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(54) Title: ENGINEERED BACTERIA FOR NON-INVASIVE IMAGING AND THERAPEUTIC APPLICATIONS

(57) Abstract: Engineered bacteria that express a protein having a nonstandard amino acid, pharmaceutical compositions comprising the bacteria, biofilms comprising a protein having a nonstandard amino acid, and methods for using the bacteria for diagnostic and therapeutic purposes are provided.

ENGINEERED BACTERIA FOR NON-INVASIVE IMAGING AND THERAPEUTIC APPLICATIONS

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/372,148, filed on August 8, 2016, the entire contents of which are expressly incorporated herein by reference.

FIELD

[0002] The technology described herein relates to engineered bacteria comprising proteins that are capable of being labeled *in vivo* and methods for using the bacteria for diagnostic and therapeutic purposes.

BACKGROUND

[0003] Currently-available methods for diagnosing and or monitoring disease progression, particularly diseases of the gastrointestinal tract are often invasive and do not readily allow for continuous monitoring. Thus there is a need for alternative approaches that would allow for non-invasive continuous monitoring of a subject's gastrointestinal tract.

[0004] Advances in microbiome biology have led to increased interest in the use of live microbes to deliver drugs and treat diseases. The use of engineered microbes for diagnosing and/or monitoring disease state progression represents an alternative non-invasive method that would be of substantial benefit to patient diagnosis and treatment. Attempts to deliver traceable microbes require that the microbes be labeled with labile detectable compound prior to administration to the subject. Unfortunately, over a prolonged period of time, the detectable compounds are no longer traceable rendering the known techniques of little use for prolonged monitoring of a subject. Accordingly, there is a need in the art for methods of producing microbes that are traceable for a prolonged period of time after administration to a subject that can be used for a variety of medical uses (*e.g.*, diagnostics and therapeutics).

SUMMARY

[0005] Embodiments of the present disclosure are directed to engineered or genetically altered microbes, such as *Escherichia coli*, that produce proteins comprising a nonstandard amino acid. The nonstandard amino acid can be labeled with a detectable substance before or

after administration to the subject which allows for monitoring of the microbe over a prolonged period of time.

[0006] In one aspect, the present invention provides an engineered bacterium comprising an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and a heterologous nucleic acid comprising a labeling codon, wherein the heterologous nucleic acid comprises a heterologous gene encoding a protein selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein; wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with a nonstandard amino acid; and wherein the orthogonal tRNA incorporates the nonstandard amino acid at the residue corresponding to the labeling codon during translation.

[0007] In some embodiments, the heterologous nucleic acid further encodes a polypeptide linker sequence. In some embodiments, the polypeptide linker sequence is flexible. In some embodiments, the heterologous nucleic acid further encodes a polypeptide tag.

[0008] In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous gene encoding the protein. In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide linker sequence. In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide tag.

[0009] In some embodiments, the heterologous nucleic acid comprises 2, 3, 4, 5, 6, 7, or 8 labeling codons.

[0010] In one aspect, the present invention provides an engineered bacterium comprising an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and a heterologous nucleic acid encoding a protein, wherein the protein comprises a nonstandard amino acid, and wherein the protein is selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein; wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid; and wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation.

[0011] In some embodiments, the protein further comprises a polypeptide linker. In some embodiment, the polypeptide linker is flexible.

[0012] In some embodiments, the protein further comprises a polypeptide tag. In some embodiments, the polypeptide tag is selected from the group consisting of a poly-histidine tag, a myc tag, a FLAG tag, a hemagglutinin (HA) tag, and a V5 tag.

[0013] In some embodiments, the protein comprises 2, 3, 4, 5, 6, 7, or 8 nonstandard amino acids.

[0014] In some embodiments, the bacterial extracellular matrix protein is a fiber protein, a flagellar protein, or a pilin protein.

[0015] In some embodiments, the protein is selected from the group consisting of InaV, Trat, and CsgA. In some embodiments, the protein is CsgA. In some embodiments, the protein is CsgA and the nonstandard amino acid is located at an amino acid residue corresponding to residue 89 of wild-type CsgA. In some embodiments, the protein is InaV and the nonstandard amino acid is located at an amino acid residue corresponding to residue 174 of wild-type InaV. In some embodiments, the protein is Trat and the nonstandard amino acid is located at an amino acid residue corresponding to residue 200 of wild-type Trat.

[0016] In some embodiments, the labeling codon is TAG.

[0017] In some embodiments, the orthogonal tRNA comprises an anticodon loop that specifically recognizes the codon UAG.

[0018] In some embodiments, the nonstandard amino acid comprises a bioorthogonal reactive functional group. In some embodiments, the bioorthogonal reactive functional group is selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a tetrazine moiety, or a norbornene moiety.

[0019] In some embodiments, the nonstandard amino acid is selected from the group consisting of *p*-azidophenylalanine (*p*-AzF), azidohomoalanine (Aha), azidolysine, homopropargylglycine (Hpg), homoallylglycine (Hag), oxonorvaline (Onv), *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine (*p*-EtF), *para*-acetylphenylalanine (*p*-AcF), azidonorleucine (Anl), *trans*-crotylglycine (Tcg), selenomethionine (Se-Met), 2-aminooctynoic acid (Aoa), and propargylglycine (Pra). In some embodiments, the nonstandard amino acid is *p*-AzF.

[0020] In some embodiments, the engineered bacterium is non-pathogenic. In some embodiments, the engineered bacterium is of the species *Escherichia coli*. In some embodiments, the engineered bacterium is of an *Escherichia coli* strain selected from the group consisting of C321, C321.ΔA and C321.ΔA.exp. In some embodiments, the engineered bacterium is of the *Escherichia coli* Nissle 1917 strain.

[0021] In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a codon. In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a TAG codon. In some embodiments, the engineered bacterium comprises a genome that has not been recoded to reduce or eliminate a codon. In some embodiments, the engineered bacterium further comprises a *prfA* gene deletion.

[0022] In some embodiments, the orthogonal aminoacyl tRNA synthetase is an Archea aminoacyl tRNA synthetase or a bacterial aminoacyl tRNA synthetase. In some embodiments, the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina*, *Desufitobacterium*, *Pyrococcus* or *Methanocaldococcus* aminoacyl tRNA synthetase gene. In some embodiments, the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina mazei*, *Methanosarcina barkeri*, *Desulfitobacterium hafniense, Pyrococcus horikoshii*, or *Methanocaldococcus jannaschii* aminoacyl tRNA synthetase gene.

[0023] In some embodiments, the orthogonal translation system is located in the bacterial genome. In some embodiments, the orthogonal translation system is located in a plasmid. In some embodiments, the plasmid is pEVOL-pAzF.

[0024] In some embodiments, the expression of the heterologous nucleic acid is under the control of a constitutive promoter. In some embodiments, the expression of the heterologous nucleic acid is under the control of an inducible promoter. In some embodiments, the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline. In some embodiments, the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the heterologous gene encoding the orthogonal tRNA is under the control of a constitutive promoter.

[0025] In some embodiments, the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the heterologous gene encoding the orthogonal tRNA is under the control of an inducible promoter. In some embodiments, the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

[0026] In one aspect the present invention provides a biofilm comprising an engineered bacterium described herein.

[0027] In one aspect, the present invention provides a pharmaceutical composition comprising an engineered bacterium described herein and a pharmaceutically-acceptable

excipient. In some embodiments, the composition is formulated for oral administration to a subject. In some embodiments, the composition is formulated for rectal administration to a subject. In some embodiments, the pharmaceutical composition is formulated as a pill, a capsule, a lozenge, or a suppository. In some embodiments, the subject is a mammalian subject. In some embodiments, the mammalian subject is a human subject.

[0028] In another aspect, the present invention provides a method for labeling of an engineered bacterium in a subject *in vivo*, the method comprising: (a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group; and (b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid of the protein, thereby labeling the engineered bacterium in the subject *in vivo*. In some embodiments, step (a) is performed concurrently with step (b). In some embodiments, step (a) is performed before step (b).

[0029] In yet another aspect, the present invention provides a method for detecting the distribution of an engineered bacterium in a subject *in vivo*, the method comprising: (a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group; (b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid; and (c) detecting the detectable compound in the subject, thereby detecting the distribution of the engineered bacterium in the subject *in vivo*. In some embodiments, step (a) is performed concurrently with step (b). In some embodiments, step (a) is performed before step (b).

[0030] In another aspect, the present invention provides a method for diagnosing a gastrointestinal disease in a subject, the method comprising: (a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group; (b) administering to the subject a detectable compound comprising a second

bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid; and (c) detecting the distribution of the detectable compound in the gastrointestinal tract of the subject, thereby diagnosing a gastrointestinal disease in the subject. In some embodiments, step (a) is performed concurrently with step (b). In some embodiments, step (a) is performed before step (b).

[0031] In yet another aspect, the present invention provides a method for detecting the distribution of a biofilm produced by an engineered bacterium in a subject, the method comprising: (a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid to a subject, and wherein the nonstandard amino acid comprises a first bioorthogonal reactive functional group; (b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo,* thereby attaching the detectable compound to the nonstandard amino acid; and (c) detecting the detectable compound in the subject, thereby determining the distribution of the biofilm produced by the engineered bacterium in the subject. In some embodiments, step (a) is performed concurrently with step (b). In some embodiments, step (a) is performed before step (b).

[0032] In another aspect, the present invention provides a method for delivering a drug to a subject in need thereof, the method comprising: (a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group; (b) administering to the subject a drug comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the drug to the nonstandard amino acid, thereby delivering the drug to the subject in need thereof. In some embodiments, step (a) is performed concurrently with step (b). In some embodiments, step (a) is performed before step (b). In some embodiments, the drug is delivered to the gastrointestinal tract of the subject. In some embodiments, the drug is delivered to the mouth, esophagus, stomach, large intestine, small intestine, rectum, colon, or anal canal of the subject. In some embodiments, the drug is delivered to the heart, lung, hair, or skin of the subject.

[0033] In some embodiments, the engineered bacterium comprises an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and a heterologous nucleic acid encoding the protein having the at least one nonstandard amino acid; wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid; and wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation.

[0034] In some embodiments, the methods further comprise administering a nonstandard amino acid to the subject. In some embodiments, the nonstandard amino acid is administered to the subject prior to administering the detectable compound or the drug to the subject.

[0035] In some embodiments, the first bioorthogonal reactive functional group is selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a tetrazine moiety, and a norbornene moiety. In some embodiments, the second bioorthogonal reactive functional group is selected from the group consisting of a hydrazine moiety, a hydroxylamine moiety, an alkyne moiety, an azide moiety, a tetrazine moiety, and a norbornene moiety. In some embodiments, the first bioorthogonal reactive functional group is a ketone moiety, and the second bioorthogonal reactive functional group is either a hydrazine moiety or a hydroxylamine moiety. In some embodiments, the first bioorthogonal reactive functional group is an azide moiety, and the second bioorthogonal reactive functional group is an alkyne moiety. In some embodiments, the first bioorthogonal reactive functional group is an alkyne moiety, and the second bioorthogonal reactive functional group is an azide moiety. In some embodiments, the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group via a click chemistry reaction.

[0036] In some embodiments, the reaction between the first bioorthogonal reactive functional group and the second bioorthogonal reactive functional group requires a catalytic agent. In some embodiments, the reaction between the first bioorthogonal reactive functional group and the second bioorthogonal reactive functional group does not require a catalytic agent. In some embodiments, the methods further comprise administering a catalytic agent to the subject. In some embodiments, the catalytic agent is copper or nickel.

[0037] In some embodiments, the detectable compound comprises a fluorescent moiety, a radioactive moiety, a colorimetric dye, a fluorescent dye, a luminescent dye, a magnetic resonance imaging (MRI) contrast agent, a CT contrast agent, a PET contrast agent, or an ultrasound contrast agent.

[0038] In some embodiments, the protein further comprises a polypeptide linker. In some embodiments, the polypeptide linker is flexible. In some embodiments, the protein further comprises a polypeptide tag. In some embodiments, the polypeptide tag is selected from the group consisting of a poly-histidine tag, a myc tag, a FLAG tag, a hemagglutinin (HA) tag, and a V5 tag.

