPKAKLEARASYTQSRWLTY
NTQDYRIERNAKNAQAVSFTWNRQQYATAESLLNRSTDALWVNTYPVDVNRISPLS
YASFVPKMDVIYKASATETGSTDFIIDSSVNIRPIYNGAYKHYYVVGHAHQFYHGFEDT
PRRRITKSASFTVDWDHPVFTGGRPVNLQLASFNNRCIQVDAQGRLAANTCDSQQSA
QSFIYDQLGRYVSASNTKLCLDGEALDALQPCNQNLQTRWEWRKGTDELTVNYSGE
SLGHDKQTGELGLYASSNDAVSLRTITAYTDVFNAQESSPILGYTQGKMNQQRVGQ
DHRLYVRAGAAIDALGSASDLLVGGNGGSLSSVDLSGVKSITATSGDFQYGGQQLV
ALTFTYQDGRQQTVGSKAYVTNAHEDRFDLPAAAKITQLKIWSDDWLVKGVQFDL
N (SEQ ID NO: 4)

Signal peptide: residues 1-24

Hemolysin N domain: residues 2-187

Leukocidin domain: residues 215-477

Ricin-type beta trefoil domain (a lectin domain): residues 484-599

Jacalin-like=beta prism domain(a lectin domain): residues 620-741

[0097] The term *protein* is used herein interchangeably with the term *polypeptide*, and refers to a polymer of amino acid residues linked together by peptide bonds. The term refers to proteins, polypeptides, and peptides of any size, structure, or function. A protein may be a naturally occurring protein, a fragment of a naturally occurring protein, or an engineered protein, for example, a recombinant protein, or a protein in which one or more amino acid residues are non-naturally occurring residues, *e.g.*, modified amino acid residues, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A protein may also be a single molecule or may be a multi-molecular complex. In some embodiments, the term *protein*

refers to a polymer of three or more amino acids linked by a peptide bond, *e.g.*, between 3 and 30, 12 and 60, or 30 and 300, or over 300 amino acids in length. In some embodiments, the protein includes one or more amino acids that does not occur in nature. In some embodiments, the polypeptide includes only natural amino acids. In some embodiments, a protein includes one or more post-translational or post-synthesis modifications, *e.g.*, a glycosylation, amidation, phosphorylation, SUMOylation, PEGylation, or nitrosylation.

[0098] The terms *conjugating*, *conjugated*, and *conjugation* refer to an association of two entities, for example, of two molecules (*e.g.*, two proteins), two domains (*e.g.*, a binding domain and a catalytic domain), or a protein and an agent, *e.g.*, a protein binding domain and a small molecule. The association can be, for example, via a direct or indirect (*e.g.*, via a linker) covalent linkage or via non-covalent interactions. In some embodiments, the association is covalent. In some embodiments, two molecules are conjugated via a linker connecting both molecules. For example, in some embodiments where two proteins are conjugated to each other, *e.g.*, an exopolysaccharide-associated protein and an antigen or enzyme, to form a protein fusion, the two proteins may be conjugated via a polypeptide linker, *e.g.*, an amino acid sequence connecting the C-terminus of one protein to the N-terminus of the other protein.

[0099] The term *fusion protein* refers to a protein comprising two heterologous proteins, protein domains, or protein fragments, that are covalently bound to each other, either directly or indirectly (*e.g.*, via a linker), via a peptide bond. In some embodiments, a fusion protein is encoded by a nucleic acid comprising the coding region of a protein in frame with a coding region of an additional protein, without intervening stop codon, thus resulting in the translation of a single protein in which the proteins are fused together.

[00100] The term *heterologous*, in the context of molecules, *e.g.*, of proteins, peptides, nucleic acids, and small molecules, refers to a molecule that is not in its natural context, *e.g.*, in that it is conjugated with another molecule that it is not naturally conjugated to, or it is expressed in a cell that does not naturally express the molecule. For example, a heterologous protein in the context of a fusion protein is a protein that does not naturally occur as a fusion with the specific fusion partner. For example, an antigen that is not naturally conjugated to, or fused with, an exopolysaccharide-associated protein, *e.g.*, Bpa1, RbmA, RbmC, or HlyA, is a heterologous protein in the context of conjugates or fusion proteins comprising the antigen and the exopolysaccharide-associated protein. Similarly, a nucleic acid that is not typically operably linked to another nucleic acid is heterologous in the context of a nucleic acid construct comprising both nucleic acids operably linked together.

[00101] The term *secreted protein* refers to a protein that is secreted from a cell, for example, from a bacterium. Accordingly, in some embodiments, a secreted protein is a protein that is synthesized within the cell and then released into the extracellular space surrounding the cell. In some embodiments, the protein comprises a signaling peptide, *e.g.*, at the N-terminus of the protein, that targets the protein for secretion. Such signaling peptides, also sometimes referred to as signal peptides or signal sequences, are well known to those of skill in the art. Exemplary signal peptides include, but are not limited to those disclosed by the SPdb Signal Peptide Resource (see, *e.g.*, Choo KH, Tan TW, Ranganathan S. 2005. *SPdb - a signal peptide database*. BMC Bioinformatics 6:249, accessible at “proline(.)bic(.)nus(.)edu(.)sg/spdb” – last accessed on March 13, 2013; the entire contents of each of which are incorporated herein by reference); the Signal Peptide Database (see, *e.g.*, Katja Kapp, *Signal Peptide Database*. Heidelberg & Thpr.net, accessible at “www(.)signalpeptide(.)de/index.php?m=listspdb_bacteria” - last accessed on March 13, 2013, the entire contents of which are incorporated herein by reference). Exemplary signal suitable peptide sequences include, without limitation, signal peptide sequences from Lectin-like protein BA14k (MNIFKQTCVGAFAVIFGATSIAPTMA, SEQ ID NO: 6); Antigen 85-C (MKFLQQMRKLFGLAAKFPARLTIAVIGTALLAGLVGVGDTAIAVA, SEQ ID NO: 7); Alginate biosynthesis protein algF (MNPMTRRHTWTRLACALSLGVAAFAAQA, SEQ ID NO: 8); Probable N-acetylmuramoyl-L-alanine amidase amiA (MSTFKLLKTLTSRRQVLKTGLAALTLSGMSHAHA, SEQ ID NO: 9); Alpha-amylase (MKLAACFLTLLPGFAVA, SEQ ID NO: 10); Beta-lactamase (MHPSTSRPSRRTLLTATAGAALAAATLVPGTAHASSGGR; SEQ ID NO: 11); and Chitinase 63 (MRFRHKAAALAATLALPLAGLVGLASPAQA, SEQ ID NO: 12). Additional suitable signal peptide sequences will be apparent to those of skill in the art, and the disclosure is not limited in this respect.

[00102] In some embodiments, a secreted protein is released from a cell into the extracellular space in a manner that no association between the cell and the protein remains after release. In some embodiments, a secreted protein is released from a cell into the extracellular space, but remains attached to the surface of the cell via non-covalent interaction.

[00103] The term *extracytoplasmic protein* refers to a protein of a cell that is expressed on the surface of the cell and abuts or protrudes into the surrounding extracellular space. In some embodiments, an extracytoplasmic protein is not a secreted protein. For

example, in some embodiments, an extracytoplasmic protein comprises a transmembrane domain that spans the cell membrane, thus anchoring the protein on the surface of the cell.

[00104] The term *protein domain* refers to a conserved part of a protein sequence and structure that can evolve, fold, function, and/or exist independently of the rest of the protein chain. A domain typically forms a characteristic three-dimensional structure and often can fold independently and stably regardless of its sequence context. Many proteins consist of several structural domains. Typically, protein domains vary in length from between about 25 amino acids up to 500 amino acids in length, even though longer and shorter protein domains exist. Some of the shortest protein domains such as zinc fingers, are stabilized by metal ions or disulfide bridges. Protein domains often form structural or functional units, such as the calcium-binding EF hand domain of calmodulin. Independently stable protein domains can be recombined or fused to produce chimeric proteins having the characteristics of the fused protein domains. For example, an exopolysaccharide binding protein domain can be fused with a binding domain of an antibody to produce a divalent binding protein.

[00105] The term *β -prism lectin domain* is used interchangeably with the term *jacalin-like lectin domain*, and refers to a mannose-binding lectin domain with a beta-prism fold consisting of three 4-stranded beta-sheets, with an internal pseudo 3-fold symmetry. Some lectins comprising this domain stimulate distinct T- and B-cell functions, such as Jacalin, which binds to the T-antigen and acts as an agglutinin. This domain is found in 1 to 6 copies in lectins. The domain is also found in the salt-stress induced protein from rice and an animal prostatic spermine-binding protein. Proteins containing this domain include: Jacalin, a tetrameric plant seed lectin and agglutinin from *Artocarpus heterophyllus* (jackfruit), which is specific for galactose; Artocarpin, a tetrameric plant seed lectin from *A. heterophyllus*; Lectin MPA, a tetrameric plant seed lectin and agglutinin from *Maclura pomifera* (Osage orange); Heltuba lectin, a plant seed lectin and agglutinin from *Helianthus tuberosus* (Jerusalem artichoke); Agglutinin from *Calystegia sepium* (Hedge bindweed); and Griffithsin, an anti-viral lectin from red algae (*Griffithsia* species). See, e.g., Jeyaprakash et al., (2002) "Crystal structure of the jacalin-T-antigen complex and a comparative study of lectin-T-antigen complexes". *J. Mol. Biol.* 321 (4): 637–45; Jeyaprakash et al., (2004) "Structural basis for the carbohydrate specificities of artocarpin: variation in the length of a loop as a strategy for generating ligand specificity". *J. Mol. Biol.* 338 (4): 757–70; Lee et al., (1998) "Structure of the complex of *Maclura pomifera* agglutinin and the T-antigen disaccharide, Galbeta1,3GalNAc". *J. Biol. Chem.* 273 (11): 6312–8; Bourne et al., (1999) "*Helianthus tuberosus* lectin reveals a widespread scaffold for mannose-binding lectins". *Structure* 7 (12):

1473–82; Bourne et al., (2004) "The crystal structure of the Calystegia sepium agglutinin reveals a novel quaternary arrangement of lectin subunits with a beta-prism fold". J. Biol. Chem. 279 (1): 527–33; and Ziolkowska et al., (2006) "Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding". Structure 14 (7): 1127–35; the entire contents of each of which are incorporated herein by reference.

Representative sequences and consensus sequences of β -prism lectin domains are well known to those of skill in the art, *e.g.*, as published in Raval et al., *A database analysis of jacalin-like lectins: sequence–structure–function relationships* Glycobiology (2004) 14(12): 1247-1263; the entire contents of which are incorporated by reference. Based on the knowledge in the art, those of skill in the art will be able to ascertain whether a protein domain is a β -prism lectin domain and whether a protein comprises a β -prism lectin domain. Methods and algorithms for protein domain analysis and alignment are well known to those of skill in the art, and the disclosure is not limited in this respect.

[00106] The term *FG-GAP domain* is used interchangeably with the term *FG-GAP repeat*, and refers to an extracellular repeat that is found in up to seven copies in alpha integrins. The FG-GAP repeat has been predicted to fold into a beta propeller structure, and is called the FG-GAP repeat after two conserved motifs in the repeat. FG-GAP repeats are found in the N terminus of integrin alpha chains, a region that has been shown to be important for ligand binding. A putative Ca^{2+} binding motif is found in some of the repeats. Representative sequences and consensus sequences of β -prism lectin domains are well known to those of skill in the art. See, *e.g.*, Springer TA et al., Proc Natl Acad Sci U S A 1997;94:65-72. Folding of the N-terminal, ligand-binding region of integrin alpha- subunits into a beta-propeller domain; and Loftus JC, Smith JW, Ginsberg MH; J Biol Chem 1994;269:25235-25238.: Integrin-mediated cell adhesion: the extracellular face; the entire contents of each of which are incorporated herein by reference. Based on the knowledge in the art, those of skill in the art will be able to ascertain whether a protein domain is an FG-GAP domain and whether a protein comprises an FG-GAP domain. Methods and algorithms for protein domain analysis and alignment are well known to those of skill in the art, and the disclosure is not limited in this respect.

[00107] The term *linker* refers to a chemical group or a molecule linking two adjacent molecules or moieties, *e.g.*, a binding domain and a cleavage domain of a nuclease. Typically, the linker is positioned between, or flanked by, two groups, molecules, or other moieties and connected to each one via a covalent bond, thus connecting the two. In some embodiments, the linker is an amino acid or a plurality of amino acids (*e.g.*, a peptide or

protein). In some embodiments, the linker is an organic molecule, group, polymer, or chemical moiety.

[00108] The terms *nucleic acid* and *nucleic acid molecule* refer to a compound comprising a nucleobase and an acidic moiety, *e.g.*, a nucleoside, a nucleotide, or a polymer of nucleotides. Typically, polymeric nucleic acids, *e.g.*, nucleic acid molecules comprising three or more nucleotides are linear molecules, in which adjacent nucleotides are linked to each other via a phosphodiester linkage. In some embodiments, “nucleic acid” refers to individual nucleic acid residues (*e.g.* nucleotides and/or nucleosides). In some embodiments, “nucleic acid” refers to an oligonucleotide chain comprising three or more individual nucleotide residues. The terms *oligonucleotide* and *polynucleotide* can be used interchangeably to refer to a polymer of nucleotides (*e.g.*, a string of at least three nucleotides). In some embodiments, a nucleic acid encompasses RNA as well as single and/or double-stranded DNA. Nucleic acids may be naturally occurring, for example, in the context of a genome, a transcript, an mRNA, tRNA, rRNA, siRNA, snRNA, a plasmid, cosmid, chromosome, chromatid, or other naturally occurring nucleic acid molecule. On the other hand, a nucleic acid molecule may be a non-naturally occurring molecule, *e.g.*, a recombinant DNA or RNA, an artificial chromosome, an engineered genome, or fragment thereof, or a synthetic DNA, RNA, DNA/RNA hybrid, or including non-naturally occurring nucleotides or nucleosides. Furthermore, the terms *nucleic acid*, *DNA*, *RNA*, and/or similar terms include nucleic acid analogs, *e.g.*, analogs having other than a phosphodiester backbone. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, *etc.* Where appropriate, *e.g.*, in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone modifications. A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a nucleic acid is or comprises natural nucleosides (*e.g.* adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose);

and/or modified phosphate groups (*e.g.*, phosphorothioates and 5'-*N*-phosphoramidite linkages).

[00109] The term *recombinant nucleic acid* refers to a nucleic acid molecule that does not occur in nature, but has been engineered, *e.g.*, in that it has been artificially synthesized, or produced from recombining or otherwise altering the nucleotide sequence of a naturally occurring nucleic acid. Suitable nucleic acid synthesis and engineering methods are well known to those of skill in the art.

[00110] The terms *small molecule* refers to an organic compound, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that has a relatively low molecular weight. Typically, an organic compound contains carbon. An organic compound may contain multiple carbon-carbon bonds, stereocenters, and other functional groups (*e.g.*, amines, hydroxyl, carbonyls, or heterocyclic rings). In some embodiments, small molecules are monomeric organic compounds that have a molecular weight of less than about 1500 g/mol. In certain embodiments, the molecular weight of the small molecule is less than about 1000 g/mol or less than about 500 g/mol. In certain embodiments, the small molecule is a drug, for example, a drug that has already been deemed safe and effective for use in humans or animals by the appropriate governmental agency or regulatory body. In certain embodiments, the organic molecule is known to bind and/or cleave a nucleic acid. In some embodiments, the organic compound is an enediyne. In some embodiments, the organic compound is an antibiotic drug, for example, an anticancer antibiotic such as dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin, or a derivative thereof.

[00111] The term *covalent bond* refers to a form of chemical bonding that is characterized by the sharing of one or more pairs of electrons between atoms. A covalent bond formed between two reactive moieties may, for example, be an amide bond, an acyl bond, a disulfide bond, an alkyl bond, an ether bond, or an ester bond. A covalent bond formed between two reactive moieties may be, for example, a carbon-carbon bond, a carbon-oxygen bond, a carbon-nitrogen bond, a carbon-sulfur bond, a sulfur-sulfur bond, a carbon-phosphorus bond, a phosphorus-oxygen bond, or a phosphorus-nitrogen bond.

[00112] The term *non-covalent bond* is used interchangeably with the term *non-covalent interaction*, and refers to a type of interaction between two molecules that does not involve the sharing of electrons between the molecules, but involves variations of electromagnetic, electrostatic, or hydrophobic interactions.

[00113] The term *enzyme* refers to a molecule, for example, a peptide, a protein, or a nucleic acid (for example, a ribozyme or DNAzyme) that catalyzes a chemical reaction. An

enzyme may be a biomolecule (a molecule made by a living organism), a derivative of a biomolecule (*e.g.*, a mutated biomolecule, a fragment of a biomolecule, and/or a fusion product of a biomolecule, or fragment thereof, with a second molecule), or an artificially made molecule (*e.g.*, a synthetic protein or nucleic acid). An enzyme may be an oxidoreductase, transferase, polymerase, hydrolase lyase, synthase, isomerase, or ligase. Accordingly, a protease and a nuclease are non-limiting examples of enzymes. In certain embodiments, the enzyme is a protein. In certain embodiments, the enzyme is a nucleic acid. In certain embodiments, the enzyme is an RNA enzyme, also referred as a ribozyme. In certain embodiments, the enzyme is a DNA enzyme, also referred to as a DNAzyme.

[00114] The term *enzyme substrate* refers to a molecule upon which an enzyme acts. An enzyme substrate is bound by an enzyme and transformed into one or more products in a chemical reaction catalyzed by the enzyme. The reaction product or products are usually released from the enzyme. For example, a protease catalyzes the hydrolysis of an amide bond in a protease substrate peptide or protein. The substrate peptide of a protease is generally bound specifically, meaning that only a peptide of a certain amino acid sequence or with a sequence similar to a consensus sequence is bound by the protease and cleaved into two or more fragments in a hydrolysis reaction.

[00115] The term *binding agent* refers to a molecule that binds to another molecule. In some embodiments, the binding is through non-covalent interaction. In some embodiments, the binding is specific, meaning that the binding agent binds only one particular type of molecule, or a narrow class of highly similar molecules with high affinity. Non-limiting examples of binding agents are antibodies, antibody fragments, aptamers, and adnectins. In some embodiments, the term binding agent, refers to a molecule, for example, a protein, nucleic acid, carbohydrate, or small molecule, that binds another molecule, referred to herein as a target molecule, with high affinity, *e.g.*, with a dissociation constant (K_D) of less than 10^{-6} M, of less than 10^{-7} M, of less than 10^{-8} M, of less than 10^{-9} M, or of less than 10^{-10} M. In some embodiments, a binding agent is or comprises a protein, a peptide, an antibody, an antibody fragment, a ligand, a receptor, or a small molecule, that binds to a target molecule with a K_D as specified above. In some embodiments, the binding agent binds to the target molecule with high affinity, *e.g.* with a K_D of less than 10^{-8} , of less than 10^{-9} M, of less than 10^{-10} M, of less than 10^{-11} M, or of less than 10^{-12} M. In some embodiments, the binding agent binds to the target molecule with high specificity, *e.g.*, in that it does not bind to molecules other than the target molecule with a K_D of less than 10^{-6} M, of less than 10^{-7} M, or of less than 10^{-8} M.

[00116] The term *antibody* refers to a protein that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence. For example, an antibody can include a heavy (H) chain variable region (abbreviated herein as VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term *antibody* encompasses *antigen-binding fragments* of antibodies (*e.g.*, single chain antibodies; Fab, Fab', and sFab fragments; F(ab')₂ fragments; Fd fragments; Fv fragments; single-chain Fv antibodies (scFv); dAb fragments, and nanobodies) as well as complete antibodies.

[00117] The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" ("FR"). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. *et al.* (1987) *J. Mol. Biol.* 196:901-917, see also www.hgmp.mrc.ac.uk). Kabat definitions are used herein. Each VH and VL is typically composed of three CDR's and four FR's, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[00118] The term *antibody fragment* refers to any derivative of an antibody which is less than full-length. Preferably, the antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')₂, scFv, Fv, dsFv, diabody, single variable domain, and Fd fragments. The antibody fragment may be produced by any means. For instance, the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody or it may be recombinantly produced from a gene encoding the partial antibody sequence. Alternatively, the antibody fragment may be wholly or partially synthetically produced. The antibody fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multi-molecular complex. A functional antibody fragment will typically comprise at least about 50 amino acids and more typically will comprise at least about 200 amino acids. Single-chain Fvs (scFvs) are recombinant antibody fragments consisting of only the variable light chain (VL) and variable heavy chain (VH) covalently connected to one another by a polypeptide

linker. Either VL or VH may be the NH₂-terminal domain. The polypeptide linker may be of variable length and composition so long as the two variable domains are bridged without serious steric interference. Typically, the linkers are comprised primarily of stretches of glycine and serine residues with some glutamic acid or lysine residues interspersed for solubility. Diabodies are dimeric scFvs. The components of diabodies typically have shorter peptide linkers than most scFvs, and they show a preference for associating as dimers. An Fv fragment is an antibody fragment which consists of one VH and one VL domain held together by non-covalent interactions. The term dsFv is used herein to refer to an Fv with an engineered intermolecular disulfide bond to stabilize the VH-VL pair. An F(ab')₂ fragment is an antibody fragment essentially equivalent to that obtained from immunoglobulins (typically IgG) by digestion with an enzyme pepsin at pH 4.0-4.5. The fragment may be recombinantly produced. A Fab' fragment is an antibody fragment essentially equivalent to that obtained by reduction of the disulfide bridge or bridges joining the two heavy chain pieces in the F(ab')₂ fragment. The Fab' fragment may be recombinantly produced. A Fab fragment is an antibody fragment essentially equivalent to that obtained by digestion of immunoglobulins (typically IgG) with the enzyme papain. The heavy chain segment of the Fab fragment is the Fd piece.

[00119] The term *antigen-binding fragment* of an antibody refers to one or more antibody fragments that retain the ability to specifically bind to a target of interest. Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) that retains functionality. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules known as single chain Fv (scFv). See *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883.

[00120] Antibody fragments can be obtained using any appropriate technique including conventional techniques known to those with skill in the art. The term

“monospecific antibody” refers to an antibody that displays a single binding specificity and affinity for a particular target, *e.g.*, epitope. This term includes a monoclonal antibody or monoclonal antibody composition, which refer to a preparation of antibodies or fragments thereof of single molecular composition. The term *isotype* refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by heavy chain constant region genes.

[00121] The term *binding affinity* refers to the apparent association constant or K_A . The K_A is the reciprocal of the dissociation constant (K_D). A binding agent may, for example, have a binding affinity for a particular target molecule that is associated with a K_D of $<10^{-6}$ M, $<10^{-7}$ M, $<10^{-8}$ M, $<10^{-9}$ M, $<10^{-10}$ M, or $<10^{-11}$ M, with lower K_D values being associated with higher affinity. Higher affinity binding of a binding agent to a first target relative to a second target can be indicated by a higher K_A (or a smaller numerical value K_D) for binding the first target than the K_A (or numerical value K_D) for binding the second target. In such cases, the binding agent specifically binds the first target relative to the second target. Differences in binding affinity (*e.g.*, for specificity or other comparisons) can be at least 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, 1000, 10000, 100000, or 1000000 -fold.

[00122] Binding affinity can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (*e.g.*, using a fluorescence assay). Exemplary conditions for evaluating binding affinity are in PBS (phosphate buffered saline) at pH 7.2 at 30°C. These techniques can be used to measure the concentration of bound and free binding protein as a function of binding protein (or target) concentration. The concentration of bound binding protein ([Bound]) is related to the concentration of free binding protein ([Free]) and the concentration of binding sites for the binding protein on the target where (N) is the number of binding sites per target molecule by the following equation:

$$[\text{Bound}] = N \cdot [\text{Free}] / ((1/K_A) + [\text{Free}]).$$

[00123] It is not always necessary to make an exact determination of K_A , though, since sometimes it is sufficient to obtain a quantitative measurement of affinity, *e.g.*, determined using a method such as ELISA or FACS analysis, is proportional to K_A , and thus can be used for comparisons, such as determining whether a higher affinity is, *e.g.*, 2-fold higher, to obtain a qualitative measurement of affinity, or to obtain an inference of affinity, *e.g.*, by activity in a functional assay, *e.g.*, an *in vitro* or *in vivo* assay.

[00124] The term *epitope* refers to the site on a target molecule that is bound by a binding agent (*e.g.*, an antibody such as a Fab or full length antibody). In the case where the target molecule is a protein, the site can be entirely composed of amino acid components,

entirely composed of chemical modifications of amino acids of the protein (*e.g.*, glycosyl moieties), or composed of combinations thereof. Overlapping epitopes include at least one common amino acid residue.

[00125] One or more regions of an antibody can be human or effectively human. For example, one or more of the variable regions can be human or effectively human. For example, one or more of the CDRs can be human, *e.g.*, HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3. Each of the light chain CDRs can be human. HC CDR3 can be human. One or more of the framework regions can be human, *e.g.*, FR1, FR2, FR3, and FR4 of the HC or LC. In one embodiment, all the framework regions are human, *e.g.*, derived from a human somatic cell, *e.g.*, a hematopoietic cell that produces immunoglobulins or a non-hematopoietic cell. In one embodiment, the human sequences are germline sequences, *e.g.*, encoded by a germline nucleic acid. One or more of the constant regions can be human or effectively human. In one embodiment, at least 70, 75, 80, 85, 90, 92, 95, or 98% of, or the entire of, the antibody can be human or effectively human.

[00126] All or part of an antibody can be encoded by an immunoglobulin gene or a segment thereof. Exemplary human immunoglobulin genes include the kappa, lambda, alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin “light chains” (about 25 KDa or 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH--terminus. Full-length immunoglobulin “heavy chains” (about 50 KDa or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, *e.g.*, gamma (encoding about 330 amino acids).

[00127] The term *ligand* refers to a binding molecule that binds non-covalently to a second binding molecule with high affinity. In some embodiments, a high-affinity bond is characterized by a $K_D < 10^{-6}$ M, a $K_D < 10^{-7}$ M, a $K_D < 10^{-8}$ M, a $K_D < 10^{-9}$ M, a $K_D < 10^{-10}$ M, a $K_D < 10^{-11}$ M, or a $K_D < 10^{-12}$ M. In some embodiments, the ligand is a small molecule. In some embodiments, the ligand is a peptide or protein. In some embodiments, the ligand is a nucleic acid.

[00128] The term *isolated*, in the context of a composition refers to a composition that is removed from at least 90% of at least one component of a natural sample from which the isolated composition can be obtained. In some embodiments, compositions produced artificially or engineered are also encompassed within the scope of the term *isolated*.

[00129] The terms *therapeutically effective dosage*, *therapeutically effective amount*, or *effective amount* is an amount that, when administered to a subject, results in a desired clinical effect, *e.g.*, in the vaccination or immunization of a subject, or in an amelioration of a symptom that is clinically manifest in the subject.

[00130] The term *affinity tag* refers to a tag, for example, a peptide tag that is N-terminally or C-terminally fused to a protein, *e.g.*, an exopolysaccharide-associated protein, that binds to a ligand with high affinity and thus allows for the detection and/or isolation of the tagged protein. Affinity tags are well known to those of skill in the art and examples of suitable affinity tags include, but are not limited to, biotin carboxylase carrier protein (BCCP) tags, myc-tags, calmodulin-tags, FLAG-tags, hemagglutinin (HA)-tags, polyhistidine tags, also referred to as histidine tags or His-tags, maltose binding protein (MBP)-tags, nus-tags, glutathione-S-transferase (GST)-tags, green fluorescent protein (GFP)-tags, thioredoxin-tags, S-tags, Softags (*e.g.*, Softag 1, Softag 3), strep-tags, biotin ligase tags, FAsH tags, V5 tags, and SBP-tags. In some embodiments, the tag is a biotin tag or a biotin variant tag, for example, desthiobiotin (DTB). Some affinity tags and methods for the use of affinity tags are described herein and other suitable tags and methods will be apparent to those of skill in the art, *e.g.*, as described in Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S. Comparison of affinity tags for protein purification. *Protein Expr Purif.* 2005;41:98-105; and Kimple, M.E., and Sondek, J. *Overview of affinity tags for protein purification.* *Curr Protoc Protein Sci.* 2004 Sep; Chapter 9:Unit 9.9; the contents of each of which are incorporated in their entirety herein for disclosure of affinity tags and related methods. Those of skill in the art will appreciate that the disclosure is not limited in this respect.

[00131] The term *detection agent* is used interchangeably with the term *detectable label*, and refers to a moiety that has at least one element, isotope, or a structural or functional group incorporated that enables detection of a molecule, *e.g.*, a protein or polypeptide, or other entity, to which the detection agent is attached. A detection agent can be directly attached (*e.g.*, via a bond) or can be attached by a tether or linker. A detection agent can also be conjugated to the molecule, *e.g.*, via non-covalent interactions. It will be appreciated that a detection agent may be attached to or incorporated into a molecule, for example, an exopolysaccharide-associated protein, a fusion protein, a polypeptide, or other entity, at any position, but preferably in a manner that does not interfere with the structural or functional characteristics of the molecule, *e.g.*, the binding of the molecule (*e.g.*, a protein) to a binding partner (*e.g.*, an exopolysaccharide). In general, a detection agent can fall into any one (or more) of five classes: a) an agent which contains isotopic moieties, which may be radioactive

or heavy isotopes, including, but not limited to, ^2H , ^3H , ^{13}C , ^{14}C , ^{15}N , ^{18}F , ^{31}P , ^{32}P , ^{35}S , ^{67}Ga , ^{76}Br , $^{99\text{m}}\text{Tc}$ (Tc-99m), ^{111}In , ^{123}I , ^{125}I , ^{131}I , ^{153}Gd , ^{169}Yb , and ^{186}Re ; b) an agent which contains an immune moiety, which may be an antibody or antigen, which may be bound to an enzyme (*e.g.*, such as horseradish peroxidase); c) an agent comprising a colored, luminescent, phosphorescent, or fluorescent moiety (*e.g.*, such as the fluorescent label fluorescein isothiocyanate (FITC)); d) an agent which has one or more photo affinity moieties; and e) an agent which is a ligand for one or more known binding partners (*e.g.*, biotin-streptavidin, His -NiTNAFK506-FKBP). In certain embodiments, a detection agent comprises a radioactive isotope, preferably an isotope which emits detectable particles, such as β particles. In certain embodiments, a detection agent comprises a fluorescent moiety. In certain embodiments, the detection agent comprises a dye, *e.g.*, a fluorescent dye, *e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, Cy3, Cy5, Cy5.5, Alexa 647 and derivatives. In certain embodiments, the detection agent comprises a ligand moiety with one or more known binding partners. In certain embodiments, the detection agent comprises biotin. In some embodiments, a detection agent is a fluorescent polypeptide (*e.g.*, GFP or a derivative thereof such as enhanced GFP (EGFP)) or a luciferase (*e.g.*, a firefly, Renilla, or Gaussia luciferase). It will be appreciated that, in certain embodiments, a detection agent may react with a suitable substrate (*e.g.*, a luciferin) to generate a detectable signal. Non-limiting examples of fluorescent proteins include GFP and derivatives thereof, proteins comprising chromophores that emit light of different colors such as red, yellow, and cyan fluorescent proteins, etc. Exemplary fluorescent proteins include, *e.g.*, Sirius, Azurite, EBFP2, TagBFP, mTurquoise, ECFP, Cerulean, TagCFP, mTFP1, mUkG1, mAG1, AcGFP1, TagGFP2, EGFP, mWasabi, EmGFP, TagYPF, EYFP, Topaz, SYFP2, Venus, Citrine, mKO, mKO2, mOrange, mOrange2, TagRFP, TagRFP-T, mStrawberry, mRuby, mCherry, mRaspberry, mKate2, mPlum, mNeptune, T-Sapphire, mAmetrine, mKeima. See, *e.g.*, Chalfie, M. and Kain, SR (eds.) Green fluorescent protein: properties, applications, and protocols (Methods of biochemical analysis, v. 47). Wiley-Interscience, Hoboken, N.J., 2006, and/or Chudakov, DM, et al., *Physiol Rev.* 90(3):1103-63, 2010 for discussion of GFP and numerous other fluorescent or luminescent proteins. In some embodiments, a detection agent comprises a dark quencher, *e.g.*, a substance that absorbs excitation energy from a fluorophore and dissipates the energy as heat.

[00132] The term *antigen* refers to a molecule or part of a molecule that is bound by the antigen-binding site of an antibody. In some embodiments, an antigen is a molecule or moiety that, when administered to a subject, activates or increases the production of

antibodies that specifically bind the antigen. In some embodiments, an antigen is a protein or a polysaccharide. Antigens of pathogens are well known to those of skill in the art and include, but are not limited to parts (coats, capsules, cell walls, flagella, fimbriae, and toxins) of bacteria, viruses, and other microorganisms. A vaccine typically comprises an antigen, and is intentionally administered to a subject to induce an immune response in the recipient subject.

[00133] The term *adjuvant* refers to a pharmacological or immunological agent that modifies the effect of other agents, for example, of an antigen in a vaccine. Adjuvants are typically included in vaccines to enhance the recipient subject's immune response to an antigen. The use of adjuvants allows the induction of a greater immune response in a subject with the same dose of antigen, or the induction of a similar level of immune response with a lower dose of injected antigen. Many adjuvants suitable for use in the context of embodiments of this disclosure are known to those of skill in the art, including, but not limited to, aluminum salts, liposomes, lipopolysaccharide (LPS), molecular cages for antigen, components of bacterial cell walls, endocytosed nucleic acids such as double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), and unmethylated CpG dinucleotide-containing DNA.

[00134] The term *vaccine* refers to a composition that activates or enhances a subject's immune response to an antigen after the vaccine is administered to the subject. In some embodiments, a vaccine typically contains an antigen characteristic for a pathogen, *e.g.*, a pathogenic microorganism, such as a pathogenic bacterium. In some embodiments, a vaccine comprises a weakened (attenuated), inactivated, or killed pathogen. In some embodiments, a vaccine comprises an antigen found on a toxin or a surface protein of a pathogen. In some embodiments, a vaccine stimulates the subject's immune system to recognize the antigen as foreign, and enhances the subject's immune response if the subject is later exposed to the pathogen, whether attenuated, inactivated, killed, or not. Vaccines may be prophylactic, for example, preventing or ameliorating a detrimental effect of a future exposure to a pathogen, or therapeutic, for example, activating the subject's immune response to a pathogen after the subject has been exposed to the pathogen.

[00135] The terms *immunizing* and *vaccinating* a subject refer to a process of administering an immunogen, typically an antigen formulated into a vaccine, to the subject in an amount effective to increase or activate an immune response against the antigen and, thus, against a pathogen displaying the antigen. In some embodiments, the terms do not require the creation of complete immunity against the pathogen. In some embodiments, the terms

encompass a clinically favorable enhancement of an immune response toward the antigen or pathogen. Methods for immunization, including formulation of a vaccine composition and selection of doses, routes of administration and the schedule of administration (*e.g.* primary dose and one or more booster doses), are well known in the art. In some cases, an evaluation of vaccine compositions can be performed in a subject, *e.g.*, in human subjects. An immune response can, for example, be detected by an increased titer of circulating antibodies or by the presence of enhanced levels of circulating CTLs against bacterial cells bearing the antigen.