[0039] In some embodiments, the protein is selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein. In some embodiments, the bacterial extracellular matrix protein is a curli fiber protein, a flagellar protein, or a pilin protein.

[0040] In some embodiments, the protein is selected from the group consisting of InaV, Trat, and CsgA. In some embodiments, the protein is CsgA. In some embodiments, the protein is CsgA and the nonstandard amino acid is located at an amino acid residue corresponding to residue 89 of wild-type CsgA. In some embodiments, the protein is InaV and the nonstandard amino acid is located at an amino acid residue corresponding to residue 174 of wild-type InaV. In some embodiments, the protein is Trat and the nonstandard amino acid is located at an amino acid residue corresponding to residue 200 of wild-type Trat.

[0041] In some embodiments, the nonstandard amino acid is selected from the group consisting of *p*-azidophenylalanine (*p*-AzF), azidohomoalanine (Aha), azidolysine, homopropargylglycine (Hpg), homoallylglycine (Hag), oxonorvaline (Onv), *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine (*p*-EtF), *para*-acetylphenylalanine (*p*-AcF), azidonorleucine (Anl), trans-crotylglycine (Tcg), selenomethionine (Se-Met), 2-aminooctynoic acid (Aoa), and propargylglycine (Pra). In some embodiments, the nonstandard amino acid is *p*-AzF.

[0042] In some embodiments, the detectable compound is DBCO-Cy5.

[0043] In some embodiments, the engineered bacterium is non-pathogenic. In some embodiments, the engineered bacterium is of the species *Escherichia coli*. In some embodiments, the engineered bacterium is of an *Escherichia coli* strain selected from the group consisting of C321, C321.ΔA and C321.ΔA.exp. In some embodiments, the engineered bacterium is of the *Escherichia coli* Nissle 1917 strain.

[0044] In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a codon. In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a TAG codon. In some embodiments, the engineered bacterium comprises a genome that has not been recoded to

reduce or eliminate a codon. In some embodiments, the engineered bacterium further comprises a *prfA* gene deletion.

[0045] In some embodiments, the orthogonal aminoacyl tRNA synthetase is an Archea aminoacyl tRNA synthetase or a bacterial aminoacyl tRNA synthetase. In some embodiments, the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina, Desufitobacterium, Pyrococcus* or *Methanocaldococcus* aminoacyl tRNA synthetase gene. In some embodiments, the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina mazei, Methanosarcina barkeri, Desulfitobacterium hafniense, Pyrococcus horikoshii,* or *Methanocaldococcus jannaschii* aminoacyl tRNA synthetase gene.

[0046] In some embodiments, the orthogonal translation system is located in the bacterial genome. In some embodiments, the orthogonal translation system is located in a plasmid. In some embodiments, the plasmid is pEVOL-pAzF.

[0047] In some embodiments, the expression of the heterologous nucleic acid is under the control of a constitutive promoter. In some embodiments, the expression of the heterologous nucleic acid is under the control of an inducible promoter. In some embodiments, the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline. In some embodiments, the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the orthogonal tRNA is under the control of a constitutive promoter.

[0048] In some embodiments, the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the orthogonal tRNA is under the control of an inducible promoter. In some embodiments, the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

[0049] In some embodiments, the methods further comprise administering to the subject an inducer that regulates the inducible promoter. In some embodiments, the inducer is administered to the subject prior to administering the detectable compound or the drug to the subject. In some embodiments, the inducer is comprised in a pharmaceutical composition.

[0050] In some embodiments, the engineered bacterium is administered to the subject orally or rectally. In some embodiments, the engineered bacterium is comprised in a pharmaceutical composition.

[0051] In some embodiments, the detectable compound or the drug is administered to the subject orally, subcutaneously, intravenously, rectally, or transdermally. In some

embodiments, the detectable compound or the drug is comprised in a pharmaceutical composition. In some embodiments, the detectable compound or the drug further comprises a drug delivery vehicle selected from the group consisting of a nanocarrier, a nanoparticle, a liposome, a dendrimer, a carbon nanotube, a micelle, and a protein.

[0052] The present invention is further illustrated by the following detailed description and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0053] The foregoing and other features and advantages of the present embodiments will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings in which:

[0054] FIG. 1A is a schematic showing plasmid maps indicating exemplary locations of a labeling codon (UAG) in mRNA transcribed from the open reading frames of the modified genes encoding the maltose-binding protein (MBP) (position 67), CsgA (position 89), the ice nucleation protein (InaV) (position 174), or the TraT protein (position 200).

[0055] FIG. 1B is a schematic showing plasmid maps indicating exemplary locations of a labeling codon (UAG) in mRNA transcribed from open reading of the genes encoding the maltose-binding protein (MBP), CsgA, the ice nucleation protein (InaV), or the TraT protein appended to a flexible linker and affinity tags. Affinity tags (*i.e.*, a myc tag and a poly-his tag) were introduced before and after the labeling codon to assess of the incorporation of the nonstandard amino acid or translation termination.

[0056] FIG. 2A depicts the structure of two nonstandard amino acids, p-acetylphenylalanine and p-azidophenylalanine.

[0057] FIGs. 2B and **2C** are two schematics showing exemplary recombinant bacteria engineered for bioorthogonal reactions that may be used to attach a detectable moiety or drug to a protein comprising a nonstandard amino acid.

[0058] FIG. 3 shows an SDS-PAGE gel stained with Coomassie Blue showing the Ni NTA affinity purification of maltose-binding protein (MBP) expressed in *E. coli* Nissle that has not been recoded to remove TAG codons. Purification eluates from three different bacterial strains are shown: cells transformed with a single plasmid to express MBP without a nonstandard amino acid, where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express MBP without a nonstandard amino acid expressed and a plasmid comprising an orthogonal translation system ("No

mutation/With OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS").

[0059] FIG. 4A depicts the structure of DBCO-Cy5.

[0060] FIGs. 4B and **4C** show an SDS-PAGE gel stained with Coomassie Blue (**FIG. 4B**), and the corresponding fluorescence detection image (**FIG. 4C**) of eluates from a Ni NTA affinity purification of maltose-binding protein (MBP) comprising the nonstandard amino acid *p*-AzF, that were reacted with DBCO-Cy5. MBP was expressed in *E. coli* Nissle that has not been recoded to remove TAG codons using three different transformation conditions: cells transformed with a single plasmid to express MBP without a nonstandard amino acid, where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express MBP without a nonstandard amino acid expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS"). Concentrated 500 mM eluates of purified MBP were incubated with DBCO-Cy5 at increasing molar ratios ranging from 1:10 to 10:1 MBP:DBCO-Cy5.

[0061] FIG. 5 shows an SDS-PAGE gel stained with Coomassie Blue showing the Ni NTA affinity purification of CsgA expressed in *E. coli* Nissle that has not been recoded to remove TAG codons using three different transformation conditions: cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon, where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS").

[0062] FIGs. 6A and **6B** show an SDS-PAGE gel stained with Coomassie Blue (**FIG. 6A**), and the corresponding fluorescence detection image (**FIG. 6B**), of eluates from a Ni NTA affinity purification of CsgA comprising the nonstandard amino acid AzF, that were reacted with DBCO-Cy5. CsgA was expressed in *E. coli* Nissle that has not been recoded to remove TAG codons using three different transformation conditions: cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon, where the bacteria

lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS"). Concentrated 500 mM eluates of purified CsgA protein were incubated with DBCO-Cy5.

[0063] FIG. 7 shows fluorescence detection imaging of cell pellets from *E. coli* Nissle cells under three different transformation conditions: cells transformed with a single plasmid to express MBP without a nonstandard amino acid, where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express MBP without a nonstandard amino acid expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS"). After exposure to DBCO-Cy5, cell pellets were washed three times with phosphate buffer. MBP is soluble and was washed away from the cell pellets, and low specific binding of DBCO-Cy5 to other proteins was observed.

[0064] FIG. 8 shows Congo Red dye-monitoring of the expression of CsgA comprising *p*-AzF at different concentrations of arabinose to induce the expression of the orthogonal translation system that incorporates *p*-AzF into the expressed protein. Congo Red dye binding to curli fiber (CsgA) was determined using spectrophotometry at OD490/OD600, a measure of bound Congo Red dye over cell density. Increased Congo Red binding signifies increased curli fiber (CsgA) production. Optimal expression of CsgA comprising *p*-AzF was observed at 0.05% arabinose induction. *E. coli* Nissle having a deletion in the endogenous curli operons were used. Data for cells expressing a heterologous gene encoding CsgA comprising *p*-AzF (as part of the synthetic curli operon) are shown in red ("Curli operon"). Data for cells expressing a heterologous gene encoding MBP comprising *p*-AzF are shown in grey (control; "MBP"). Experiments were performed using three different transformation conditions: cells transformed with a single plasmid to express wild-type protein (CsgA or MBP), where the bacteria lacked an orthogonal translation system (no label); cells transformed with a plasmid to express wild-type protein (CsgA or MBP) and a plasmid comprising an orthogonal translation system ("OTS"); and cells transformed with a plasmid

to express protein (CsgA or MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("OTS/mut"). Background signal could be due to Congo Red binding to other extracellular appendages of *ECN* (cellulose for example). Only background signal was observed for cells expressing MBP.

[0065] FIG. 9 shows scanning electron microscopy images of curli (CsgA) expression in *E. coli* Nissle expressing either MBP or CsgA as indicated. The protein of interest (MBP or CsgA) was expressed in *E. coli* Nissle that has not been recoded to remove TAG codons using three different transformation conditions: cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon, where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS").

[0066] FIGs. 10A and **10B** show bacterial growth curves of *E. coli* Nissle cells either: lacking plasmid ("No plasmid"); comprising an orthogonal translation system (encoded by the pEVOL-pAzF plasmid) ("OTS"); cells transformed with a single plasmid to express wild-type MBP (without a nonstandard amino acid), where the bacteria lacked an orthogonal translation system ("MBP"); cells transformed with a plasmid to express wild-type MBP (without a nonstandard amino acid) expressed and a plasmid comprising an orthogonal translation system ("MBP + OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("MBP (mutation) + OTS"); cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon), where the bacteria lacked an orthogonal translation system ("Curli operon"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("Curli operon + OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("Curli operon (mutation) + OTS"). *E. coli* Nissle having a deletion in the endogenous curli operons were used. **FIG. 10A** shows growth curves in the absence of an inducer for the orthogonal translation system (arabinose).

[0067] FIG. 10B shows growth curves in the presence of 1 mM *p*-AzF.

[0068] FIGs. 11A and **11B** shows bacterial growth curves of *E. coli* Nissle cells either: lacking plasmid ("No plasmid"); comprising an orthogonal translation system (encoded by the pEVOL-pAzF plasmid) ("OTS"); cells transformed with a single plasmid to express wild-type MBP (without a nonstandard amino acid), where the bacteria lacked an orthogonal translation system ("MBP"); cells transformed with a plasmid to express wild-type MBP (without a nonstandard amino acid) expressed and a plasmid comprising an orthogonal translation system ("MBP + OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("MBP (mutation) + OTS"); cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon), where the bacteria lacked an orthogonal translation system ("Curli operon"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("Curli operon + OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthologous translation system ("Curli operon (mutation) + OTS"). *E. coli* Nissle having a deletion in the endogenous curli operons were used. **FIG. 11A** shows growth curves in the presence of 0.05% arabinose, the inducer for the orthogonal translation system and the MBP protein. **FIG. 11B** shows growth curves in the presence of in the presence of 0.05% arabinose and 1 mM *p*-AzF.

[0069] FIG. 12 shows an SDS-PAGE gel stained with Coomassie Blue, and the corresponding fluorescence detection image of purified MBP comprising *p-AzF*, that was reacted with DBCO-Cy5 at varying molar ratios.

[0070] FIG. 13 shows a fluorescence detection image of an SDS-PAGE gel of purified CsgA comprising *p*-AzF that was reacted with DBCO-Cy5. Cells were grown in media containing varying concentrations of the nonstandard amino acid (*p*-AzF; labeled "NSAA"). CsgA was expressed in *E. coli* Nissle that has not been recoded to remove TAG codons using three different transformation conditions: cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon), where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard

amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS").

[0071] FIGs. 14A and **14B** shows an SDS-PAGE gel stained with Coomassie Blue (**FIG. 14A**), and a Western Blot (**FIG. 14B**) using anti-His antibody to detect the expression of TraT surface protein having a poly-His tag in cell lysates from *Escherichia coli* Nissle. Cells were lysed with three different lysing buffers, as indicated (Buffer 1: 50 mM Tris-HCl pH 8.0, 2% Triton X-100; Buffer 2: 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 150 mM NaCl; and Buffer 3: 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate). Ladder in **FIG. 14A**: Precision Plus Protein™ Dual Color Standards; ladder in **FIG. 14B:** Novex® Sharp Pre-stained Protein Standard.

[0072] FIGs. 15A, **15B**, **15C**, and **15D** show the expression of wild-type and mutant TraT in lysates from cells lacking or having an orthogonal translation system (OTS) in the presence or absence of the NSAA pAzF. **FIG. 15A** and **15B** each show a Western Blot using anti-His antibody (**FIG. 15A**) or anti-myc antibody (**FIG. 15B**) from cell lysates of either a) bacterial cells transformed with a plasmid to express wild-type TraT and lacking a plasmid expressing an OTS, grown in the absence of the NSAA pAzF (Mutation "-"; OTS "+"; NSAA "-"); b) bacterial cells transformed with a plasmid to express wild-type TraT and a plasmid expressing an OTS, grown in the presence of the NSAA pAzF (Mutation "-"; OTS "+"; NSAA "+"); c) bacterial cells transformed with a plasmid to express mutant TraT comprising a NSAA, and lacking a plasmid expressing an OTS, grown in the absence of the NSAA pAzF (Mutation "+"; OTS "-"; NSAA "-"); d) bacterial cells transformed with a plasmid to express mutant TraT comprising a NSAA and a plasmid expressing an OTS, grown in the absence of the NSAA pAzF (Mutation "+"; OTS "+"; NSAA "-"); or e) bacterial cells transformed with a plasmid to express mutant TraT comprising a NSAA and a plasmid expressing an OTS, grown in the presence of the NSAA pAzF (Mutation "+"; OTS "+"; NSAA "+"). **FIG. 15C** is a bar graph quantitatively depicting the percentage of TraT protein detected with anti-His antibody corresponding to the approximately 37 kDa full length TraT protein in each of the lanes of the Western Blot shown in **FIG. 15A**. **FIG. 15D** is a bar graph quantitatively depicting the intensity (in absolute intensity units) of the approximately 37 kDa full length TraT protein in each of the lanes of the Western Blot shown in **FIG. 15A**.

[0073] FIG. 16 shows an SDS-PAGE of either wild-type TraT, mutant TraT comprising pAzF, wild-type CsgA or mutant TraT comprising pAzF, labelled with DBCO-Cy5.

[0074] FIGs. 17A, 17B and **17C** show the optimization of labeling of mutant TraT comprising pAzF with DBCO-Cy5. **FIG. 17A** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and mutant TraT comprising pAzF ("TraT") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with increasing concentrations of DBCO-Cy5 for labelling. **FIG. 17B** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and mutant TraT comprising pAzF ("TraT") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with 200 μM DBCO-Cy5 at 37°C for 1-4 hours. **FIG. 17C** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and mutant TraT comprising pAzF ("TraT") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with 100 μM or 200 μM DBCO-Cy5 at 25 °C or 37 °C for labelling. N=5 for all experiments.

[0075] FIGs. 18A, 18B, 18C, and **18D** show the optimization of labeling of mutant TraT comprising pAzF or mutant CsgA comprising pAzF with DBCO-Cy5. **FIG. 18A** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and either mutant CsgA comprising pAzF ("CsgA") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with increasing concentrations (200 μM - 400 μM) of DBCO-Cy5 for labelling. **FIG. 18B** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and either mutant TraT comprising pAzF ("TraT") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with increasing concentrations of DBCO-Cy5 for labelling. **FIG. 18C** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and mutant CsgA comprising pAzF ("CsgA") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with 200 μM DBCO-Cy5 at 37°C for 1-4 hours. **FIG. 18D** shows the fluorescence intensity of the *E. coli* PBP8 strain transformed to express the OTS and mutant TraT comprising pAzF ("TraT") or untransformed PBP8 cells ("sham"), grown in the presence of 5 mM pAzF. Cells were incubated with 200 μM DBCO-Cy5 at 37°C for 1-4 hours. N=5 for all experiments.

[0076] FIG. 19 depicts flow cytometry data for *E. coli* PBP8 cells alone ("Control cells"), cells transformed to express wild-type CsgA ("Wild-type fibers"), cells transformed to wild-type CsgA and the orthogonal translation system (OTS) ("Wild-type fibers with OTS"), cells transformed to express mutant CsgA comprising pAzF and the OTS in the absence of the expression inducer arabinose ("Mutant fibers with OTS, no induction"), or cells transformed

to express mutant CsgA comprising pAzF and the OTS in the presence of the expression inducer arabinose ("Mutant fibers with OTS"). Cells were labelled with 300 μM DBCO-Cy5 for 2 hours at 37°C.

[0077] FIG. 20 depicts flow cytometry data for untransformed *E. coli* PBP8 cells ("sham"), cells transformed to express wild-type TraT ("wt TraT"), cells transformed to wild-type TraT and the orthogonal translation system (OTS) ("wt TraT + OTS + NSAA"), cells transformed to express mutant TraT comprising pAzF and the OTS grown in the absence of the expression inducer arabinose ("mut TraT + OTS + NSAA (non induction)"), or cells transformed to express mutant TraT comprising pAzF and the OTS grown in the presence of the expression inducer arabinose ("mut TraT + OTS + NSAA"). Cells were labelled with 300 μM DBCO-Cy5 for 2 hours at 37°C.

[0078] Figs. 21A, 21B, 21C, and **21D** show confocal microscopy images of *E. coli* PBP8 cells transformed to express either mutant CsgA comprising pAzF and the OTS, or mutant TraT comprising pAzF and the OTS. Cells were labeled with Cy5-DBCO and Hoechst dye. **FIG. 21A** shows a confocal microscopy image of cells expressing mutant CsgA comprising pAzF labelled with Cy5-DBCO and Hoechst dye. **FIG. 21B** shows a confocal microscopy image of single cells expressing mutant CsgA comprising pAzF labelled with Cy5-DBCO and Hoechst dye. **FIG. 21C** shows confocal microscopy images of cells expressing mutant TraT comprising pAzF labelled with Cy5-DBCO and Hoechst dye. **FIG. 21D** shows a confocal microscopy image of single cells expressing mutant TraT comprising pAzF labelled with Cy5-DBCO and Hoechst dye.

[0079] FIG. 22 show fluorescence detection images of Cy5-DBCO labelled bacteria in mice administered either no bacteria (control), or either 1×10^6 or 1×10^9 PBP8 cells expressing mutant CsgA comprising pAzF and the OTS labeled *in vitro* with Cy5-DBCO. Cy5 fluorescence detected *in vivo* using a IVIS Lumina II system (PERKIN ELMER).

[0080] FIG. 23 shows fluorescence detection images of Cy5-DBCO in mice administered either 5 μM, 50 μM, 100 μM, or 200 μM of Cy5-DBCO. Cy5 fluorescence detected *in vivo* using a IVIS Lumina II system (PERKIN ELMER).

[0081] FIG. 24 shows fluorescence detection images of mice administered either Cy5-DBCO alone or mice administered *E. coli* PBP8 cells transformed to express either mutant CsgA comprising pAzF and the OTS and labeled with Cy5-DBCO at -1 hour (prior to administration), 0 hours, 6 hours, 11 hours, 24 hours, 35 hours, 48 hours, 75 hours, and 100 hours. Cy5 fluorescence detected *in vivo* using a IVIS Lumina II system (PERKIN

ELMER). Epifluorescence subtracted images: 675-500 nm excitation, 698 nm emission. Scaled to background: 0% = background at -1 hour, 100%= max. signal after gavage at 0 hours.

[0082] FIG. 25 is a line graph depicting the normalized radiant efficiency analysis of the fluorescence detected in the mice depicted in **FIG. 24**. Normalized total radiant efficiency of subtracted imaged detected according to the following equation:

$$Y = \frac{TRE(X) - TRE\ (background)}{TRE(0) - TRE\ (background)}$$

[0083] FIGs. 26A and **26B** show the detection of mutant CsgA comprising pAzF in cells lysates from two bacterial colonies isolated from fecal samples of mice administered *E. coli* PBP8 cells transformed to express either mutant CsgA comprising pAzF and the OTS. **FIG. 26A** shows a fluorescence image of an SDS-PAGE gel stained with Cy5-DBCO of either His-tag purified or unpurified cell lysates from bacterial cultures derived from two separate bacterial colonies that were either grown in the absence of pAzF ("Mut w/o NSAA"), presence of pAzF ("Mut with NSAA"). **FIG. 26B** shows a Coomassie Blue stain of the SDS-PAGE gel shown in **FIG. 26A**.

[0084] FIGs. 27A and **27B** show fluorescence detection images of Cy5-DBCO-labelled bacteria in the gastrointestinal tracts of mice administered either untransformed PBP8 cells ("control") or PBP8 cells transformed to express mutant CsgA comprising pAzF and the OTS, and Cy5-DBCO. **FIG. 27C** are bar graphs depicting the normalized radiant efficiency analysis of the fluorescence detected in **FIGs. 27A** and **27B.**

[0085] FIG. 28 shows e*x vivo* fluorescence detection imaging of the gastrointestinal tract of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("(+) NSAA, inducer Mutant EcN") or untreated mice ("control"), 24 hours after treatment with Cy5-DBCO.

[0086] FIG. 29 shows e*x vivo* fluorescence detection imaging of the gastrointestinal tract of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("(+) NSAA, inducer Mutant EcN") or untreated mice ("control"), 36 hours after treatment with Cy5-DBCO.

[0087] FIG. 30A shows fluorescence detection images of the cecum of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("Mutant") or untreated mice ("(-)"), 36 hours after treatment with Cy5-DBCO.

[0088] **FIGs. 30B** and **30C** depict fluorescence detection images of extracted supernatants and pellets, respectively, derived from the cecum of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("Mutant") or untreated mice ("(-)"), 36 hours after treatment with Cy5-DBCO.

[0089] **FIGs. 31A** and **31B** are images of SDS-PAGE gels from soluble fraction (supernatant) and pellets derived from ceca of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("Mutant") or untreated mice ("(-)"), 36 hours after treatment with Cy5-DBCO. **FIG. 31A**, the left panel, shows the Coomassie Blue-stained SDS-PAGE gel of the soluble fractions (supernatant) before ("Sup1") and after ("Sup2") HFIP/TFA extraction, and the right panel depicts a fluorescent image of the Cy5-DBCO stained gel at a 710 nm excitation wavelength. **FIG. 31B**, the left panel, shows the Coomassie Blue-stained SDS-PAGE gel with the pellet fractions before ("Pel1") and after ("Pel2") HFIP/TFA extraction, and the right panel depicts a fluorescent image of the Cy5-DBCO stained gel at a 710 nm excitation wavelength.

[0090] **FIGs. 32A** and **32B** are images of SDS-PAGE gels from soluble fraction (supernatant) and pellets derived from ceca of mice administered either PBP8 bacteria expressing mutant curli fibers supplemented with NSAA, arabinose inducers and appropriate antibiotics ("Mutant") or untreated mice ("(-)"), 36 hours after treatment with Cy5-DBCO. **FIG. 32A**, the left panel, shows the Coomassie Blue-stained SDS-PAGE gel of the soluble fractions (supernatant) before ("Sup1") and after ("Sup2") HFIP/TFA extraction, and the right panel depicts a fluorescent image of the Cy5-DBCO stained gel at a 710 nm excitation wavelength. Samples are at a 1:20 dilution of the samples run in the SDS-PAGE gels of **FIGs. 31A** and **31B**. **FIG. 32B**, the left panel, shows the Coomassie Blue-stained SDS-PAGE gel with the pellet fractions before ("Pel1") and after ("Pel2") HFIP/TFA extraction, and the right panel depicts a fluorescent image of the Cy5-DBCO stained gel at a 710 nm excitation wavelength.