[00136] The term *pharmaceutical composition* refers to a composition that can be administered to a subject in the context of treatment of a disease or disorder. In some embodiments, a pharmaceutical composition comprises an active ingredient, *e.g.* a nuclease or a nucleic acid encoding a nuclease, and a pharmaceutically acceptable excipient. In some embodiments, a pharmaceutical composition is sterile. In some embodiments, a pharmaceutical composition is free of undesired toxins, undesired allergens, undesired infectious agents, and/or undesired pathogens.

[00137] The term *effective amount* refers to an amount of a biologically active agent that is sufficient to elicit a desired biological response. For example, in some embodiments, an effective amount of a nuclease may refer to the amount of the nuclease that is sufficient to induce cleavage of a target site specifically bound and cleaved by the nuclease. As will be appreciated by the skilled artisan, the effective amount of an agent, *e.g.*, a nuclease, a hybrid protein, or a polynucleotide, may vary depending on various factors as, for example, on the desired biological response, the specific allele, genome, target site, cell, or tissue being targeted, and the agent being used.

[00138] The term *subject* refers to an individual organism. In some embodiments, is a mammal, for example, a human, a non-human primate, a mouse, a rat, a cat, a dog, a cattle, a goat, a pig, or a sheep. In some embodiments, the subject is a vertebrate.

[00139] The term *target site* refers to a site to which delivery of a molecule is desired, or that is targeted for delivery of a molecule. In some embodiments, the target site is an organ or a site of disease in a subject. In some embodiments, the target site is the gastrointestinal tract of a subject, *e.g.*, a human. In some embodiments, the target site is the surface of a bioreactor or culture vessel adjacent to a culture medium within the bioreactor or the culture vessel. In some embodiments, the target site is a surface, *e.g.*, a solid surface of a bioreactor or culture vessel that borders on a liquid phase, *e.g.*, a culture medium in the bioreactor or culture vessel, or the surface of a body of water, *e.g.*, a water/air interface.

[00140] The term *surface*, in the context of materials or target sites, refers to the exterior boundary or the outermost layer of a material. A surface typically constitutes the interface of a material with a different material and/or a different phase. For example, a surface may be the exterior boundary of a liquid material, for example, a culture medium in a bioreactor or culture dish, or liquid comprised in a body of water (*e.g.*, a pond, a lake, an ocean), that is in contact with a solid material, *e.g.*, a solid material comprised in the bioreactor or culture vessel, or a gaseous material, *e.g.*, a gaseous phase overlaying the culture medium (*e.g.*, air, oxygen, nitrogen, carbon dioxide, or a controlled mix of different gases (*e.g.*, 5% Oxygen, 5% carbon dioxide, and 90% nitrogen), or overlaying the body of water (*e.g.*, air). In some embodiments, a surface is an air/water interface, *e.g.*, the region in which the outer layer of a body of water meets the outer layer of an adjacent body of air, or any material within 1 μ m, 1 mm, 5 mm, 10 cm, 50 cm, 1 m, or 10m from the air/water interface.

Engineered exopolysaccharide-associated proteins

[00141] Some aspects of this disclosure provide engineered exopolysaccharide-associated proteins. Such proteins can embrace, for example, on secreted bacterial proteins that are retained in the bacterial biofilm matrix by association with the biofilm exopolysaccharide scaffold, as previously identified [1], or other exopolysaccharide-associated proteins, for example, as identified herein, or otherwise known to those of skill in the art.

[00142] Some aspects of this disclosure provide a composition comprising an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof; and a heterologous molecule conjugated to the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof. The exopolysaccharide-associated protein may be any exopolysaccharide-associated protein described herein or known to those of skill in the art. Similarly, the exopolysaccharide-binding fragment may be any such fragment described herein or known to those of skill in the art.

[00143] In some embodiments, the exopolysaccharide-associated protein is a secreted protein or an extracytoplasmic protein. In some embodiments, the exopolysaccharide associated protein remains attached to the cell after synthesis and cellular processing, for example, via noncovalent interactions with molecules on the surface of the cell in the case of a secreted protein, or by a tethering of a transmembrane domain to the cell

membrane in the case of an extracytoplasmic protein. Accordingly, an engineered exopolysaccharide associated protein is provided herein, also remains attached to the cell after synthesis and cellular processing in such embodiments. In the case of an engineered secreted exopolysaccharide associated protein, the heterologous molecule conjugated to the exopolysaccharide associated protein will typically also be secreted. In the case of an engineered extracytoplasmic exopolysaccharide associated protein, it is preferable that the protein is engineered so that the heterologous molecule conjugated to the exopolysaccharide associated protein protrudes into the extracytoplasmic space after cellular processing of the engineered protein.

[00144] In some embodiments, an engineered exopolysaccharide-associated protein, as provided herein, comprises a known exopolysaccharide-associated protein, or fragment thereof, conjugated to a heterologous molecule. In some embodiments, the exopolysaccharide-associated protein is a type I β -prism lectin domain-containing protein, or an exopolysaccharide-binding fragment thereof. For example, in some embodiments, the exopolysaccharide-associated protein is Bap1 (SEQ ID NO: 1), RbmA (SEQ ID NO: 2), RbmC (SEQ ID NO: 3), or HlyA (SEQ ID NO:4). In some embodiments, the exopolysaccharide associated protein is an exopolysaccharide-binding fragment of Bap1 (SEQ ID NO: 1), RbmA (SEQ ID NO: 2), RbmC (SEQ ID NO: 3), or HlyA (SEQ ID NO:4). In some such embodiments, the fragment comprises a type I β -prism lectin domain contained in the respective full-length protein.

[00145] Exemplary known exopolysaccharide-associated proteins from *V. cholerae* are described in **Table 1** and **Table 2** below. Other exopolysaccharide-associated proteins from other bacteria strains, *e.g.*, from other *Vibrio sp.* strains, other diarrheal pathogens, other pathogens, and other nonpathogenic organisms or molecules, are also useful according to some aspects of this disclosure. Homologous exopolysaccharide-associated proteins can be determined by those of skill in the art via homology determinations, *e.g.*, based on sequence alignments. The sequences of some exemplary exopolysaccharide-associated proteins are provided elsewhere herein, including, the sequences of *V. cholerae* Bap1, RbmA, RbmC, and HlyA. Those of skill in the art will be able to identify homologous sequences in other *Vibrio* strains, other bacterial strains, and non-bacterial sources based on this sequence information, and will also be able to determine which of the identified proteins bind exopolysaccharides, *e.g.*, based on the identified proteins comprising a polysaccharide-binding domain, such as, for example, a β -prism lectin domain or an FG-GAP domain.

Table 1. Secreted proteins identified in preparations of biofilm matrix.

Genomic Locus	Annotation
VC0409	MshA
VC0928	RbmA
VC0930	RbmC
VC2142	FlaB
VC2143	FlaD
VC2187	FlaC
VC2188	FlaA
VCA0027	ChiA-2
VCA0219	HlyA
VCA0865	HAP

Table 2. Extracytoplasmic proteins of unknown location.

Genomic Locus	Annotation
VC0174	hypothetical
VC0430	immunogenic protein
VC0483	hypothetical
VC1101	hypothetical
VC1154	hypothetical
VC1334	hypothetical
VC1384	hypothetical
VC1523	hypothetical
VC1834	hypothetical
VC1853	hypothetical
VC1887	hypothetical
VC1894	hypothetical
VC2168	hypothetical
VC2517	hypothetical
VCA0026	hypothetical
VCA0058	conserved, hypothetical
VCA0144	immunogenic protein
VCA0900	hypothetical

[00146] Additional exopolysaccharide-associated proteins found in bacterial biofilms are well known to those of skill in the art and include, for example, those described in

Oliver-Kozup et al., *The group A streptococcal collagen-like protein-1, Scl1, mediates biofilm formation by targeting the extra domain A-containing variant of cellular fibronectin expressed in wounded tissue*. Mol Microbiol. 2013;87(3):672-89; Diggle et al., *The galactophilic lectin, LecA, contributes to biofilm development in Pseudomonas aeruginosa*. Environ Microbiol. 2006 Jun;8(6):1095-104; Johansson et al., *Inhibition and dispersion of Pseudomonas aeruginosa biofilms by glycopeptide dendrimers targeting the fucose-specific lectin LecB*. Chem Biol. 2008 Dec 22;15(12):1249-57; and Abdian et al., *RapA2 Is a Calcium-binding Lectin Composed of Two Highly Conserved Cadherin-like Domains That Specifically Recognize Rhizobium leguminosarum Acidic Exopolysaccharides*. J Biol Chem. 2013 25;288(4):2893-904; the entire contents of each of which are incorporated herein by reference.

[00147] In some embodiments, an engineered exopolysaccharide-associated protein as provided herein comprises a β -prism lectin domain, or a fragment thereof, or an FG-GAP domain, or fragment thereof. In some embodiments, an exopolysaccharide-associated protein as provided herein comprises a β -prism lectin domain flanked by an FG-GAP domain. For example, in some embodiments, an engineered exopolysaccharide-associated protein provided herein comprises a structure

N-[β -prism lectin domain]_x-[FG-GAP domain]_y-C;

N-[FG-GAP domain]_n-[β -prism lectin domain]_m-C;

N-[β -prism lectin domain]_i-[FG-GAP domain]_{ii}-[β -prism lectin domain]_{iii}-C

N-[FG-GAP]_a-[β -prism lectin domain]_b-[FG-GAP domain]_c-C;

wherein N: N-terminus; C: C-terminus; and x, y, n, m, i, ii, iii, a, b, and c representing, independently, an integer between 0 and 25, and preferably an integer between 1 and 10. In some such embodiments, a heterologous molecule is conjugated to either the N terminus or the C terminus of the provided structure. In some embodiments, the β -prism lectin domain and/or the FG-GAP domain comprises or is encoded by a sequence found in or derived from a naturally occurring exopolysaccharide-associated protein, *e.g.*, from RbMC, Bap1, or HlyA, which are described in more detail elsewhere herein. In some such embodiments, the engineered exopolysaccharide-associated protein can be generated by recombinant methods in which the naturally occurring coding sequences are recombined to form their respective structure. In some embodiments, the β -prism lectin domain and/or the FG-GAP domain comprise a non-naturally occurring sequence, for example a sequence determined to be the minimal sequence required for exopolysaccharide binding. In some embodiments, exopolysaccharide binding its binding to a known exopolysaccharide, for example, an N-

glycan, with an affinity characterized by a K_D of $\leq 10^{-6}$ M, $\leq 10^{-7}$ M, $\leq 10^{-8}$ M, $\leq 10^{-9}$ M, $\leq 10^{-10}$ M, $\leq 10^{-11}$ M, or $\leq 10^{-12}$ M. Some such minimal sequences are provided herein, and others are known to those of skill in the art, will be apparent to those of skill in the art based on the instant disclosure, or can be ascertained by those of skill in the art with no more than routine experimentation. For methods useful for the determination of the binding affinity of an exopolysaccharide-associated proteins or fragments thereof to an exopolysaccharide, see, *e.g.*, Levan et al., *Vibrio cholerae Cytolysin Recognizes the Heptasaccharide Core of Complex N-Glycans with Nanomolar Affinity*. J Mol Biol. 2012 S0022-2836(12)00949-7; the entire contents of which are Incorporated herein by reference.

[00148] In some embodiments, the exopolysaccharide-associated protein of this disclosure is a RbmA protein from *Vibrio Cholerae*. In mature *V. Cholerae* biofilms, RbmA is present throughout the biofilms and retains daughter cells in the biofilm following division. Further, RbmA also facilitates micro-colony and cell cluster formation by stabilizing both cell-cell and cell-exopolysaccharide interactions. RbmA is expressed in *V. cholerae* cells and secreted to the matrix of the biofilm, where it remains attached to the cell surface. As illustrated in the drawings and examples of this disclosure, by fusing a heterologous molecule to RbmA, RbmA was able to deliver the heterologous molecule to the cell surface. A fusion protein comprising RbmA and cholera toxin B (CtxB) induced antigen-specific immune response in the host.

[00149] As used herein, the term “a RbmA protein,” encompasses wild type RbmA protein and variants thereof. In some embodiments, the *V. Cholerae* RbmA protein comprises an amino acid sequence of SEQ ID NO: 2. Further provided herein are RbmA variants that may be used as the exopolysaccharide protein of this disclosure. A “variant” of a protein, *e.g.*, RbmA, as used herein, refers to a protein that shares homology to the wild type protein, *e.g.*, wild type RbmA, or a fragment thereof. For example, a RbmA variant is at least about 70% identical, at least about 80% identical, at least about 90% identical, at least about 95% identical, at least about 96% identical, at least about 97% identical, at least about 98% identical, at least about 99% identical, at least about 99.5% identical, or at least about 99.9% to wild type RbmA. A RbmA variant may be made by deletion, insertion, amino acid substitution, or other recombinant protein techniques known by one skilled in the art. In some embodiments, the RbmA variants of this disclosure comprise amino acid substitution mutations.

[00150] As used herein, “substitution mutation” without the reference to a specific amino acid, may include any amino acid other than the wild type residue normally found at

that position. Such substitutions may be replacement with non-polar (hydrophobic) amino acids, such as glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, and proline. Substitutions may be replacement with polar (hydrophylic) amino acids such as serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Substitutions may be replacement with electrically charged amino acids, *e.g.*, negatively electrically charged amino acids such as aspartic acid and glutamic acid and positively electrically charged amino acids such as lysine, arginine, and histidine.

[00151] The substitution mutations described herein will typically be replacement with a different naturally occurring amino acid residue, but in some cases non-naturally occurring amino acid residues may also be substituted. Non-natural amino acids, as the term is used herein, are non-proteinogenic (*i.e.*, non-protein coding) amino acids that either occur naturally or are chemically synthesized. Examples include but are not limited to β -amino acids ($\beta 3$ and $\beta 2$), homo-amino acids, proline and pyruvic acid derivatives, 3 -substituted alanine derivatives, glycine derivatives, ring-substituted phenylalanine and tyrosine derivatives, linear core amino acids, diamino acids, D-amino acids, and N-methyl amino acids. In some embodiments, the amino acid can be substituted or unsubstituted. The substituted amino acid or substituent can be a halogenated aromatic or aliphatic amino acid, a halogenated aliphatic or aromatic modification on the hydrophobic side chain, or an aliphatic or aromatic modification.

[00152] In some embodiments, the RbmA variants comprise amino acid substitution mutations in a surface groove that is lined with several positively charged residues, *e.g.*, lysine. Such surface groove is formed at the interface of a RbmA dimer, as described in Giglio et al., Journal of Bacteriology, July 2013 Volume 195 Number 14, p. 3277-3286, the entire contents of which is herein incorporated by reference. In some embodiments, the RbmA variant comprises one or more substitution mutations in positions R234, R219, or R116 in SEQ ID NO: 2. In some embodiments, the RbmA variant comprises one or more substitution mutations selected from R234A, R219A, or R116A in SEQ ID NO: 2. Possible combination of the mutations include: R234A, R219A, R116A, R234A/R219A, R234A/R116A, and R219A/R116A. “/” indicates double mutations. In some embodiments, the RbmA variants comprise an amino acid sequence of any of SEQ ID NOs: 38-43.

[00153] RbmA R234A

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV

TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
DTYSKVAGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 38)

Signal peptide: residues 1-30

[00154] RbmA R219A

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAAEKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 39)

Signal peptide: residues 1-30

[00155] RbmA R116A

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TAGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 40)

Signal peptide: residues 1-30

[00156] RbmA R234A/R219A

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAAEKWVIASG
DTYSKVAGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 41)

Signal peptide: residues 1-30

[00157] RbmA R234A/R116A

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TAGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG

DTYSKVAGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 42)

Signal peptide: residues 1-30

[00158] RbmA R219A/R116A

MSNFKGSIMNKRHYYLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TAGFVWPPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIPVNAAEKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKK (SEQ ID NO: 43)

Signal peptide: residues 1-30

[00159] Both β -prism lectin domain and FG-GAP domain mediate protein adhesion to exopolysaccharides. In some embodiments, the heterologous molecule is conjugated to a fragment of an exopolysaccharide-associated protein that comprises a minimal exopolysaccharide-binding sequence. Exemplary minimal exopolysaccharide-binding sequences are described in more detail elsewhere herein, and additional minimal exopolysaccharide-binding sequences will be apparent to those of skill in the art based on the instant disclosure, or can be ascertained by those of skill in the art with no more than routine experimentation.

[00160] The heterologous molecule may be any molecule that can be conjugated to an exopolysaccharide-associated protein, for example, a protein, a polypeptide, a nucleic acid, a lipid, a polysaccharide, a small molecule, a binding agent, or a detection agent. The heterologous molecule may be conjugated to the exopolysaccharide-associated protein, or fragment thereof, via covalent linkage, for example, direct covalent linkage, or indirectly via a linker. In some embodiments, the linker comprises a cleavage site, for example, a protease cleavage site. Inclusion of a cleavage site allows the controlled release of the heterologous molecule from the exopolysaccharide-associated protein, which is useful, among other instances, for the controlled release of the heterologous molecule from the exopolysaccharide-associated protein, for example, for isolation and/or purification of the heterologous molecule.

[00161] In some embodiments, the heterologous molecule comprises a heterologous protein, for example, an enzyme, or an antigen. In some such embodiments, the heterologous protein is fused to the exopolysaccharide-associated protein, or to the exopolysaccharide-binding fragment thereof, thus forming a fusion protein. The methods and compositions for

the generation of fusion proteins are well known to those of skill in the art. In general, a fusion protein of an exopolysaccharide associated protein, or fragment thereof, and a heterologous protein can be created by generating a nucleic acid construct encoding both proteins in frame, optionally with a sequence encoding a linker sequence separating both protein-encoding sequences. Typically, such fusion protein encoding nucleic acid constructs are generated by recombinant technologies, which are well known to those of skill in the art. In some embodiments, a nucleic acid encoding a fusion protein, or a fusion protein itself, may also be synthesized de novo. Methods for synthesizing nucleic acids and proteins are also well known to those of skill in the art.

[00162] In some embodiments, the heterologous molecule comprises an antigen, for example, an antigen of a pathogen. Antigens, and, in particular, antigens of pathogens, are well known to those of skill in the art. Some antigens that are of particular interest in the context of some aspects of this disclosure, are antigens of diarrheal pathogens. Diarrheal pathogens include, without limitation, *V. cholerae*, *Salmonella sp.* (e.g., *Salmonella paratyphi*), *Campylobacter sp.* (e.g., *C. jejuni*) enterotoxigenic and enteropathogenic *E. coli* (e.g., EHEC, EPEC), and *Norovirus*. Immunogenic proteins and encoding genes or ORFs from various pathogens, including the diarrheal pathogens listed above, are well known to those of skill in the art, and include, without limitation, the antigens described in Adkins et al., *Bacteria and their toxins tamed for immunotherapy*. Curr Pharm Biotechnol. 2012 Jun;13(8):1446-73; Nielsen et al., *Identification of immunogenic and virulence-associated Campylobacter jejuni proteins*. Clin Vaccine Immunol. 2012 Feb;19(2):113-9; Yang et al., *Screening of the Salmonella paratyphi A CMCC 50973 strain outer membrane proteins for the identification of potential vaccine targets*. Mol Med Report. 2012 Jan;5(1):78-83; Harro et al., *A combination vaccine consisting of three live attenuated enterotoxigenic Escherichia coli strains expressing a range of colonization factors and heat-labile toxin subunit B is well tolerated and immunogenic in a placebo-controlled double-blind phase I trial in healthy adults*. Clin Vaccine Immunol. 2011 Dec;18(12):2118-27; Vasconcellos et al., *Generation of recombinant bacillus Calmette-Guérin and Mycobacterium smegmatis expressing BfpA and intimin as vaccine vectors against enteropathogenic Escherichia coli*. Vaccine. 2012 Sep 7;30(41):5999-6005; and Tan et al., *Norovirus P particle, a novel platform for vaccine development and antibody production*. J Virol. 2011 Jan;85(2):753-64; the entire contents of each of which are incorporated herein by reference. Some exemplary antigens suitable as heterologous molecules according to some aspects of this disclosure are provided in **Tables 3** and **4** below.

ORF (NCTC 11168) ^a	Annotation
Cj0014c	Putative integral membrane protein
Cj0034c (4)	Putative periplasmic protein
Cj0111 (3)	Putative periplasmic protein
Cj0203	Putative transmembrane transport protein
Cj0383c	<i>ribH</i> , 6,7-dimethyl-8-ribityllumazine synthase
Cj0404 (3)	Putative transmembrane protein
Cj0408	<i>frdC</i> , fumarate reductase cytochrome B subunit
Cj0477	<i>rplL</i> , 50S ribosomal protein
Cj0525c (3)	<i>pbpB</i> , putative penicillin binding protein
Cj0645 (2)	Putative secreted transglycosylase
Cj0774c (5)	ABC transport system ATP binding protein
Cj0811	<i>lpxK</i> , tetrasaccharide 4'-kinase
Cj0917c	<i>cstA</i> , carbon starvation protein A homolog
Cj0965c	Putative acyl coenzyme A thioester hydrolase
Cj1092c	<i>secF</i> , protein export membrane protein
Cj1094c	<i>yajC</i> , preprotein translocase subunit
Cj1163c (4)	Putative cation transport protein
Cj1174 (3)	Putative efflux protein
Cj1292	<i>dcd</i> , dCTP deaminase
Cj1364c	<i>fumC</i> , fumarate hydratase
Cj1371 (2)	Putative periplasmic protein (<i>vacJ</i> homolog)
Cj1382c (4)	<i>fldA</i> , flavodoxin
Cj1529c (5)	<i>purM</i> , phosphoribosylaminoimidazole synthase
Cj1628	<i>exbB2</i> , putative <i>exbB/tolQ</i> family transport protein
Cj1632c	Putative periplasmic protein

Table 3: highly immunogenic ORFs cloned from *Campylobacter jejuni* (see Nielsen et al., *Identification of immunogenic and virulence-associated Campylobacter jejuni proteins*. Clin Vaccine Immunol. 2012 Feb;19(2):113-9, the entire contents of which are incorporated herein by reference).

NCBI GI identifier	Mass (Da)	pI (calc)	Protein	Gene
56416031	50640	4.87	Maltoporin precursor	LamB
56415127	553649	5.43	Outer membrane channel precursor protein	tolC
56412835	41214	4.63	Outer membrane protein C	ompC
56412712	47675	4.90	Long-chain fatty acid transport protein precursor	fadL
56415967	68470	5.40	Vitamin B12 receptor protein	btuB
56412364	89861	5.30	Organic solvent tolerance protein	imp
56413481	39655	4.66	New outer membrane protein: predicted bacterial porin	nmpC
56413763	20090	6.28	Outer membrane invasion protein	pagC
56413343	22990	5.64	Putative outer membrane protein	ompW
56413933	37583	5.47	Outer membrane protein A	OmpA
56413728	28035	5.51	Putative outer membrane protein	mipA
56414068	18540	5.74	Outer membrane protein x precursor	OmpX

Table 4: immunogenic proteins from *Salmonella paratyphi* (from Yang et al., *Screening of the Salmonella paratyphi A CMCC 50973 strain outer membrane proteins for the identification of potential vaccine targets*. Mol Med Report. 2012 Jan;5(1):78-83, the entire contents of which are incorporated herein by reference).

[00163] In some embodiments, the heterologous molecule is a bacterial toxin, for example, *Bordetella pertussis* adenylate cyclase toxin, *Bacillus anthracis* lethal and edema toxins, *Shigella dysenteriae* shiga toxin, *Escherichia coli* shiga-like toxin, *E. coli* α -hemolysin, *Vibrio cholerae* cholera toxin (e.g., cholera toxin B), *E. coli* heat-labile enterotoxin, *Bordetella pertussis* pertussis toxin, *Bacillus thuringiensis* Cry1A protein, *Clostridium perfringens* perfringolysin O, *Streptococcus intermedius* intermedilysin, *Streptococcus pneumoniae* pneumolysin, *Corynebacterium diphtheriae* diphtheria toxin, or a *Pseudomonas aeruginosa* exotoxin A-based immunotoxin. Bacterial toxins are well known to those of skill in the art, and while some exemplary suitable bacterial toxins are disclosed herein, this disclosure is not limited in this respect. Additional suitable toxins will be apparent to the skilled artisan based on the instant disclosure and the knowledge in the art, including, but not limited to, the toxins described in Adkins et al., *Bacteria and their toxins tamed for immunotherapy*. Curr Pharm Biotechnol. 2012 Jun;13(8):1446-73, the entire contents of which are incorporated herein by reference.

[00164] In some embodiments, the heterologous molecule is a bacteria colonization factor antigen. A “bacteria colonization factor antigen,” as used herein, are bacterial proteins that confer adhesive and colonizing properties for enteropathogenic strains of *E. coli* (ETEC) in the intestinal epithelium a host. Non-limiting examples of colonization factor antigens include ETEC exoproteins (e.g., EtpA), CFA-1 (fimbrial), KS 71A fimbrial antigen,

colonization factor CS19, colonization factor CSS, colonization factor antigen-1, colonization factor antigen-2, colonization factor antigen-3, fimbrial antigen F7(1), and fimbrial colonization factor antigen-1. In some embodiments, the heterologous molecule is EtpA protein, or a fragment thereof. The EtpA protein was identified in ETEC H10407 in a recent search for candidate immunogens. The EtpA protein is a large glycosylated exoprotein secreted via two-partner secretion (TPS). Similar to structurally related molecules, EtpA functions in vitro as an adhesion (Fleckenstein *et al.*, Infection and Immunity, 74:2245-2258.). The amino acid sequence of EtpA is provided in SEQ ID NO: 44.

[00165] ETEC EtpA

MNRIYKLKFDKRRNELVVVSEITTGVGNAKATGSVEGEKSPRRGVRAMALSLLSGM
MIMAHFAMSANLPTGGQIVAGSGSIQTSGNQMNHQNSQNMVANWNSFDIGKGN
VQFDQPSSSAVALNRVVGGSQIMGNLKGQVFLVNPNGVLFEGASVSTSGFV
ASTRDIKNDDFMNRRYTFSGGQKAGAAIVNQGELTTNAGGYIVLAADRVSNSGTIRT
PGGKTVLAASERITLQLDNGGLMSVQVTGDVVNALVENRGLVSARDGQVYLTALG
RGMLMNTVLNVSGVVEASGMHRQDGNIVLDGGDSGVVHLSGTLQADNASGQGGK
VVVQGKNILLDKGSNITATGGQGGGEVYVGGGWQKDSNIRNADKVVMQGGARID
VSATQQGNGGTAVLWSDSYTNFHGQISAKGGETGGNGGRVETSSHGNLQAFGTVSA
SAKKGKAGNWLLDSADITVNGSNVSKTETTQSPPHQTQFAPTAAGSAVSNTSINNRL
NNGTSVTILTHRTRTGTAQGGNITVNAAINKSNGSDVNLTLQAGGNITVNNSITSTEG
KLVNLSGARTSNGSITISNNANITNGGDITVGTNTSNRVNISINNTTLNASNGNIQ
LTGTGTDGILFAGNNRLTASNIALTGNSTSGNAINLTGTATLNATNNITLTGSSTSGN
AINLKGNNTLTASNITLTGESTSGNAINLTDTTGTTTLNATNNITMQGTRVQIKHSNIT
AGNFALNATVAGSEISNTTLTATNNINLAAKTNSASSGVYLKDARITSTNGSITANGT
ATANGKATHLDGNVTNLNASNGRIKLTGNHGHSASGILFAGNNRLTASNIALTGNSTS
GNAINLTGTATLNATNDITLTGSSTSGNAINLTGTATLNATNNITLTGSSTSGNAINLK
GNNTLTASNITLTGESTSGNAINLTDTTGTTTLNATNNITMQGTRVQIKHSNITAGNF
ALNATVAGSEISNTTLTATNNINLAAKTNSASSGVYLKDARITSTNGSITANGTATAN
GKATHLDGNVTNLNASNGRIKLTGNHGHSASGILFAGNNRLTASNIALTGNSTSGNAI
NLTGTATLNATNDITLTGSSTSGNAINLTGTATLNATNNITLTGSSTSGNAINLKGNNT
LTASNITLTGESTSGNAINLTDTTGTTTLNATNNITMQGTRVQIKHSNITAGNFALNAT
VAGSEISNTTLTATNNINLAAKTNSASSGVYLKDARITSTNGSITANGTATANGKATH
LDGNVTNLNASNGRIKLTGNHGHSASGILFAGNNRLTASNIALTGNSTSGNAINLTGTA
TLNATNDITLTGSSTSGNAINLTGTATLNATNNITLTGSSTSGNAINLKGNNTLTASNI
TLTGESTSGNAINLTDTTGTTTLNATNNITMQGTRVQIKHSNITAGNFALNATVAGSE

ISNTTLTATNNINLAAKTNSASSGVYLKDARITSTNGSITTNGTATANGKATHLDGNV
 TLNASNGRIKLTGNGHGSASGILFAGNNRLTASNIALTGNSTSGNAINLTGTATLNAT
 NDITLTGSSTSGNAINLTGTATLNATNNITLTGSSTSGNAINLKGNNTLTASNITLTGES
 TSGNAINLTDTTGTTTLNATNNITMQGTRVQIKHSNITAGNFALNATVAGSEISNTTL
 TATNNINLAAKTNSASSGVYLKDARITSTNGSITANGTAPANDNATYLDGNVTLNAS
 NGSIKLTGNGNGSTSGILFAGNNLTASNITLTGNSEVYWQ (SEQ ID NO: 44)

[00166] In some embodiments, the bacterial toxin is a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC). STa and heat-labile toxin (LT) contribute to the virulence of ETEC diarrhea caused by ETEC. LT antigens are often used in vaccine development, but STa has not been included because of its poor immunogenicity and potent toxicity. Toxic STa is not safe for vaccines, but only STa possessing toxicity is believed to be able to induce neutralizing antibodies.

[00167] However, recent studies demonstrated that nontoxic STa derivatives (toxoids) induced neutralizing antibodies, after being fused to an LT protein. Further, it has been shown that a A14H mutation in STa (the STa^{A14H} variant) reduces its toxicity and improves its immunogenicity. As such, in some embodiments, the antigen used in the vaccines described herein is a STa^{A14H} toxoid fused to subunit B of the LT protein (LTB).

[00168] In some embodiments, the STa^{A14H} and LTB antigens are fused to RmbA to form a fusion protein having an order of RmbA-LTB-STa^{A14H}. In some embodiments, the fusion protein is encoded by a polynucleotide comprising the nucleotide sequence of SEQ ID No: 48 in Table 8. In some embodiments, the nucleotide sequence encoding the RmbA-LTB-STaA14H fusion protein is placed under control of the native RmbA promoter.

[00169] In some embodiments, the heterologous molecule is a bacterial lipopolysaccharide (LPS). “Lipopolysaccharides (LPS),” also known as lipoglycans and endotoxins, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. LPS molecules are found in the outer membrane of Gram-negative bacteria, and elicit strong immune responses in animals. In some embodiments, the LPS is from *Vibrio cholerae*.

[00170] In some embodiments, the heterologous molecule is a virulence factor. A “virulence factor,” as used herein, refers to a molecule produced by a pathogen (e.g., bacteria, virus, fungi, and protozoa) that contributes to the pathogenicity of the organism and enables them to colonize, evade and/or suppress host immune system, enter host cells, etc.. Some virulence factors are enzymes that interfere with host cellular process and cause damage to

host tissues, e.g., proteases, lipases, DNases, hemolysins, etc.. In some embodiments, the virulence factor is a bacterial virulence factor. In some embodiments, the bacterial virulence factor is from *Shigella*. In some embodiments, the virulence factor is VirG from *Shigella*. The amino acid sequence of VirG is provided in SEQ ID NO: 45.

[00171] *Shigella* VirG

SFSPFVVGASLLLGGPIAFAIPLSGTQELHFSEDNYEKLLTPVDGLSPLGAGEDGMDA
WYITSSNPISHASRTKLRINSDIMISAGHGGAGDNDGNSCGGNGGDSITGSDLSIINQ
GMILGGSGGSGADHNGDGGEAVTGDNLFIINGEIISSGGHGGDSYSDSDGGNGGDAVT
GVNLPINKGTISGGNGGNNYGEDGGNGGDAITGSSLSVINKGTFAGGNGGAAYGY
GYDGYGGNAITGDNLIIINNGAILGGNGGHWGDAINGSNMTIANSGYIISGKEDDGT
QNVAGNAIHITGGNNSLILHEGSVITGDVQVNNSSILKIINNDYTGTTPTEIGDLCAGD
CTTVSLSGNKFTVSGDVSGENSSNLLAGISSLEASGNMSFGNNVKVEAIINNWAQK
DYKLLSADKGITSNISIINPLLTTGAIDYTKSYISDQNKLIYGLSWNDTDGDSHGEFNL
KENAELTVSTILADNLSHHNINSWDGKSLTKSGEGTLILAEKNTYSGFTNINAGILKM
GTVEAMTRTAGVIVNKGATLNFSGMNQTVNSLLNSGTVLINNINAPFLPDPVIVTGN
MTLEKNGHVILNNSSSNVGQTYVQKGNWHGKGGILSLGAVLGNDNSKTDRLEITGH
ASGITYVAVTNEGGSGDKTLEGVQIISTDSSDKNAFIQKGRIVAGSYDYRLKQGTVSG
LNTNKWYLT SQMDNQESKQMSNQESTQMSSRRASSQLVSSLNLGEGSIHTWRPEAG
SYIANLIAMNTMFSPSLYDRHGSTIVDPTTGQLSETTMWIRTVGGHNEHNLADRQLK
TTANRMVYQIGGDILKTNFTDHDGLHVGIMGAYGYQDSKTHNKYTSYSSRGTVSGY
TAGLYSSWFQDEKERTGLYMDAWLQYGWFNNTVKGDGLTGEKYSSKGITGALEAG
YIYPTIRWTAHNNIDNALYLN PQVQITRHGVKANDYIEHNGTMVTSSGVNNIQAKLG
LRTSLISQSCIDKETLRKFEPFLEVNWKWSSKQYGVIMNGMSNHQIGNRNVIELKTG
VGGRLADNLSIWGNVSQQLV (SEQ ID NO: 45)

[00172] In some embodiments, the heterologous molecule comprises an enzyme. Engineered exopolysaccharide-associated proteins comprising enzymes are useful for the generation of bacteria in biofilms that exhibits novel or increased enzymatic activities. Suitable enzymes for some embodiments of this disclosure, for example, oxidoreductases, transferases, polymerases, hydrolases, lyases, synthases, isomerases, and ligases. In some embodiments, the enzyme is a protein that is conjugated to the exopolysaccharide-associated protein by a covalent bond, thus forming a fusion protein. In certain embodiments, however, the enzyme is a non-protein enzyme, for example, a nucleic acid (e.g., a ribozyme or a DNAzyme). In some embodiments, the enzyme is a therapeutic enzyme. Therapeutic enzymes are well known to those of skill in the art and include, for example, digestive

enzymes (*e.g.*, proteases, lipases, carbohydrases, and nucleases). In some embodiments, the enzyme is selected from the group consisting of lactase, beta-galactosidase, a pancreatic enzyme, an oil-degrading enzyme, mucinase, cellulase, isomaltase, or alginase. In some embodiments, an engineered exopolysaccharide-associated protein is provided that comprises an exopolysaccharide-associated protein conjugated to a digestive enzyme expressed in the mammalian digestive tract. Suitable digestive enzymes for conjugation to exopolysaccharide-associated proteins include, but are not limited to, digestive lipases (*e.g.*, lingual lipase, pancreatic lipase, phospholipase), amylases, cellulases, lysozyme, proteases (*e.g.*, pepsin, trypsin, chymotrypsin, carboxypeptidase, elastase,), esterases (*e.g.* sterol esterase), disaccharidases (*e.g.*, sucrase, lactase, beta-galactosidase, maltase, isomaltase), DNases, and RNases. Enzymes that are particularly suitable as heterologous molecules in some embodiments of this disclosure are enzymes expressed throughout the lining of the small intestine, which are also referred to sometimes as “brush border enzymes.” This brush border enzymes include, but are not limited to the disaccharidases listed above. Other suitable digestive enzymes that can be used according to some aspects of the instant disclosure will be apparent to those of skill in the art and the disclosure is not limited in this respect.