DETAILED DESCRIPTION

[0091] The disclosure includes engineered bacteria, pharmaceutical compositions thereof, methods of labeling an engineered bacterial cell, and methods of using engineered bacteria for the diagnosis and treatment of a disease or disorder.

[0092] In one aspect, the engineered bacterium has been genetically modified to comprise an orthogonal translation system. The orthogonal translation system allows the engineered bacterium to incorporate one or more nonstandard amino acids into a protein encoded by a nucleic acid having a labeling codon, and being expressed by the bacterium. The orthogonal translational system can be designed to particularly target specific codons (*i.e.*, labeling codons) present in a gene encoding a protein of interest in order to incorporate one or more nonstandard amino acids into the protein during translation. Proteins having a nonstandard amino acid bearing a bioorthogonal reactive functional group can be reacted with detectable moiety which allow for the tracking, labeling and/or visualization of the protein. Further, bacterial cells comprising proteins having a nonstandard amino acid bearing a bioorthogonal reactive functional group can also be labeled with a detectable moiety, after being administered to a subject, which allows for the tracking, labeling and/or visualization of the bacterium in the subject.

[0093] The genetic modification of a bacterium to include an orthogonal translation system allows for the incorporation of nonstandard amino acids into any protein expressed by the bacterium, a concept known as genetic code expansion (see, *e.g.*, Young *et al.* (2010) *J. Mol. Biol.* 395: 361-374). Genetic code expansion includes the genetic modification of a microorganism to express an orthogonal aminoacyl tRNA synthetase/tRNA (aaRS/tRNA) pair that is specific for a particular nonstandard amino acid and that does not function with standard amino acids, endogenous tRNAs or endogenous aminoacyl tRNA synthetases.

[0094] At a minimum, genetic code expansion requires the use of both an orthogonal aminoacyl aaRS/tRNA pair, as well as a labeling codon that can be decoded by the tRNA of the aaRS/tRNA pair, providing a mechanism for incorporating a nonstandard amino acid into any protein of interest. In some embodiments, the amber stop codon TAG (UAG on mRNA) is used as the labeling codon. Multiple aaRS/tRNA pairs have been developed thus far, allowing for the incorporation of over 100 nonstandard amino acids into proteins (see, *e.g.*, Liu *et al.* (2010) *Annu. Rev. Biochem.* 79: 413-444). Further, many nonstandard amino acids having a variety of different chemical structures (*e.g.*, bioorthogonal reactive functional groups, metal chelation moieties, photo-crosslinking side chains, photo-caged side chains, post-translational modifications, redox reactive agents, and infrared, NMR and fluorescent probes) have also been developed (see, *e.g.*, Wang, *et al.* (2006) *Annu. Rev. Biomol. Struct.* 35: 225-249).

[0095] It has been surprisingly discovered by the present inventors that bacteria can be genetically modified to be able to express a protein having a nonstandard amino acid and can be used for *in vivo* diagnostic and therapeutic methods which allow for the prolonged monitoring of the bacteria, as well as for the use of the bacteria in multiple therapeutic and diagnostic methods. The engineered bacteria described herein are capable of expressing a protein of interest having at least one non-standard amino acid comprises a bioorthogonal reactive functional group. By using the engineered bacteria and methods described herein, the engineered bacteria can be safely administered to a subject, allowed to colonize and/or engraft in the subject, and subsequently be triggered (*e.g.*, with an inducer) to express a protein having a nonstandard amino acid bearing a bioorthogonal reactive functional group. These proteins can then be reacted with a variety of molecular entities (*e.g.*, detectable probes and/or drugs) for a multitude of clinical applications in a non-invasive manner. For example, multiple nonstandard amino acids having distinct bioorthogonal reactive functional groups have been developed and may be used in the present invention (*see*, *e.g.*, Lang and Chin (2014) *Chem. Rev.* 114: 4764-4806; hereby incorporated by reference in its entirety). In some embodiments, the engineered bacteria described herein are labeled by reacting the bioorthogonal reactive functional group of the nonstandard amino acid with a bioorthogonal reactive functional group of a detectable moiety, such as a dye or MRI contrast agent. Thus, in one embodiment of the present invention the engineered bacteria described herein can be administered to a subject, modified to comprise a detectable moiety and/or drug and be used for variety of diagnostic and/or therapeutic methods to, for example, image tissues and organs of a subject.

[0096] In order that the disclosure may be more readily understood, certain terms are first defined. These definitions should be read in light of the remainder of the disclosure and as understood by a person of ordinary skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. Additional definitions are set forth throughout the detailed description.

Definitions

[0097] As used herein, the term "engineered bacterium" or "engineered bacterial cell" refers to a bacterial cell that has been genetically modified from their native state. For instance, an engineered bacterial cell may have nucleotide insertions, nucleotide deletions, nucleotide rearrangements, and nucleotide modifications introduced into their DNA. These genetic

modifications may be present in the chromosome of the bacteria or bacterial cell, or on a plasmid in the bacteria or bacterial cell. Engineered bacterial cells of the disclosure may comprise exogenous nucleotide sequences on plasmids. Alternatively, recombinant bacterial cells may comprise exogenous nucleotide sequences stably incorporated into their chromosome. In some embodiments, the engineered bacterium is non-pathogenic. In some embodiments, the engineered bacterium is pathogenic.

[0098] "Probiotic", as used herein, refers to a live, non-pathogenic microorganisms, *e.g.,* bacteria, which can confer health benefits to a host organism. In some embodiments, the host organism is a mammal. In some embodiments, the host organism is a human. Some species, strains, and/or subtypes of non-pathogenic bacteria are currently recognized as probiotic bacteria. Examples of probiotic bacteria include, but are not limited to, *Bifidobacteria*, *Escherichia coli*, *Lactobacillus,* and *Saccharomyces, e.g., Bifidobacterium bifidum, Enterococcus faecium, Escherichia coli* strain Nissle, *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus paracasei*, and *Lactobacillus plantarum.* The probiotic may be a variant or a mutant strain of bacterium. Non-pathogenic bacteria may be genetically engineered to enhance or improve desired biological properties, *e.g.,* survivability. Non-pathogenic bacteria may be genetically engineered to provide probiotic properties. Probiotic bacteria may be genetically engineered to enhance or improve probiotic properties.

[0099] As used herein, the term "gene" refers to a nucleic acid fragment that encodes a protein or fragment thereof, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. In one embodiment, a "gene" does not include regulatory sequences preceding and following the coding sequence.

[00100] As used herein, a "heterologous" gene or "heterologous sequence" refers to a nucleotide sequence that is not normally found in a given cell in nature. As used herein, a heterologous sequence encompasses a nucleic acid sequence that is exogenously introduced into a given cell. "Heterologous gene" includes a native gene, or fragment thereof, that has been introduced into the host cell in a form that is different from the corresponding native gene. A heterologous gene may include a native gene, or fragment thereof, introduced into a non-native host cell. Thus, a heterologous gene may be foreign or native to the recipient cell; a nucleic acid sequence that is naturally found in a given cell but expresses an unnatural amount of the nucleic acid and/or the polypeptide which it encodes; and/or two or more

nucleic acid sequences that are not found in the same relationship to each other in nature. As used herein, the term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. As used herein, the term "transgene" refers to a gene that has been introduced into the host organism, *e.g.,* host bacterial cell's genome.

[00101] A "labeling codon", as used herein in reference to DNA, refers to an assembly of three or more nucleotides in a deoxyribonucleic acid molecule which are transcribed to three or more nucleosides of a ribonucleic acid molecule that are specifically recognized and/or decoded by an orthogonal tRNA, but are not recognized and/or decoded by an endogenous tRNA. A "labeling codon", as used herein in reference to RNA, refers to an assembly of three or more nucleosides in a ribonucleic acid molecule that are specifically recognized and/or decoded by an orthogonal tRNA, but are not recognized and/or decoded by an endogenous tRNA. It will be readily understood to one of skill in the art that when transcribed, the thymine ("T") nucleotides of a DNA molecule are converted to uracil ("U") nucleosides. Thus, a labeling codon in a DNA molecule comprising one or more thymines will comprise uracils at the corresponding position when transcribed to RNA (*e.g.*, mRNA).

[00102] A "promoter" as used herein, refers to a nucleotide sequence that is capable of controlling the expression of a coding sequence or gene. Promoters are generally located 5' of the sequence that they regulate. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from promoters found in nature, and/or comprise synthetic nucleotide segments. Those skilled in the art will readily ascertain that different promoters may regulate expression of a coding sequence or gene in response to a particular stimulus, *e.g.,* in a cell-specific or tissue-specific manner, in response to different environmental or physiological conditions, or in response to specific compounds. Prokaryotic promoters are typically classified into two classes: inducible and constitutive.

[00103] "Constitutive promoter" refers to a promoter that is capable of facilitating continuous transcription of a coding sequence or gene under its control and/or to which it is operably linked. Constitutive promoters and variants are well known in the art and include, but are not limited to, a constitutive *Escherichia coli* σ^S promoter, a constitutive *Escherichia coli* σ^{32} promoter, a constitutive *Escherichia coli* σ^{70} promoter, a constitutive *Bacillus subtilis* σ^A promoter, a constitutive *Bacillus subtilis* σ^B promoter, and a bacteriophage T7 promoter.

[00104] An "inducible promoter" refers to a promoter that initiates increased levels of transcription of the coding sequence or gene under its control in response to a stimulus or an exogenous environmental condition. A "directly inducible promoter" refers to a regulatory

region, wherein the regulatory region is operably linked to a gene encoding a protein or polypeptide, where, in the presence of an inducer of said regulatory region, the protein or polypeptide is expressed. An "indirectly inducible promoter" refers to a regulatory system comprising two or more regulatory regions, for example, a first regulatory region that is operably linked to a first gene encoding a first protein, polypeptide, or factor, *e.g.,* a transcriptional regulator, which is capable of regulating a second regulatory region that is operably linked to a second gene, the second regulatory region may be activated or repressed, thereby activating or repressing expression of the second gene. Both a directly inducible promoter and an indirectly inducible promoter are encompassed by "inducible promoter."

[00105] As used herein the term "bioorthogonal reactive functional group" refers to a chemical moiety that mediates a chemical reaction with another chemical moiety, whereby the chemical reaction can occur inside of a living system with minimal interference of native biochemical processes. A "bioorthogonal reaction", as used herein, is a reaction between two bioorthogonal reactive functional groups typically proceed under physiological conditions that results in a covalent bond that is stable under physiological conditions.

[00106] As used herein, "click chemistry reaction" refers to a chemical reaction occurring between a pair of terminal reactive moieties that rapidly and selectively react ("click") with each other to form a targeting or effector moiety conjugated binding polypeptide. Click chemistry reactions can be categorized into two separate groups: copper (Cu(I))-catalyzed and copper-free. The Cu(I)-catalyzed azide-alkyne click chemistry reaction relies on the presence of Cu(I) ions whereas the copper-free reactions proceed without metal catalysis (*e.g.*, the Staudinger Ligation or the azide-phosphine reaction).

[00107] A "nonstandard amino acid", as used herein refers to any amino acid, other than the standard amino acids that are the building blocks of polypeptides of most organisms, including alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In some embodiments, the nonstandard amino acid comprises a bioorthogonal reactive functional group. As used herein, the term "nonstandard biorthogonal functional amino acid" refers to any nonstandard amino acid which comprises a bioorthogonal reactive functional group.