[00173] Engineered exopolysaccharide-associated proteins comprising a therapeutic enzyme as the heterologous molecule are useful for various therapeutic applications. For example, such engineered proteins allow the targeted delivery of digestive enzymes to specific target sites within the gastrointestinal tract. For example, an engineered exopolysaccharide-associated protein comprising an exopolysaccharide-associated protein that binds to the biofilm matrix in the small intestine may be conjugated to a digestive enzyme catalyzing a digestive reaction taking place in the small intestine. The engineered protein, or a bacterium expressing the engineer protein, may then be administered to a subject having a deficiency in the respective digestive enzyme. For example, a bacterium expressing a fusion protein of exopolysaccharide associated protein and the digestive enzyme lactase may be administered to a subject with a lactose intolerance to improve lactose digestion and the small intestine.

[00174] Importantly, this disclosure is not limited to embodiments of enzyme replacement, but the technology disclosed herein can also be used to transfer entirely new functionalities to biofilms, for example biofilms within the gastrointestinal tract. For example, engineered exopolysaccharide-associated proteins comprising a digestive enzyme not naturally occurring in the intestinal tract of a subject, for example, cellulase, may be deliberate to the gastrointestinal tract of the subject to enable the subject to digest a

previously unknown digestible food source, in this case, cellulose. In an exemplary embodiment, a human subject may be administered a bacterium that can colonize the gastrointestinal tract of the subject, and that expresses an engineered exopolysaccharide-associated protein fused to a cellulase. Upon colonization of the gastrointestinal tract of the subject with the engineered bacteria, the subject will be able to digest cellulose. In preferred embodiments, the bacterium and/or the exopolysaccharide-associated protein are present, and their non-modified form, in the intestinal tract of the subject.

[00175] In some embodiments, the heterologous molecule comprises an enzyme that can digest an environmental pollutant. Engineered exopolysaccharide-associated proteins comprising such enzymes allow for the generation of engineered bacteria and biofilms that can help in the cleanup of environmental pollutants. For example, in some embodiments, the heterologous molecule comprises an enzyme that can digest mineral oil contaminations in the environment, for example, in the form of an oil spill into the ocean. Such enzymes include, for example, alkane 1-monooxygenase, naphthalene 1,2-dioxygenase, E-phenylitaconyl-CoA hydratase, benzylsuccinyl-CoA dehydrogenase, methane monooxygenase. In some embodiments, the heterologous molecule comprises an enzyme that protects a bacterium from detrimental effects of an environmental pollutant, or that catalyzes a reaction that increases the capability of a bacterium to break down an environmental pollutant. Such enzymes may include, in some embodiments, enzymes that catalyze sulfite, phosphorus, or iron reduction, or enzymes that confer metal resistance to a bacterium. Such enzymes include, without limitation, sulfite reductases, exopolyphosphatases, and metal reductases. The structure and sequence of many enzymes that can digest or help in the digestion of environmental pollutants, or confer protection against environmental pollutants, are well known to those of skill in the art. Such enzymes include, for example, those described in Hazen et al., *Deep-sea oil plume enriches indigenous oil-degrading bacteria*. Science. 2010 Oct 8;330(6001):204-8, including supplemental content (see, e.g., Table S6 of Hazen et al.); Lu et al., *Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume*. ISME J. 2012 Feb;6(2):451-60; Kostka et al., *Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill*. Appl Environ Microbiol. 2011 Nov;77(22):7962-74; and Wood et al., *Engineering biofilm formation and dispersal*. Trends Biotechnol. 2011 Feb;29(2):87-94; the entire contents of each of which are incorporated herein by reference.

[00176] In some embodiments, the heterologous molecule comprises a binding agent. Engineered exopolysaccharide-associated proteins comprising a binding agent are

useful for the generation of bacteria and biofilms that exhibits novel or improved binding capabilities. In some embodiments, In some embodiments, this allows for the specific delivery of bacteria or biofilms to a target site of interest, which may be, for example an abiotic surface, such as the surface of a bioreactor, a solid support, a liquid-gas interface, the surface of an environmental pollutant, a biotic surface, *e.g.*, the surface of a living cell or tissue, for example, of a cell or tissue exhibiting a structural or functional deficiency, or a diseased cell or tissue, or a specific region within the gastrointestinal tract. Suitable binding agents are well known to those of skill in the art, and include, but are not limited to, antibodies and antibody fragments. For the preparation of fusion proteins comprising an exopolysaccharide-associated protein and a binding agent specifically binding to antigen, single chain antibodies, such as nanobodies and scFvs are particularly useful. In some embodiments, the binding agent is an adnectin, a lectin, a ligand, or an affinity tag. In embodiments, where an engineered bacterium or an engineered by of time is to be targeted to a specific target site, the binding agent is chosen to bind a molecule or moiety present at the target site. Accordingly, the nature and specificity of the binding agent will depend on the nature and structural characteristics of the target site. For example, in embodiments where an engineered bacterium expressing the fusion protein comprising an exopolysaccharide-associated protein and an antigen-binding antibody fragment is to be delivered to a specific cell or tissue, the binding agent will be chosen to bind an antigen expressed on the surface of the respective cell or tissue.

[00177] In some embodiments, the heterologous molecule comprises a detection agent. The generation of engineered exopolysaccharide-associated proteins comprising a detection agent is useful for the detection of engineered bacteria or engineered biofilms expressing such an engineered protein. While any detection agent that can be conjugated to an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, is suitable, the use of detectable proteins, *e.g.*, a fluorescent proteins, allows for the generation of fusion proteins comprising an exopolysaccharide-associated protein, or fragment thereof and the detectable protein. The use of detection agents, such as fluorescent proteins or affinity tags, is preferable in some embodiments, because the respective fusion proteins with exopolysaccharide-associated proteins or fragments thereof can easily be expressed in a host cell, *e.g.*, a bacterial cell.

[00178] In some embodiments, the compositions further comprises a signal peptide fused to the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, or to the heterologous molecule, wherein the signal peptide targets the

exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, conjugated to the heterologous molecule for secretion. In some embodiments, the exopolysaccharide-associated protein or fragment thereof that is conjugated to the heterologous molecule comprises an endogenous signal peptide that is retained in the engineered protein, *e.g.*, an engineered fusion protein comprising the respective exopolysaccharide-associated protein or fragment thereof fused to a heterologous protein. In particular, secreted exopolysaccharide-associated proteins comprise such signal peptides in their native sequences. In other embodiments, a heterologous signal peptide is fused to the engineered exopolysaccharide-associated protein, for example, at the N-terminus of the respective fusion protein. Suitable signal peptide sequences for expression of secreted proteins in bacteria are well known to those of skill in the art. Some exemplary signal peptides are described herein, and additional suitable signal peptide will be apparent to the skilled artisan based on the instant disclosure. The disclosure is not limited in this respect. Exemplary fusion proteins according to some aspects of this disclosure comprise a structure according to the general formula [signal peptide]-[exopolysaccharide-associated protein (or fragment)]-[heterologous molecule], wherein the hyphens represent a peptide bond or a linker. Methods and strategies for preparing such fusion proteins will be apparent to those of skill in the art in view of this disclosure, and exemplary suitable strategies for generating such fusion proteins are described in Absalon et al., *The Bacterial Biofilm Matrix as a Platform for Protein Delivery* . mBio 3(4): e00127-12, the entire contents of which are incorporated herein by reference.

Engineered bacteria and biofilms

[00179] Some aspects of this disclosure provide bacteria and biofilms comprising, or associated with, engineered exopolysaccharide-associated proteins. Some aspects of this disclosure provide a composition comprising a bacterium associated with an exopolysaccharide; an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof; and a heterologous molecule conjugated to the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof. For example, some aspects of this disclosure provide a bacterium associated with an exopolysaccharide, wherein the bacterium expresses or is associated with an engineered exopolysaccharide-associated protein, *e.g.*, an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof conjugated to a heterologous molecule. In some embodiments, the

bacterium expresses the engineered exopolysaccharide-associated protein, for example, from an expression construct encoding the engineered protein that is comprised within the bacterium, *e.g.*, in the form of an expression vector, such as a plasmid. In some embodiments, a bacterium may be contacted with an isolated engineered exopolysaccharide-associated protein under conditions suitable for the protein to associate with the bacterium. In some embodiments, it is advantageous to express the engineered exopolysaccharide-associated protein from an expression vector within the bacterium, because this allows for constant or inducible production of the engineered exopolysaccharide-associated protein, which, in turn, circumvents the need for exogenous replacement of protein over time. This is particularly useful in embodiments where a bacterium is used to deliver a heterologous protein to a target site, *e.g.*, to deliver a digestive enzyme to a cell, tissue, organ, or environmental target site. In such cases, a bacterium expressing the engineered exopolysaccharide-associated protein can, once at the target site, proliferate and replenish the engineered exopolysaccharide-associated protein, either continually or upon exposure to a molecular or environmental cue.

[00180] In some embodiments, an engineered exopolysaccharide-associated protein or exopolysaccharide-associated protein fragment described herein is expressed in a bacterium, for example, by contacting the bacterium with an expression construct encoding the engineered exopolysaccharide-associated protein or exopolysaccharide-associated protein fragment. Expression of such engineered proteins in bacteria will confer the engineered characteristics, *e.g.*, engineered structural or functional characteristics, to the expressing bacteria. For example, if an engineered exopolysaccharide-associated protein or protein fragment comprising a fusion protein of an exopolysaccharide-associated protein and a digestive enzyme, *e.g.*, lactase, is expressed in a bacterium, the bacterium will retain the fusion protein on its surface, and thus display lactase functionality. In some embodiments, the expression of an engineered exopolysaccharide-associated protein in a bacterium confers a structural or functional characteristic upon the bacterium that was not natively present in the bacterium, *e.g.*, in the case of expression of the lactase-comprising fusion protein described above in a lactase-deficient bacterium. If such a bacterium can ferment the products of lactase digestion, the acquisition of lactase functionality will result in the bacterium being able to utilize lactose as a food source. Similarly, if the bacterium colonizes a site together with other bacteria that can ferment the products of lactase digestion, but cannot ferment lactose, the bacterial community can now utilize lactose as a food source. The same principle

applies to other digestive enzymes, *e.g.*, cellulases, amylases, disaccharidases, oil-degrading enzymes, and so forth.

[00181] The engineered exopolysaccharide-associated protein expressed by or contacted with the bacteria can be any engineered exopolysaccharide-associated protein provided herein, *e.g.*, an engineered exopolysaccharide-associated protein comprising a heterologous protein, enzyme, antigen, binding agent, detection agent, small molecule, and so forth; an engineered exopolysaccharide-associated protein comprising an exopolysaccharide-associated protein or exopolysaccharide-binding fragment thereof, as described herein, *e.g.*, as described in Table 1 or 2.

[00182] In some embodiments, the engineered bacterium can be any bacterium that can express an engineered exopolysaccharide-associated protein is suitable for use according to some aspects of this disclosure. In some embodiments, the bacterium is In some embodiments, the bacterium is a gram-negative bacterium. In some embodiments, the bacterium is a gram-positive bacterium. Gram-positive and gram-negative bacteria are well known to those of skill in the art. In addition, whether or not a bacterium is gram-positive or gram-negative can easily be determined without more than routine experimentation, *e.g.*, by performing a routine gram staining procedure. In some embodiments, the bacterium is a non-pathogenic bacterium. The use of non-pathogenic bacteria is particularly useful in embodiments that include the delivery of a therapeutic heterologous molecule to a subject, and also in embodiments related to bioremediation, *e.g.*, in embodiments that involve the cleanup of an environmental pollutant. In some embodiments, the bacterium is a pathogenic bacterium. The use of pathogenic bacteria is of particular use in some embodiments that involve or are related to the induction of an immune response, *e.g.*, in embodiments involving the generation of a vaccine or a vaccination of a subject. Non-pathogenic and pathogenic bacteria are well known to those of skill in the art. Exemplary, non-limiting bacterial taxa, species, and strains, suitable for use in some embodiments of this disclosure, *e.g.*, suitable for contacting with or for expression of an engineered exopolysaccharide-associated protein disclosed herein, are provided herein and include, without limitation, *Escherichia sp.*, *Enterobacter sp.* (*e.g.*, *Enterobacter cloacae*), *Salmonella sp.* (*e.g.*, *Salmonella enteritidis*, *Salmonella typhi*), *Shigella sp.*, *Pseudomonas sp.* (*e.g.*, *Pseudomonas aeruginosa*, *Pseudomonas pachastrellae*, *Pseudomonas stutzeri*), *Moraxella sp.* (*e.g.*, *Moraxella catarrhalis*), *Neisseria sp.* (*e.g.*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*), *Helicobacter sp.*, (*e.g.*, *Helicobacter pylori*) *Stenotrophomonas sp.*, *Vibrio sp.* (*e.g.*, *Vibrio cholerae*), *Legionella sp.* (*Legionella pneumophila*), *Hemophilus sp.* (*e.g.*, *Hemophilus*

influenzae), *Klebsiella* sp. (e.g., *Klebsiella pneumoniae*), *Proteus* sp. (e.g., *Proteus mirabilis*), *Serratia* sp. (*Serratia marcescens*), *Streptococcus* sp., *Staphylococcus* sp., *Corynebacterium* sp., *Listeria* sp., and *Clostridium* sp., *Bacillus* sp. (e.g., *Bacillus anthracis*) *Bordetella* sp. (e.g., *Bordetella pertussis*); *Borrelia* sp. (e.g., *Borrelia burgdorferi*); *Brucella* sp. (e.g., *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*); *Campylobacter* sp. (e.g., *Campylobacter jejuni*); *Chlamydia* sp. and *Chlamydophila* sp. (e.g., *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila psittaci*); *Clostridium* sp. (e.g., *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*); *Corynebacterium* sp. (e.g., *Corynebacterium diphtheriae*); *Enterococcus* sp. (e.g., *Enterococcus faecalis*, *Enterococcus faecium*); *Escherichia* sp. (e.g., *Escherichia coli*, Enterotoxigenic *E. coli*, enteropathogenic *E. coli*; *E. coli* O157:H7); *Francisella* sp. (e.g., *Francisella tularensis*); *Haemophilus* sp. (e.g., *Haemophilus influenzae*); *Helicobacter* sp. (e.g., *Helicobacter pylori*); *Legionella* sp. (e.g., *Legionella pneumophila*); *Leptospira* sp. (e.g., *Leptospira interrogans*); *Listeria* sp. (e.g., *Listeria monocytogenes*); *Mycobacterium* sp. (e.g., *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*); *Mycoplasma* sp. (e.g., *Mycoplasma pneumoniae*); *Neisseria* sp. (e.g., *Neisseria gonorrhoeae*, *Neisseria meningitidis*); *Pseudomonas* sp. (e.g., *Pseudomonas aeruginosa*); *Rickettsia* sp. (e.g., *Rickettsia rickettsii*); *Salmonella* sp. (e.g., *Salmonella typhi*, *Salmonella typhimurium*); *Shigella* sp. (e.g., *Shigella sonnei*); *Staphylococcus* sp. (e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*); *Streptococcus* sp. (e.g., *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*); *Treponema* sp. (e.g., *Treponema pallidum*); *Pseudodimeria* sp. (e.g., *P. maritima*); *Marinobacter* sp. (e.g., *Marinobacter hydrocarbonoclasticus*, *Marinobacter vinifirmus*) *Alcanivorax* sp. (e.g., *alcanivorax dieselolei*); *Acetivibrio* sp. (e.g., *A. venetianus*); *Halomonas* sp. (e.g., *H. shengliensis*); *Labrenzia* sp.; *Microbulifer* sp. (e.g., *M. schleiferi*); *Shewanella* sp. (e.g., *S. algae*); *Vibrio* sp. (e.g., *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio hepaticus*); and *Yersinia* sp. (e.g., *Yersinia pestis*). In some embodiments, the bacterium is a *Vibrio* sp. bacterium. In some embodiments, the bacterium is a *Vibrio cholerae* bacterium. In some embodiments, the bacterium is an *E. coli* bacterium. Other bacterial taxa and strains that are suitable in embodiments of this disclosure will be apparent to those of skill in the art.

[00183] Some aspects of this disclosure provide a bacterial biofilm comprising an engineered bacterium as described herein, for example, a bacterium associated with an exopolysaccharide that is bound by an exopolysaccharide-associated protein (or an exopolysaccharide-binding fragment thereof) conjugated to a heterologous molecule.

Depending on the structure and function of the heterologous molecule, such engineered biofilms exhibit novel functional or structural characteristics as compared to the native, non-engineered biofilms.

[00184] For example, if the heterologous molecule comprises a binding agent and is conjugated to an exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof that is expressed at the surface of the biofilm, the biofilm can bind to a molecule or surface comprising a moiety bound by the binding agent. This allows the engineering of bacterial biofilms to bind to, *e.g.*, surfaces that the respective native biofilm cannot or does not bind to. For another example, if the heterologous molecule comprises an enzyme and is conjugated to an exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof that is expressed at the surface of the biofilm, the biofilm will be able to digest the substrate of the enzyme, *e.g.*, a substrate that was previously inaccessible to the bacteria within the biofilm, or an environmental pollutant.

[00185] In some embodiments, an engineered biofilm comprises a combination of engineered exopolysaccharide-associated proteins, *e.g.*, an engineered protein in which the heterologous molecule is a binding agent and an engineered protein in which the heterologous molecule is an enzyme, allowing targeted delivery of the enzyme to a surface bound by the binding agent. In some embodiments, the biofilm comprises a detection agent, *e.g.*, as part of an engineered exopolysaccharide-associated protein expressed by bacteria comprised in the biofilm. This allows the engineering of bacterial biofilms that can be detected, *e.g.*, when bound to a surface, for example, if the detection agent is a fluorescent agent, by fluorescent imaging.

Vaccines

[00186] Some aspects of this disclosure provide that engineered exopolysaccharide-associated proteins, as provided herein, are useful for the generation of vaccines. A vaccine typically comprises an agent that mimics or comprises an antigen of a pathogen and, when administered to the subject, and uses an immune response in the subject. When subsequently exposed to the real pathogen, the subject's vaccine-primed immune system is able to recognize and destroy the pathogen with increased efficiency as compared to the immune system of a non-vaccinated subject.

[00187] Some aspects of this disclosure provide vaccines that comprise engineered exopolysaccharide-associated proteins that are conjugated to an antigen. In some

embodiments, this disclosure provides vaccines that comprise a bacterium associated with an exopolysaccharide; an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, that binds the exopolysaccharide; and an antigen conjugated to the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof. In some such embodiments, the bacterium expresses the engineered exopolysaccharide-associated protein. In some embodiments, the vaccines provided herein are advantageous over currently available vaccines, in that they are easier and cheaper to produce, particularly in a resource-poor setting.

[00188] For example, one licensed cholera vaccine comprises killed whole *V. cholerae* bacterial cells combined with the purified B subunit of cholera toxin (CtxB), which serves as both an antigen and an adjuvant [7]. While the purified CtxB component improves short term protection, its production and isolation is cost-intensive, and the use of isolated CtxB negatively affects ease of delivery of the vaccine. As a result, cholera vaccines manufactured in resource-poor settings typically omit this component [8,9], resulting in less-than-optimal vaccinations.

[00189] Some aspects of this disclosure provide an engineered vaccine, for example, an engineered *V. cholerae* vaccine, in which an engineered exopolysaccharide-associated protein conjugated to an antigen or adjuvant is expressed in a bacterial cell, resulting in a decoration of the bacterial cell's exopolysaccharide with the antigen or adjuvant. In some such embodiments, the bacterium comprises a recombinant nucleic acid encoding the engineered exopolysaccharide-associated protein conjugated to the antigen or adjuvant.

[00190] For example, in some embodiments, a *V. cholerae* vaccine is provided that comprises a bacterium, for example, a *Vibrio sp.* bacterium (*e.g.*, *V. cholerae*) that expresses an engineered exopolysaccharide-associated protein, *e.g.*, an isolated *V. cholerae* exopolysaccharide-associated protein (*e.g.*, Bap1, RbmA, RbmC, or HlyA), or an exopolysaccharide-binding fragment thereof, conjugated to an antigen or adjuvant, *e.g.*, *V. cholerae* CtxB. One advantage of this type of vaccine over conventional vaccines is its ease of production. Instead of having to grow the bacteria and, separately, having to produce the antigen and/or adjuvant, the bacteria in this type of vaccine express the antigen and/or adjuvant, display it on their surface, and retain it based on the conjugation of the antigen and/or adjuvant to the exopolysaccharide-associated protein. In some embodiments, the antigen and/or adjuvant is a protein or polypeptide that is expressed as a fusion protein with the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof.

[00191] As described in more detail in the Examples section, expression of an engineered exopolysaccharide-associated protein comprising CtxB (the B subunit of cholera toxin) as the heterologous agent in *V. cholerae* resulted in a decoration of the bacterial cell's biofilm exopolysaccharide scaffold with CtxB. This integrated decoration avoids the need for a separate production and isolation of CtxB, and could greatly simplify the production and administration of a vaccine including CtxB and also potentially improve vaccine efficacy. In the a proof of principle experiment, wild-type *V. cholerae* was engineered to express an engineered exopolysaccharide-associated protein comprising RbmA with either the CtxB subunit or a FLAG affinity tag fused to its C-terminus. Biofilms were formed with these strains and the RbmA-CtxB fusion protein was observed to be sequestered to the biofilm matrix.

[00192] In some embodiments, a vaccine is provided that comprises a bacterium that is not genetically engineered, *e.g.*, wild-type bacterium such as a wild-type *Vibrio sp.* bacterium (*e.g.*, a wild-type *V. cholerae* bacterium), but that is contacted with an isolated engineered exopolysaccharide-associated protein conjugated to an antigen and/or adjuvant, *e.g.*, an isolated *V. cholerae* exopolysaccharide-associated protein (*e.g.*, Bap1, RbmA, RbmC, or HlyA), or an exopolysaccharide-binding fragment thereof, that is conjugated to an antigen or adjuvant, *e.g.*, *V. cholerae* CtxB. In some such embodiments, the engineered exopolysaccharide-associated protein conjugated to the antigen and/or adjuvant can be contacted or combined with the bacterium in a biofilm, and will associate with the exopolysaccharide scaffold of the biofilm. The biofilm can then be dispersed and used for the production of a vaccine. Alternatively, the engineered exopolysaccharide-associated protein conjugated to the antigen and/or adjuvant can be contacted or combined with the bacterium in planktonic form, and will associate with the surface of the bacterium based on the expression of exopolysaccharides on the surface of the bacterium.

[00193] The vaccines provided herein, comprising an exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, conjugated to an antigen and/or adjuvant can be produced according to methods of vaccine production known to those of skill in the art. In some embodiments, the bacterium is killed or attenuated, while in other embodiments, the bacterium is alive and/or not attenuated. In some embodiments, the vaccine comprises a pathogenic bacterium, for example, the pathogenic bacterium described herein. In some embodiments, the vaccine comprises a nonpathogenic bacterium, for example a nonpathogenic bacterium as described herein.

[00194] In some embodiments, the vaccine comprises a pathogenic bacterium, *e.g.*, a pathogenic *Vibrio sp.* bacterium, such as *Vibrio cholerae* or *Vibrio haemolyticus*, and an engineered exopolysaccharide associated protein comprising an antigen expressed by the pathogenic bacterium as the heterologous molecule. In some embodiments, the vaccine comprises a nonpathogenic bacterium and engineered exopolysaccharide-associated protein comprising an antigen expressed by a pathogenic bacterium as the heterologous molecule. For example, in some embodiments, the vaccine comprises a nonpathogenic *Vibrio sp.* strain, such as *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio anguillarum*, or *Vibrio fluvialis*, and an engineered exopolysaccharide associated protein comprising an antigen expressed by a pathogenic bacterium, *e.g.*, *Vibrio cholerae* or *Vibrio haemolyticus*, as the heterologous molecule. In some embodiments, the antigen expressed by a pathogenic bacterium is *Vibrio cholerae* CtxB. In some embodiments, the pathogenic bacterium in the vaccine of this disclosure, *e.g.*, a pathogenic *Vibrio sp.* bacterium, such as *Vibrio cholerae* or *Vibrio haemolyticus*, may be genetically modified. In some embodiments, the genetically modified pathogenic bacterium has reduced pathogenicity, *i.e.*, is attenuated. In some embodiments, the genetically modified pathogenic bacterium is a *Vibrio Cholerae* with inactivated CtxA. In some embodiments, the CtxA gene is deleted from the *Vibrio Cholerae* genome (Δ ctxA strain). Such Δ ctxA strain may be used in the live attenuated vaccines of this disclosure.

[00195] In some embodiments, the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof, is fused to the antigen and/or adjuvant, thus forming a fusion protein. In some embodiments, the fusion protein is expressed by the bacterium, *e.g.*, from a recombinant nucleic acid construct. In some embodiments, the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof that is conjugated to the antigen and/or adjuvant comprises a β -prism lectin domain and/or an FG-GAP domain. For example, in some embodiments, the exopolysaccharide-associated protein, or exopolysaccharide-binding fragment thereof that is conjugated to the antigen and/or adjuvant comprises a β -prism lectin domain flanked on one or both sides by an FG-GAP domain, and/or an FG-GAP domain flanked on one or both sides by a β -prism lectin domain. In some embodiments, the exopolysaccharide-associated protein is Bap1 (SEQ ID NO: 1), RbmA (SEQ ID NO: 2), RbmC (SEQ ID NO:3), or HlyA (SEQ ID NO: 4).

[00196] In some embodiments, the vaccine further comprises an adjuvant that is not comprised in the engineered exopolysaccharide-associated protein. Suitable adjuvants are known to those of skill in the art, include, without limitation, any adjuvants that are in use for vaccines known in the art. Exemplary suitable adjuvants include, without limitation,

inorganic adjuvants, such as aluminium salts or gels (*e.g.*, aluminium phosphate, aluminium hydroxide), alum, organic adjuvants, such as squalene (*e.g.*, ASO₃), QS21, oil-based adjuvants (*e.g.*, MF95), and virosomes (*e.g.*, containing a membrane-bound hemagglutinin and neuraminidase derived from an influenza virus). Other adjuvants that are useful according to some aspects of this disclosure include imidazoquinoline amines, imidazopyridine amines, 6,7-fused cycloalkylimidazopyridine amines, 1,2-bridged imidazoquinoline amines, imiquimod and resiquimod, dsRNA, poly I:C, bacterial lipopolysaccharide (LPS), VSV-G, and HMGB-1. Some aspects of this disclosure provide vaccines that comprise engineered exopolysaccharide-associated proteins that are conjugated to an antigen and that also comprise an adjuvant. Some aspects of this disclosure provide vaccines that comprise engineered exopolysaccharide-associated proteins that are conjugated to an adjuvant. Some aspects of this disclosure provide vaccines that comprise engineered exopolysaccharide-associated proteins that are conjugated to an antigen and an adjuvant. For example, in some embodiments, a vaccine is provided that comprises a fusion protein of the general structure [exopolysaccharide-associated protein (or fragment thereof)]-[antigen] and an adjuvant. In some embodiments, a vaccine is provided that comprises a fusion protein of the general structure [exopolysaccharide-associated protein (or fragment thereof)]-[antigen]-[adjuvant] or [exopolysaccharide-associated protein (or fragment thereof)]-[adjuvant]-[antigen]. In some embodiments, a composition, *e.g.*, a vaccine, is provided that comprises a fusion protein of the general structure [exopolysaccharide-associated protein (or fragment thereof)]-[adjuvant]. In some embodiments, the adjuvant comprises a peptide. For example, in some embodiments, the adjuvant comprises a cholera toxin B peptide, or a fragment thereof. In some embodiments, the adjuvant comprises a host defense peptide, *e.g.*, a host defense peptide or other immunostimulatory sequence as described in U.S. Patent Application Publication US20100239611, entitled *Combination Adjuvant Formulation*, published on September 23, 2010; or in Hancock et al., *Synthetic peptides as antigens for antibody production*. Methods Mol Biol. 2005;295:13-26; the entire contents of each of which are incorporated herein by reference. Exemplary suitable peptide adjuvants include, without limitation, ILPWKWPWWPWRR (SEQ ID NO: 13); VFLRRIRVIVIR (SEQ ID NO: 14); VFWRRIRVWVIR (SEQ ID NO: 15); VQLRAIRVRVIR (SEQ ID NO: 16); VQLRRIRVWVIR (SEQ ID NO: 17); VQWRAIRVRVIR (SEQ ID NO: 18); VQWRRIRVWVIR (SEQ ID NO: 19); GRFKRFRKKFKKLFKKLSPVIPLHLG (SEQ ID NO: 20); GGLRSLGRKILRAWKKYGPIIVPIIRIG (SEQ ID NO: 21); RLARIVVIRVAR

LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES (SEQ ID NO: 22);
 VQLRIRVAVIRA (SEQ ID NO: 23); VQRWLIVWRIRK (SEQ ID NO: 24);
 VRLIVAVRIWRR (SEQ ID NO: 25); IWVIWRR (SEQ ID NO: 26);
 I(Dhb)AI(Dha)LA(Abu)PGAK(Abu)GALMGANMK(Abu)A(Abu)ANASINV(Dha)L (SEQ
 ID NO: 27, Dhb: dehydrobutyrine, Dha: dehydroalanine, Abu: 2-aminobutyric acid);
 VXXRXIRVXVIR (SEQ ID NO: 28); ILKWKPWWPWRR (SEQ ID NO: 29);
 ILPWKKPWPPWRR (SEQ ID NO: 30); ILKWKPWWKWRR (SEQ ID NO: 31); and
 ILRWKWRWWRR (SEQ ID NO: 32). For example, an exemplary fusion protein may
 comprise a structure as follows: [RbmA]-[CtxB]-[ILPWKPWWPWRR (SEQ ID NO: 33)]
 or [RbmC]-[ILKWKPWWPWRR (SEQ ID NO: 34)]. These two examples are merely to
 illustrate possible structures of fusion proteins provided herein, but are not limiting, as
 additional suitable combinations of exopolysaccharide-associated proteins or
 exopolysaccharide-binding fragments thereof with antigens and/or peptide adjuvants as
 provided herein will be apparent to those of skill in the art based on the instant disclosure.

[00197] In some embodiments, the adjuvant is a cholera toxin subunit A (CtxA)
 variant. The use of cholera toxin subunit A variants as vaccine adjuvants to harness their
 immunomodulating properties have been described in the art, e.g., in PCT publications
 WO2004083251 and WO2015004105, the entire contents of which are herein incorporated by
 reference. Wild-type CtxA amino acid sequence is provided as SEQ ID NO: 46. CtxA
 variants that have reduced activity are particular suitable for use as adjuvants. For example,
 CtxA variants comprising substitution mutations between amino acids corresponding to 189-
 211 in SEQ ID NO: 46 have significantly reduced activity, e.g., reduced by at least 2-100
 fold. For example, the CtxA variant's activity may have been reduced by at least 2 fold, at
 least 3 fold, at least 4 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 30 fold, at
 least 40 fold, at least 50 fold, at least 60 fold, at least 70 fold, at least 80 fold, at least 90 fold,
 at least 100 fold, or more. Suitable mutations include mutations in positions correspond to
 N189, A190, P191, R192, S193, S194, M195, S196, N197, T198, C199, D200, or L211 of
 SEQ ID NO: 46. In some embodiments, the mutations correspond to N189D, A190S, P191S,
 R192G, S193T, S194I, M195T, S196G, N197I, N197D, or L211A in SEQ ID NO: 46. The
 exemplary mutations are not meant to be limiting. Any CtxA variants that are known in the
 art to be suitable for use as an adjuvant may be used herein.

[00198] Cholera toxin subunit A (CtxA)
 NDDKLYRADSRPPDEIKQSGGLMPRGQSEYFDRGTQMNNLYDHARGTQTGFVRHD
 DGYVSTSLRSAHLVGQTILSGHSTYYIYVIATAPNMFNVNDVLGAYSPHPDEQEVS

ALGGIPYSQIYGWYRVHFGVLDEQLHRNRGYRDRYYSNLDIAPAADGYGLAGFPPE
HRAWREEPWIIHHAPPGCGNAPRSSMSNTCDEKTQSLGVKFLDEYQSKVKRQIFSGY
QSDIDTHNRIKDEL (SEQ ID NO: 46)

[00199] In some embodiments, the CtxA variant adjuvant is encoded by a nucleic acid molecule and expressed in the bacterium in the vaccine of this disclosure. In some embodiments, the CtxA variant adjuvant is constitutively expressed by the bacterium of the vaccine. In some embodiments, the CtxA variant adjuvant is added to the vaccine after it is made.

[00200] In some embodiments, the adjuvant is a multiple-mutated Cholera toxin (mmCT). The mmCT adjuvant has been described in the art, e.g., in Lebens et al., Vaccine. 2016 Apr 19;34(18):2121-8, incorporated herein by reference. In some embodiments, the mmCT adjuvant is produced by the bacterium associated with RmbA and the antigen (e.g., the LTB-STa^{A14H} antigen). For example, the nucleotide sequence encoding the mmCT gene may be delivered to the bacterium, e.g., by any known methods of delivering nucleic acids to bacterial cells such as transformation. In some embodiments, the nucleotide sequence encoding the mmCT adjuvant may be integrated into the genome of the bacteria. In some embodiments, the nucleotide sequence encoding the mmCT adjuvant is placed under control of the LacZ promoter.

[00201] In some embodiments, the vaccine is formulated into a pharmaceutical composition for administration (e.g., orally, sublingually, intramuscularly, intradermally, or rectally) to a subject. In some embodiments, the vaccine is administered to a subject. Accordingly, some embodiments provide a method of vaccinating a subject against a pathogen. Typically, the method comprises administering to the subject an effective amount of a vaccine described herein. An effective amount, in some embodiments, is an amount that induces an immune reaction in the subject. For example, in some embodiments, an effective amount of a vaccine is an amount that, when administered to a subject, results in a measurable increase in immunity of the subject against a pathogen expressing an antigen comprised in the vaccine. In some embodiments, an effective amount of a vaccine is an amount that, when administered to a subject, results in a measurable immune response in the subject against a pathogen expressing an antigen comprised in the vaccine. For example, a measurable immune response may include an increased number or level of immunoglobulins (IgG and/or IgA) specifically binding the antigen, or an increased number of immune cells recognizing or producing antibodies directed towards the antigen. In some embodiments, the vaccine is administered in an amount sufficient to elicit an immune response against the

bacterium and/or against the antigen. In some embodiments, the vaccine is administered in an amount sufficient to immunize the subject against the bacterium and/or against the antigen. While total immunization against the antigen may be desirable and feasible in some embodiment, partial immunization is also beneficial in many cases. For example, such partial immunization may ameliorate the clinical manifestation of an exposure to a pathogen, *e.g.*, as evident in a shortened period of sickness or a decrease in the symptoms associated with the pathogen, as compared to an average subject within a population or to a non-vaccinated subject.

[00202] The inventive vaccines may be administered by a variety of routes of administration, including but not limited to parenteral (such as subcutaneous, intramuscular, intravenous, or intradermal); oral; transnasal, transmucosal, rectal; ophthalmic, or transdermal. The vaccines of this disclosure may induce systemic immune response or mucosal immune response. "Mucosal immune response," as used herein, refers the immune response elicited an organism's various mucous membranes upon invasion by potentially pathogenic microbes. Mucosal immune response provides three main functions: protecting the mucous membrane against infection; preventing the uptake of antigens, microorganisms, and other foreign materials; and moderating the organism's immune response to that material. At birth, the neonate's mucosal immune system is relatively undeveloped, but the colonization of intestinal flora accelerates its development. Because of its front-line status within the immune system, the mucosal immune system is being investigated for use in vaccines for various afflictions, including AIDS and allergies.

[00203] "Systemic immune response," as used herein, refers to the response elicited by the host's defense system, which may be categorized into two subsystems: the innate immune response and the adaptive immune response. The innate response is usually triggered when microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms, or when damaged, injured or stressed cells send out alarm signals, many of which (but not all) are recognized by the same receptors as those that recognize pathogens. Innate immune defenses are non-specific, meaning these systems respond to pathogens in a generic way. This system does not confer long-lasting immunity against a pathogen. The innate immune system is the dominant system of host defense in most organisms. The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The ability to mount these tailored responses is

maintained in the body by "memory cells". Should a pathogen infect the body more than once, these specific memory cells are used to quickly eliminate it.

[00204] Since most enteric infections begin at mucosal surfaces, the first line of protection is likely provided by locally produced secretory IgA. Mucosal and systemic immune systems are, to a large extent, independent of one another. For example, monomeric IgA or polymeric IgA are produced independently by mucosal or systemic immune response, respectively. Adult levels of systemic IgA reach its maximum at adolescence, but younger children can respond well to exposure to bacterial and viral vaccines delivered mucosally. Thus, the vaccines of this disclosure may be particularly efficacious for young children, e.g., children before they reach adolescence. In some embodiments, the children may be 0-13 years of age. For example, the vaccines of this disclosure may be administered to children 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 years of age. In addition, the vaccines of this disclosure may also be administered to subjects more than 13 years of age, e.g., adolescents and adults.

Methods

[00205] Some aspects of this disclosure provide methods for delivering a molecule to a target site. In some embodiments, the method comprises delivering to the target site a bacterium associated with an exopolysaccharide that binds an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, wherein the exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, binds the molecule. In some embodiments, the molecule is a heterologous molecule. In some embodiments, the molecule is conjugated to the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof, for example, via covalent bond or via non-covalent interaction. In some embodiments, the molecule is bound to the exopolysaccharide-associated protein, or the exopolysaccharide-binding fragment thereof, via a linker, for example via a linker comprising a sequence of immune acids that can be cleaved by a protease. In some embodiments, the molecule comprises a heterologous protein or polypeptide. In some embodiments, the polypeptide is fused to the exopolysaccharide-associated protein or the exopolysaccharide binding protein fragment. In some embodiments, the bacterium being delivered to the target site comprises a recombinant nucleic acid encoding the polypeptide fused to the exopolysaccharide-associated protein or the exopolysaccharide binding protein fragment.

[00206] In some embodiments, a method of delivering a protein of interest to a target site is disclosed herein can be used to deliver the protein to a target site that can be colonized by a bacterium expressing a fusion protein comprising the protein of interest and an exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof. In some embodiments, the protein of interest is a heterologous protein in relation to the exopolysaccharide-associated protein, or the exopolysaccharide-binding fragment thereof. In some embodiments, the heterologous protein comprises an antigen, an enzyme, a binding agent, a detection agent, a therapeutic agent, or an antibiotic agent. Any protein comprising an antigen, an enzyme, a binding agent, a detection agent, a therapeutic agent, or antibiotic agent described herein or otherwise known to those of skill in the art that can be fused to a exopolysaccharide-associated protein, or an exopolysaccharide-binding fragment thereof, can be delivered to a target site using the methods provided herein.

[00207] In some embodiments, the target site is a site within a subject that can be colonized by the bacterium. For example, in some embodiments, the target site is within the gastrointestinal tract, for example, within the stomach, the duodenum, the small intestine, the cecum, the appendix, or the colon, or within the oral cavity. In some embodiments, the target site is within the respiratory tract of the subject, for example, within the lung, within a primary bronchus, within a secondary bronchus, within a tertiary bronchus, within a bronchiole, within the trachea, or within the larynx. In some such embodiments, the method comprises administering to a subject a bacterium expressing a fusion protein comprising the protein of interest fused to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof, in an amount sufficient for the bacterium to colonize the respective target site within the subject. In some embodiments, bacterial colonization of the target site refers to bacteria reaching the target site and proliferating and/or persisting at the target site for at least one day, at least two days, at least three days, at least four days, at least five days, at least six days, at least one week, at least two weeks, at least three weeks, at least four weeks, at least one month, at least two months, at least three months, at least four months, at least six months, at least one year, or at least two years.

[00208] For example, in some embodiments, a method is provided that comprises administering to a subject having a deficiency in and a digestive enzyme, for example, a lactase deficiency, a nonpathogenic bacterium that is able to colonize the gastrointestinal tract of the subject, for example, the small intestine, and that expresses a fusion protein comprising a digestive enzyme that the subject is deficient in, *e.g.*, lactase, fused to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof, in

an amount sufficient for the bacterium to colonize the small intestines of the subject. In some embodiments, the bacterium is a bacterium that, in wild type form, colonizes the target site, for example the small intestine, of the subject, or is isolated from the target site, for example, from the small intestines, of the subject. In some embodiments, the subject is lactose intolerant, and the enzyme is lactase. In some embodiments, the subject has cystic fibrosis, and the enzyme is a pancreatic enzyme. In some embodiments, the bacterium is administered to the subject in the form of a probiotic. In some embodiments, administration route is oral or rectal.

[00209] In some embodiments, a method is provided that comprises administering to a subject having cystic fibrosis a nonpathogenic bacterium that is able to colonize the subject's respiratory tract, for example, the lung, the bronchi, the bronchioles, or the trachea, and that expresses a fusion protein comprising a mucus digesting enzyme, for example, a mucinase or alginase, fused to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof, in an amount sufficient for the bacterium to colonize the respiratory tract of the subject. In some embodiments, the bacterium is a bacterium that, in wild type form, colonizes the respiratory tract, or part of the respiratory tract of the subject, or is a bacterium that is isolated from the pulmonary airway of a subject. In some embodiments, the bacterium is a nonpathogenic bacterium that can colonize the respiratory tract of a subject. In some embodiments, the bacterium is administered directly to the respiratory tract of the subject, *e.g.*, via an aerosol or an inhalant. In some embodiments, the bacterium is of a nonpathogenic species that is found in the respiratory tract of a healthy subject, *e.g.*, *Prevotella sp.*, *Mesorhizobium sp.*, *Microbacterium sp.*, *Micrococcus sp.*, *Veillonella sp.*, *Rhizobium sp.*, *Stenotrophomonas sp.*, or *Lactococcus sp.* In some embodiments, the bacterium is a *Pseudomonas sp.* bacterium. In some embodiments, the bacterium is a nonpathogenic *Pseudomonas* bacterium, *e.g.*, *Pseudomonas chlororaphis* or *Pseudomonas putida*.

[00210] In some embodiments, the target site is a surface. In some embodiments, the target site is an abiotic surface. In some embodiments, the surface is a liquid-gas interface, *e.g.*, a water-air interface, or a culture media-air interface, for example, in a body of water, such as an ocean, a lake, or a pond, or in a bioreactor or culture vessel holding liquid media. In some embodiments, the target site is a liquid-solid interface, *e.g.*, a water-soil interface, a water-metal interface, or a water-plastic interface. In some embodiments, the molecule to be delivered to the target site is an enzyme that can digest a substrate present at the target site. In some embodiments, the substrate is a contaminant that is present at the

target site. For example, in some embodiments, the target site is a water-air interface and the substrate is an environmental pollutant, such as a toxic chemical or oil, for example, in the context of an oil spill. In some such embodiments, the enzyme expressed as a fusion protein with an exopolysaccharide-associated protein or exopolysaccharide-binding fragment thereof by a bacterium that forms a biofilm. The bacterium is then delivered to the target site, where it expresses the fusion protein and forms or integrates into a biofilm. The biofilm formed retains the fusion protein, and, thus, exhibits the functionality of the fusion protein, here an enzymatic activity directed towards the enzyme's substrate, *e.g.*, a contaminant or an environmental pollutant.

[00211] Accordingly, the instantly disclosed technology can be used in the context of bioremediation, referring to the use of bacteria to remove, or aid in the removal of, pollutants from the environment. Engineered bacteria as provided herein can be delivered to sites of contamination and pollution and, through expression of an engineered exopolysaccharide-associated protein, *e.g.*, a fusion protein comprising an enzyme that can break down the pollutant or contaminant.

[00212] In some embodiments related to bioremediation, the target site is an environmental surface. For example, in some embodiments, the target site is a polluted water-air interface, a polluted water-soil interface, or a polluted soil-air interface. In some embodiments, the molecule to be delivered to the target site is an enzyme that can break down a pollutant present at the target site. In some such embodiments, the enzyme is expressed as a fusion protein with an exopolysaccharide-associated protein or exopolysaccharide-binding fragment thereof by a bacterium that forms a biofilm. The bacterium is then delivered to the polluted target site, where it expresses the fusion protein and forms or integrates into a biofilm. The biofilm retains the fusion protein, and, thus, exhibits the functionality of the fusion protein, here an enzymatic activity that breaks down the pollutant.

[00213] For example, in some embodiments in the context of a marine oil spill, an engineered bacterium expressing an oil-degrading enzyme, *e.g.*, an oil-degrading enzyme as described elsewhere herein, is delivered to the water-air interface of the contaminated body of water, where it proliferates and forms a biofilm expressing the oil-degrading enzyme functionality. Suitable enzymes that are useful in such embodiments include, without limitation, alkane 1-monooxygenase, naphthalene 1,2-dioxygenase, E-phenylitaconyl-CoA hydratase, benzylsuccinyl-CoA dehydrogenase, methane monooxygenase, and the enzymes described in Hazen et al., *Deep-sea oil plume enriches indigenous oil-degrading bacteria*.

Science. 2010 Oct 8;330(6001):204-8, including supplemental content (see, *e.g.*, Table S6 of Hazen et al.); Lu et al., *Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume*. ISME J. 2012 Feb;6(2):451-60; Kostka et al., *Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill*. Appl Environ Microbiol. 2011 Nov;77(22):7962-74; and Wood et al., *Engineering biofilm formation and dispersal*. Trends Biotechnol. 2011 Feb;29(2):87-94; the entire contents of each of which are incorporated herein by reference. Suitable bacterial genera and strains that are useful for expressing engineered exopolysaccharide-associated proteins as provided herein in the context of bioremediation scenarios, *e.g.*, in the context of a marine oil spill, are known to those of skill in the art and include, without limitations, the bacterial taxa, genera, species, and strains described in Hazen et al., *Deep-sea oil plume enriches indigenous oil-degrading bacteria*. Science. 2010 Oct 8;330(6001):204-8; Kostka et al., *Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill*. Appl Environ Microbiol. 2011 Nov;77(22):7962-74; and Wood et al., *Engineering biofilm formation and dispersal*. Trends Biotechnol. 2011 Feb;29(2):87-94; the entire contents of each of which are incorporated herein by reference. Additional suitable enzymes and bacterial genera will be apparent to those of skill in the art, and the disclosure is not limited in this respect.

[00214] In some embodiments related to bioremediation, or otherwise to the release of engineered bacteria into the environment, it is preferable that the bacteria also comprise a safeguard measure that prevents uncontrolled proliferation in the environment. For example, in some embodiments, the bacteria exhibit a dependency on a nutrient that does not exist or is extremely rare in the environment. This nutrient can be dispersed at the target site, *e.g.*, at the water-air interface affected by a marine oil spill, to allow the bacteria to survive, proliferate, and form biofilms. Once the bioremediation task is done, dispersal of the nutrient is discontinued, resulting in the death of the released bacteria. In some embodiments, the bacteria used are auxotrophs that require a nutrient not typically found at the site of release, or the environment exposed to the bacteria, for example, an amino acid (*e.g.*, histidine), a vitamin (*e.g.*, biotin), or a cell wall component (*e.g.*, diaminopimelic acid).

[00215] Some aspects of this disclosure provide methods for using an engineered exopolysaccharide-associated protein, an engineered bacterium, or an engineered biofilm as provided herein for the purification of a product generated in a cell culture or in a bioreactor. In some such embodiments, engineered bacteria or biofilms function as a biocolumn,

retaining and/or concentrating the product on their cell surface or within the biofilm. The bacteria or the biofilm can then be retrieved and the product isolated from the bacteria or the biofilm. The use of engineered bacteria or biofilms as provided herein as biocolumns is advantageous as compared to conventional strategies. Such conventional strategies typically rely on the desired product of a biofermentation being secreted by cells within a bioreactor into a liquid medium, separation of the cells from the liquid medium, and isolation of the product from the liquid medium. In many instances, this strategy required the processing of large amounts of liquid medium containing small amounts of the desired product. If the product is soluble, the subsequent isolation typically requires the use of binding agents conjugated to a solid support or the precipitation of the product from the liquid media.

[00216] In contrast, the methods for purifying a product from a bioreactor by using the engineered cells or biofilms provided herein as biocolumns allow for the separation of the product via a method that includes separating the bacteria or the biofilm from the liquid media in the bioreactor, and then isolating the product from the bacteria or biofilm. This obviates the use of binding agents for the isolation of the product from liquid media, and allows for a one-step purification method.

[00217] For example, in some embodiments, the product is a protein. In some such embodiments, the protein is expressed as a fusion protein with an exopolysaccharide-associated protein, or with an exopolysaccharide-binding fragment thereof. In some embodiments, the method of purifying the protein involves culturing a bacterium expressing the protein as a fusion protein with an exopolysaccharide-associated protein, or with an exopolysaccharide-binding fragment thereof in a liquid medium. In some embodiments, the fusion protein comprises a cleavable linker, *e.g.*, a protease- or photocleavable linker, connecting the product protein to the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof. In some embodiments, the bacteria express the fusion protein. In some embodiments, the protein product is fused to the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof in manner that results in the product protein being located in the extracellular space. In some embodiments, the fusion protein is secreted by the bacteria. In some embodiments, the bacteria are in planktonic form and retain the product protein on their cell surface based on its fusion to an exopolysaccharide-associated protein, or with an exopolysaccharide-binding fragment thereof. In other embodiments, the bacteria form a biofilm and the product protein is retained within the biofilm based on its fusion to an exopolysaccharide-associated protein. In some embodiments, the method includes isolating the bacteria, *e.g.*, by pelleting planktonic bacteria

(*e.g.*, by centrifugation), or by retrieving a biofilm from a surface within a bioreactor. In some embodiments, the method further includes separating the protein product from its fusion partner, *e.g.*, the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof, for example, by cleavage of the cleavable linker. In some embodiments, the cleaved-off protein product is then eluted from the bacteria, which will retain the cleaved-off portion of the fusion protein that comprises the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof.

[00218] For example, in some embodiments, the product is a secreted molecule. In some such embodiments, the bacteria express the secreted molecule and a fusion protein comprising a protein binding agent that binds the secreted product molecule (*e.g.*, an affinity tag, antibody fragment, adnectin, or aptamer) fused to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof. In some embodiments, the method of purifying the protein involves culturing a bacterium expressing the secreted product and a protein binding agent that binds the secreted product molecule (*e.g.*, an affinity tag, antibody fragment, adnectin, or aptamer) fused to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof in a liquid medium. In some embodiments, the fusion protein comprises a cleavable linker, *e.g.*, a protease- or photocleavable linker, connecting the binding agent to the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof. The binding agent in such embodiments is fused to the exopolysaccharide-associated protein or the exopolysaccharide-binding fragment thereof in manner that results in the binding agent being located in the extracellular space. In some embodiments, the fusion protein is secreted by the bacteria. In some embodiments, the bacteria are in planktonic form and retain the binding agent on their cell surface based on its fusion to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof. In other embodiments, the bacteria form a biofilm and the binding agent is retained within the biofilm based on its fusion to an exopolysaccharide-associated protein or an exopolysaccharide-binding fragment thereof. The bacteria are cultured under conditions that permit the binding agent on the surface of the bacteria or within the biofilm to bind the product molecule. In some embodiments, the method includes isolating the bacteria, *e.g.*, by pelleting planktonic bacteria (*e.g.*, by centrifugation), or by retrieving a biofilm from a surface within a bioreactor. In some embodiments, the method further includes separating the product molecule from the binding agent, *e.g.*, by eluting the product molecule. In some embodiments, the method includes cleaving the binding agent from its fusion partner, *e.g.*, the exopolysaccharide-associated

protein or the exopolysaccharide-binding fragment thereof, for example, by cleavage of the cleavable linker. In some embodiments, the cleaved-off binding agent, bound to the product molecule, is then separated from the bacteria, *e.g.*, by elution, and subsequently the product molecule is isolated, *e.g.*, eluted from the binding agent. The nature of the binding agent will depend on the product molecule to be purified. Binding agents for various product molecules are well known to those of skill in the art, and the disclosure is not limited in this respect.

[00219] Some of the embodiments, advantages, features, and uses of the technology disclosed herein will be more fully understood from the Examples below. The Examples are intended to illustrate some of the benefits of the present disclosure and to describe particular embodiments, but are not intended to exemplify the full scope of the disclosure and, accordingly, do not limit the scope of the disclosure.

EXAMPLES

Materials and Methods

[00220] Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 5:

<u>Bacterial Strains</u>	<u>Genotype and/or phenotype</u>	<u>Ref.</u>
<i>E. coli</i> strains		
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::MuλpirR6K;Km^r</i>	[1]
<i>V. cholerae</i> strains		
PW249	MO10 ; Sm ^r	[2]
PW328	MO10 Δ <i>vpsL</i> , Sm ^r	[3]
PW357	MO10 <i>lacZ::vpsLp</i> → <i>lacZ</i> ; Sm ^r	[3]
PW454	MO10 Δ <i>vpsL lacZ::gfp</i> ; Sm ^r	
PW707	MO10 Δ <i>bap1</i> Δ <i>rbmC</i> ; <i>lacZ::vpsLp</i> → <i>lacZ</i> ; Sm ^r	
PW1085	MO10 <i>rbmA-flag</i> ; <i>lacZ::vpsLp</i> → <i>lacZ</i> ; Sm ^r Ap ^r	
PW1086	MO10 <i>bap1-flag</i> ; <i>lacZ::vpsLp</i> → <i>lacZ</i> ; Sm ^r Ap ^r	
PW1087	MO10 Δ <i>rbmA</i> ; <i>lacZ::vpsLp</i> → <i>lacZ</i> ; Sm ^r	
PW1088-90	MO10 Δ <i>bap1</i> Δ <i>rbmC</i> , Sm ^r	
<u>Plasmids</u>		
pWM91	<i>oriR6KmobRP4 lacI pTac tnp miniTn10Km</i> ; Ap ^r	[4]
pWM91 Δ <i>bap1</i>	pWM91 carrying a fragment of <i>bap1</i> harboring an internal, unmarked deletion; Ap ^r	[5]

pWM91 Δ <i>rbmC</i>	pWM91 carrying a fragment of <i>rbmC</i> harboring an internal, unmarked deletion; Ap ^r	
pWM91 Δ <i>rbmA</i>	pWM91 carrying a fragment of <i>rbmA</i> harboring an internal, unmarked deletion; Ap ^r	
pGP704:: <i>bap1</i> - <i>flag</i>	pGP704 carrying 3' end of <i>bap1</i> fused to a <i>flag</i> tag; Ap ^r	
pGP704:: <i>rbmA</i> - <i>flag</i>	pGP704 carrying 3' end of <i>rbmA</i> fused to a <i>flag</i> tag; Ap ^r	
pJZ111	<i>Plac</i> :: <i>gfp</i> :: <i>lacZ</i> in pCVD442; Ap ^r	[6]
pBAD-TOPO- <i>rbmA</i>	pBAD-TOPO carrying the gene at locus VC0928 (<i>rbmA</i>); Ap ^r	
pFLAG- <i>chiA</i> -2	pFLAG-CTC carrying the gene at locus VCA0027 (<i>chiA</i> -2); Ap ^r	
pFLAG- <i>hlyA</i>	pFLAG-CTC carrying the gene at locus VCA0219 (<i>hlyA</i>); Ap ^r	
pFLAG- <i>hapA</i>	pFLAG-CTC carrying the gene at locus VCA0865 (<i>hapA</i>); Ap ^r	
pFLAG- <i>tcpG</i>	pFLAG-CTC carrying the gene at locus VC0034 (<i>tcpG</i>); Ap ^r	
pFLAG- <i>mshA</i>	pFLAG-CTC carrying the gene at locus VC0409 (<i>mshA</i>); Ap ^r	
pFLAG- <i>rbmA</i>	pFLAG-CTC carrying the gene at locus VC0928 (<i>rbmA</i>); Ap ^r	
pFLAG- <i>crr</i>	pFLAG-CTC carrying the gene at locus VC0964 (<i>crr</i>); Ap ^r	
pFLAG- <i>bap1</i>	pFLAG-CTC carrying the gene at locus VC1888 (<i>bap1</i>); Ap ^r	

Table 5: bacterial strains and plasmids. All listed references (Ref.) are incorporated herein by reference in their entirety.

[00221] Vectors used for protein expression included an IPTG inducible promoter driving expression of the protein of interest with a C-terminal FLAGtag (pFLAG-CTC, Sigma-Aldrich). Bacteria were cultivated in Luria-Bertani broth (LB) supplemented with ampicillin (100 µg/ml). Because adequate protein expression was observed without induction, the growth medium was not supplemented with IPTG.

[00222] *V. cholerae* strain MO10 (serotype O139, streptomycin resistant) inactivated in *ctxA* and carrying a plasmid encoding the RbmA-CtxB fusion protein as previously described (Δ *ctxA*/*prbmA*-*ctxB*) was maintained in Luria-Bertani medium (LB) with 25% glycerol at -80°C. Broth cultures were inoculated from the frozen stocks into LB and grown overnight for 18 hr at 37°C, 200 rpm. Ampicillin (100 µg/mL) was included at all times for plasmid maintenance.

[00223] Construction of plasmids for protein expression. The ORFs of interest were amplified by PCR using primers including the start and stop codons of each gene of interest. Kpn I restriction sites were included in the primers used for amplification of *chiA*-2. These restriction sites were used to insert *chiA*-2 between *rbmA* and the FLAG tag in expression vector pFLAG-*rbmA* [1]. Kpn I and Sal I restriction sites were used to fuse *ctxB* to the C-terminal end of *rbmA* pFLAG-*rbmA*. In this case, the FLAG tag was removed. All insertions were confirmed by sequence analysis. Before use, we confirmed that the RbmA-CtxB and

RbmA-ChiA-2-FLAG fusion proteins did not interfere with formation of the wild-type *V. cholerae* biofilm.

[00224] Mutant construction. The *V. cholerae* Δ bap1 Δ rbmC Δ rbmA mutant was constructed as previously described [14] using the strain *V. cholerae* Δ bap1 Δ rbmC mutant (PW707) and the suicide plasmid pWM91 Δ rbmA [1].

[00225] Generation of the live-attenuated vaccine. Bacterial cultures grown overnight in LB were centrifuged for 5 min at 6,000 x g, washed once with phosphate-buffered saline (PBS), and inoculated at a 1:100 dilution into 25 mL of fresh LB containing ampicillin. Protein production is induced with 0.1 mM of β -d-1-thiogalactopyranoside (IPTG). Following induction, cells were incubated for 8 hr at 37°C and 200 rpm, then harvested by centrifuging for 20 min at 6000 x g. Cell pellets were washed twice in PBS and finally resuspended in 2 mL of PBS. This constituted the vaccine suspension. From this suspension, 10 μ L was removed for viable counts of colony forming units (CFU), 20 μ L was removed and added to 180 μ L of 4 x Laemmli buffer. For each immunization, the vaccine suspension was prepared fresh and administered within 2 hr of preparation.

[00226] Generation of vaccines. Bacterial cultures were grown overnight from FS in 2 mL with ampicillin (100 μ g/ml). They were shaken at 27°C at 180 rpm for 14 hours. A 25 mL culture was inoculated with 1:100 dilution of the overnight culture, including ampicillin and IPTG (100 μ g/ml ampicillin + 0.5mM IPTG added from start, in 250mL-volume flasks). Following inoculation, the cultures were shaken at 27°C at 180 rpm until the OD₆₀₀ reached 0.6. The cells were placed on ice for 10 minutes. The cells were then pelleted at 5000xG for 20 minutes at 4°C and 100 μ L of the supernatant was removed and added to 100 μ L 2X Laemmli sample buffer for Western blot sample. The rest of the supernatant was discarded and the pellet was resuspended in 10 mL of PBS. The cells were pelleted at 5000xG for 20 minutes at 4°C, and the resuspension and pelleting process was repeated for a total of three washes. The washed pellet was then resuspended in 5mL of PBS and then 20 μ L to 180 μ L 2X Laemmli sample buffer were added for Western blot samples. Both Western blot samples were boiled for 5 minutes. Then, the samples were sonicated 15s x 2, with a 15s rest in between. The samples were stored at -20°C. The vaccine was then stored on ice until administration. The vaccines were prepared fresh on the day of administration and were not stored overnight.

[00227] Once the vaccine was prepared, it was screened. Sterility was analyzed by adding LB culture to the vaccine and then streaking on a LB plate and measuring the OD at 600 nm. Cells were also counted using a hemocytometer and a BSA assay was performed to

determine the protein concentration. Then, the presence of CtxB was assayed after formaldehyde; it was quantified by Western blot (see below) and the CtxB per cell was calculated.

[00228] Before the vaccine is shipped, it is diluted to match the cell levels required for the vaccine study. Purified CtxB is added to WT/pFLAG samples to match the concentration found in fusion protein samples.

[00229] Quantitative Western blot. Samples were boiled for 5 min, briefly centrifuged, sonicated twice for 15 s with one 10 s pulse (output approximately 10 W), and collected by brief centrifugation. Protein samples were resolved by 4-20% gradient Tris-glycine SDS-PAGE (BioRad). Aliquots of purified cholera toxin B subunit (List Laboratories) at known concentrations were included as standards for quantification. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer method (BioRad). The membrane was incubated in a blocking solution consisting of Tris-buffered saline containing 0.1% Tween 20 (TBS-Tween) and 5% skim milk with gentle mixing for 2 hr at room temperature. Fresh blocking solution containing horseradish peroxidase (HRP)-conjugated antibody against CtxB (1:400) (Thermo Fisher) was added. Following overnight incubation at 4°C with gentle mixing, the membrane was washed three times with TBS-Tween and developed using ECL Western blotting substrate (Thermo Fisher). ImageJ was used to generate a standard curve based on CtxB standards. Concentration of the fusion protein in the sample was determined using the linear portion of the standard curve.

[00230] Biofilm assays. A single colony of *V. cholerae* was inoculated into 1 ml of LB broth and allowed to grow to mid exponential phase. The culture was then diluted in LB broth to yield an OD₆₅₅ of 0.05 and divided into three disposable glass culture tubes (10mm X 75 mm). These tubes were incubated statically at 27 °C. After 24 hrs, planktonic cells were removed, and the OD₆₅₅ of the cells was measured. Remaining biofilms were washed with 0.1 M phosphate-buffered saline solution (PBS) (pH 7.0) and then disrupted with 1 mm beads (Biospec). The OD₆₅₅ of the resulting cell suspension was measured. For assays of biofilm integrity, biofilms were formed as described above in 24 well plates and then vortexed.

[00231] Immunofluorescence. Immunofluorescence experiments were performed as previously described with the following modifications [1]. To detect the RbmA-CtxB fusion protein, an anti-CtxB antibody (Sigma) (1:1000 dilution) followed with an Alexa Fluor 488 Goat Anti-Rabbit Antibody (Invitrogen) was used. To detect ChiA-2-FLAG and RbmA-ChiA-2-FLAG, an anti-FLAG M2 antibody (1:1000 dilution) (Sigma-Aldrich) was used followed

by an incubation with DyLight 549 AffiniPure Rabbit Anti-Mouse IgG H+L (1:1000 dilution) (Jackson ImmunoResearch). Confocal images were acquired at the Children's Hospital, Boston Imaging Core with a LSM700 microscope (Zeiss) equipped with a 63X objective and 405, 488, and 555 nm laser lines. A computer equipped with ZEN 2009 software was used to acquire and process images.

[00232] Chitinase assays. For assays of protein activity, cells were cultured in LB broth supplemented with ampicillin at 27 °C for approximately 5 hours and then back-diluted in the same medium to yield an OD₆₅₅ of 0.05. For assays of activity within the biofilm, three 80 µl aliquots of each culture were transferred to the wells of a 96 well, black microtiter dish and three to wells of a polystyrene 96 well plate. Both plates were incubated statically at 27 °C for 24 hours. The planktonic fractions of the resulting cultures were removed, fractions from one well of the black plate and one well of the polystyrene plate were pooled, an OD₆₅₅ was recorded, and the cell suspensions were centrifuged. A 5 µl volume of the supernatant was removed and assayed for chitinase activity. Biofilms remaining in the black 96 well plates were rinsed twice with PBS and assayed directly for chitinase activity. For assays of the Δ bap1 Δ rbmA Δ rbmC mutant, cells were cultured in 1 ml of LB broth at 27 °C with shaking overnight. An OD₆₅₅ was recorded. The cell suspension was pelleted, and the supernatant was removed. Cells were rinsed once with PBS and then resuspended in an equal volume of PBS. 5 µl of the cell suspension and supernatant were assayed for chitinase activity. Chitinase activity was measured using a fluorometric chitinase assay kit (Sigma-Aldrich) according to the manufacturer's protocol including the following steps. Bacterial cells, biofilms, or supernatants were incubated in substrate buffer containing 0.2 mg/ml 4-methylumbelliferyl N, N'-diacetylchitobioside hydrate chitobiose for 20 minutes at 37 °C in the dark prior to measurement of fluorescence with an Infinite 200 spectrophotometer (Tecan).

[00233] Sublingual immunization of mice. Female specific pathogen-free BALB/c mice (6-8 week) were obtained from Charles River Laboratories and housed in groups of five. The mice were given food and water *ad libitum*. Experimental procedures performed here have been previously approved by the Institutional Animal Care and Use Committee. To establish baseline values of antibodies reacting with CtxB prior to immunization, stool pellets were collected one day prior to the first vaccination and sera were obtained immediately before the first vaccination. The mice were immunized three times at 2 week intervals. Sera and stool samples were collected every 2 weeks after the first vaccination and analyzed with ELISA for the presence of CtxB-specific antibodies. For sublingual immunization, mice were

anesthetized by intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg), then held upright as 10 μ L of the vaccine suspension is delivered under the tongue by a micropipette directed toward the floor of the mouth. The mice were held in the upright position for at least 2 min, then rested ventral side down for 30 min.

[00234] Measurement of antigen-specific antibodies. Blood was collected from the tail vein of the mice. Sera was obtained by incubating the samples for 45 min at room temperature, then centrifuging at 1,500 rpm for 15 min. Supernatant was removed into a new microcentrifuge tube and spun at 1,500 rpm for 5 min. Sera was stored at -20°C until use. Stool pellets were resuspended in cold PBS containing 25mM EDTA and 0.01% (w/v) soybean trypsin inhibitor. The suspension was centrifuged at 1,800 rpm for 10 min. The supernatant was removed and centrifuged once more at 12,000 rpm for 10 min at 4°C. Stool extracts were stored at -80°C with 2 mM PMSF until use. Antibodies were detected by ELISA using monosialotetrahexosylganglioside G_{M1} (10 μ g/mL in 50 mM carbonate buffer, pH 9.5) followed by recombinant CtxB (1 μ g/mL) for CtxB-specific antibodies and goat anti-mouse IgA (1 μ g/mL) (Bethyl Laboratories) coating for total IgA in the stool samples. Presence of bound antibodies were detected with HRP-conjugated anti-IgA or anti-IgG (1 μ g/mL) (Bethyl Laboratories). Plates were developed with 1-Step Ultra ELISA Turbo Substrate (Thermo Fisher) using a kinetic ELISA protocol, reading for 7 min at 10 s intervals with 3 s mixing. Standard curves were generated with mouse IgA (Bethyl Laboratories) or mouse anti-CtxB-IgG (Thermo Fisher).

[00235] Serum vibriocidal responses. Serum vibriocidal antibody titer was determined as previously described, with some modifications. Sera were serially diluted 2-fold in 5 μ L with PBS. Wild-type MO10 was used as the indicator strain and grown to mid-logarithmic phase in brain heart infusion broth (BHI) containing streptomycin (100 μ g/mL). The bacterial culture was diluted in PBS containing 10% guinea pig complement to 2×10^6 CFU/mL, and 5 μ L of this suspension was added to the sera. The combination was incubated for 1 hr at 37°C with shaking at 200 rpm. Viable cells were enumerated from sera dilution by CFU counts. Bactericidal titer was determined as the reciprocal of the sera dilution capable of killing 50% or more of the indicator strain compared with a control containing pre-immune sera.

[00236] Statistical analysis. Three experimental replicates were included in all quantitative experiments, and each experiment was repeated at least twice. Reported values represent the mean of the three experimental replicates, error bars represent the standard deviation, and statistical significance was calculated using a student's t-test.

Example 1

[00237] The bacterial biofilm matrix is comprised of exopolysaccharide, proteins, and DNA [2]. A model is emerging in which the biofilm exopolysaccharide is a scaffold to which adhesive proteins are anchored rather than the glue that holds the biofilm structure together [1,3]. We and others recently identified three such adhesive *V. cholerae* proteins, Bap1, RbmA, and RbmC [1,4-6]. Here we demonstrate the feasibility of using RbmA as a biofilm matrix targeting moiety for proteins of biological significance.

[00238] One licensed cholera vaccine includes killed whole *V. cholerae* cells combined with the purified B subunit of cholera toxin (CtxB), which serves as both an antigen and an adjuvant [7]. The purified CtxB component improves short term protection but also decreases affordability and ease of delivery. As a result, cholera vaccines manufactured in resource-poor settings have omitted this component [8,9]. We reasoned that engineering *V. cholerae* to decorate its biofilm exopolysaccharide scaffold with CtxB could greatly simplify the production and administration of a vaccine including CtxB and also potentially improve vaccine efficacy. In a proof of principle experiment, we engineered wild-type *V. cholerae* to express RbmA with either the CtxB subunit or a FLAG affinity tag fused to its C-terminus. Biofilms were formed with these strains. To determine if the RbmA-CtxB fusion protein was sequestered to the biofilm matrix, we subjected these biofilms to immunofluorescence using an antibody recognizing CtxB.