[00108] As used herein, the term "expression" refers to the transcription and stable accumulation of sense (mRNA) or anti-sense RNA derived from a nucleic acid, and/or to translation of an mRNA into a polypeptide

[00109] The term "genetic modification," as used herein, refers to any genetic change. Exemplary genetic modifications include those that increase, decrease, or abolish the expression of a gene, including, for example, modifications of native chromosomal or extrachromosomal genetic material. Exemplary genetic modifications also include the introduction of at least one plasmid, modification, mutation, base deletion, base addition, and/or codon modification of chromosomal or extrachromosomal genetic sequence(s), gene over-expression, gene amplification, gene suppression, promoter modification or substitution, gene addition (either single or multi-copy), antisense expression or suppression, or any other change to the genetic elements of a host cell, whether the change produces a change in phenotype or not. Genetic modification can include the introduction of a plasmid, *e.g.*, a plasmid comprising at least one amino acid catabolism enzyme operably linked to a promoter, into a bacterial cell. Genetic modification can also involve a targeted replacement in the chromosome, *e.g.*, to replace a native gene promoter with an inducible promoter, regulated promoter, strong promoter, or constitutive promoter. Genetic modification can also involve gene amplification, *e.g.*, introduction of at least one additional copy of a native gene into the chromosome of the cell. Alternatively, chromosomal genetic modification can involve a genetic mutation.

[00110] A "pharmaceutical composition," as used herein, refers to a composition comprising an active ingredient (*e.g.*, a bacterial cell, an inducer, a drug, or a detectable compound) with other components such as a physiologically suitable carrier and/or excipient.

[00111] As used herein, the term "pharmaceutically acceptable" or "pharmacologically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Moreover, for animal (*e.g.*, human) administration, it will be understood that compositions should meet sterility, pyrogenicity, general safety and purity standards as required by the FDA Office of Biological Standards.

[00112] As used herein, the term "pharmaceutically acceptable excipient" means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of

the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C_2-C_{12} alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, disintegrating agents, binders, sweetening agents, flavoring agents, perfuming agents, protease inhibitors, plasticizers, emulsifiers, stabilizing agents, viscosity increasing agents, film forming agents, solubilizing agents, surfactants, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable excipient" or the like are used interchangeably herein.

[00113] The term "orthogonal", as used herein, when referring to a heterologous translation system, or to a heterologous component of the translation system (*e.g.*, a heterologous amino acyl synthetase), refers to the ability of an aminoacyl synthetase and/or tRNA to function independently of the aminoacyl synthetases and tRNAs endogenous to the host bacterium.

[00114] As used herein, the term "orthogonal aminoacyl tRNA synthetase" refers to an enzyme that does not specifically aminoacylate endogenous tRNAs of the host bacterium, but specifically aminoacylates its cognate orthogonal tRNA.

[00115] As used herein, the term "orthogonal tRNA" refers to a tRNA that is not a substrate for the endogenous aminoacyl tRNA synthetases of a host bacterium, but

specifically interacts with an orthogonal aminoacyl tRNA synthetase. In some embodiments, the orthogonal tRNA directs the translation incorporation of a nonstandard amino acid of an orthogonal aminoacyl tRNA synthetase in response to a specific codon (*e.g.*, the UAG codon, also known as the amber stop codon).

[00116] A “plasmid” or “vector" includes a nucleic acid construct designed for delivery to a host cell or transfer between different host cell. An "expression plasmid" or “expression vector” can be a plasmid that has the ability to incorporate and express heterologous nucleic acid fragments in a cell. An expression plasmid may comprise additional elements, for example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms. The nucleic acid incorporated into the plasmid can be operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence.

[00117] As used herein, a “biofilm” refers to a mass of microorganisms which can adhere or is adhering to a surface. A biofilm comprises a matrix of extracellular polymeric substances, including, but not limited to extracellular DNA, proteins, glycopeptides, and polysaccharides. The nature of a biofilm, such as its structure and composition, can depend on the particular species of bacteria present in the biofilm. Bacteria present in a biofilm are commonly genetically or phenotypically different than corresponding bacteria not in a biofilm, such as isolated bacteria or bacteria in a colony. Conditions suitable for the production of a biofilm can include, but are not limited to, conditions under which the microbial cell is capable of logarithmic growth and/or polypeptide synthesis. Conditions may vary depending upon the species and strain of microbial cell selected. Conditions for the culture of microbial cells are well known in the art. Biofilm production can also be induced and/or enhanced by methods well known in the art, *e.g.* contacting cells with subinhibitory concentrations of beta-lactam or aminoglycoside antibiotics, exposing cells to fluid flow, contacting cells with exogenous poly-N-acetylglucosamine (PNAG), or contacting cells with quorum sensing signal molecules. In some embodiments, conditions suitable for the production of a biofilm can also include conditions which increase the expression and secretion of CsgA, *e.g.* by exogenously expressing CsgD.

[00118] As used herein, the terms “protein" and “polypeptide" are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The

terms "protein", and "polypeptide" refer to a polymer of amino acids, including modified amino acids (*e.g.*, phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms "protein" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[00119] A "nucleic acid" or "nucleic acid sequence" may be any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one nucleic acid strand of a denatured double- stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA. In another aspect, the nucleic acid can be RNA. Suitable nucleic acid molecules are DNA, including genomic DNA or cDNA. Other suitable nucleic acid molecules are RNA, including mRNA.

[00120] The term "non-pathogenic" as used herein to refer to bacteria refers to bacteria that are not capable of causing disease or harmful responses in a host. In some embodiments, non-pathogenic bacteria are commensal bacteria. Examples of non-pathogenic bacteria include, but are not limited to *Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Clostridium, Enterococcus, Escherichia coli, e.g., Escherichia coli Nissle 1917, Lactobacillus, Lactococcus, Saccharomyces,* and *Staphylococcus, e.g., Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium butyricum, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus,* and *Lactococcus lactis*. Naturally pathogenic bacteria may be genetically engineered to provide reduce or eliminate pathogenicity.

[00121] The articles "a" and "an," as used herein, should be understood to mean "at least one," unless clearly indicated to the contrary. For example, as used herein, "a heterologous gene encoding an amino acid catabolism enzyme" should be understood to

mean "at least one heterologous gene encoding at least one amino acid catabolism enzyme." Similarly, as used herein, "a heterologous gene encoding an amino acid importer" should be understood to mean "at least one heterologous gene encoding at least one amino acid importer."

[00122] The phrase "and/or," when used between elements in a list, is intended to mean either (1) that only a single listed element is present, or (2) that more than one element of the list is present. For example, "A, B, and/or C" indicates that the selection may be A alone; B alone; C alone; A and B; A and C; B and C; or A, B, and C. The phrase "and/or" may be used interchangeably with "at least one of" or "one or more of" the elements in a list.

[00123] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Engineered Bacteria of the Invention

[00124] In one aspect, the engineered bacterium has been genetically modified to comprise an orthogonal translation system. In some embodiment, the orthogonal translation system is composed of a heterologous gene encoding an orthogonal aminoacyl synthetase and a heterologous gene encoding an orthogonal tRNA. The orthogonal translation system allows the engineered bacterium to incorporate one or more nonstandard amino acids into a protein expressed by the bacterium.

[00125] In some embodiments, the orthogonal translation system is located in the bacterial genome. In some embodiments, the orthogonal translation system is located in a plasmid. In some embodiments, the orthogonal translation system is located in plasmid pEVOL-pAzF (described, *e.g.,* in Chin *et al.* (2002) *J. Am. Chem. Soc.* 124(31): 9026-7; Rovner *et al.* (2015) *Nature* 518(7537): 89-93, which are incorporated herein by reference). In some embodiments, the heterologous gene encoding a component of the orthogonal translation system is located in the bacterial genome. In some embodiments, the heterologous gene encoding a component of the orthogonal translation system is located in a plasmid. In some embodiments, the heterologous gene encoding an orthogonal aminoacyl synthetase is located in the bacterial genome. In some embodiments, the heterologous gene encoding an orthogonal aminoacyl synthetase is located in a plasmid. In some embodiments, the heterologous gene encoding an orthogonal tRNA is located in the bacterial genome. In

some embodiments, the heterologous gene encoding an orthogonal tRNA is located in a plasmid.

[00126] In some embodiments, the orthogonal translation system includes a tRNA that decodes or recognizes a specific codon (*i.e.*, a labeling codon) that is not decoded and/or recognized by any endogenous tRNA, and as aminoacyl tRNA synthetase that preferentially aminoacylates a cognate tRNA with a specific nonstandard amino acid. For example, if the bacterium expressing the orthogonal translation system is of the species *Lactobacillus lactis*, the orthogonal translation system will comprise an aminoacyl tRNA synthetase that does not preferentially aminoacylate endogenous *L. lactis* tRNAs. The orthogonal translation system will further comprise an orthogonal tRNA that is minimally, or not at all, aminoacylated by an endogenous *L. lactis* aminoacyl tRNA synthetase.

[00127] In some embodiments, the orthogonal translation system is from, or is derived from, an Archea translational system. In some embodiments, the orthogonal translation system is from, or is derived from, an bacterial translation system. In some embodiments, the orthogonal translations system is an engineered translation system. In some embodiments, the orthogonal translation system is a naturally-occurring translation system. In some embodiments, the orthogonal translations system is from, or is derived from, a *Methanosarcina*, *Desufitobacterium*, *Pyrococcus* or *Methanocaldococcus* translation system. In some embodiments, the orthogonal translation system is from, or is derived from, a *Methanosarcina mazei*, *Methanosarcina barkeri*, *Desulfitobacterium hafniense, Pyrococcus horikoshii*, or *Methanocaldococcus jannaschii* translation system.

[00128] In some embodiments, the orthogonal aminoacyl synthetase is from, or is derived from, an Archea aminoacyl synthetase. In some embodiments, the orthogonal aminoacyl synthetase is from, or is derived from, an bacterial aminoacyl synthetase. In some embodiments, the orthogonal aminoacyl synthetase is an engineered aminoacyl synthetase. In some embodiments, the orthogonal aminoacyl synthetase is a naturally-occurring aminoacyl synthetase. In some embodiments, the orthogonal aminoacyl synthetase is from, or is derived from, a *Methanosarcina*, *Desufitobacterium*, *Pyrococcus* or *Methanocaldococcus* aminoacyl synthetase. In some embodiments, the orthogonal aminoacyl synthetase is from, or is derived from, a *Methanosarcina mazei*, *Methanosarcina barkeri*, *Desulfitobacterium hafniense, Pyrococcus horikoshii*, or *Methanocaldococcus jannaschii* aminoacyl synthetase.

[00129] In some embodiments, the orthogonal tRNA is from, or is derived from, an Archea tRNA. In some embodiments, the orthogonal tRNA is from, or is derived from, a

bacterial tRNA. In some embodiments, the orthogonal tRNA is an engineered tRNA. In some embodiments, the orthogonal tRNA is a naturally-occurring tRNA. In some embodiments, the orthogonal tRNA is from, or is derived from, a *Methanosarcina*, *Desufitobacterium*, *Pyrococcus* or *Methanocaldococcus* tRNA. In some embodiments, the orthogonal tRNA is from, or is derived from, a *Methanosarcina mazei*, *Methanosarcina barkeri*, *Desulfitobacterium hafniense, Pyrococcus horikoshii*, or *Methanocaldococcus jannaschii* tRNA.

[00130] Orthogonal translation systems, orthogonal aminoacyl synthetases and/or an orthogonal tRNAs for use in the present invention may be selected based on their ability to incorporate a specific nonstandard amino acid and/or based on their ability to recognize a specific labeling codon. In some embodiments, the orthogonal aminoacyl synthetase and the orthogonal tRNA are from, or are derived from, the same organism. In some embodiments, the orthogonal aminoacyl synthetase and the orthogonal tRNA are from, or are derived from, different organisms.

[00131] In some embodiments, the orthogonal aminoacyl synthetase and/or orthogonal tRNA is from, or is derived from, an organism selected from the group consisting of *Methanosarcina mazei, Methanosarcina acetovorans, Methanosarcina barken, Methanosarcina frisia, Methanosarcina thermophila, Methanosarcina vacolata, Desulfitobacterium hqfhiense, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Haloferax volcanii, Archaeoglobus fulgidus, Pyrococcus furiosus, Pyrococcus horikoshii, Aeuropyrum pernix, Methanococcus maripaludis, Methanopyrus kandleri, Methanosarcina mazei, Pyrobaculum aerophilum, Pyrococcus abyssi, Sulfolobus solfataricus, Sulfolobus tokodaii, Thermoplasma acidophilum,* and *Thermoplasma volcanium.* Multiple orthogonal translation systems, and methods for producing orthogonal translation systems, are known in the art and can be used as described herein (see, *e.g.*, PCT Publication Numbers WO 2005/007624; WO 2002/086075; WO 2002/085923; WO 2004/094593; WO 2005/019415; WO 2005/007870; WO 2006/110182; and WO 2007/103490; which are all incorporated by reference herein in their entireties).