[00239] As a control, a biofilm formed by wild-type *V. cholerae* carrying an empty vector was also subjected to immunofluorescence using an anti-CtxB antibody. As shown in **Figure 1**, the RbmA-CtxB fusion was visualized in the biofilm in a distribution similar to that of RbmA-FLAG. However, the anti-CtxB antibody did not associate with the biofilm in the absence of the RbmA-CtxB fusion protein. This result suggests that bacteria can be engineered to deliver and concentrate antigens or adjuvants at their surfaces by fusion to biofilm matrix-associated proteins. Such engineered bacterial strains could be candidates for live-attenuated or killed whole cell vaccines. Exemplary sequences of the respective fusions are provided below:

[00240] RbmA-FLAG:

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESCE TVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN

QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKKDYKDDDDK (SEQ
ID NO: 35)

[00241] RbmA-CtxB

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKKMIKKFGVFFTVL
LSSAYAHGTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKREMAITFKNGAIFQVE
VPGSQHIDSQKKAIERMKDTRLRIAYLTEAKVEKLCVWNNKTPHAIAAISMAN (SEQ
ID NO: 36)

[00242] RbmA-ChiA-FLAG

MSNFKGSIMNKRHYLLASCLALLFSTASYAEVDCELQPVIEANLSLNQNQLASNGGY
ISSQLGIRNESECETVKFKYWLSIKGPEGIYFPAKAVVGVDTAQQESDALTDGRMLNV
TRGFWVPEYMADGKYTVSLQVVAENGKVFKANQEFVKGVDLNSLPENGLTIDIKN
QFGINSVESTGGFVPFTVDLNNGREGEANVEFWMTAVGPDGLIIPVNAREKWVIASG
DTYSKVRGINFDKSYPAGEYTINAQVVDIVSGERVEQSMTVVKKMNRMTLCAASIA
CALASTAMAAPSAPSDVYGSNNLQFSKIELAMETTAGYNQMVKYHEEAPITLKFN
QWSGVTGNTYKIYFDGVEVATGPISGSQTTAQFTYKGGVYQLVIEACDATGCTKSA
PSEITIADTDGSHLKPLKMNVDPNKSYTIPQNTVIGTYFVEWSIYDRKFTVDNIPGQ
NLTHILYGFIPICGPNESLKS VGGNSFNALQTACKGVPDFEVVIHDPWAAYQKSFPQA
GHQYSSPIKGN YAMLMALKKTY PDLKIIPSIGGWTLSDPFFSFTDKAKRDVFVASVK
RFLKTKWKFYDGDIDWEYPGGGGQAADLGDPVKDGPAYVALMAELRAMLDELEA
ETGRKYELTSAIGVGHDKIEDVNYGQAVQYMDYIFAMTYDFYGGWNNVLGHQTAL
YCGSFM R PGQCDGKGVDENGEPYKGPAYTTDNGIQLLLAQGVPPSKLVVGAAMYG
RGWEGVTPASLKDPNDPMTGVGNGKLKGTTAQGVWEAGVIDYKGVKNFMLGANK
TGVNGFEYGYDEQAEAPWVWNRTTGQLVTFDDDRSVKAKGAYVRNLGLAGLFSW
EIDADNGDILNAMHEGLAGGTTTPPVNKAPVANAGADITVTGPAAVSLDGSASKDS
DGSIASYLWEQTAGPAVTLTGANSASAFNAEVEKQTFTFKLTVDNKGATATD
TVVVTVNPKSTTPVNTAPVAALSAPASVKAGATVVVDASASSDADQDPLSFTWDL P
VGVNATVQGAKVTFVAGEYTQD TTLDFTVTVSDGKATSKASASVLVEKKAGTGGD
ACTNLWNAESIYTGGQQVTWAGKTWEAKWWTRGEDPSKSGQWGVWKDLGAASC
STH DYKDDDDK (SEQ ID NO: 37)

[00243] Additionally, we reasoned that this technology might be used to deliver a functional enzyme to a surface. To model this, we inserted a secreted *V. cholerae* chitinase between RbmA and a C-terminal FLAG tag (RbmA-ChiA-2-FLAG) and expressed this fusion protein from a plasmid in wild-type *V. cholerae*. As controls, we also generated wild-type *V. cholerae* carrying an empty vector or the same vector encoding ChiA-2-FLAG. Biofilms were formed with these three strains, and immunofluorescence was performed using an anti-FLAG antibody. As shown in **Figure 2A** and **B**, the RbmAChiA-2-FLAG fusion was concentrated in the biofilm, whereas the ChiA-2-FLAG protein alone was not.

[00244] To be useful in surface modification, enzymes directed to the biofilm matrix must retain their activity. Therefore, we assessed whether the biofilm-associated RbmA-ChiA-2 fusion protein retained enzymatic activity. We formed biofilms with wild-type *V. cholerae* expressing RbmA-CtxB, ChiA-2-FLAG, or RbmA-ChiA-2-FLAG from a plasmid. As an additional control, a wild-type strain carrying an empty vector was included. Planktonic cells were removed, the biofilms were rinsed, and the chitinase activity of the biofilms and cell supernatants was tested. As shown in **Figure 2C**, chitinase activity was approximately 15 times greater in the biofilm formed by the strain expressing the RbmAChiA-2-FLAG fusion than in any of the other biofilms tested. Because chitinase is a secreted protein native to *V. cholerae*, chitinase activity was high in all the cell supernatants, particularly those expressing either ChiA-2-FLAG alone or the RbmAChiA-2-FLAG fusion protein (**Figure 2D**). These experiments show that the biofilm matrix can be used to deliver active enzymes to surfaces.

[00245] For some applications, it may be advantageous to anchor proteins to planktonic cells. Bap1 and RbmC are found at the biofilm-surface interface and are important for anchoring the biofilm to surfaces. When biofilms are formed under static growth conditions, a *V. cholerae* Δ bap1 Δ rbmC mutant forms a multicellular structure or pellicle at the air-water interface, but this structure does not adhere to the walls of the well.

[00246] Therefore, in quantitative assays of biofilm association, the Δ bap1 Δ rbmC mutant pellicle is easily dislodged, and the resulting measurement is indistinguishable from that of an exopolysaccharide mutant [1,5]. In contrast, RbmA is distributed throughout the biofilm and cements intercellular interactions. The Δ rbmA mutant biofilm forms a pellicle at the air-water interface that remains strongly attached to the surface. However, the pellicle is easily dispersed by vortexing [1,4]. The biofilm defects of both the Δ bap1 Δ rbmC mutant and the Δ rbmA mutant can be rescued by addition of these purified proteins to the culture medium 1.

This suggests that the exopolysaccharide scaffold is synthesized and exported in the absence of matrix protein synthesis.

[00247] We hypothesized that a Δ bap1 Δ rbmA Δ rbmC mutant would be defective in cell-surface and cell-cell interactions and, therefore, unable to form a biofilm. To test this, we created this triple mutant and assessed biofilm formation. As shown in **Figure 3A** and **B**, the triple mutant did not associate with the surfaces in quantitative assays and did not form a pellicle. Pellicle formation was rescued, however, by a plasmid encoding RbmA-FLAG or RbmA-CtxB (**Figure 3B**). Expression of ChiA-2-FLAG and the RbmAChiA-2-FLAG from a plasmid did not rescue pellicle formation by the triple mutant. This suggests that, unlike RbmA and RbmA-CtxB, the RbmA-ChiA-2-FLAG protein is unable to mediate intercellular interactions.

[00248] To determine whether the RbmA-ChiA2-FLAG fusion remained associated with cells in the absence of biofilm formation, a triple mutant expressing RbmA-ChiA-2-FLAG was cultured with agitation, cells were pelleted, and the chitinase activity of both the cellular fraction and the supernatant was assayed. As shown in **Figure 3C** and **D**, chitinase activity was sequestered to cells expressing the RbmA-ChiA-2-FLAG fusion but not to cells expressing ChiA-2-FLAG or RbmA-CtxB. This suggests that RbmA-ChiA-2-FLAG is cell-associated even in the absence of a biofilm structure. We hypothesize that the biofilm matrix exopolysaccharide is produced and exported by the triple mutant.

[00249] Furthermore, although RbmA-ChiA-2-FLAG is unable to mediate intercellular interactions, it likely retains the ability to associate with this matrix exopolysaccharide and remains functional on the cell surface. These experiments suggest that this protein presentation platform can also be adapted for use in planktonic bacterial cells.

Example 2

[00250] Like other bacteria, *Vibrio cholerae* synthesizes a highly regulated extracellular matrix that enables attachment to surfaces in a complex, three-dimensional multicellular structure known as a biofilm. Components of the biofilm matrix include exopolysaccharide, protein, and DNA. The polysaccharide component of the biofilm matrix has often been thought of as the glue that stabilizes the biofilm structure. We have recently completed the first proteomic analysis of a Gram-negative bacterial biofilm matrix [1]. Our findings suggest that the *V. cholerae* biofilm exopolysaccharide is tightly associated with the cell of origin, while secreted matrix-associated proteins are a communal resource required for

cell-cell and cell-surface interactions. Furthermore, once secreted, these proteins segregate to distinct regions of the biofilm matrix to maintain the structural integrity of the biofilm.

[00251] Biofilm matrix-associated proteins can be harnessed to target enzymes or antigens of interest to specific regions of the biofilm matrix of Gram-negative bacteria. Engineered peptides that attach to the biofilm matrix in distinct distributions are provided herein. Proof of principle experiments in which a heterologous molecule (*e.g.*, the enzyme chitinase or the cholera toxin B subunit) conjugated to an exopolysaccharide-associated protein or protein fragment is delivered to a biofilm comprising the exopolysaccharide, either uniformly or in a specific spatial distribution are also described herein. As described in more detail herein, engineered biofilm matrix-associated proteins for antigen presentation and targeting of enzymes to surfaces can be used for antigen presentation in vaccine development and to expand the enzymatic functionality of the bacterial biofilm matrix for therapeutic and bioengineering applications.

[00252] Proteins associated with the biofilm matrix. A recently published study represents the first comprehensive analysis of proteins associated with the Gram-negative biofilm matrix. [1] The experiments described here will advance the understanding of the mechanisms by which proteins are retained in the biofilm matrix, the spectrum of functions carried out by biofilm matrix-associated proteins, and the mechanisms by which proteins maintain spatial segregation in the biofilm.

[00253] In contrast to current views of the biofilm matrix proteins as the “glue” that holds the biofilm together, in which the biofilm matrix polysaccharide is depicted as a secreted, continuous matrix in which biofilm-associated bacteria are embedded, the preliminary results provided herein suggest that the *V. cholerae* matrix exopolysaccharide is tightly associated with cells and provides a scaffold for biofilm matrix-associated proteins. It is these proteins that mediate the intercellular and cell-surface contacts that provide structure to the biofilm.

[00254] Based on this understanding, different uses of bacterial biofilm matrix proteins and of biofilm matrix-associated proteins or protein fragments in novel applications is envisioned. For example, as described herein, biofilm matrix-associated proteins are spatially localized. For another example, the use of the biofilm matrix as a platform for presentation of antigens and delivery of functional enzymes to surfaces is envisioned. Heterologous molecules of interest, *e.g.*, heterologous antigens or enzymes, can be strategically targeted to specific parts of a biofilm by conjugating them to proteins or protein fragments that associate with particular regions of the biofilm matrix.

[00255] *Bacterial biofilm formation.* Biofilm formation is the process by which bacteria attach to a living or non-living surface. In biofilms comprised of multiple layers of bacteria, cell-cell and cell-surface interactions are mediated by a secreted matrix of natural polymers that may include exopolysaccharides, protein, and DNA. This matrix is secreted in response to specific environmental cues.

[00256] *The Vibrio cholerae multilayer biofilm matrix.* *V. cholerae* is an epidemic diarrheal pathogen of humans and a natural inhabitant of estuarine environments. When environmental conditions are favorable, *V. cholerae* forms a multilayer biofilm by elaborating a matrix that contains several proteins as well as the VPS polysaccharide, whose synthesis is largely encoded by the vps genes. It was recently demonstrated that the VPS polysaccharide is tightly associated with cells. Preliminary results suggest that it does not mediate biofilm formation directly. Rather, secreted biofilm matrix-associated proteins mediate the cell-cell and cell-surface interactions. [1].

[00257] *Proteins in the V. cholerae biofilm matrix.* The first complete proteomic analysis of a bacterial biofilm matrix was recently published [1]. In that study, 10 proteins that are known to be secreted into the extracellular space were identified (**Table 1**) as well as 18 proteins that are predicted to be extracytoplasmic (**Table 2**).

[00258] *Type I β -prism domain-containing proteins in the V. cholerae biofilm matrix.* Bap1, RbmC, and HlyA, three biofilm matrix-associated proteins identified, form a paralogous family of Type I β -prism lectin domain-containing proteins. Type I β -prism domains were first described in jacalin, a lectin found in the seeds of the Jack Fruit [15, 16]. Bap1 and RbmC are highly similar proteins with overlapping functions that are essential for biofilm formation [1, 6, 5]. These proteins contain multiple, conserved FG-GAP domains surrounding a β -prism domain (**Figure 4A**). As a result, the β -prism domain is predicted to be part of a larger, β -propeller-like structure. While RbmC has conserved N and C terminal domains not found in Bap1, Bap1 represents the minimal peptide required to rescue the biofilm defect of a Δ bap1 Δ rbmC mutant. HlyA, a pore-forming toxin, is secreted as a protoxin and is activated by cleavage of the 15 kDa N-terminal chaperone-like domain. Activated HlyA consists of spatially separated cytolysin, β -trefoil, and β -prism domains (**Figure 4B**) [17]. This protein forms heptameric pores in cholesterol and sphingolipid-rich cell membranes leading to membrane depolarization and/or hemolysis [18, 19, 20]. There is evidence that HlyA associates with the *V. cholerae* biofilm matrix [1]. RbmA is a novel biofilm matrix-associated protein. RbmA is a secreted protein encoded in the VPS island. It

has no conserved domains. Deletion of RbmA was originally noted to result in a weakened biofilm structure [4].

[00259] Bap1 reinforces the attachment of the biofilm to the surface, while RbmA strengthens intercellular contacts. The results shown herein suggest that RbmA plays a role in biofilm formation that is distinct from that of Bap1 and RbmC. As shown in **Figure 5**, the $\Delta bap1\Delta rbmC$ mutant is able to form a pellicle, which is a biofilm structure found at the air-water interface. However, this pellicle is only weakly attached to the surrounding solid surface and, therefore, can be easily dissociated from the surface by gentle shaking. In contrast, $\Delta RbmA$ mutants form strong attachments to the surface that are not disrupted by shaking. However, when vortexed, the $\Delta rbmA$ mutant biofilm disperses into much smaller particles than the $\Delta bap1\Delta rbmC$ mutant biofilm, suggesting that RbmA reinforces intercellular contacts. The biofilm made by a $\Delta bap1\Delta rbmA\Delta rbmC$ mutant is indistinguishable from that made by a $\Delta vpsL$ mutant (data not shown).

[00260] Bap1 and RbmC localize to the base of the biofilm, while RbmA is distributed throughout the biofilm. To correlate the observed biofilm formation defects of $\Delta bap1\Delta rbmC$ and $\Delta rbmA$ mutants with the distribution of these proteins in native biofilms, we added a FLAG affinity tag to the C-terminal end of Bap1, RbmC, and RbmA expressed from their native promoters and performed immunofluorescence microscopy on intact, viable biofilms (**Figure 6A and B**). To quantify the protein distribution, we measured the total fluorescence in each transverse section as a function of distance from the substratum (**Figure 6C**). Controls were performed to demonstrate that: (i) all proteins were well-expressed and secreted, (ii) FLAG-tagged proteins did not disrupt biofilm formation, and (iii) cytoplasmic, periplasmic proteins, and other unrelated secreted proteins were NOT visible by this method.

[00261] Bap1 and RbmC were noted to localize to the biofilm-surface interface, while RbmA was distributed throughout the biofilm ([1] and data not shown). A similar protein distribution was observed when expression of these proteins was driven by a constitutive promoter on a plasmid, suggesting that spatial localization of proteins in the biofilm is not the result of differential transcription [1].

[00262] Addition of purified Bap1 or RbmA can restore wild-type biofilm formation to a $\Delta bap1\Delta rbmC$ or $\Delta rbmA$ mutant, respectively. Affinity-tagged Bap1 and RbmA were purified from the supernatants of *V. cholerae* strains. Each mutant was allowed to form a biofilm in the presence of the purified protein that it lacked. Addition of purified Bap1 or RbmA rescued the biofilm defect of the corresponding mutant [1].

[00263] Four biofilm matrix-associated proteins, Bap1, RbmA, RbmC, and HlyA were characterized. The resulting data suggest that these proteins are spatially segregated in the biofilm and adhere to the exopolysaccharide component of the matrix. Accordingly, these proteins can be used to target heterologous molecules, such as functional proteins to specific regions of the biofilm matrix.

Example 3

[00264] *Structure-Function analysis of HlyA.* Of the β -prism domain-containing proteins specifically discussed herein, HlyA has been most intensively studied because of its hemolytic activity [17, 18, 19]. Although it has not previously been implicated in biofilm formation, researchers have postulated a role for HlyA in the environment because it is well-represented in environmental strains of *V. cholerae* [21, 22]. In this experiment, the role of HlyA in biofilm formation is characterized and the minimal HlyA peptide required for association with the biofilm, or, in other words, the minimal exopolysaccharide-binding HlyA fragment, is identified.

[00265] *Characterization of the role of full length HlyA in biofilm formation.* First, an in-frame deletion of HlyA in a wild-type genetic background is constructed to determine if HlyA plays a role in biofilm surface attachment and/or structure. The impact of HlyA deletion in a Δ bap1 Δ rbmC mutant background is also assessed in order to determine if the function of HlyA overlaps at all with that of Bap1 and RbmC. Surface attachment is quantified and the resistance of Δ hlyA and Δ hlyA Δ bap1 Δ rbmC biofilms to shaking and vortexing is analyzed in analogy to the analyses performed for Δ rbmA and Δ bap1 Δ rbmC biofilms. If a biofilm phenotype is observed, rescue experiments are performed with an HlyA-FLAG construct encoded on a plasmid. Adequate protein expression is confirmed by Western analysis.

[00266] *Distribution of HlyA in the biofilm matrix.* In this experiment, the transverse and vertical distributions of HlyA in the *V. cholerae* biofilm matrix are defined. The goal of this experiment is two-fold: first, to better understand the roles of the conserved domains of HlyA in spatial localization within the biofilm; and second, to identify additional proteins with distinct matrix distributions within the biofilm that may be useful to target proteins to different regions within the biofilm matrix. To this end, strains are constructed that express HlyA-FLAG both from its native promoter on the chromosome and from a constitutive promoter on a plasmid. After these tagged HlyA constructs are confirmed to be well

expressed by Western analysis, the distribution of these proteins in the biofilm are examined by coupling confocal microscopy with immunofluorescence. The distribution of HlyA in the biofilm matrix is compared to that of Bap1, RbmA, and RbmC.

[00267] *Role of the HlyA cytolyisin, β -trefoil, and β -prism domains in biofilm matrix association.* As illustrated in the crystal structure shown in **Figure 4**, the domains of HlyA are spatially separated. The minimal peptide required for attachment to the *V. cholerae* biofilm matrix is identified. From a plasmid, a series of six FLAG-tagged, HlyA-based peptides are constitutively expressed. These engineered peptides include either one or two conserved domains of HlyA. After establishing expression and stability, association of these peptides with the biofilm matrix is assessed by immunofluorescence. A negative result is not informative as it might reflect severe compromise of the three-dimensional domain structure. However, a positive result allows to assign roles in biofilm association to one or more domains of HlyA. If HlyA plays an essential role in biofilm formation, these experiments are performed in a wild-type genetic background only. Otherwise, these experiments are performed in Δ hlyA and Δ bap1 Δ rbmC mutant g backgrounds as well as in wild-type *V. cholerae*.

[00268] *Functional analysis of HlyA point mutants.* If the β -trefoil and β -prism domains are required for biofilm matrix association, mutation of the conserved sugar-binding residues within these domains will decrease protein retention within the biofilm. In this experiment, residues in the β -trefoil and β -prism lectin domains of HlyA that are predicted to be critical for binding to the polysaccharide component of the biofilm matrix are mutated.

[00269] The β -trefoil domain of HlyA contains two out of three QXW sugar-binding motifs that are conserved in ricin-like lectins (Q537/F539 and Q574/W576). In the crystal structures of β -prism domains of jacalin-like lectins, several conserved amino acids are known to make contacts with the bound sugar. These include an aspartate in a GXXXD motif that forms a hydrogen bond with the sugar and two aromatic residues that form stacking interactions with the bound sugar [23]. The β -prism domain of HlyA contains these conserved putative sugar binding residues (G647, D651, Y698, and Y723). FLAG-tagged versions of full length HlyA and HlyA peptides found to be matrix-associated in the experiment above are constructed with one or more of the conserved residues replaced by alanine. Mutant proteins are expressed from a plasmid along with a C-terminal FLAG-tag in wild-type *V. cholerae* as well as in Δ hlyA and Δ bap1 Δ rbmC mutants. After ascertaining that these mutant proteins are adequately expressed and stable by Western analysis, their biofilm retention and spatial localization within the pellicle or biofilm matrix is assessed by immunofluorescence.

The creation of point mutations by alanine substitution is unlikely to disturb the protein structure. If a mutation of a residue has no effect on matrix binding, it can be concluded that this residue is not essential for matrix binding function.

Example 4

[00270] *Identification of additional biofilm matrix-binding proteins.* Eighteen additional candidate matrix-associated proteins that were identified in a preliminary proteomic analysis are characterized (see **Table 2**). While all of these proteins are predicted to be secreted, their function and role in biofilm formation has not been characterized. The preliminary results are validated by characterizing the role and distribution of confirmed biofilm matrix-associated proteins using an approach that has been successful in characterization of Bap1, RbmA, and RbmC [1].

[00271] *Confirmation of protein secretion.* To establish that the proteins listed in Table 2 are secreted outside the cell, the relevant gene is cloned along with its native signal sequence and a C-terminal FLAG affinity tag into an IPTG-inducible expression vector. These vectors are electroporated into wild-type *V. cholerae*. The engineered strains are grown to mid-log phase in the presence and absence of inducer (IPTG). Cells are pelleted and separated from the supernatant. Western analysis is carried out on both the cell pellet and supernatant to determine if the proteins are expressed and secreted. Similarly treated strains expressing FLAG-tagged cytoplasmic and periplasmic proteins are used as controls to establish that the experimental treatment does not release cytoplasmic and periplasmic contents into the medium. Importantly, the controls behaved appropriately in previous experiments that are now published [1].

[00272] *Assessment of biofilm matrix association.* To determine whether secreted proteins are selectively retained in the matrix, biofilms are formed with strains expressing the FLAG-tagged proteins and the proteins are visualized in the biofilm matrix using immunofluorescence. Similarly expressed cytoplasmic and periplasmic proteins are used as controls. Only proteins that are secreted and visualized in the biofilm matrix are pursued further.

[00273] *Native distribution of proteins in the biofilm matrix.* The native distributions of proteins found to associate with the biofilm matrix is characterized. Strains are constructed in which the protein of interest is expressed from its native promoter with the addition of a C-

terminal FLAG affinity tag. Biofilms are formed with these strains, and immunofluorescence is used to image the distribution of the protein in the biofilm.

[00274] *Role of biofilm matrix-associated proteins in biofilm formation.* In order to determine the function of biofilm matrix-associated proteins in biofilm formation, *V. cholerae* carrying in-frame deletions in the genes encoding these proteins are created. Biofilm formation by these mutants is quantified and compared to that by wild-type *V. cholerae*. The resistance of mutant biofilms to surface dissociation and dispersal is also tested by shaking and vortexing. If no phenotype is observed, it is concluded that the respective protein does not play a structural role in biofilm formation.

[00275] *Identification of minimum biofilm-association peptides.* For any multi-domain proteins that are identified in these experiments, experiments similar to those described for HlyA are performed to identify the minimal peptide required for association with the biofilm.

Example 5

[00276] There is evidence that matrix-associated proteins fulfill similar roles in the biofilms of other organisms [12, 13, 24, 25]. While *V. cholerae* may not be a suitable host for some applications, the “proof of principle” experiments described herein serve as a model for application development in other bacteria.

[00277] *Targeting a secreted, active enzyme to a specific location within the biofilm matrix.* ChiA-2 is an efficiently secreted chitinase that is not concentrated in the biofilm matrix [1]. In this experiment, ChiA-2 is targeted to the biofilm matrix via conjugation to an exopolysaccharide-associated protein. The tagged versions of each of the exopolysaccharide-associated proteins described herein are fused to the C-terminus of ChiA-2 via recombinant technology, and the recombinant nucleic acids encoding the resulting chimeric proteins are placed in a neutral chromosomal location such as the lacZ gene of wild-type *V. cholerae*. Immunofluorescence is used to compare the levels and distribution of ChiA-2 in the biofilm matrix in the presence and absence of the recombinant proteins. For all chimeric proteins that successfully localize ChiA-2 in the biofilm matrix, a fluorescent chitinase substrate is used to compare biofilm-associated chitinase activity in the presence and absence of matrix-associated ChiA-2.

[00278] It is expected that it is possible to target a protein, *e.g.*, ChiA-2 to either the substrate-biofilm interface or the biofilm apex by using specific exopolysaccharide-

associated proteins as fusion partners for the protein. In some embodiments, surface-active proteins (*e.g.*, proteins with antibacterial, glycolytic, lipolytic, or proteolytic activity) are targeted to concentrate at the biofilm matrix-surface interface for purposes of medical treatment or bioremediation.

[00279] *Targeting a secreted antigen to the biofilm matrix.* The oral cholera vaccine currently licensed in Europe consists of whole cell, killed *V. cholerae* strains combined with the purified cholera toxin B subunit (CtxB) [26]. This vaccine affords some protection against cholera and traveler's diarrhea but is expensive to produce and administer. Therefore, simpler whole cell, killed vaccines have been developed in Vietnam and India [9].

[00280] A whole cell, killed vaccine strain that concentrates CtxB in the biofilm matrix would be less expensive to administer and produce and could be more immunogenic. Furthermore, one could engineer a strain that also incorporated protein antigens affording protection against other diarrheal diseases, thus generating a broad spectrum diarrheal vaccine.

[00281] To explore this application, a strain of *V. cholerae* is engineered in which CtxB is targeted to the biofilm matrix via fusion to an exopolysaccharide-associated protein. The resulting biofilm is processed into a killed-cell vaccine according to methods known to those of skill in the art. The vaccine is administered to a human subject, resulting in an immune response similar to that of a currently licensed *V. cholerae* vaccine.

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Example 6 A self-assembling, self-adjuvanting whole cell vaccine targeting diarrheal disease
[00282]

In low-income countries, severe diarrhea caused by intestinal pathogens is the primary cause of morbidity in children under the age of five. Vaccines are a cost-effective means of disease prevention. However, vaccines targeting the most common intestinal pathogens are lacking, and the highest incidence of childhood diarrheal disease occurs in regions that are unable to fund immunization campaigns without financial assistance. Therefore, there is a great need for a multi-valent diarrheal vaccine that is inexpensive to prepare and simple to administer. Described herein is a self-assembling vaccine platform based on *Vibrio cholera*. A multi-valent, live-attenuated vaccine was developed based on this

platform that elicits a robust immune response against enterotoxigenic *E. coli* and *Vibrio cholerae* antigens when administered sublingually.

[00283] In low-income countries, severe diarrhea caused by intestinal pathogens is the primary cause of morbidity, leading to 1.7 billion cases of childhood disease annually with profound consequences for physical and mental development¹. Treatment of infection is essential for survival, but prevention is preferable as it allows these children to escape life-altering developmental sequelae. In the absence of improved sanitation, vaccination is an excellent means of prevention. The *Vibrio cholerae* biofilm matrix has previously been proposed to be able to be used to link secreted proteins to the cell surface². It is demonstrated herein that fusion of an enterotoxigenic *E. coli*-derived heat-stable toxoid (STa^{A14H}) and the B subunit of the heat-labile toxin (LTB) to the *Vibrio cholerae* biofilm matrix protein RbmA results in decoration of whole cells with these antigens and a robust immune response when administered sublingually as a live-attenuated, whole cell vaccine. The immune response to this vaccine can be boosted by inclusion of a chromosomally-encoded, constitutively expressed, non-toxic cholera toxin variant mmCT^{3, 4}. A self-assembling, flexible protein antigen presentation platform was developed that can be used to create an affordable combination vaccine targeting diarrheal disease.

Results

[00284] RbmA, a lectin that spontaneously associates with the biofilm matrix polysaccharide VPS after secretion from the cell, consists of two tandem fibronectin III domains. These domains are presumed to bind to VPS as an antiparallel homodimer to mediate cell-to-cell adhesion⁵⁻⁷ (Figure 14A). While RbmA is essential for biofilm structure and development⁸, some strains of *V. cholerae* express VPS and RbmA in the free-swimming or planktonic state². It was previously shown that proteins of interest could be anchored to VPS by genetic fusion to the C terminus of RbmA and hypothesized that cells expressing RbmA-antigen fusion proteins could serve as antigen presentation vehicles for mucosal vaccination.

[00285] Dukoral, a currently licensed cholera vaccine, consists of killed, whole *V. cholerae* cells combined with the purified B subunit of cholera toxin (CTB). This vaccine is costly and must be administered with sodium bicarbonate resuspended in a large volume of potable water to ensure presentation of intact CTB to the intestinal immune system. Due to the higher cost and requirement for potable water, Dukoral is mainly a vaccine of travelers. To determine whether a vaccine presenting a comparable amount of CTB could be developed

without the need for purification or administration with bicarbonate, a prototype strain was generated harboring a plasmid expressing CTB fused to the C terminus of RbmA under the control of an inducible promoter (Vc(pR-CTB)) (Figure 14B). A similar strain harboring a plasmid encoding native CTB was used as a control. Western blot analysis was used to assess association of CTB to cells. It was found that R-CTB was efficiently anchored to the bacterial cell, whereas CTB expressed alone was found exclusively in the culture supernatant (Figure 14C). In fact, the amount of CTB associated with the cell pellet compared favorably to that incorporated in the Dukoral vaccine (Figure 14D).

[00286] Planktonic Vc(pR-CTB) was tested as a formalin-inactivated whole cell vaccine. While formalin treatment led to some protein cross-linking, CTB remained cell-associated (Figure 17). The fixed vaccine preparation was administered to mice by orogastric gavage (o.g.) with boosters 2 and 4 weeks later (Figure 15A) and measured the immune response. As a positive control, a Dukoral-like vaccine consisting of formalin-fixed wild-type *V. cholerae* combined with purified CTB was prepared. Although not required for the fixed vaccine, both Vc(pR-CTB) and the positive control were administered with bicarbonate to allow a direct comparison. Negative controls included PBS alone and formalin-fixed wild-type *V. cholerae*. While the Dukoral-like vaccine induced production of CTB-specific serum IgA and IgG, the formalin-treated Vc(pR-CTB) vaccine elicited no antigen-specific antibodies (Figures 18A-18C). It was hypothesized that this was the result of formalin fixation of CTB, which has been reported to alter protein structure and abrogate antigenicity⁹. Therefore, formalin fixation was eliminated and a live-attenuated formulation of Vc(pR-CTB) was used.

[00287] Several live-attenuated *V. cholerae* vaccines are either under development or licensed¹⁰⁻¹². However, these vaccines have disadvantages. Because *V. cholerae* is extremely acid-sensitive and colonization of the intestine is thought to be essential for a robust immune response, such vaccines must be administered with bicarbonate to maintain viability during transit through the stomach. Furthermore, the intestinal colonization required for immunogenicity increases the risk of acquisition of the phage carrying cholera toxin and dissemination into the environment¹³. To minimize these disadvantages, pR-CTB was introduced into a Δ ctxA strain (Vc Δ ctxA (pR-CTB)) and administered via the sublingual route (s.l.) according to the vaccination schedule shown in Figure 15A. Sublingual immunization with live *V. cholerae*, given without bicarbonate, resulted in low level shedding of live bacteria that ceased after 24 hours (Figure 19). Next, the antibody response to the *V. cholerae* O-antigen carbohydrate of the lipopolysaccharide (LPS) was examined.

Although generation of protective anti-*V. cholerae* O-antigen antibodies by oral vaccines is well-described, the immune response to a sublingually administered, live-attenuated *V. cholerae* vaccine has not been determined¹⁴⁻¹⁷. It was found that, for both the oral, inactivated Vc(pR-CTB) and the sublingual, live-attenuated VcΔctxA (pR-CTB) vaccine, O-antigen specific IgA and IgG antibodies were detected in serum two weeks after the final immunization (Figure 15B). Furthermore, the serum vibriocidal titers, a measurement of antibody-mediated activation of serum complement and a predictive marker for protection against disease, did not differ between the two vaccine groups (Figure 15C). Finally, the level of O-specific IgA in serum was significantly higher in the group that received the sublingual, live-attenuated vaccine as compared with the group that received the inactivated vaccine. Because robust immune responses to the O-antigen and high vibriocidal titers are correlated with protection against infection, it was tested whether the live-attenuated, sublingual vaccine afforded passive immunity using an infant mouse model. Suckling mice born to immunized or unimmunized dams were challenged with wild-type *V. cholerae* and assessed for disease outcome after 24 hours^{18, 19}. Pups born to vaccinated dams had significantly decreased *V. cholerae* colonization (Figure 15D). Furthermore, animals in the unvaccinated group exhibited signs of cholera including weight loss, fluid accumulation in the large intestine and cecum, and reduced skin turgor indicative of dehydration (Figures 15E-15G). The results show that sublingual immunization with a live-attenuated *V. cholerae* vaccine stimulates a robust immune response comparable to that provided by the currently licensed, oral inactivated vaccine and provides passive protection against cholera in a murine model.

[00288] Next, the generation of CTB-specific antibodies in serum and stool was evaluated. CTB-specific serum IgA was significantly increased six weeks after the initial vaccine dose, while serum IgG was not detected throughout the study period (Figures 15H and 15I). Secretory IgA in stool was significantly increased two weeks after the first vaccine dose and remained elevated throughout the study period (Figure 15J). This was consistent with a previous report that sublingual immunization with a live bacterial vector generated antigen-specific fecal IgA but not serum IgG²⁰. These results demonstrate for the first time that a protein antigen anchored on RbmA can be delivered sublingually to stimulate a robust antigen-specific mucosal immune response.

[00289] Inspired by the success of the VcΔctxA(pR-CTB) vaccine, the vaccine strain was engineered to produce its own adjuvant by introducing the cholera toxin-based adjuvant mmCT^{3, 4} onto the chromosome under control of the lacZ promoter (PlacZmmCT) (Figure 15K). First, the secretion of the mmCT components was confirmed by Western blot

(Figure 15K). The vaccine was then administered to mice following the immunization and sample collection scheme shown in Figure 15A. Inclusion of mmCT greatly enhanced the systemic and mucosal immune responses to the O-antigen (Figure 15L-15N, Figures 20A-20C), but abrogated the mucosal immune response to CTB (Figures 21A-21C). It was hypothesized that delivery of CTB within mmCT in addition to that affixed to RbmA might have stimulated tolerance. To assess this possibility, the amount of mmCT secreted during the approximate period the vaccine remains in the murine sublingual site and the amount of CTB delivered as R-CTB in a single vaccine dose were compared. As shown in Figure 22, mmCT contributed minimally to the total level of CTB. This suggests that mmCT may induce tolerance to protein antigens such as CTB when delivered via the sublingual route. To minimize this effect, we decreased the amount of antigen delivered in subsequent vaccine trials by expression of fusion proteins from the native RbmA promoter rather than from a multi-copy plasmid. In addition, three improvements (Figure 16A) were instituted. First, the whole cell platform was transferred to the *V. cholerae* vaccine strain Bengal-2, in which all elements of the CTX phage responsible for horizontal transfer of cholera toxin are absent²¹. Second, because the robust immune response obtained with sublingual vaccination suggested that intestinal colonization was not essential, the gene encoding the major subunit of the principal colonization factor, *tcpA* was deleted. Third, the heterologous antigens from enterotoxigenic *Escherichia coli* (ETEC), a major cause of childhood diarrhea worldwide¹, was introduced. Based on previous studies^{22, 23, 24}, the genes encoding LTB and an immunogenic STa point mutant were linked to that encoding RbmA on the chromosome at the native RbmA locus (R-LTB/STa^{A14H}).

[00290] The performance of the multi-valent, improved diarrheal vaccine was then assessed. Secretion and cell-association of R-LTB/STa^{A14H} were documented by Western blot (Figure 16B), and it was estimated that each vaccine dose delivered approximately 30 femtomoles of cell-associated R-LTB/ STa^{A14H} (Figure 23). This vaccine was administered to mice (Figure 16C). Levels of LTB- or CTB-specific IgA and IgG in the serum after one vaccine dose were comparable between the mice that received the adjuvanted vaccine with ETEC antigens and those that received the adjuvanted vaccine alone. Furthermore, STa-specific IgG was strongly induced in the serum between two to four weeks after the first vaccine dose (Figure 16H). There was also a trend towards increased STa-specific IgA in serum but this did not reach statistical significance (Figure 16G). Stool IgA was not detected (Figure 16I). STa is known to be poorly immunogenic. To determine whether it is possible to increase the immune response, a second vaccine dose was delivered after 36 days (Figure

16C). A boosting effect was seen in the group that received the control vaccine, while an immune suppressive effect was found in the R-LTB/STa^{A14H} vaccine group after the second dose (Figures 16D-16I). This supports the hypothesis that co-administration of mmCT and a protein antigen leads to immune tolerance and that delivery of smaller quantities of this antigen mitigate this effect. Taken together, the results suggest that an optimized vaccine would include mmCT alone combined with RbmA fused to additional copies of STa, which has previously been shown to improve immunogenicity²⁵.

[00291] ST-producing enterotoxigenic *E. coli*, *Shigella* spp., *Aeromonas* spp., *V. cholerae*, and *Campylobacter jejuni*, have been identified as the five most common bacterial causes of severe childhood diarrhea in the developing world¹. Yet currently a vaccine against only one of these pathogens, *V. cholerae*, is licensed for pediatric use. Described herein is the development of a self-assembling, multi-valent, self-adjuvanting sublingual vaccine that generates immunity against both *V. cholerae* and ETEC antigens. The platform described herein simplifies manufacturing and administration costs and provide a path to affordable, efficacious combination vaccines against diarrheal pathogens for use in resource-poor settings.

Table 6 Vaccine Constructs

Strains / Plasmid	Description	Reference
V. cholerae		
PW17	N16961 (El Tor, Inaba); Sm ^r	1
PW724	MO10; Sm ^r	2
PW839	MO10ΔctxA; Sm ^r	3
PW1947	MO10ΔctxA, P _{lacZ} mmCT; Sm ^r	This study
PW139 (Bengal-2)	MO10ΔattRS1; Sm ^r	2
PW1159	MO10ΔattRS1ΔtcpA; Sm ^r	This study
PW1938	MO10ΔattRS1ΔtcpA, P _{lacZ} mmCT; Sm ^r	This study
PW1934	MO10ΔattRS1ΔtcpA, P _{lacZ} mmCT, P _{rbmA} -rbmA-LTb-STa ^{A14H} ; Sm ^r	This study
E. Coli		
SM10λpir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i> (λpirR6K); Km ^r	4

Plasmids		
pWM91	oriR6K mobRP4 <i>lacI</i> pTac <i>tnp</i> mini-Tn10; Km ^r Ap ^r	5
pFLAG-CTC- <i>rbmA</i> - <i>ctxB</i>	IPTG-inducible expression of RbmA-CTB fusion protein; Ap ^r	6
pWM91- <i>lacZ</i> ::mmCT	pWM91 carrying in-frame mmCT insertion into <i>lacZ</i> for homologous recombination; Ap ^r	This study
pWM91- <i>rbmA</i> -LTb-STa ^{A14H}	pWM91 carrying in-frame insertion of LTb-STa ^{A14H} fusion protein between the 3' end of <i>rbmA</i> and downstream sequence; Ap ^r	This study
pHT3	pCVD442 carrying unmarked, in-frame deletion of <i>tcpA</i> ; Ap ^r	7

Table 7 Primer Sequences

Primer	Sequence	Note
P_{lacZ}mmCT		
mmCT_1F	GATCATTGTTGGT <u>TAATAG</u> GTATCGATTAAATA <u>AAGGAGG</u>	Stop codons bold and underlined; RBS underlined
mmCT_1R	TGCTTTATTTTCGTCGGGCGGGCGACTATC	
mmCT_2F	CCGCCCCGACGAAATAAAGCAGTCAGGTGGTCTTATGC	
mmCT_2R	ATAACCATCTGCTGCTGGAGCAATATCTAAGTTACTG	
mmCT_3F	ATTGCTCCAGCAGCAGATGGTTATGGATTGGCAGGTTTC	
mmCT_3R	AT <u>CAC</u> <u>CGT</u> <u>GAT</u> <u>GTT</u> <u>CCGCTACT</u> AT <u>CCCCACA</u> ACCCGGCGGTGCATGATG	LT189-197 bold and underlined, R192G double underlined
mmCT_4F	<u>GATAGTAGCGGAACAATCACG</u> <u>G</u> GT <u>G</u> ATACTTGCGATGAAA AAACCCAAAGTC	
mmCT_4R	GATTGGTATTCGTC <u>AGC</u> GAATTTTACACCTAGACTTTG	L211A double underlined
mmCT_5F	GTAAAATT <u>CGCT</u> GACGAATACCAATCTAAAGTTAAAAGAC	
mmCT_5R	GTATTGCACAGGTTAATTTGCCATACTAATTGCG	
lacZ_1F	GCGCGCGCGAGCTCAAGCCTTACATACAGGCCAGCG	SacI site underlined
lacZ_1R	CGATACCTATTACCAAATGATCACACAAGGGTG	Stop codons bold and underlined
lacZ_2F	GCAAATTAACCTGTGCAATACGAAGGGGGC	
lacZ_2R	GCGCGCGCG <u>GAGCTCGCT</u> GGACTTTTTTGACTTCATGTAATG	SacI site

		underlined
rbmA-LTb-STa ^{A14H}		
rbmA_1F	ATTGGGTACCGGGCCCCCCCCCTTACTGATGGTCGTATG	
rbmA_1R	CCACTGTCATTGACTGTTCC	
rbmA_2F	GTCGTATGTATAAAAAACCG	
rbmA_2R	CTTATCGATACCGTCGACCTCGATAGCATCAATGACCCAAAC	

Table 8 Adjuvant and Antigen Nucleotide Sequences

Construct	Sequence synthesized	Note
PlacZmmCT	GTATCGATTAAATAAGGAGGAATAAACCATGGTAAAGATAATC TTCGTGTTCTTCATCTTCCTGAGCAGCTTTTCGTACGCTAACGAT GATAAGCTCTATCGCGCAGATAGTCGCCCCGCCGACG (SEQ ID NO: 47)	RBS underlined
rbmA-LTBS Ta^{A14H}	GAGTGGAACAGTCAATGACAGTGGTA <u>AAGAAGATGA</u> AATAAAG TAAAATGTTATGTTTTATTTACGGCGTTACTATCCTCTCTATGTG CATACGGAGCTCCCCAGTCTATTACAGAACTATGTTCCGGAATAT CGCAACACACAAATATATACGATAAATGACAAGATACTATCAT ATACGGAATCGATGGCAGGCCAAAAGAGAAATGGTTATCATTAC ATTTAAGAGCGGCGCAACATTTTCAGGTCGAAGTCCCGGGCAGTC AACATATAGACTCCCCAAAAAAGCCATTGAAAGGATGAAGGACA CATTAAGAATCACATATCTGACCGAGACCAAAATTGATAAATT ATGTGTATGGAATAATAAAACCCCCAATTCAATTGCGGCAATCAG TATGGAAAACGATCCCCGGGTACCGAGCTCGaatagtagcaattactgctgtgaa ttgtgttgaatcct <u>CAT</u> gtaccgggtgctafTAAATTTACCTAGTCACTTAGTCG TATGTATAAAAAACCG (SEQ ID NO: 48)	AAA to AAG degenerate codon change bold and underlined Start and stop codons bold Linker between LTb and STa underlined A14H change double underlined

Methods

[00292] Bacterial strains and culture conditions. *Vibrio cholerae* strains were cultured in Luria-Bertani (LB) broth supplemented with 100 µg/mL streptomycin at 27°C, with shaking at 200 rpm. *Escherichia coli* was grown in LB broth at 37°C with shaking. Where necessary, plasmids were maintained with 100 µg/mL of ampicillin in the culture medium. Protein production was induced with 0.5 mM IPTG (β-d-1-thiogalactopyranoside). Frozen stocks were maintained in 15% glycerol at -80°C. Strains used in this study are listed in Table 6.

[00293] DNA manipulations and strain construction. All oligonucleotides were synthesized by Integrated DNA Technologies and detailed in Tables 7 and 8. Restriction enzymes were purchased from New England Biolabs (NEB) and used according to manufacturer's instructions. Gibson assembly of DNA fragments was carried out with the NEBuilder HiFi DNA Assembly kit (NEB). PCR reactions were performed with GoTaq polymerase (Promega) for screening and Q5 High-Fidelity polymerase (NEB) for cloning. Genomic DNA from wild-type MO10, a *V. cholerae* O139 serotype strain, was used as the PCR template unless otherwise noted.

[00294] Construction of the prototype vaccine expressing pR-CTB. The previously described pFLAG-CTC derivative carrying the gene encoding the B subunit of cholera toxin (CTB) fused to the gene encoding RbmA (R-CTB)₂ or CTB alone under an IPTG-inducible promoter was introduced into wild-type MO10 or Δ ctxA by electroporation. Protein production in positive transformants was verified by Western blot analysis using an anti-CTB antibody as described below.

[00295] Construction of PlacZmmCT in *V. cholerae*. The mmCT sequence was obtained by Gibson assembly of PCR fragments amplified from genomic DNA with exception of the first 97 bases, which were amplified from an oligonucleotide synthesized to contain the proximal ribosome binding motif from the Ptrc promoter and the first 97 nucleotides of the ctxA gene. Mutations of mmCT4 and stop codons distal to the ribosome binding site were introduced on primers and confirmed in the assembled product by sequencing. This was then ligated between two PCR fragments of the lacZ gene by Gibson assembly to obtain the final construct. The lacZ-mmCT construct was digested using SacI and ligated into the sacB-encoding suicide plasmid pWM91. Plasmids from positive clones were isolated, and the sequence of the inserted DNA was confirmed by sequencing with M13 primers. Homologous recombination into the chromosomal lacZ gene and positive clone selection was carried out as previously described^{26, 27}.

[00296] Construction of R-LTB/STa^{A14H} vaccine strain. A synthetic oligonucleotide containing the sequence of a fusion peptide between the B subunit of the *E. coli* heat-labile toxin (LTB) and the heat-stable toxin (STa) with an A14H point mutation that abrogated toxicity²² was custom synthesized (Integrated Gene Technologies). The two sequences were connected by a heptapeptide linker²⁸. The 5' and 3' ends of the oligonucleotide included 32 bp at the 3' end of rbmA excluding the stop codon and 37 bp just downstream of rbmA, respectively. The rbmA stop codon was removed to allow read-through translation of the ETEC antigens. The nucleotides encoding the last two amino acid residues of RbmA were

altered from AAA to the degenerate codon AAG to facilitate oligonucleotide synthesis. The STa sequence encoded only the 19 structural amino acid residues and did not include sequences encoding the N terminal residues processed in the pre-pro-peptide. Gibson assembly was used to ligate this fragment, two PCR fragments including ~500 bp encoding the C-terminus of RbmA and ~500 bp downstream of *rbmA*, respectively, and XhoI-linearized suicide plasmid pWM91. Plasmids from positive clones were confirmed by sequencing. Homologous recombination was carried out as described above. Positive clones were selected through screening by PCR.

Vaccine preparation

[00297] Protein induction. Frozen stocks were inoculated into 3 mL of LB supplemented with streptomycin and ampicillin (LB-Sm/Amp) and cultured at 27°C overnight. This starter culture was collected by centrifugation, washed once with LB-Sm/Amp, and sub-cultured into 25 mL of fresh LB-Sm/Amp in a 250-mL flask. After incubating for 6-8 h at 27°C with shaking, IPTG was added to a final concentration of 0.5 mM, and the culture was incubated for an additional 2 h at 27°C with shaking.

[00298] Trichloroacetic acid (TCA) precipitation of secreted proteins. Proteins secreted into the supernatant was precipitated with TCA and washed with acetone. Briefly, spent supernatant was passed through a 0.2 µm filter to remove bacterial cells. TCA was added to the cell-free supernatant to a final concentration of 10% and the supernatant was incubated overnight at 4°C with gentle mixing. Precipitated proteins were collected by centrifugation and washed three times with ice-cold acetone. Residual acetone was evaporated by brief incubation at 95°C. The protein pellet was resuspended in 4x Laemmli buffer containing β-mercaptoethanol and prepared for Western blot as described below.

[00299] Preparation of whole cell vaccines. After protein induction for strains carrying pR-CTB or after overnight culture for the strains encoding chromosomal vaccine constructs, the bacterial culture was centrifuged at 5,000 x g for 15 min at 4°C to collect cells. The supernatant was passed through a 0.2 µm filter, and the resulting cell-free supernatant was used for Western blot analysis. The remaining bacterial pellet was washed three times with 12 mL of sterile PBS and finally resuspended in 1 mL of PBS. This constituted the live, whole cell vaccine. For each vaccine preparation, 10 µL was removed to quantify colony forming units (CFU), and 20 µL was reserved for Western blot analysis. For each immunization, the vaccine was prepared and used within 2 h.

[00300] Preparation of the formalin-inactivated whole cell vaccine. An equal volume of freshly prepared 1% formaldehyde was added to the live vaccine suspension and gently mixed overnight at room temperature while protected from light. The following day, cells from the formaldehyde-treated suspension were collected by centrifugation at 5,000 x g for 20 min and washed 3 times with 12 mL of PBS. The final cell pellet was resuspended in 5 mL of PBS to obtain the killed whole cell vaccine. From this preparation, 20 μ L were reserved for Western blot analysis, 10 μ L were cultured in LB medium to test for sterility, and 10 μ L were used for cell count in a hemocytometer. Purified CTB was added, where necessary, to match the protein concentration in the Dukoral vaccine.

[00301] Western blot analysis. Supernatants and cell pellet samples were separated by centrifugation. TCA precipitation of the supernatant was performed prior to detection of mmCT. These samples were combined with 4x Laemmli buffer containing β -mercaptoethanol, sonicated in an ice bath, boiled for 5 min, and finally briefly centrifuged to remove particulates. Proteins were resolved on a denaturing 4-20% gradient Tris-HCl gel and then transferred onto a polyvinylidene difluoride membrane by semi-dry transfer (BioRad). The membrane was blocked in tris-buffered saline with 0.1% Tween (TBS-T) and 5% skim milk for 2 h at room temperature with gentle shaking. Fresh blocking solution containing primary antibody was added in a 1:1,000 dilution. An anti-CTB polyclonal antibody conjugated to horseradish peroxidase (HRP) (Pierce, PA1-85293) was used to detect RbmA-CTB in cell pellets and native CTB in supernatants. Rabbit-derived serum raised against both the A and B subunits of cholera toxin (Sigma) was used to detect mmCT. Anti-STa antibody (Fitzgerald Industries, clone 30) was used to detect the R-LT/ST fusion protein. After overnight incubation with primary antibodies, the membrane was washed 3 times with TBS-T. For anti-CT and anti-STa antibodies, membranes were then incubated for 2 h at room temperature with 1:5,000 diluted HRP-conjugated anti-rabbit secondary antibody (Cell Signaling) or anti-mouse antibody (Sigma). All membranes were developed using an ECL Western blotting substrate (Pierce).

[00302] Quantification of R-CTB by densitometry. Known concentrations of purified CTB (List Laboratories) were resolved by SDS-PAGE alongside R-CTB samples and used as standards for quantification. ImageJ was used to generate a standard curve fitted to the intensities of bands corresponding to the CTB standards. Concentration of R-CTB was calculated using the linear portion of the standard curve.

[00303] Quantification of R-LT/STa by Enzyme-linked immunosorbent assay (ELISA). Because unconjugated STa cannot be detected by Western blot, the amount of cell-

associated R-LTB/STa^{A14H} was determined from whole cell lysates using ELISA. Lysates of R-CTB and R-LTB/STa^{A14H} vaccines were prepared by resuspending whole cells in 50 mM sodium carbonate binding buffer (pH 9.6) and sonicating in an ice bath for 2 min with 15 s burst and 10 s pulse cycles. Duplicate samples of lysates were serially diluted in binding buffer and placed into wells of microtiter plates (Nunc, Maxisorp). Dilutions ranged from 1:2 to 1:128,000. A standard curve was generated with purified STa diluted to 1 µg/mL and serially diluted onto the plate.

[00304] The plates were incubated overnight at room temperature, washed three times in phosphate-buffered saline containing 0.1% Tween (PBS-T), and blocked with PBS containing 1% bovine serum albumin (PBS-BSA). Anti-STa antibody diluted to 1 µg/mL in PBS-T containing 0.1% BSA (PBS-T-BSA), added to the plates, and incubated overnight. Plates were washed in PBS-T, incubated for 1 h at 37°C with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, 1 µg/mL in PBS-T-BSA) (Bethyl Laboratories), and developed with TMB (3,3',5,5'-tetramethylbenzidine). Absorbance at 650 nm was recorded on a Biorad Benchmark Plus plate reader using a kinetic ELISA protocol.

[00305] Quantification of supernatant mmCT by ELISA. The amount of mmCT secreted into the supernatant by the live vaccine suspension was quantified by GM1 ELISA as previously described²⁹. The VcΔctxA(pR-CTB) vaccine was prepared and then incubated at room temperature for 30 min. The cells were collected by centrifuging at 5,000 x g for 10 min and 300 µL of the supernatant was removed for quantification. The supernatant of a wild-type strain that does not express mmCT was similarly prepared and used as a negative control.

[00306] One hundred ng of bovine monosialoganglioside GM1 (Sigma) in sodium carbonate buffer was added to each well of a 96 well microtiter plate (Nunc, Maxisorp), incubated overnight at room temperature, and then washed in PBS-T. Serial dilutions of cell-free supernatants in PBS were applied to GM1-coated wells. Purified CTB was prepared in a 1 µg/mL concentration in PBS, and serial dilutions were also applied to GM1-coated wells to generate a standard curve. The plates were incubated overnight at room temperature, washed in PBS-T, and blocked in PBS-BSA. Monoclonal anti-CTB IgG (Fisher, PIMA183519) diluted to 1 µg/mL in PBS-T-BSA was added to the plates and incubated overnight. The plates were probed with HRP-conjugated goat anti-mouse antibodies (Bethyl Laboratories) and developed as described above.

Immunization and sample collection

[00307] Animals. Female, 6-8-week old BALB/c mice were used in all immunization experiments. For sublingual immunizations, mice were purchased from Charles River Laboratories and housed in a biosafety level two facility at Boston Children's Hospital with food and water ad libitum. Mice were acclimatized for 5 days. All procedures had been previously approved by the Institutional Animal Care and Use Committee. Animals used for inactivated vaccine immunizations were housed at Cocalico Biologicals.

[00308] Orogastric administration of killed, whole cell vaccine. The formalin-inactivated whole cell vaccine was administered by orogastric gavage. Briefly, 5×10^9 cells per milliliter of the vaccine was prepared as described above and mixed with an equal volume of 6% sodium bicarbonate and administered immediately. Each inoculation delivered 200 μ L of the mixture, corresponding to 5×10^8 inactivated cells.

[00309] Sublingual administration of live, attenuated vaccine. Mice were anesthetized by intraperitoneal injection with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), then held upright while 10 μ L of the vaccine was delivered under the tongue by a micropipette directed toward the floor of the mouth. Mice were maintained in the upright position for 2 min before resting, ventral side down, for at least 30 min until regaining consciousness.

[00310] Collection of blood and stool samples. Blood and stool samples were collected one day before vaccination and at the designated time points throughout the study period. Fresh stool pellets were frozen at -80°C until use. Blood was collected from the tail vein using capillary tubes with clot activator (Sarstedt). Sera were obtained by clearing the clotted blood with centrifugation at $10,000 \times g$ for 5 min at room temperature and stored at -20°C . Stool samples were prepared as previously described²⁹. Briefly, pellets were thawed on ice, transferred to 15-mL conical tubes containing 3 mL of chilled resuspension solution (0.1 mg/mL soybean trypsin inhibitor, 3:1 mixture of PBS to 0.1 M EDTA), thoroughly homogenized, and centrifuged at $650 \times g$ for 10 min at room temperature. The supernatant was collected and centrifuged once more at $15,300 \times g$ for 10 min at 4°C . PMSF (phenylmethane sulfonyl fluoride) was added to the supernatant to a final concentration of 2 mM. Stool samples were kept at -20°C or at -80°C for long term storage.

[00311] Enumeration of *V. cholerae* in fecal pellets. A fresh stool pellet was collected from each mouse 24 h and 48 h after sublingual immunization. The pellet was weighed, homogenized in 1 mL sterile PBS, and serially diluted. One hundred μ L of the undiluted and diluted stool suspensions were plated on LB agar containing 100 μ g/mL

streptomycin and incubated at 37°C overnight. The number of colony forming units (CFU) was recorded and normalized to the weight of the pellet to calculate CFU per gram stool. Because 1/10 of the total stool suspension was plated containing approximately 22.7 mg stool/ml, the lower limit of detection was estimated to be approximately 440 CFU/g.

Enzyme-linked immunosorbent assays

[00312] Quantification of CTB- and LTB-specific antibodies by ELISA. CTB and LTB are more than 80% identical at the amino acid level and share the same mechanism of action. Anti-LTB and anti-CTB antibodies recognize both proteins. Therefore, we used the same ELISA protocol, described below, to measure anti-CTB and anti-LTB antibodies.

[00313] Standard curve: IgA anti-CTB IgA antibodies were not available for use in a standard curve. Therefore, to assess linearity, standard curves were generated by capturing IgA and IgG in reference mouse serum with goat anti-mouse IgG or IgA. Microtiter plate wells were incubated overnight with 100 µg of goat anti-mouse IgG or IgA diluted in sodium carbonate buffer. The wells were washed in PBS-T and blocked with PBS-BSA. Reference mouse serum (Bethyl Laboratory) was diluted to 1 µg/mL of total IgG or IgA and applied to the wells. The wells were washed after overnight incubation, then probed with HRP-conjugated goat anti-mouse antibodies and developed as described above for quantification of mmCT.

[00314] Test samples: Microtiter plates were coated with GM1 followed by purified CTB as described above. The plates were blocked in PBS-BSA and washed in PBS-T. Serially diluted sera or stool samples were applied to the wells and incubated overnight. Serum dilutions ranged from 1:50 to 1:6400, and stool dilutions ranged from 1:2 to 1:128. The plates were probed and developed as described above.

[00315] Quantification of STa-specific antibodies by ELISA. Antibodies to STa in the sera and stool were measured using the protocol described for CTB-specific antibodies using microtiter plates coated with 100 ng/mL of purified STa in sodium carbonate buffer in each well. Standard curves were generated using the procedure described above.

[00316] Quantification of total stool IgA by ELISA. Total fecal IgA was used to normalize antigen-specific IgA in the stool. Each well of a microtiter plate was coated with 100 ng of goat anti-mouse IgA antibody in sodium bicarbonate buffer and incubated overnight. Plates were washed in PBS-T and blocked in PBS-BSA. Stool samples were serially diluted from 1:200 to 1:25,600 in PBS-T-BSA and added to the plates. The plates

were incubated overnight and then probed and developed as described above. Standard curves were generated as described for CTB/LTB-specific antibodies.

Lipopolysaccharide extraction and O-antigen (LPS) specific antibodies. Lipopolysaccharide (LPS) was extracted from 50 mL of *Vibrio cholerae* MO10 (serotype O139) and N16961 (serotype O1) overnight cultures using a commercial kit (Bulldog Bio). Serum and stool antibodies recognizing the O-antigen of lipopolysaccharide were quantified as previously described³⁰. A 1:1,000 dilution of LPS in sodium carbonate buffer was applied to microtiter plates and incubated overnight. The plates were washed in PBS-T and blocked for 40 min at 37°C in PBS-BSA. Serum and stool samples were applied to the plates in dilutions similar to those used to measure CTB-specific antibodies. The plates were incubated for 90 min at 37°C and then washed in PBS-T. Plates were incubated for 90 min at 37°C after addition of 100 ng of HRP-conjugated goat anti-mouse antibodies per well. Plates were developed using the same protocol described for quantification of CTB-specific antibodies.

Serum vibriocidal titers.

[00317] Serum vibriocidal antibody titers were determined as previously described with the following modifications³¹. Immunized mouse sera were incubating at 56°C for 1 h to inactivate endogenous complement, serially diluted two-fold in PBS in 0.5-μL tubes, and kept on ice. Wild-type MO10 was grown to mid-logarithmic phase in brain heart infusion broth containing 100 μg/mL streptomycin and diluted in PBS containing 20% guinea pig complement to 4 x 10⁶ CFU/mL. An equal volume of this suspension was added to the serum dilutions to obtain a final concentration of 10% complement and 2 x 10⁶ CFU/mL *V. cholerae*, the mixture was incubated for 1 h at 37°C with shaking at 200 rpm, and viable cells were enumerated by plating. Bactericidal titer was determined as the reciprocal of the serum dilution capable of killing 50% or more of the indicator strain compared with a control containing pre-immune or PBS-immunized serum. Sera from mice that received the inactivated vaccine were randomly pooled into groups of three for determination of vibriocidal titers.

Infant mouse challenge model

[00318] Orogastric challenge of infant mice. At the end of the study period (between 60 to 70 days after the initial immunization), vaccinated female mice were mated with age-matched male. Non-vaccinated, non-timed pregnant mice were purchased from Charles River Laboratories and housed in the same facility as vaccinated mice. When pups were born, wild-

type MO10 was grown overnight in LB broth with 100 µg/mL streptomycin at 27°C. The cell density was adjusted to 5×10^9 CFU/mL. Bacteria were collected by centrifugation at 6,000 x g for 5 min and resuspended in 2.5% sodium bicarbonate (0.29 M). Four- to five-day-old pups were challenged with 2.5×10^7 CFU of wild-type MO10 delivered in 50 µL of sodium bicarbonate solution by oral gavage. Pups were monitored for immediate signs of distress and then returned to the dam. All pups were sacrificed 24 h after infection, and signs of disease were documented.

[00319] Quantification of bacterial colonization. The small and large intestines were weighed, added to sterile conical tubes containing 1 mL of PBS, and homogenized. The homogenates were serially spread plated on LB agar supplemented with 100 µg/mL of streptomycin. Plates were incubated overnight at 37°C. The limit of detection for each spread plate is 10 CFU per intestine.

Statistical analysis. Statistical analyses were performed in GraphPad Prism 7. Two-tailed Mann-Whitney rank-sum test was used. All vaccine groups consisted of ten mice. Error bars indicate standard deviations unless otherwise noted. Western blot images are representative of experimental triplicates.

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[00320] All publications, patents, patent applications, publication, and database entries (*e.g.*, sequence database entries) mentioned herein, *e.g.*, in the Background, Summary, Detailed Description, Examples, and/or References sections, are hereby incorporated by reference in their entirety as if each individual publication, patent, patent application, publication, and database entry was specifically and individually incorporated herein by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS AND SCOPE

[00321] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the embodiments described herein. The scope of the present disclosure is not intended to be limited to the above description, but rather is as set forth in the appended claims.

[00322] Articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between two or more members of a group are considered satisfied if one, more than one, or all of the group members are present, unless indicated to the contrary or otherwise evident from the context. The disclosure of a group that includes “or” between two or more group members provides embodiments in which exactly one member of the group is present, embodiments in which more than one members of the group are present, and embodiments in which all of the group members are present. For purposes of brevity those embodiments have not been individually spelled out herein, but it will be understood that each of these embodiments is provided herein and may be specifically claimed or disclaimed.

[00323] It is to be understood that the disclosure encompasses all variations, combinations, and permutations in which one or more limitation, element, clause, or descriptive term, from one or more of the claims or from one or more relevant portion of the description, is introduced into another claim. For example, a claim that is dependent on another claim can be modified to include one or more of the limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of making or using the composition according to any of the methods of making or using disclosed herein or according to methods known in the art, if any, are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[00324] Where elements are presented as lists, *e.g.*, in Markush group format, it is to be understood that every possible subgroup of the elements is also disclosed, and that any element or subgroup of elements can be removed from the group. It is also noted that the term “comprising” is intended to be open and permits the inclusion of additional elements or steps. It should be understood that, in general, where an embodiment, product, or method is referred to as comprising particular elements, features, or steps, embodiments, products, or methods that consist, or consist essentially of, such elements, features, or steps, are provided as well. For purposes of brevity those embodiments have not been individually spelled out herein, but it will be understood that each of these embodiments is provided herein and may be specifically claimed or disclaimed.

[00325] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in some embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. For purposes of brevity, the values in each range have not been individually spelled out herein, but it will be understood that each of these values is provided herein and may be specifically claimed or disclaimed. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

[00326] Where websites are provided, URL addresses are provided as non-browser-executable codes, with periods of the respective web address in parentheses. The actual web addresses do not contain the parentheses.

[00327] In addition, it is to be understood that any particular embodiment of the present disclosure may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the disclosure, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.

CLAIMS

What is claimed is:

1. A composition comprising
 - (i) a bacterium associated with an exopolysaccharide;
 - (ii) a RbmA protein; and
 - (iii) a heterologous molecule conjugated to the RbmA protein, or an exopolysaccharide-binding fragment thereof.
2. The composition of claim 1, wherein the heterologous molecule is a heterologous protein.
3. The composition of claim 2, wherein the heterologous protein is an enzyme, or an antigen.
4. The composition of claim 2, wherein the heterologous protein is fused to the RbmA protein, or to the exopolysaccharide-binding fragment thereof, thus forming a fusion protein.
5. The composition of claim 3, wherein the fusion protein is encoded by a recombinant nucleic acid comprised in the bacterium.
6. The composition of any one of claims 1-5, wherein the RbmA protein comprises the amino acid sequence of SEQ ID NO: 2.
7. The composition of any one of claims 1-5, wherein the RbmA protein is a RbmA variant comprising one or more substitution mutations in a surface groove of the RbmA protein.
8. The composition of claim 7, wherein the one or more substitution mutations are in positions R234, R219, or R116 in SEQ ID NO: 2.
9. The composition of claim 8, wherein the one or more mutations are R234A, R219A, or R116A in SEQ ID NO: 2.

10. The composition of claim 9, wherein the RbmA variant comprises the amino acid sequence of any one of SEQ ID NOs: 38-43.
11. The composition of any one of claims 1-10, wherein the heterologous molecule comprises an antigen.
12. The composition of claim 11, wherein the antigen comprises an antigen of a pathogen.
13. The composition of claim 12, wherein the antigen comprises an antigen of a bacterial toxin.
14. The composition of claim 13, wherein the bacterial toxin is a cholera toxin.
15. The composition of claim 14, wherein the cholera toxin is the B subunit of cholera toxin.
16. The composition of claim 15, wherein the B subunit of cholera toxin comprises the sequence of (SEQ ID NO: 5).
17. The composition of claim 12, wherein the antigen comprises a colonization factor antigen.
18. The composition of claim 17, wherein the colonization factor antigen is from Enterotoxigenic Escherichia coli (ETEC).
19. The composition of claim 18, wherein the colonization factor antigen is EtpA.
20. The composition of claim 19, wherein the EtpA comprise the amino acid sequence of SEQ ID NO: 44.
21. The composition of claim 12, wherein the antigen comprises a bacterial virulence factor antigen.

22. The composition of claim 22, wherein the bacterial virulence factor antigen is from *Shigella*.
23. The composition of claim 22, wherein the bacterial virulence factor antigen is VirG.
23. The composition of claim 23, wherein the VirG comprises the amino acid sequence of SEQ ID NO: 45.
24. The composition of any one of claims 1-10, wherein the heterologous molecule comprises an enzyme.
25. The composition of claim 24, wherein the enzyme is a therapeutic enzyme.
26. The composition of claim 24 or 25, wherein the enzyme is selected from the group consisting of lactase, a pancreatic enzyme, an oil-degrading enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase.
27. The composition of any one of claims 1-10, wherein the heterologous molecule comprises a binding agent.
28. The composition of claim 27, wherein the binding agent is selected from the group comprising an antibody, an antigen-binding antibody fragment, a nanobody, an ScFv, an adnectin, a lectin, a ligand, or an affinity tag.
29. The composition of any one of claims 1-10, wherein the heterologous molecule comprises a detection agent.
30. The composition of any one of claims 1-29, further comprising a signal peptide fused to the RbmA protein, or exopolysaccharide-binding fragment thereof, or to the heterologous molecule, wherein the signal peptide targets the RbmA protein, or exopolysaccharide-binding fragment thereof, conjugated to the heterologous molecule for secretion.
31. The composition of any one of claims 1-30, wherein the bacterium is a gram-negative bacterium.

32. The composition of any one of claims 1-30, wherein the bacterium is a gram-positive bacterium.
33. The composition of any one of claims 1-30, wherein the bacterium is a non-pathogenic bacterium.
34. The composition of any one of claims 1-30, wherein the bacterium is a *Vibrio sp.* bacterium.
35. The composition of any one of claims 1-30, wherein the bacterium is a *Vibrio cholerae* bacterium.
36. The composition of any one of claims 1-31, wherein the bacterium is an *E. coli* bacterium.
37. A composition comprising
(i) a RbmA protein; and
(ii) a heterologous molecule conjugated to the RbmA protein, or an exopolysaccharide-binding fragment thereof.
38. The composition of claim 37, wherein the heterologous molecule comprises a heterologous protein.
39. The composition of claim 30, wherein the heterologous protein is an enzyme, or an antigen.
40. The composition of claim 38 or 39, wherein the heterologous protein is fused the RbmA protein, or to the exopolysaccharide-binding fragment thereof, thus forming a fusion protein.
41. The composition of any one of claims 37-40, wherein the RbmA protein comprises the amino acid sequence of SEQ ID NO: 2.