[00132] In some embodiments, the engineered bacterium comprises more than one orthogonal translation system, thus allowing for the incorporation of more than one type of nonstandard amino acid into a protein of interest. In some embodiments, when more than one orthogonal translation system is used, each of the orthogonal translation systems may recognize the same and/or different labeling codons.

[00133] In embodiments where the engineered bacterium comprises more than one orthogonal translation system, one or more than one labeling codon(s) may be used as described herein. Thus, multiple same or different nonstandard amino acid may be incorporated into a protein of interest by using different labeling codons.

[00134] In some embodiments, the engineered bacterium described herein comprises a heterologous nucleic acid comprising a labeling codon. A labeling codon that is not recognized by the endogenous translation machinery of a bacterial cell results in a blockade of the translation of the polypeptide being produced. However, in the presence of an orthogonal translation system, the aminoacyl tRNA synthetase/tRNA pair of the system recognizes the labeling codon and loads an amino acid (*e.g.*, a nonstandard amino acid) in response to the labeling codon, thereby suppressing the blockade of translation.

[00135] Labeling codons for use in the present invention expand the genetic codon framework of the bacterium. In some embodiments, the labeling codon is a three base codon. In some embodiments, the labeling codon is a nonsense codon. In some embodiments, the labeling codon is a stop codon (*e.g.*, an amber codon (TAG), an ochre codon (TAA), or an opal codon (TGA)). In some embodiments, the labeling codon is an unnatural codon. In some embodiments, the labeling codon is an extended codon (*e.g.*, a codon comprising four or more bases). In some embodiments, the labeling codon comprises four bases (*e.g.*, CTAG, AGGA, TAGA, CCCT). In some embodiments, the labeling codon comprises five bases (*e.g.*, AGGAC, CCCCT, CCCTC, CTAGA, CTACT, TAGGC). In some embodiments, the labeling codon comprises six bases. In some embodiments, the labeling codon comprises seven or more bases.

[00136] In some embodiments, the labeling codon is a three base codon that is not used by the bacterium's translational machinery. In some embodiments, the labeling codon is a three base codon that is rarely used by the bacterium's translational machinery. In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce the abundance of the labeling codon. In some embodiments, the engineered bacterium comprises a genome that has been recoded to remove the labeling codon. In some embodiments, the engineered bacterium comprises a genome that has been recoded to reduce the abundance of the labeling codon TAG. In some embodiments, the engineered bacterium comprises a genome that has been recoded to remove the labeling codon TAG.

[00137] In embodiments where the engineered bacterium comprises more than one orthogonal translation system such that more labeling codons may be used as described

herein. Thus, multiple same or different nonstandard amino acid may be incorporated into a polypeptide of interest by using different labeling codons.

[00138] In some embodiments, the engineered bacterium described herein comprises a heterologous nucleic acid comprising a labeling codon. In some embodiments, the heterologous nucleic acid is located in the bacterial genome. In some embodiments, the heterologous gene is located in a plasmid. In some embodiments, the expression of the heterologous nucleic acid is under the control of a constitutive promoter (*e.g.*, a constitutive promoter disclosed herein). In some embodiments, the heterologous nucleic acid is under the control of an inducible promoter (*e.g.*, an inducible promoter disclosed herein). In some embodiments, the heterologous nucleic acid comprises one labeling codon. In some embodiments, the heterologous nucleic acid comprises more than one labeling codon. In some embodiments, the heterologous nucleic acid comprises one, two, three, four, five, six, seven, eight or more labeling codons. Labeling codons may be introduced into the heterologous nucleic acid using methods known in the art, including, *e.g.*, site-directed mutagenesis).

[00139] In some embodiments, the heterologous nucleic acid comprises a labeling codon and a heterologous gene encoding a protein. In some embodiments, the heterologous nucleic acid further encodes a polypeptide linker sequence. In some embodiments, the polypeptide linker sequence is flexible. In some embodiments, the heterologous nucleic acid further encodes a polypeptide tag. In some embodiments, the labeling codon is located at a nucleic acid position corresponding to the open reading frame of the protein. In some embodiments, the labeling codon is not located at a nucleic acid position corresponding to the open reading frame of the protein. In some embodiments, the labeling codon is located at a nucleic acid position corresponding to the open reading frame of the polypeptide linker sequence. In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous gene encoding the protein. In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide linker sequence. In some embodiments, the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide tag.

[00140] In some embodiments, the engineered bacterium described herein comprises a protein having a nonstandard amino acid. In some embodiments, the protein comprises one nonstandard amino acid. In one embodiment, the non standard amino acid does not interfere with the structural fold, function, enzymatic activity and/or location of the protein. In one

embodiment, the non standard amino acid interferes with the structural fold, function, enzymatic activity and/or location of the protein. In some embodiments, the protein comprises more than one nonstandard amino acid. In some embodiments where the protein comprises multiple nonstandard amino acids, one or more of the nonstandard amino acids does not interfere with the structural fold, function, enzymatic activity and/or location of the protein. In some embodiments where the protein comprises multiple nonstandard amino acids, one or more of the nonstandard amino acids interfere with the structural fold, function, enzymatic activity and/or location of the protein. In some embodiments, the protein comprises two, three, four five, six, seven, eight, or more nonstandard amino acids. Multiple nonstandard amino acids are know in the art and can be used as described herein (see, *e.g.*, Lang and Chin (2014) *Chem. Rev.* 114: 4764-806, which is incorporated herein by reference in its entirety).

[00141] In some embodiments, the nonstandard amino acid comprises a bioorthogonal reactive functional group. In some embodiments, the nonstandard amino acid is selected from the group consisting of *p*-azidophenylalanine (*p*-AzF), azidohomoalanine (Aha), azidolysine, homopropargylglycine (Hpg), homoallylglycine (Hag), oxonorvaline (Onv), *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine (*p*-EtF), *para*-acetylphenylalanine (*p*-AcF), azidonorleucine (Anl), trans-crotylglycine (Tcg), selenomethionine (Se-Met), 2-aminooctynoic acid (Aoa), and propargylglycine (Pra). In one embodiment, the non-standard amino acid is *p*-AzF.

[00142] In some embodiments, the non-standard amino acid comprises a bioorthogonal reactive functional group. In some embodiments, the detectable moiety comprises a bioorthogonal reactive functional group. Multiple bioorthogonal reactive functional groups are known in the art and can be used in the methods described herein (see, *e.g.*, Lang and Chin (2014)). In some embodiments, the bioorthogonal reactive functional group is selected from the group consisting of an azide moiety, a ketone moiety, an alkene moiety, an alkyne moiety, a tetrazine moiety, and a norbornene moiety. In some embodiments, the bioorthogonal reactive functional group is able to participate in a click chemistry reaction with a second bioorthogonal reactive functional group (*e.g.*, a bioorthogonal reactive functional group present on a drug or detectable compound). Multiple click chemistry reactions are known in the art (see, *e.g.*, Lang and Chin (2014)).

[00143] Any protein can be expressed in the engineered bacterium described herein to incorporate a nonstandard amino acid. In some embodiments, the protein is a secreted

protein. In some embodiments, the protein is a non-secreted protein. In some embodiments, the protein is a membrane protein. In some embodiments, the protein is a cytosolic protein. In some embodiments, the protein is a nuclear protein. In some embodiments, the protein is an enzyme. In some embodiments, the protein is an extracellular matrix protein. In some embodiments, the protein is a biofilm protein. In some embodiments, the protein is a fiber protein (*e.g.*, a curli fiber protein). In some embodiments, the protein is a pilin protein.

[00144] In some embodiments the protein is CsgA. CsgA is the major structural subunit of curli. The sequences of CsgA and its homologs are known in a number of species, *e.g.*, *E. coli* (NCBI Gene ID NO: 949055; NCBI Ref Seq: NP_415560). In some embodiments, the protein is CsgA and the nonstandard amino acid is located at an amino acid residue corresponding to residue 89 of wild-type CsgA.

[00145] In some embodiments, the protein is Trat. TraT is an outer membrane lipoprotein encoded by the *tra* operon. In some embodiments, the protein is Trat and the nonstandard amino acid is located at an amino acid residue corresponding to residue 200 of wild-type TraT.

[00146] In some embodiments, the protein is InaV. In some embodiments, the protein is InaV and the nonstandard amino acid is located at an amino acid residue corresponding to residue 174 of wild-type InaV.

[00147] In some embodiments, the protein is maltose binding protein (MBP). In some embodiments, the protein is MBP and the nonstandard amino acid is located at an amino acid residue corresponding to residue 67 of wild-type MBP.

[00148] In some embodiments, the protein expressed by the engineered bacterium described herein comprises a polypeptide tag. In some embodiments, the protein comprises more than one polypeptide tag. In some embodiments, the protein comprises two, three, four, or more polypeptide tags. Multiple polypeptide tags are known in the art and can be used as described herein. Exemplary polypeptide tags for use in the methods described herein include, but are not limited to, the polypeptide tags shown in Table 1. In one embodiment, the polypeptide tag is at the N-terminus of the protein. In one embodiment, the polypeptide tag is at the C-terminus of the protein. In one embodiment, the polypeptide tag is located between two domains of the protein.

Table 1. Exemplary polypeptide tags

Tag	Epitope
CBP	KRRWKKNFIAVSAANRFKKISSSGAL (SEQ ID NO: 1)
FLAG	DYKDDDD (SEQ ID NO: 2) or

	DYKDDDDK (SEQ ID NO: 3) or DYKDDDK (SEQ ID NO: 4)
HA	YPYDVPDYA (SEQ ID NO: 5) or YAYDVPDYA (SEQ ID NO: 6) or YDVPDYASL (SEQ ID NO: 7)
Myc	EQKLISEEDL (SEQ ID NO: 8)
poly His	HHHHHH (SEQ ID NO: 9)
S-tag	KETAAAKFERQHMDS (SEQ ID NO: 10)
V5	GKPIPNPLLGLDST (SEQ ID NO: 11)

[00149] In some embodiments, the protein expressed by the engineered bacterium described herein comprises a polypeptide linker. In some embodiments, the polypeptide linker is one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, twenty-five, thirty, thirty-five, forty, forty-five, fifty, or more amino acids in length. In some embodiments, the polypeptide linker is twelve, twenty-four, thirty-six, forty-eight, sixty, seventy-two, eighty-four, or ninety-six amino acids in length. In some embodiments, the polypeptide linker is N-terminus of the protein. In some embodiments, the polypeptide linker is C-terminus of the protein. In some embodiments, the polypeptide linker is located between a domain of the protein and a polypeptide tag. In some embodiments, the polypeptide linker is located between two domains of the protein. In some embodiments, the polypeptide linker is flexible. In some embodiments, the polypeptide linker is rigid. In some embodiment, the polypeptide linker comprises a nonstandard amino acid.

[00150] Many plasmids (also referred to as “vectors” herein) useful for transferring genes into bacterial cells are available. The plasmids may be episomal or may be integrated into the bacterial cell’s genome through homologous recombination or random integration. In some embodiments, is an expression vector. A plasmid can be viral or non-viral. Plasmids for use as described herein can include, but are not limited to, pEVOL (see Young *et al.* (2010) *J. Mol. Biol.* 395: 361-74, which is incorporated hereby by reference in its entirety), pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see “Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier *et al.* (1990) “Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology 185, which is hereby incorporated by reference in its entirety).