42. The composition of any one of claims 37-40, wherein the RbmA protein is a RbmA variant comprising one or more substitution mutations in a surface groove of the RbmA protein.
43. The composition of claim 42, wherein the one or more substitution mutations are in positions R234, R219, or R116 in SEQ ID NO: 2.
44. The composition of claim 8, wherein the one or more mutations are R234A, R219A, or R116A in SEQ ID NO: 2.
45. The composition of claim 44, wherein the RbmA variant comprises the amino acid sequence of any one of SEQ ID NOs: 38-43.
46. The composition of any one of claims 37-45, wherein the heterologous molecule comprises an antigen.
47. The composition of claim 46, wherein the antigen comprises an antigen of a pathogen.
48. The composition of claim 47, wherein the antigen comprises an antigen of a bacterial toxin.
49. The composition of claim 48 wherein the bacterial toxin is a cholera toxin.
50. The composition of claim 49, wherein the cholera toxin is the B subunit of cholera toxin.
51. The composition of claim 50, wherein the B subunit of cholera toxin comprises the sequence of (SEQ ID NO: 5).
52. The composition of claim 46, wherein the antigen comprises a colonization factor antigen.
53. The composition of claim 52, wherein the colonization factor antigen is from Enterotoxigenic Escherichia coli (ETEC).

54. The composition of claim 53, wherein the colonization factor antigen is EtpA.
55. The composition of claim 54, wherein the EtpA comprise the amino acid sequence of SEQ ID NO: 44.
56. The composition of claim 46, wherein the antigen comprises a bacterial virulence factor antigen.
57. The composition of claim 56, wherein the bacterial virulence factor antigen is from *Shigella*.
58. The composition of claim 57, wherein the bacterial virulence factor antigen is VirG.
59. The composition of claim 58, wherein the VirG comprises an amino acid sequence of SEQ ID NO: 45.
60. The composition of any one of claims 37-59, wherein the heterologous molecule comprises an enzyme.
61. The fusion protein of claim 60, wherein the enzyme is a therapeutic enzyme.
62. The composition of claim 60 or 61, wherein the enzyme is selected from the group consisting of lactase, a pancreatic enzyme, an oil-degrading enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase.
63. The composition of any one of claims 37-59, wherein the heterologous molecule comprises a binding agent.
64. The composition of claim 63, wherein the binding agent is selected from the group comprising an antibody, an antigen-binding antibody fragment, a nanobody, an ScFv, an adnectin, a lectin, a ligand, or an affinity tag.

65. The composition of any one of claims 37-59, wherein the heterologous molecule comprises a detection agent.
66. The composition of any one of claims 37-65, further comprising a signal peptide fused to the RbmA protein, or exopolysaccharide-binding fragment thereof, or to the heterologous molecule, wherein the signal peptide targets the RbmA protein, or exopolysaccharide-binding fragment thereof, conjugated to the heterologous molecule for secretion.
67. A vaccine, the vaccine comprising the composition of any one of claims 11-36.
68. The vaccine of claim 67, wherein the fusion protein is expressed by the bacterium.
69. The vaccine claims 67 or 68, wherein the exopolysaccharide is an exopolysaccharide secreted by the bacterium.
70. The vaccine of any one of claims 67-69, wherein the exopolysaccharide is bound by the bacterium.
71. The vaccine of any one of claims 67-70, wherein the fusion protein is bound by the exopolysaccharide.
72. The vaccine of any one of claims 48-54, wherein the antigen is an antigen of a pathogen.
73. The vaccine of any one of claims 67-73, wherein the vaccine further comprises an adjuvant.
74. The vaccine of claim 73, wherein the adjuvant is a Cholera toxin subunit A (CtxA) variant.
75. The vaccine of claim 74, wherein the CtxA variant comprises mutations corresponding to N189D, A190S, P191S, R192G, S193T, S194I, M195T, S196G, N197I, N197D, or L211A in SEQ ID NO: 46.

76. The vaccine of claims 74 or 75, wherein the CtxA variant has reduced activity.
77. The vaccine of claim 76, wherein the CtxA variant activity is reduced by at least 10-100 fold.
78. The vaccine of any of claims 74-77, wherein the CtxA variant is expressed by the bacterium.
79. The vaccine of claim 78, wherein the CtxA variant is constitutively expressed.
80. The vaccine of any one of claims 67-79, wherein the vaccine is a killed whole cell vaccine.
81. The vaccine of any one of claims 67-79, wherein the vaccine is a live attenuated vaccine.
82. The vaccine of any one of claims 67-80, wherein the bacterium is a pathogenic bacterium.
83. The vaccine of any one of claims 67-82, wherein the bacterium is *V. Cholerae*.
84. The vaccine of any one of claims 67-82, wherein the bacterium is *E. Coli*.
85. The vaccine of claim 83, wherein the *V. cholerae* is inactivated in CtxA.
86. The vaccine of claim 85, wherein the CtxA is deleted from the *C. cholerae* genome.
87. A method of vaccinating a subject against a pathogen, the method comprising administering to the subject an effective amount of the vaccine of any one of claims 67-86.
88. The method of claim 87, wherein the vaccine is administered in an amount sufficient to elicit an immune response against the bacterium and/or against the antigen.

89. The method of claim 87 or 88, wherein the vaccine is administered in an amount sufficient to immunize the subject against the bacterium and/or against the antigen.
90. The method of any one of claims 87-89, wherein the vaccine is administered sublingually.
91. The method of any one of claims 87-89, wherein the vaccine is administered orally.
92. The method of any one of claims 87-90, wherein the subject is 0-13 years of age.
93. The method of any one of claims 87-93, wherein the vaccine stimulates systemic immune response to the antigen.
94. The method of any one of claims 87-93, wherein the vaccine stimulates mucosal immune response to the antigen.
95. The method of any one of claims 87-94, wherein the vaccine elicits antigen-specific antibodies in the subject.
96. The method of claim 95, wherein the antigen-specific antibody is IgG.
97. The method of claim 95, wherein the antigen-specific antibody is IgA.
98. A method for delivering a molecule to a target site, the method comprising delivering to the target site a bacterium associated with an exopolysaccharide that binds a RbmA protein, wherein the RbmA protein, or an exopolysaccharide-binding fragment thereof, binds the molecule.
99. The method of claim 98, wherein the target site is a surface.
100. The method of claim 99, wherein the target site is an air/water interface.
101. The method of any one of claims 98-100, wherein the heterologous molecule comprises a polypeptide.

102. The method of claim 101, wherein the polypeptide is fused to the RbmA protein or the exopolysaccharide binding protein fragment.

103. The method of claim 102, wherein the bacterium comprises a recombinant nucleic acid encoding the polypeptide fused to the RbmA protein or the exopolysaccharide binding protein fragment.

104. The method of any one of claims 98-103, wherein the molecule comprises a therapeutic protein.

105. The method of claim 104, wherein the method comprises administering an effective amount of the bacterium to a subject.

106. The method of claim 105, wherein the bacterium is a non-pathogenic bacterium.

107. The method of any one of claims 104-106, wherein the molecule is an enzyme and wherein the target site is a digestive organ of the subject.

108. The method of claim 107, wherein the target site is the intestinal tract of the subject.

109. The method of claims 107 or 108, wherein the molecule is lactase, a pancreatic enzyme, beta-galactosidase, mucinase, cellulase, isomaltase, or alginase.

110. The method of any one of claims 107-109, wherein the bacterium can colonize the intestinal tract of the subject.

111. The method of any one of claims 107-110, wherein the bacterium is administered orally.

112. The method of any one of claims 101-103, wherein the molecule is a mucus-digesting enzyme, and wherein the target site is the lung of the subject.

113. The method of claim 112, wherein the mucus-digesting enzyme is mucinase or alginase.
114. The method of claims 112 or 113, wherein the bacterium can colonize the lung of the subject.
115. The method of any one of claims 112-114, wherein the bacterium is administered via an aerosol.
116. The method of any one of claims 101-103, wherein the molecule is an enzyme that can digest a contaminant.
117. The method of claim 116, wherein the contaminant is oil.
118. The method of claims 116 or 117, wherein the molecule is an oil-digesting enzyme.
119. The method of any one of claims 116-118, wherein the target site is an environmental water-air interface.
120. A bacterial biofilm comprising the bacterium of any one of claims 1-36.
121. A method for modifying the surface of a bacterium, the method comprising contacting a bacterium associated with an exopolysaccharide with a composition comprising a heterologous molecule conjugated to a RbmA protein, wherein the RbmA protein binds to the exopolysaccharide.
122. The method of claim 121, wherein the composition is the composition of any one of claims 37-66.
123. A method of targeting a molecule to a biofilm, the method comprising contacting a biofilm that comprises an exopolysaccharide with a RbmA protein, conjugated to the molecule.

124. The method of claim 123, wherein the biofilm comprises a pathogen and the molecule comprises a polypeptide toxic to the pathogen.

125. The composition of claim 13, wherein the bacteria toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC).

126. The composition of claim 125, wherein the non-toxic STa variant comprises a A14H mutation (STa^{A14H}).

127. The composition of claim 125, wherein the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}.

128. The composition of claim 127, wherein the fusion protein is encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.

129. The composition of claim 48, wherein the bacteria toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic Escherichia coli (ETEC).

130. The composition of claim 129, wherein the non-toxic STa variant comprises a A14H mutation (STa^{A14H}).

131. The composition of claim 130, wherein the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}.

132. The composition of claim 131, wherein the fusion protein is encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.

133. A vaccine comprising the composition of any one of claims 125-132.

134. The vaccine of claim 133, wherein the vaccine further comprises an adjuvant.

135. The vaccine of claim 134, wherein the adjuvant is a multiple-mutated Cholera toxin (mmCT).
136. The vaccine of claim 135, wherein the mmCT is encoded by a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 47.
137. A vaccine, the vaccine comprising:
- (i) a *Vibrio cholerae* bacterium associated with an exopolysaccharide;
 - (ii) a RbmA protein; and
 - (iii) an antigen conjugated to the RbmA protein, or an exopolysaccharide-binding fragment thereof.
138. The vaccine of claim 137, wherein the RbmA protein comprises the amino acid sequence of SEQ ID NO: 2.
139. The vaccine of claim 137 or claim 138, wherein the antigen is a bacterial toxin.
140. The vaccine of claim 139, wherein the bacterial toxin is a cholera toxin.
141. The vaccine of claim 140, wherein the cholera toxin is the B subunit of cholera toxin (CtxB).
142. The vaccine of claim 15, wherein the CtxB comprises the amino acid sequence of (SEQ ID NO: 5).
143. The vaccine of claim 139, wherein the bacterial toxin comprises a non-toxic heat-stable toxoid (STa) variant and a B subunit of heat-labile toxin (LTB) from Enterotoxigenic *Escherichia coli* (ETEC).
144. The vaccine of claim 143, wherein the non-toxic STa variant comprises a A14H mutation (STa^{A14H}).
145. The vaccine of claim 144, wherein the STa^{A14H}, LTB, and RbmA forms a fusion protein in an order of RbmA-LTB-STa^{A14H}.

146. The vaccine of claim 145, wherein the fusion protein is encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID NO: 48.
147. The vaccine of claim 137, wherein the antigen is bacterial lipopolysaccharide (LPS).
148. The vaccine of claim 147, wherein the LPS is from *Vibrio cholerae*.
149. The vaccine of any one of claims 137-146, wherein the vaccine further comprises an adjuvant.
150. The vaccine of claim 149, wherein the adjuvant is a Cholera toxin subunit A (CtxA) variant.
151. The vaccine of claim 150, wherein the CtxA variant comprises mutations corresponding to N189D, A190S, P191S, R192G, S193T, S194I, M195T, S196G, N197I, N197D, or L211A in SEQ ID NO: 46.
152. The vaccine of claims 150 or claim 151, wherein the CtxA variant has reduced activity.
153. The vaccine of claim 149, wherein the adjuvant is a multiple-mutated Cholera toxin (mmCT).
154. The vaccine of claim 153, wherein the mmCT is encoded by a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 47.
155. The vaccine of any of claims 149-154, wherein the adjuvant is expressed by the *Vibrio cholera* bacterium.
156. The vaccine of claim 155, wherein the adjuvant is constitutively expressed.
157. The vaccine of claim 155, wherein the adjuvant is under the control of a LacZ promoter.

158. The vaccine of any one of claims 137-157, wherein the RbmA protein and the antigen conjugated to the RbmA protein are under the control of a native RbmA promoter.
159. The vaccine of any one of claims 137-158, wherein the *Vibrio cholera* bacterium lacks endogenous CtxA.
160. The vaccine of any one of claims 137-159, wherein the vaccine is a live attenuated vaccine.
161. A method of vaccinating a subject against a pathogen, the method comprising administering to the subject an effective amount of the vaccine of any one of claims 137-160.
162. The method of claim 161, wherein the vaccine is administered in an amount sufficient to elicit an immune response against the bacterium and/or against the antigen.
163. The method of claim 161 or claim 162, wherein the vaccine is administered sublingually.
164. The method of claim 161 or claim 162, wherein the vaccine is administered orally.
165. The method of any one of claims 161-164, wherein the subject is 0-13 years of age.
166. The method of any one of claims 161-165, wherein the vaccine stimulates systemic immune response to the antigen.
167. The method of any one of claims 161-166, wherein the vaccine stimulates mucosal immune response to the antigen.
168. The method of any one of claims 161-167, wherein the vaccine elicits antigen-specific antibodies in the subject.
169. The method of claim 168, wherein the antigen-specific antibody is IgG.

170. The method of claim 168, wherein the antigen-specific antibody is IgA.

FIGURE 1A

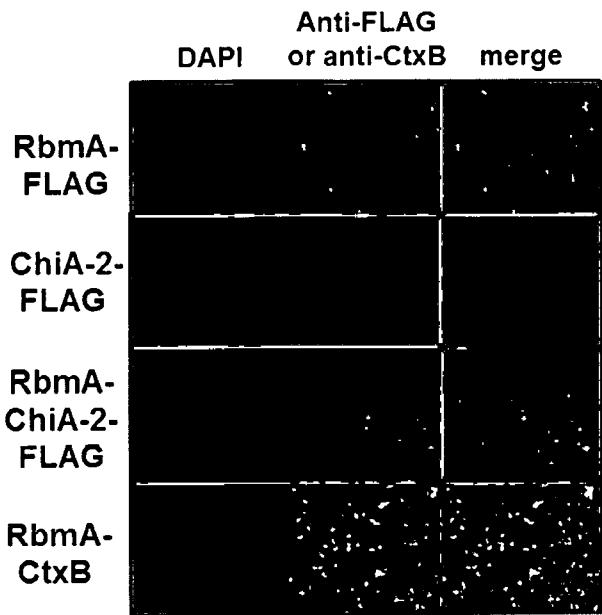
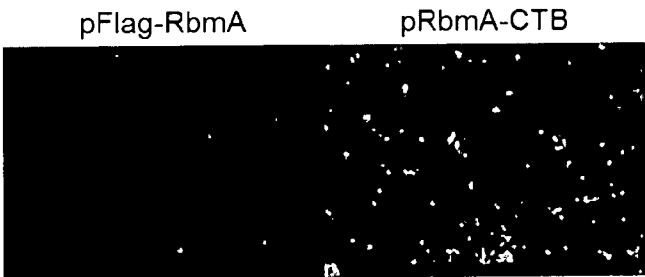


FIGURE 1B



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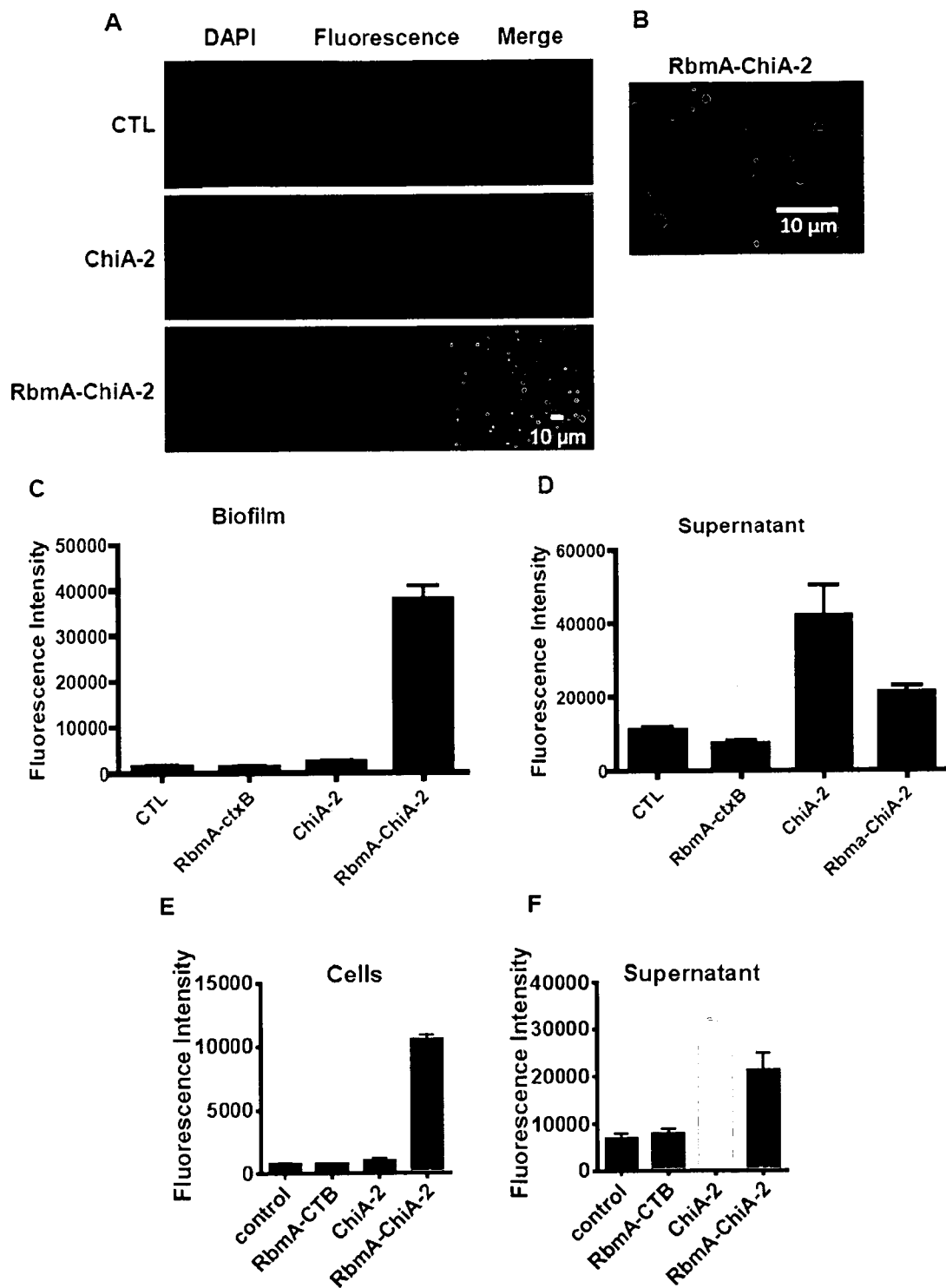


FIGURE 2

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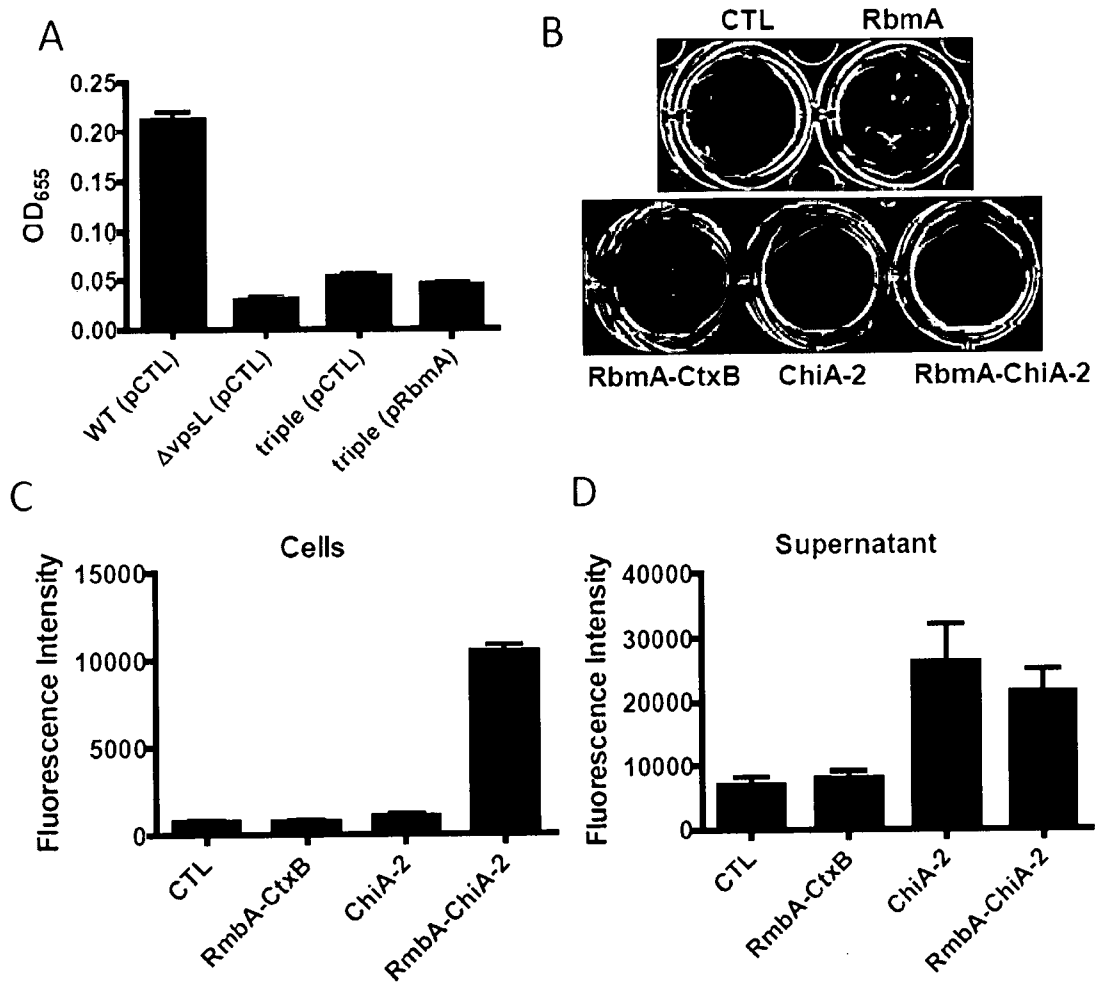


FIGURE 3

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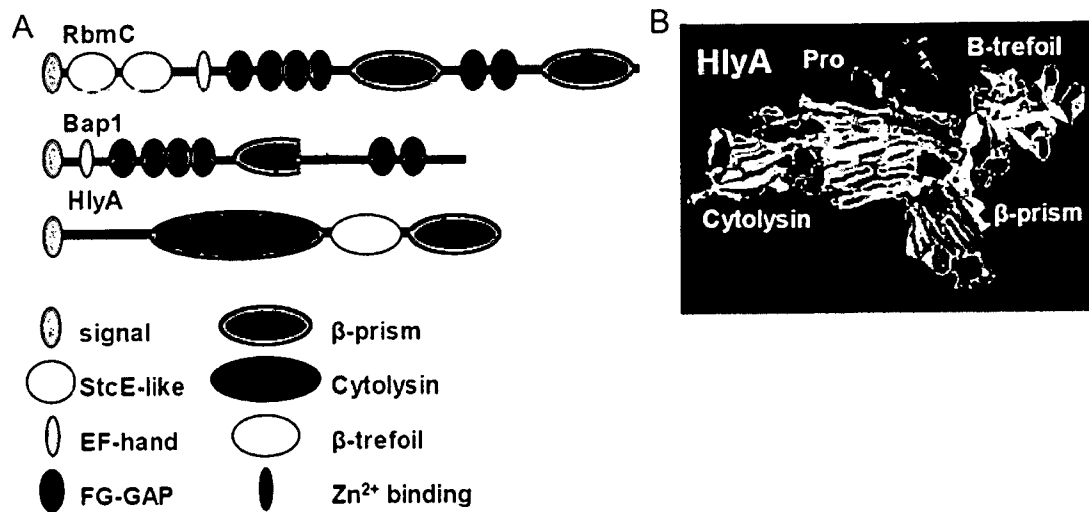


FIGURE 4

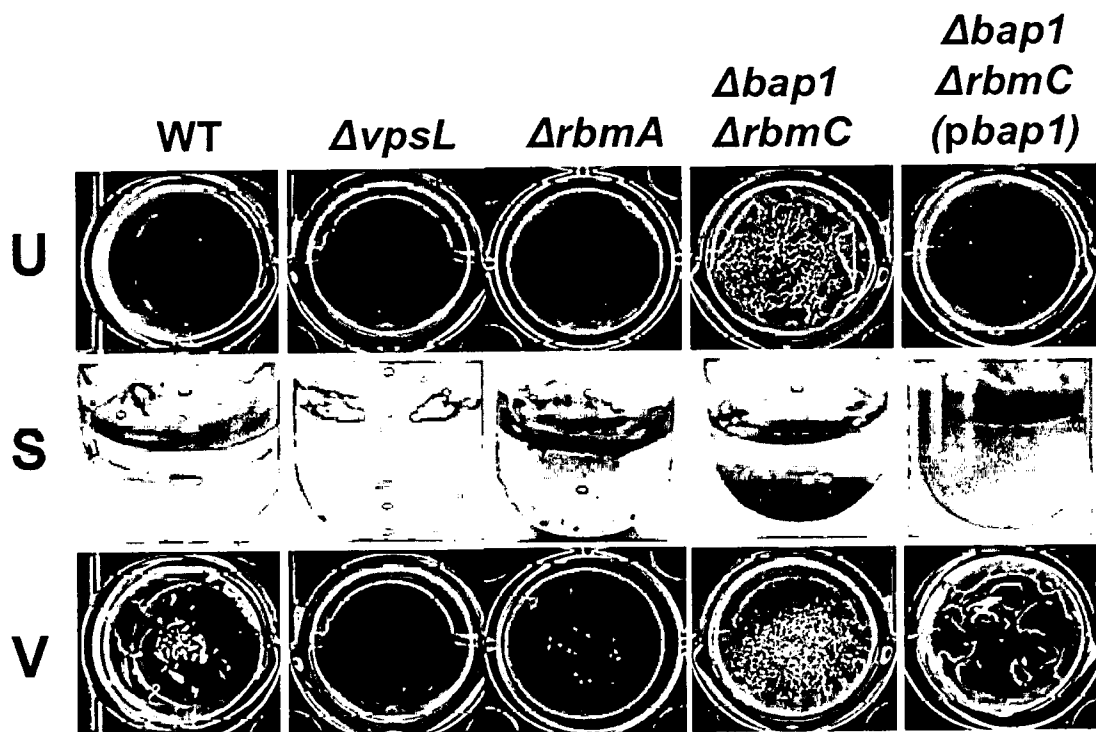


FIGURE 5

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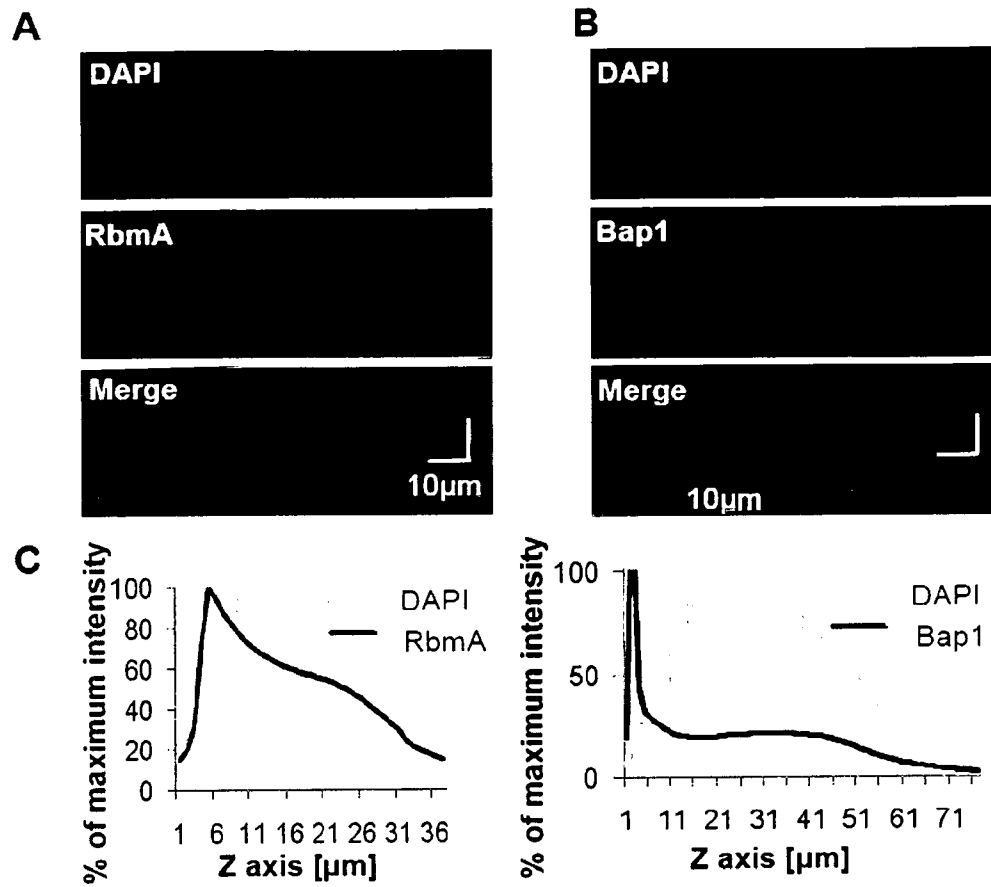


FIGURE 6

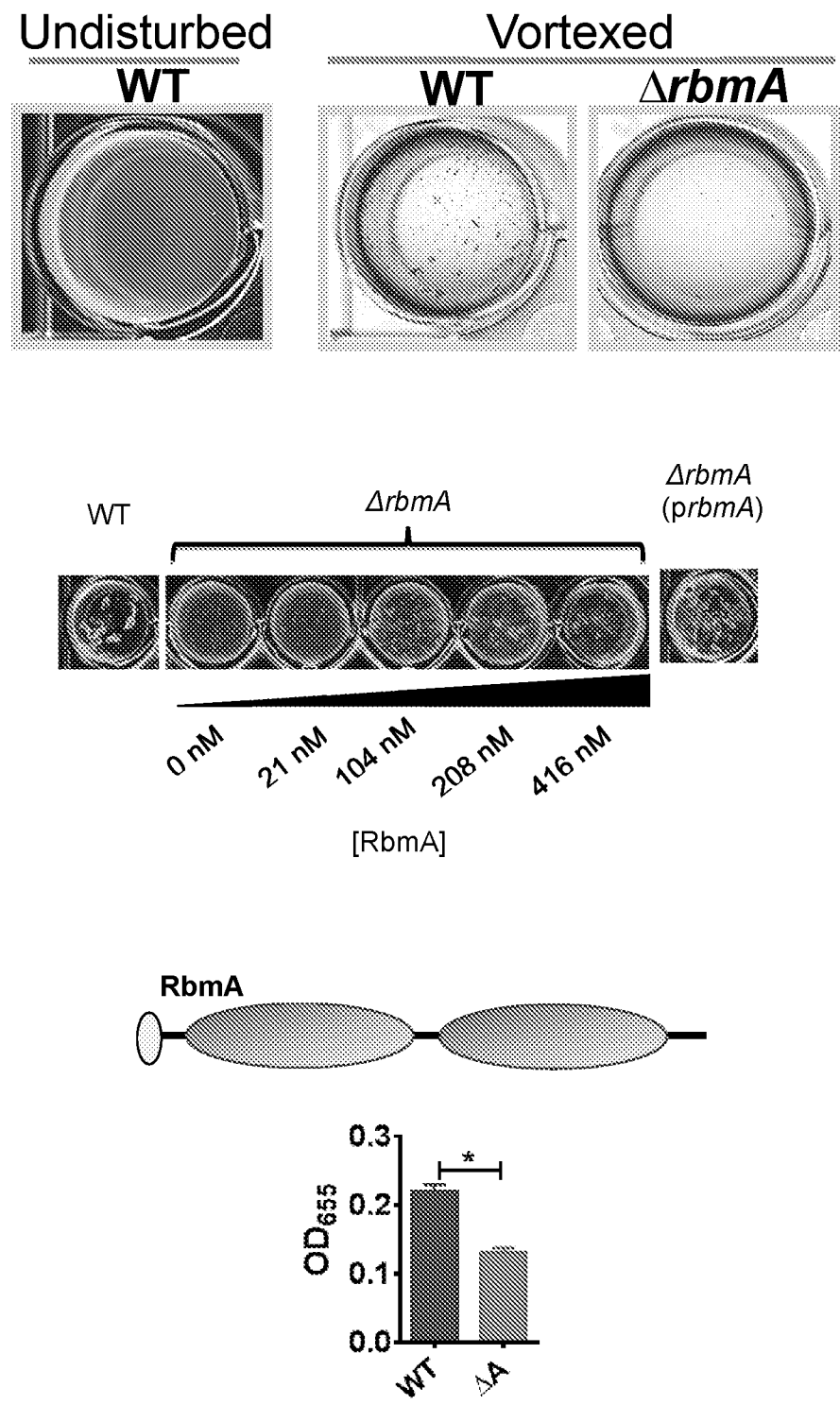
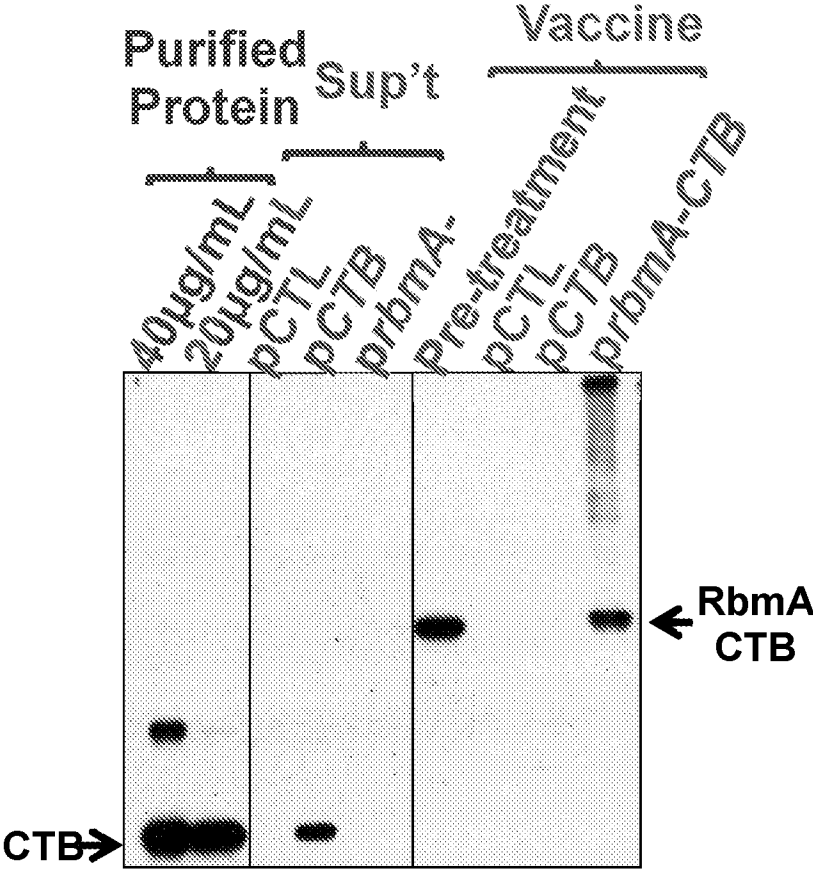