[00151] The expression of any of the genes incorporated into the engineered bacterium described herein may be regulated using any promoter known in the art. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the promoter is an inducible promoter. An "inducible promoter" may be one that is characterized by initiating or enhancing transcriptional activity when in the presence of, influenced by, or contacted by an inducer or inducing agent than when not in the presence of, under the influence of, or in contact with the inducer or inducing agent. An "inducer" or "inducing agent" may be endogenous, or a normally exogenous compound or protein that is administered in such a way as to be active in inducing transcriptional activity from the inducible promoter. In some embodiments, the inducer or inducing agent, *e.g.*, a chemical, a compound or a protein, can itself be the result of transcription or expression of a nucleic acid sequence (*e.g.*, an inducer can be a transcriptional repressor protein), which itself may be under the control or an inducible promoter. Non-limiting examples of inducible promoters include but are not limited to, the lac operon promoter, a nitrogen-sensitive promoter, an IPTG-inducible promoter, a salt-inducible promoter, tetracycline-inducible promoter, the lambda phage *pL* promoter, steroid-responsive promoters, rapamycin responsive promoters and the like. Inducible promoters for use in prokaryotic systems are well known in the art, see, *e.g.* the β-lactamase and lactose promoter systems (Chang *et al.* (1978) *Nature* 275: 615, which is incorporated herein by reference; Goeddel *et al.* (1979) *Nature* 281: 544, which is incorporated herein by reference), the arabinose promoter system, including the araBAD promoter (Guzman *et al.* (1992) *J . Bacteriol.* 174: 7716-28, which is incorporated herein by reference; Guzman *et al.* (1995) *J. Bacteriol.*, 177: 4121-30, which is incorporated herein by reference; Siegele and Hu (1997) *Proc. Natl. Acad. Sci. USA* 94: 8168-72, which is incorporated herein by reference), the rhamnose promoter (Haldimann *et al.* (1998) *J. Bacteriol.* 180: 1277-86, which is incorporated herein by reference), the alkaline phosphatase promoter, a tryptophan (trp) promoter system (Goeddel (1980) *Nucleic Acids Res.*, 8: 4057, which is incorporated herein by reference), the PLtetO-1 and Plac/are-1 promoters (Lutz and Bujard (1997) *Nucleic Acids Res.* 25: 1203-10, which is incorporated herein by reference), the phage lambda pL promoter system and pR promoter system (Simmons *et al.* (1984) *Gene* 28(1): 55-64; Gilman and Love (2016) *Biochem. Soc. Trans.* 44(3): 731-7; and Valdez-Cruz *et al.*(2010) *Microb. Cell Fact.* 9: 18. which are incorporated herein by reference), and hybrid promoters such as the tac promoter (see deBoer *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80: 21-5, which is incorporated herein by reference).

[00152] An inducible promoter useful in the methods and systems as disclosed herein can be induced by one or more physiological conditions, such as changes in pH, temperature, radiation, osmotic pressure, saline gradients, cell surface binding, and the concentration of one or more extrinsic or intrinsic inducing agents. The extrinsic inducer or inducing agent may comprise amino acids and amino acid analogs, saccharides and polysaccharides, nucleic acids, protein transcriptional activators and repressors, cytokines, toxins, petroleum-based compounds, metal containing compounds, salts, ions, enzyme substrate analogs, hormones, and combinations thereof. In specific embodiments, the inducible promoter is activated or repressed in response to a change of an environmental condition, such as the change in concentration of a chemical, metal, temperature, radiation, nutrient or change in pH. Thus, an inducible promoter useful in the methods and systems as disclosed herein can be a phage inducible promoter, nutrient inducible promoter, temperature inducible promoter, radiation inducible promoter, metal inducible promoter, hormone inducible promoter, steroid inducible promoter, and/or hybrids and combinations thereof. Appropriate environmental inducers can include, but are not limited to, exposure to heat (*i.e.,* thermal pulses or constant heat exposure), various steroidal compounds, divalent cations (including Cu^{2+}, Ca^{2+}, and Zn^{2+}), galactose, arabinose, anhydrotetracycline (ATc), tetracycline, IPTG (isopropyl-β-D thiogalactoside), as well as other naturally occurring and synthetic inducing agents and gratuitous inducers.

[00153] Inducible promoters useful in the methods and systems as disclosed herein also include those that are repressed by “transcriptional repressors" that are subject to inactivation by the action of environmental, external agents, or the product of another gene. Such inducible promoters may also be termed “repressible promoters" where it is required to distinguish between other types of promoters in a given module or component of the biological switch converters described herein. Preferred repressors for use in the present invention are sensitive to inactivation by physiologically benign agent. Thus, where a lac repressor protein is used to control the expression of a promoter sequence that has been engineered to contain a lacO operator sequence, treatment of the host cell with IPTG will cause the dissociation of the lac repressor from the engineered promoter containing a lacO operator sequence and allow transcription to occur. Similarly, where a tet repressor is used to control the expression of a promoter sequence that has been engineered to contain a tetO operator sequence, treatment of the host cell with tetracycline, or the tetracycline analog anhydrotetracycline (ATc), will cause the dissociation of the tet repressor from the

engineered promoter and allow transcription of the sequence downstream of the engineered promoter to occur.

[00154] Bacteria for use in the methods and compositions described herein can be of any species. Preferably, the bacteria are of a species and/or strain which is amenable to culture and genetic manipulation. In some embodiments, the bacterium is a gram-positive bacterium. In some embodiments, the bacterium is a gram-negative bacterium. In some embodiments, the bacterium is a probiotic. In some embodiments, the bacterium is Generally Recognized as Safe (GRAS) by a regulatory authority. In some embodiments, the bacterium is pathogenic. In some embodiments, the bacterium is non-pathogenic. In some embodiments, the bacterium is an attenuated pathogenic bacterium. In some embodiments, the parental strain of the bacterium used herein is of a strain optimized for protein expression.

[00155] Non-limiting examples of bacterial species and strains suitable for use in the present technologies include *Escherichia coli*, *E. coli* BL21, *E. coli* Tuner, *E. coli* Rosetta, *E. coli* JM101, and derivatives of any of the foregoing. In one embodiment, the bacterium is of the *E. coli* Nissle 1917 strain. Other non-pathogenic bacterial strains known to those of skill in the art such as MG1655, K12-derived strains, and the like, may also be used.

[00156] In some embodiments, the parental strains of the bacterium described herein is optimized for use with an orthogonal translation system. In some embodiments, the bacterium is genetically modified (*e.g.*, recoded) to reduce or eliminate a codon that is specifically recognized by the orthogonal translation system. Any codon may be reduced or eliminated from the genome of a bacterium described herein using methods known in the art. In some embodiments, the bacterium is genetically modified (*e.g.*, recoded) to reduce or eliminate the TGA codon (see, *e.g.*, Lajoie *et al.* (2013) *Science* 342(6156):357-60. In some embodiments, the bacterium is genetically modified (*e.g.*, recoded) to reduce the codon TGA in the bacterial genome. In some embodiments, the bacterium is genetically modified (*e.g.*, recoded) to eliminate the codon TGA in the bacterial genome. In some embodiments, the bacterium for use in the present invention comprise a deletion in the gene encoding peptide chain release factor 1 or a homolog thereof. In some embodiments, the bacterium for use in the present invention comprise a deletion of the *prfA* gene (see, *e.g.*, Lajoie *et al.* (2013)). In some embodiments, the bacterium is of the *Escherichia coli* strain C321 (Addgene bacterial strain #48999). In some embodiments, the bacterium is of the *Escherichia coli* strain C321.ΔA (Addgene bacterial strain #48998). In some embodiments, the bacterium is of the *Escherichia coli* strain C321.ΔA.exp (Addgene bacterial strain #49018).

[00157] In some embodiments, the bacterium for use in the present invention comprise a deletion of the endogenous gene encoding the protein in which a nonstandard amino acid is being incorporated. In some embodiments, the bacterium comprises a deletion in the *csgA* gene. In some embodiment, the bacterium comprises a deletion of a curli operon. In some embodiments, the bacterium comprises a deletion of one or more endogenous Csg genes. In some embodiments, the bacterium comprises a deletion of *csgA*. In some embodiments, the bacterium comprises a deletion of *csgB*. In some embodiments, the bacterium comprises a deletion of *csgC*. In some embodiments, the bacterium comprises a deletion of *csgD*. In some embodiments, the bacterium comprises a deletion of *csgE*. In some embodiments, the bacterium comprises a deletion of *csgF*. In some embodiments, the bacterium comprises a deletion of *csgG*. In some embodiments, the bacterium comprises a deletion of the *csgBAC* operon. In some embodiments, the bacterium comprises a deletion of the *csgDEFG* operon. In some embodiments, the bacterium comprises a deletion of the *csgBAC* and *csgDEFG* operons. In some embodiments, the bacterium comprises a deletion in the *inaV* gene. In some embodiments, the bacterium comprises a deletion in the *traT* gene. In some embodiments, the bacterium comprise a deletion in the *malE* gene.

Biofilms of the Invention

[00158] In one aspect, biofilms comprising the engineered bacterium described herein are also provided. In one aspect, the biofilm comprises an engineered bacterium described herein and a protein comprising a nonstandard amino acid expressed by an engineered bacterium described herein. In some embodiments, the biofilm comprises a protein comprising a nonstandard amino acid expressed by an engineered bacterium described herein. In some embodiments, the biofilm comprises a bacterium (*e.g.*, an engineered bacterial cell described herein). In some embodiments, the biofilm does not comprise a bacterium (*e.g.*, an engineered bacterial cell described herein). In some embodiments, the biofilm comprises a curli fiber composed of bacterial extracellular matrix protein having a nonstandard amino acid. In some embodiments, the biofilm comprises curli fiber comprising CsgA protein having a nonstandard amino acid. In some embodiments, the nonstandard amino acid has been reacted with a detectable compound or drug as described herein.

Pharmaceutical Compositions of the Invention

[00159] Pharmaceutical compositions comprising the engineered bacterium described herein may be used to diagnose, treat, manage, ameliorate, and/or prevent a disease or disorder, as described herein Pharmaceutical compositions comprising one or more

engineered bacteria, alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers are provided.

[00160] In certain embodiments, the pharmaceutical composition comprises one species, strain, or subtype of bacteria that are engineered to comprise the genetic modifications described herein, *e.g.*, to express at least one orthogonal translation system and at least one protein comprising a nonstandard amino acid. In alternate embodiments, the pharmaceutical composition comprises two or more species, strains, and/or subtypes of bacteria that are each engineered to comprise the genetic modifications described herein, *e.g.*, to express at least one orthogonal translation system and at least one protein comprising a nonstandard amino acid.

[00161] The pharmaceutical compositions described herein may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into compositions for pharmaceutical use. Methods of formulating pharmaceutical compositions are known in the art (see, *e.g.*, "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA). In some embodiments, the pharmaceutical compositions are subjected to tableting, lyophilizing, direct compression, conventional mixing, dissolving, granulating, levigating, emulsifying, encapsulating, entrapping, or spray drying to form tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. Appropriate formulation depends on the route of administration.

[00162] The engineered bacteria described herein may be formulated into pharmaceutical compositions in any suitable dosage form (*e.g.*, liquids, capsules, sachet, hard capsules, soft capsules, tablets, enteric coated tablets, suspension powders, granules, or matrix sustained release formations for oral administration) and for any suitable type of administration (*e.g.*, oral, topical, injectable, immediate-release, pulsatile-release, delayed-release, or sustained release). Suitable dosage amounts for the engineered bacteria may range from about 10^5 to 10^{12} bacteria, *e.g.*, approximately 10^5 bacteria, approximately 10^6 bacteria, approximately 10^7 bacteria, approximately 10^8 bacteria, approximately 10^9 bacteria, approximately 10^{10} bacteria, approximately 10^{11} bacteria, or approximately 10^{12} bacteria. The composition may be administered once or more daily, weekly, or monthly.

[00163] The engineered bacterium may be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers,

buffering agents, surface active agents, neutral or cationic lipids, lipid complexes, nanoparticles, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or agents. Further, any one of the detectable compound, inducer, and/or nonstandard amino acid may also be formulated into pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers, thickeners, diluents, buffers, buffering agents, surface active agents, neutral or cationic lipids, lipid complexes, nanoparticles, liposomes, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or agents. For example, the pharmaceutical composition may include, but is not limited to, the addition of calcium bicarbonate, sodium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20. The engineered bacterium may be administered and formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. The engineered bacterium disclosed herein may be administered topically and formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form well-known to one of skill in the art. See, *e.g.*, "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. In an embodiment, for non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity greater than water are employed. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, etc., which may be sterilized or mixed with auxiliary agents (*e.g.*, preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, *e.g.*, osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations wherein the active ingredient in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (*e.g.*, a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms. Examples of such additional ingredients are well known in the art.