FIGURE 7



Quantification

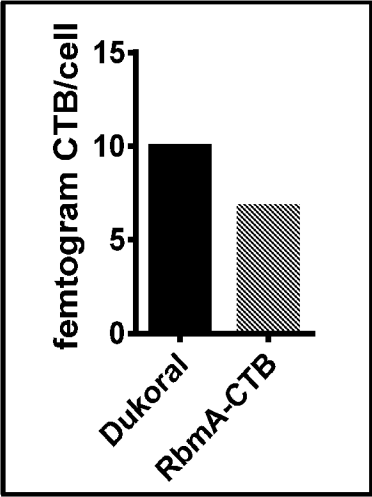


FIGURE 8

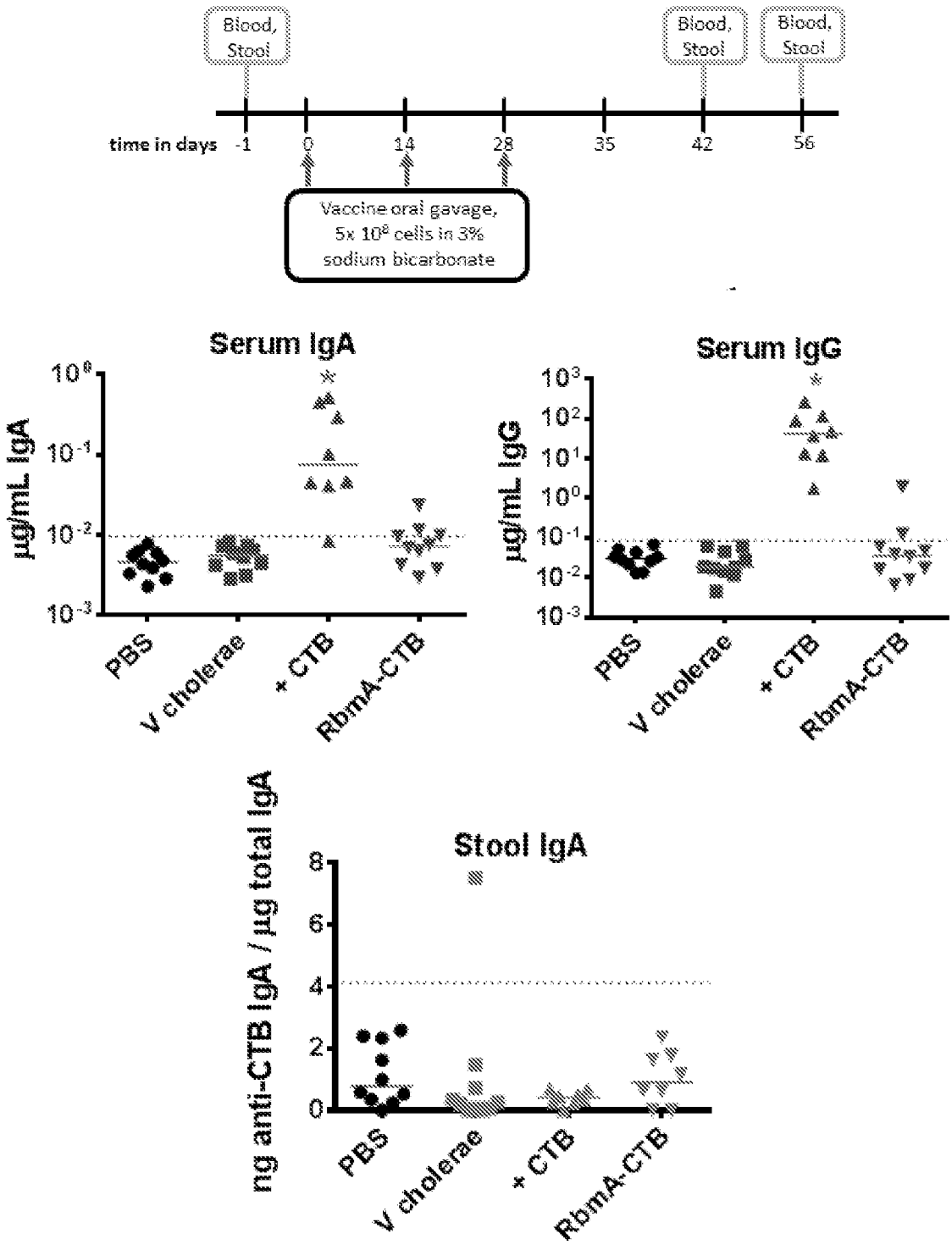


FIGURE 9

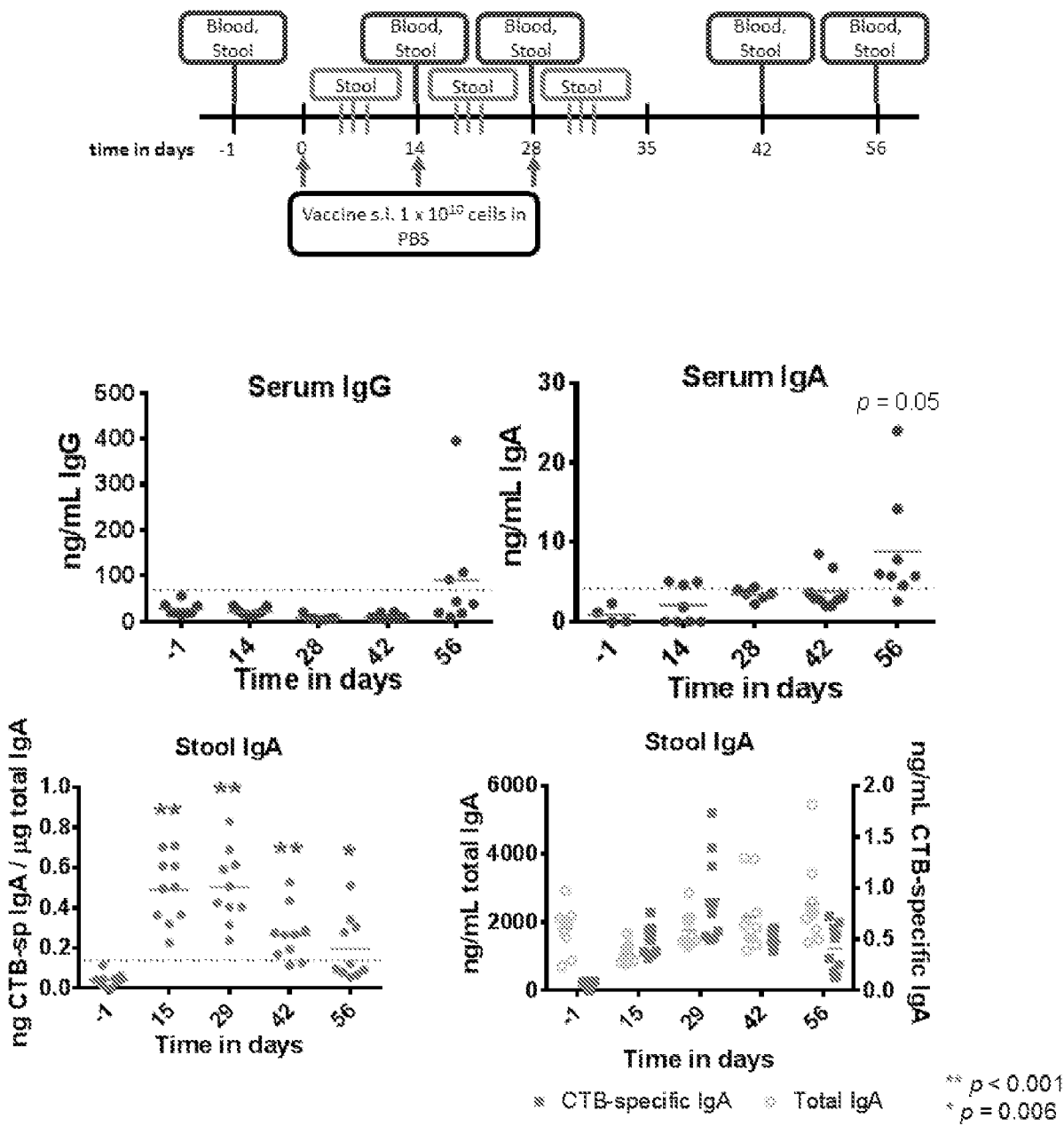


FIGURE 10

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Vibriocidal titer				
	<u>Condition</u>	<u>Day 14</u>	<u>Day 28</u>	<u>Day 56</u>
Killed whole-cell vaccine	PBS	n.d.	n.d.	< 2
	<i>V. cholerae</i>	n.d.	n.d.	64
	+ CTB	n.d.	n.d.	64
Live-attenuated	RbmA-CtxB	2	16	64

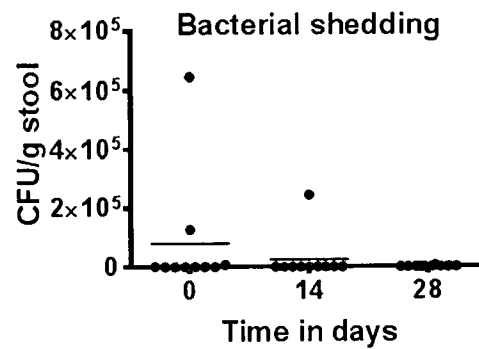


FIGURE 11

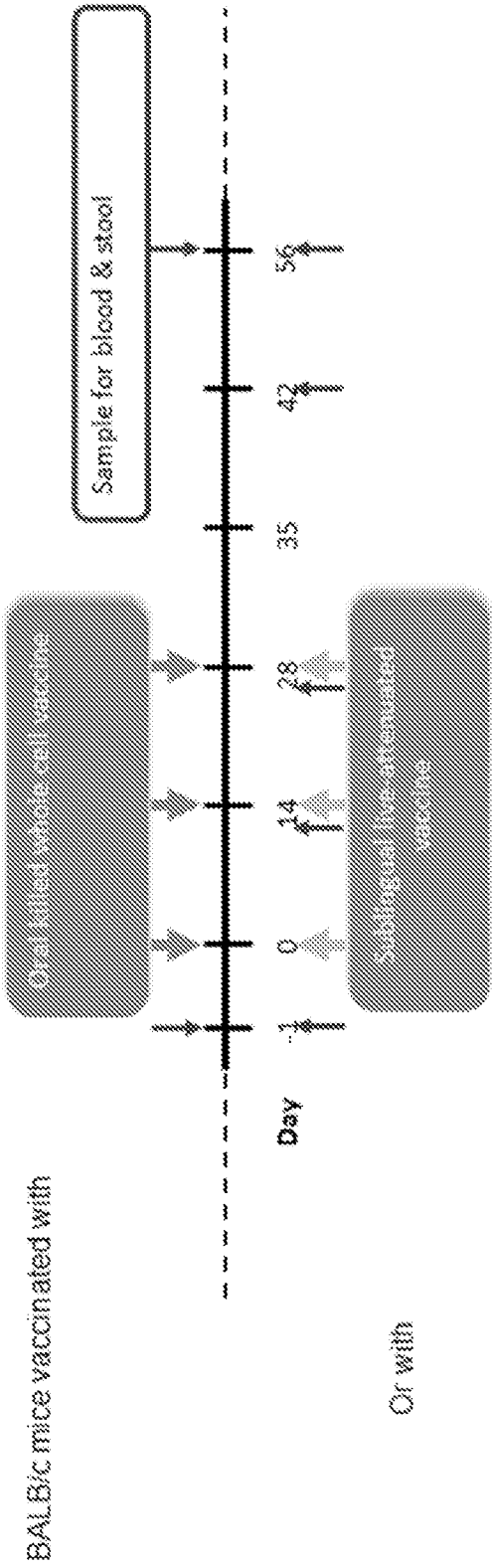


FIGURE 12

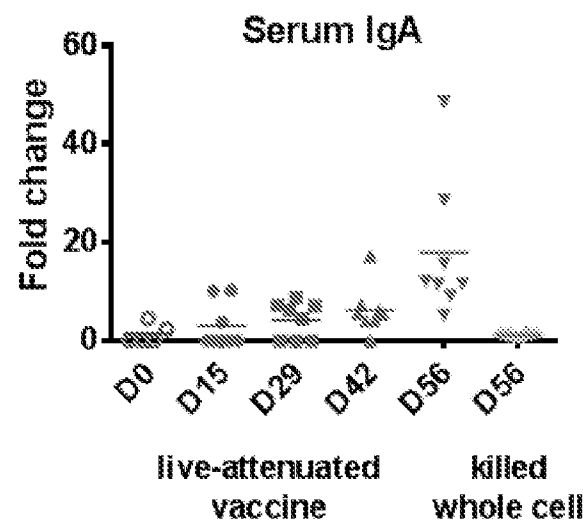


FIGURE 13A

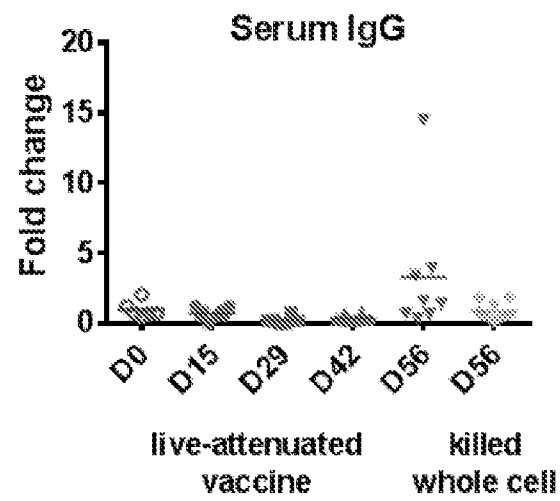


FIGURE 13B

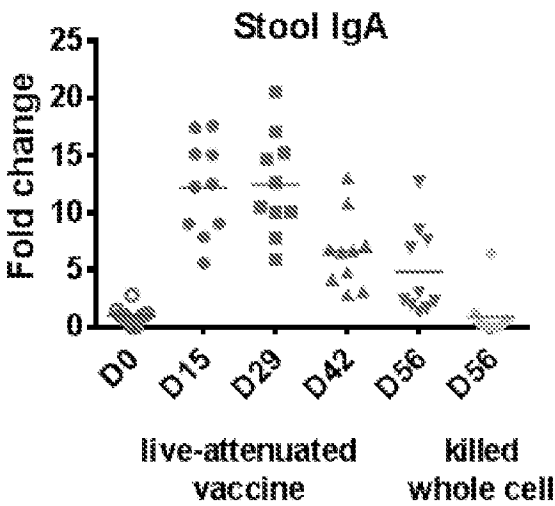


FIGURE 13C

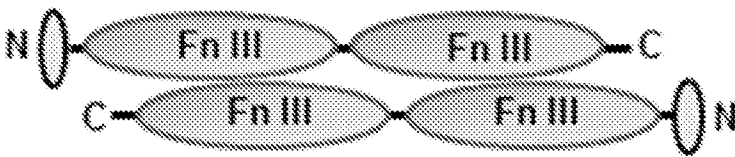


FIGURE 14A

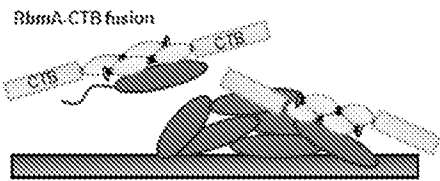


FIGURE 14B

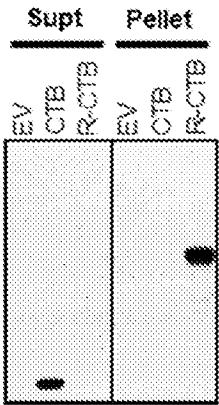


FIGURE 14C

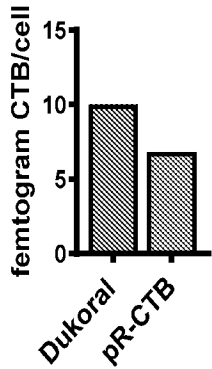


FIGURE 14D

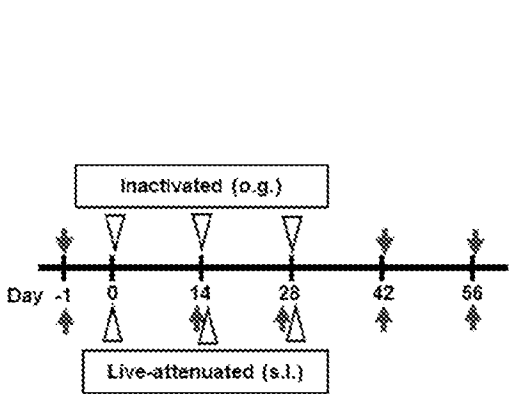


FIGURE 15A

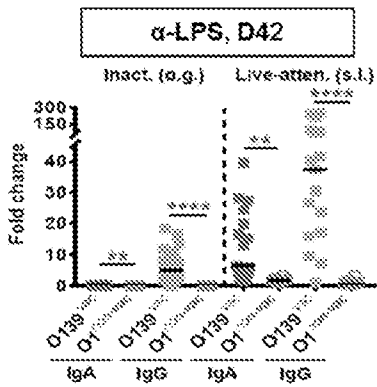


FIGURE 15B

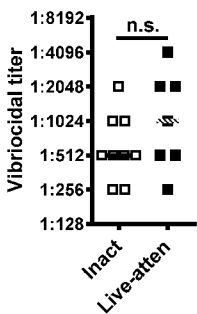


FIGURE 15C

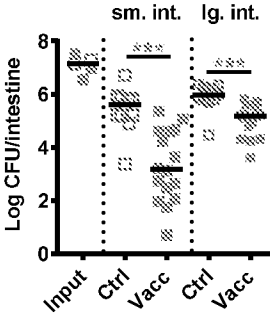


FIGURE 15D

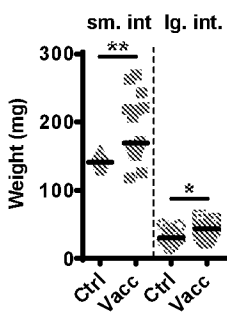


FIGURE 15E

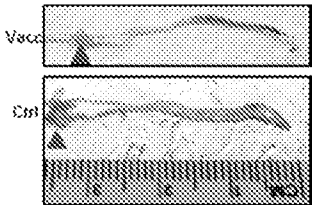


FIGURE 15F

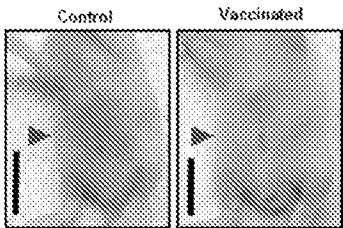
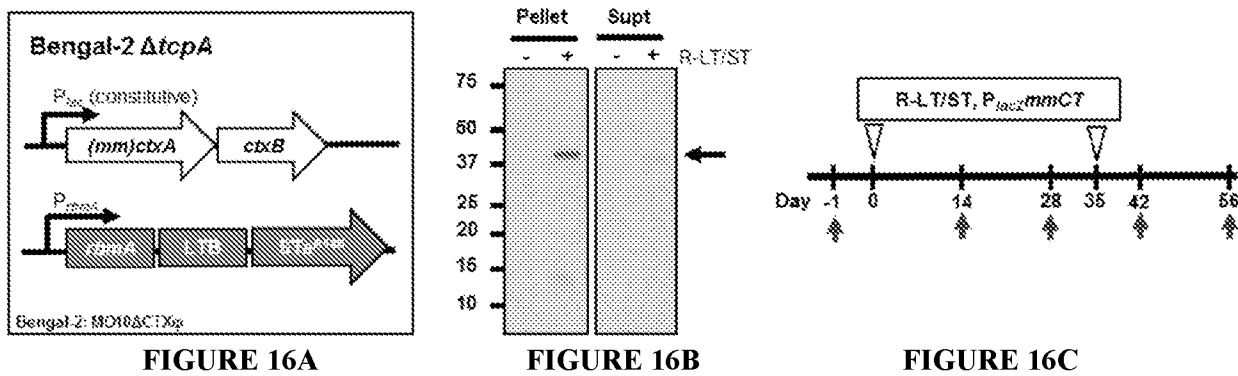
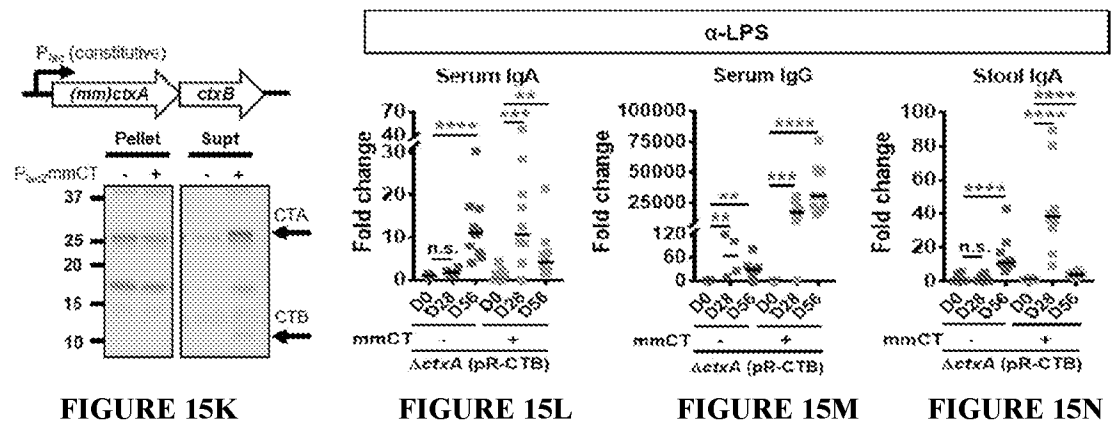
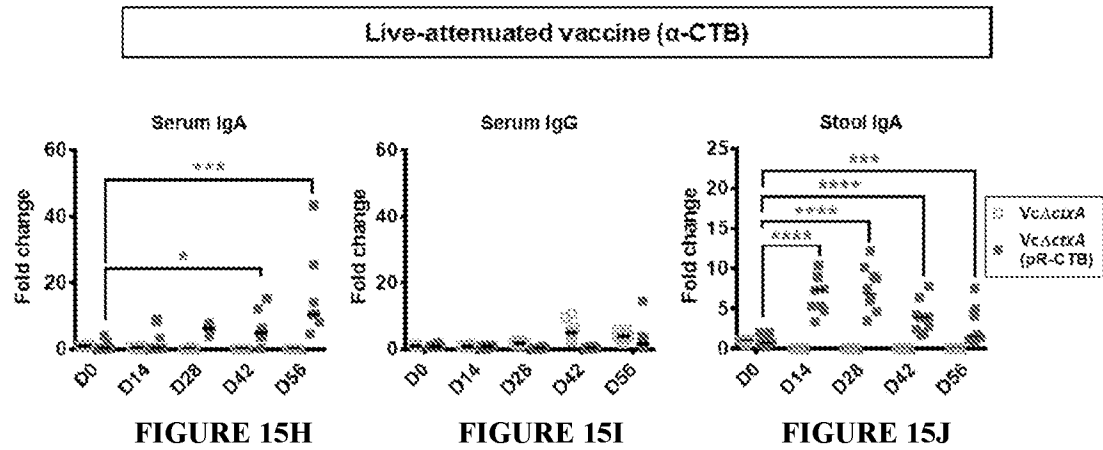


FIGURE 15G



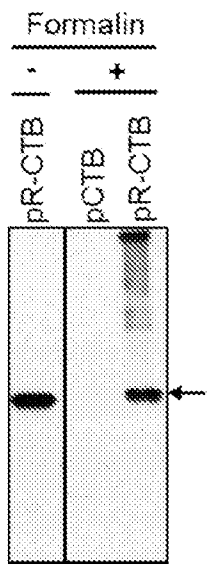
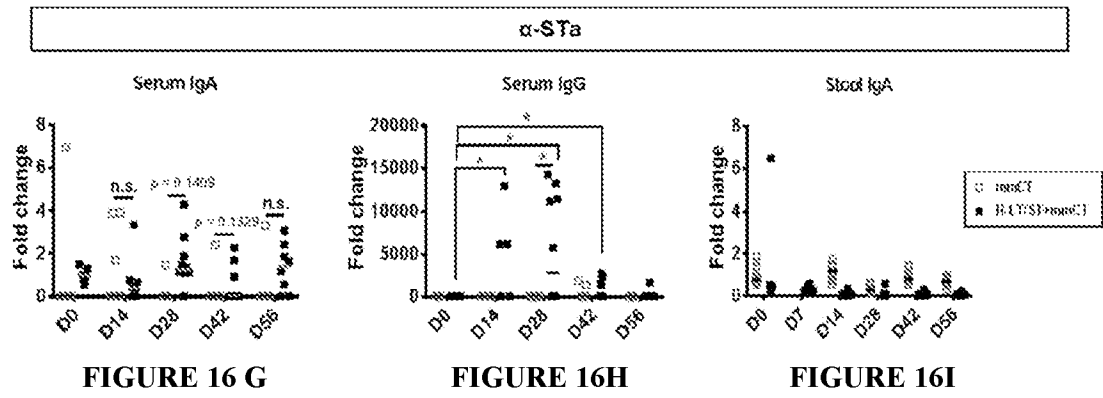
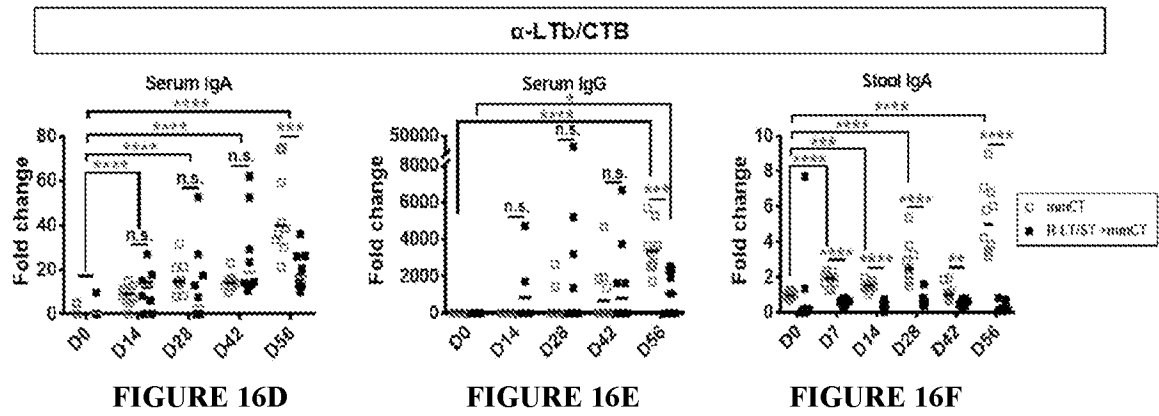


FIGURE 17

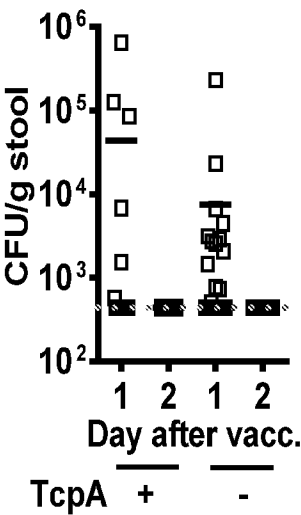
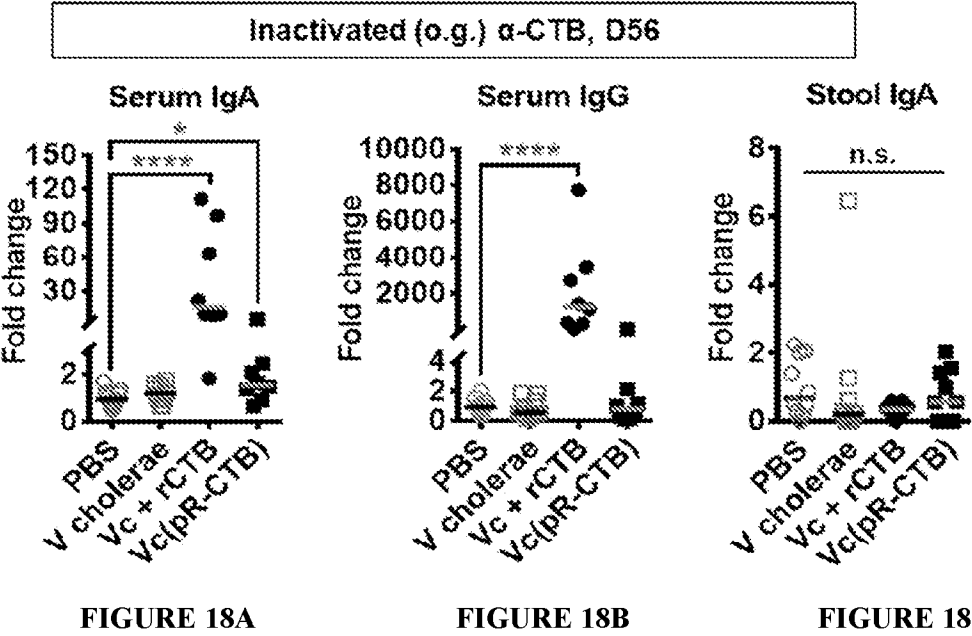
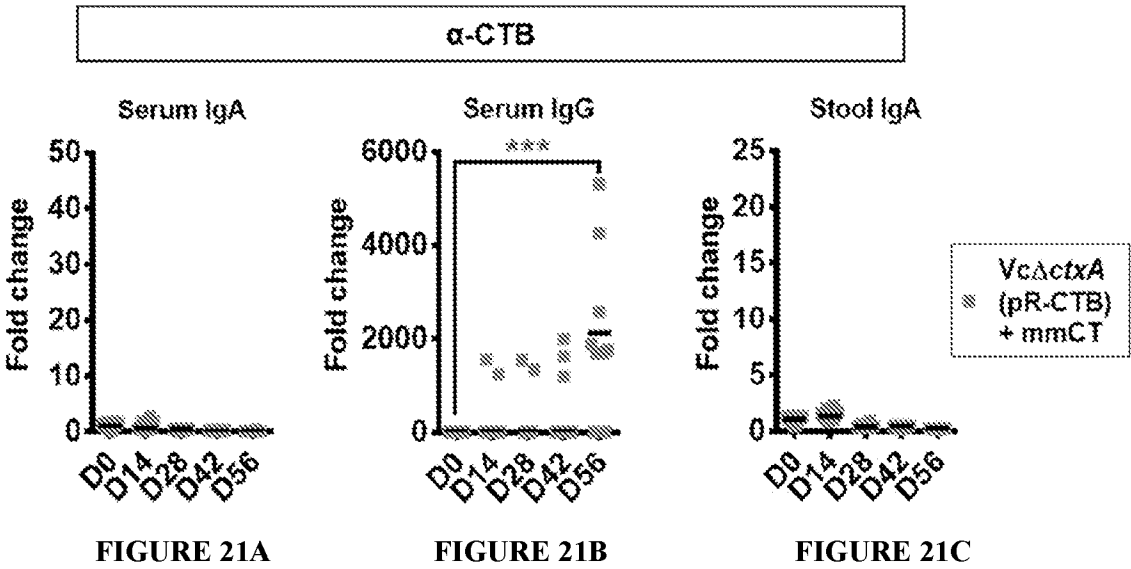
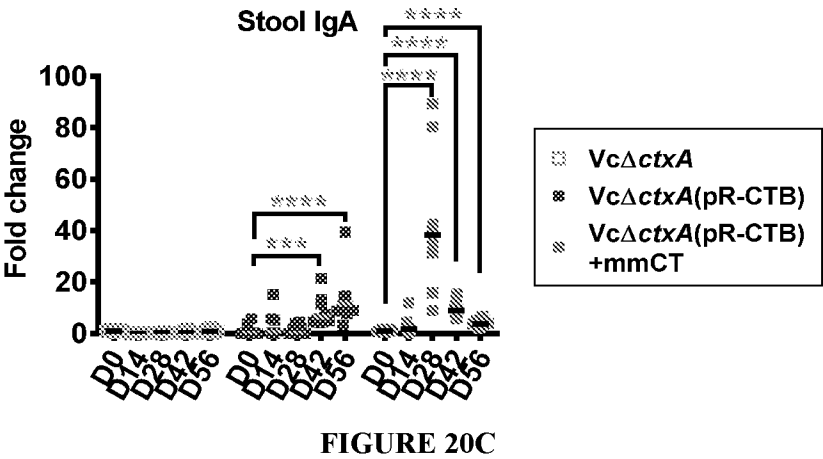
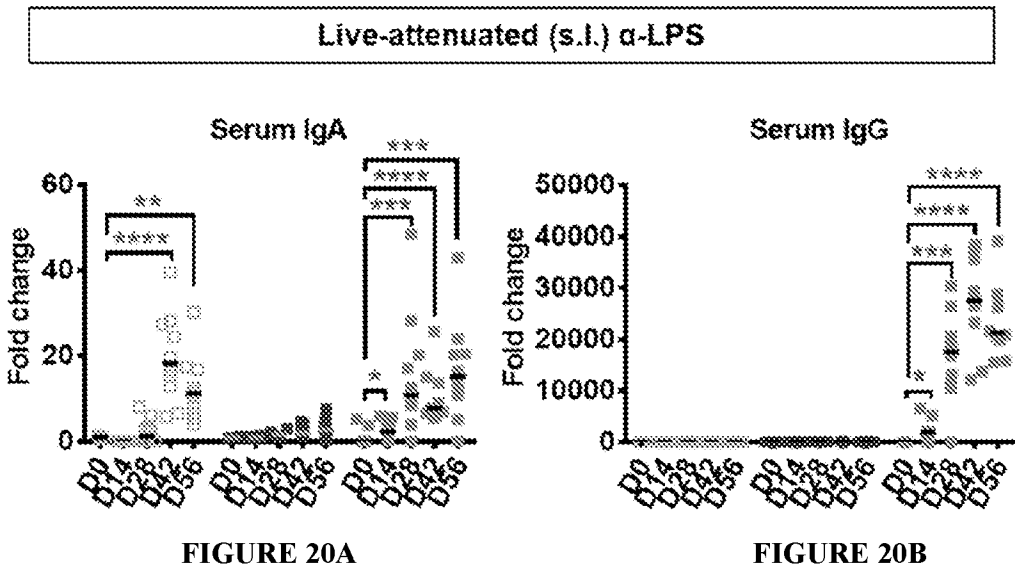


FIGURE 19



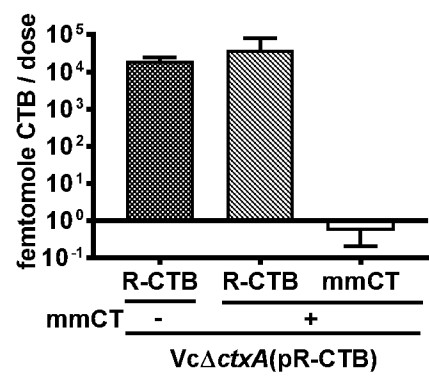


FIGURE 22

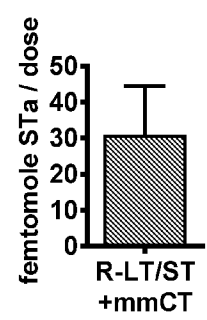


FIGURE 23