[00164] The pharmaceutical compositions disclosed herein may be administered orally and formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc.

Pharmacological compositions for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients include, but are not limited to, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose compositions such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP) or polyethylene glycol (PEG). Disintegrating agents may also be added, such as cross-linked polyvinylpyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

[00165] Tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone, hydroxypropyl methylcellulose, carboxymethylcellulose, polyethylene glycol, sucrose, glucose, sorbitol, starch, gum, kaolin, and tragacanth); fillers (*e.g.*, lactose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.*, calcium, aluminum, zinc, stearic acid, polyethylene glycol, sodium lauryl sulfate, starch, sodium benzoate, L-leucine, magnesium stearate, talc, or silica); disintegrants (*e.g.*, starch, potato starch, sodium starch glycolate, sugars, cellulose derivatives, silica powders); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. A coating shell may be present, and common membranes include, but are not limited to, polylactide, polyglycolic acid, polyanhydride, other biodegradable polymers, alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroymethylacrylate-methyl methacrylate (HEMA-MMA), multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS), poly N,N- dimethyl acrylamide (PDMAAm), siliceous encapsulates, cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG), cellulose acetate phthalate, calcium alginate, k-carrageenan-locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carrageenan, starch poly-anhydrides, starch polymethacrylates, polyamino acids, and enteric coating polymers.

[00166] In some embodiments, the pharmaceutical compositions may be enterically coated for release into the gut or a particular region of the gut, for example, the large intestine. The typical pH profile from the stomach to the colon is about 1-4 (stomach), 5.5-6 (duodenum),

7.3-8.0 (ileum), and 5.5-6.5 (colon). In some diseases, the pH profile may be modified. In some embodiments, the coating is degraded in specific pH environments in order to specify the site of release. In some embodiments, at least two coatings are used. In some embodiments, the outside coating and the inside coating are degraded at different pH levels.

[00167] Liquid preparations for oral administration may take the form of solutions, syrups, suspensions, or a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable agents such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated for slow release, controlled release, or sustained release of the engineered bacterium described herein.

[00168] In some embodiments, the pharmaceutical composition is formulated for intraintestinal administration, intrajejunal administration, intraduodenal administration, intraileal administration, gastric shunt administration, or intracolic administration, via nanoparticles, nanocapsules, microcapsules, or microtablets, which are enterically coated or uncoated. The pharmaceutical compositions may also be formulated in rectal compositions such as suppositories or retention enemas, using, *e.g.*, conventional suppository bases such as cocoa butter or other glycerides. The compositions may be suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain suspending, stabilizing and/or dispersing agents.

[00169] The pharmaceutical compositions described herein may be administered intranasally, formulated in an aerosol form, spray, mist, or in the form of drops, and conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant (*e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). Pressurized aerosol dosage units may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (*e.g.*, of gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[00170] The pharmaceutical compositions may be administered and formulated as depot preparations. Such long acting formulations may be administered by implantation or by injection, including intravenous injection, subcutaneous injection, local injection, direct injection, or infusion. For example, the compositions may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (*e.g.*, as a sparingly soluble salt).

[00171] In some embodiments, disclosed herein are pharmaceutically acceptable compositions in single dosage forms. Single dosage forms may be in a liquid or a solid form. Single dosage forms may be administered directly to a patient without modification or may be diluted or reconstituted prior to administration. In certain embodiments, a single dosage form may be administered in bolus form, *e.g.*, single injection, single oral dose, including an oral dose that comprises multiple tablets, capsule, pills, lozenge, etc. In alternate embodiments, a single dosage form may be administered over a period of time, *e.g.*, by infusion.

[00172] Single dosage forms of the pharmaceutical composition may be prepared by portioning the pharmaceutical composition into smaller aliquots, single dose containers, single dose liquid forms, or single dose solid forms, such as tablets, granulates, nanoparticles, nanocapsules, microcapsules, microtablets, pellets, or powders, which may be enterically coated or uncoated. A single dose in a solid form may be reconstituted by adding liquid, typically sterile water or saline solution, prior to administration to a patient.

[00173] In other embodiments, the composition can be delivered in a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release. In another embodiment, polymeric materials can be used to achieve controlled or sustained release of the therapies of the present disclosure (see *e.g.*, U.S. Patent No. 5,989,463). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N- vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. The polymer used in a sustained release formulation may be inert, free of leachable impurities, stable on storage, sterile, and biodegradable. In some embodiments, a controlled or sustained release system can be placed in proximity of the prophylactic or

therapeutic target, thus requiring only a fraction of the systemic dose. Any suitable technique known to one of skill in the art may be used.

Methods of the Invention

[00174] Further disclosed herein are methods for labeling an engineered bacterium disclosed herein comprising a protein having a nonstandard amino acid. The engineered bacterium described herein comprise a protein having a nonstandard amino acid, wherein the nonstandard amino acid comprises a bioorthogonal reactive functional group. The engineered bacterium can be exposed to a labeling reagent or detectable compound also having a bioorthogonal reactive functional group that will react the bioorthogonal reactive functional group of the nonstandard amino acid.

[00175] In one aspect, methods for labeling a protein having a nonstandard amino acid expressed by an engineered bacterium described herein are provided. The protein comprises a nonstandard amino acid, wherein the nonstandard amino acid comprises a bioorthogonal reactive functional group. In one embodiment, the protein can be reacted with a labeling reagent or detectable compound having a bioorthogonal reactive functional group to attach the labeling reagent or detectable compound to the bioorthogonal reactive functional group of the nonstandard amino acid.

[00176] In some embodiments, the method is performed *in vitro*. In some embodiments, the method is performed *in vivo*. Thus, in some embodiments, the method comprises administering to the subject an engineered bacterium having a protein comprising at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group, and administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid of the protein, thereby labeling the engineered bacterium in the subject *in vivo*.

[00177] In some embodiments, the labeling reagent or detectable compound comprises a fluorescent moiety, a radioactive moiety, a colorimetric dye, a fluorescent dye, a luminescent dye, or a magnetic resonance imaging (MRI) contrast agent, a CT contrast agent, a PET contrast agent, or an ultrasound contrast agent. In some embodiments, the labeling reagent or detectable compound comprises a drug delivery vehicle selected from the group consisting of a nanocarrier (*e.g.*, a polymer), a nanoparticle (*e.g.*, a metal gold, silver, or iron nanoparticle), a liposome, a dendrimer, a carbon nanotube, a micelle, and a protein. Thus,

the bioorthogonal reactive functional group may be present on the drug delivery vehicle. In some embodiments, the labeling reagent or detectable compound comprises a bioorthogonal reactive functional group selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a hydrazine moiety, a tetrazine moiety, a norbornene moiety and a hydroxylamine moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a ketone moiety, and the bioorthogonal reactive functional group of the labeling reagent or detectable compound comprises a hydrazine moiety or a hydroxylamine moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises an azide moiety, and the bioorthogonal reactive functional group of the labeling reagent or detectable compound comprises an alkyne moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises an alkyne moiety, and the bioorthogonal reactive functional group of the labeling reagent or detectable compound comprises an azide moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a tetrazine moiety, and the bioorthogonal reactive functional group of the labeling reagent or detectable compound comprises a norbornene moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a norbornene moiety, and the bioorthogonal reactive functional group of the labeling reagent or detectable compound comprises a tetrazine moiety. In some embodiments, the labeling reagent or detectable compound is a detectable or traceable compound. In some embodiments, the labeling reagent or detectable compound is selected from the group consisting of a fluorescent moiety, a radioactive moiety, a colorimetric dye, a fluorescent dye, a luminescent dye, a magnetic resonance imaging (MRI) contrast agent, a CT contrast agent, a PET contrast agent, and an ultrasound contrast agent. Examples of labeling reagents or detectable compounds include, but are not limited to, radioisotopes, dyes, enzymes, contrast agents, fluorescent compounds or molecules such as a fluorescent dye, MRI contrast agents (*e.g.*, paramagnetic ions such as Gadolinium(III)-based MRI contrast agents, , superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO) MRI contrast agents, superparamagnetic iron platinum particle (SIPPs) MRI contrast agents, and Mn-based nanoparticle MRI contrast agents, and ^{19}F MRI contrasting agents), positron emission tomography (PET) contrast agents (*e.g.*, ^{18}F-based contrast agents such as [^{18}F]maltose, [^{18}F]-maltohexaose and [^{18}F]2-fluorodeoxysorbitol (FDS) and ^{64}Cu-based contrast agents), CT contrast agents (*e.g.*, iodine-

based contrast agents), ultrasound contrast agents, and small molecules including both inorganic and organic small molecules. The labeling reagent or detectable compound may include any suitable label or detectable group detectable by optical, spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means including but not limited to biotin, dyes, fluorophores, antigens, porphyrins, chromophores, and radioactive isotopes. Diagnostic agents include, but are not limited to, radiolabels (*e.g.*, ^{35}S, ^{125}I, ^{32}P, ^{3}H,^{14}C), radioacoustic labels, enzyme labels (*e.g.*, horseradish peroxidase, alkaline phosphatase), gold beads, chemiluminescence labels, ligands (*e.g.*, biotin, digoxin) and/or fluorescence labels (*e.g.*, rhodamine, phycoerythrin, fluorescein, fluorescent proteins), a fluorescent protein including, but not limited to, a green fluorescent protein or one of its many modified forms, energy absorbing and energy emitting agents, fluorescent dyes (*e.g.*, fluorescein, fluorescein-isothiocyanate (FITC), Texas red, rhodamine, green fluorescent protein, enhanced green fluorescent protein, lissamine, phycoerythrin, near-IR dye, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX (Amersham), and SyBR Green I & II (Molecular Probes)), chemiluminescent groups, and chromogenic agents. In some embodiments, the labeling reagent or detectable compound comprises a dibenzocyclooctyne group (DBCO). In some embodiments, the labeling reagent or detectable compound is selected from the group consisting of BDP-FL-PEG4-DBCO, DBCO-PEG4-5/6-FAM, DBCO-PEG4-ATTO-488, DBCO-PEG4-5/6-Carboxyrhodamine 110, DBCO-PEG4-5/6-TAMRA, DBCO-Sulfo-Cy3, DBCO-5/6-Sulforhodamine B, DBCO-PEG4-5/6-Sulforhodamine B, DBCO-5/6-Texas Red, DBCO-PEG4-5/6-Texas Red, DBCO-Sulfo-Cy5, DBCO-Cy5.5, DBCO-Cy7. In some embodiments, the labeling reagent or detectable compound is dibenzyl cyclooctyne-conjugated Cy5 (DBCO-Cy5). In some embodiments, the labeling reagent or detectable compound is dibenzyl cyclooctyne-conjugated Cy3 (DBCO-Cy3).

[00178] Methods for detection of the detectable compound are known in the art and include, for example, microscopy (*e.g.*, confocal microscopy), x-rays, ultrasound, X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) scan, ultrasound imaging (tomography), optical imaging, scintigraphy (including PET scintigraphy and gamma scintigraphy), and near infrared imaging.

[00179] In some embodiments, disclosed herein are methods for detecting the distribution of an engineered bacterium disclosed herein in a subject. In some embodiments, the method comprises administering to a subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first

bioorthogonal reactive functional group, and administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid and detecting the detectable compound in the subject, thereby detecting the distribution of the engineered bacterium in the subject. In some embodiments, the engineered bacterium is administered to the gastrointestinal tract of the subject. In some embodiments, the engineered bacterium is administered to the respiratory tract of the subject. In some embodiments, the engineered bacterium is administered to the circulatory system of the subject. In some embodiments, the engineered bacterium is administered to an organ or tissue selected from the group consisting of mouth, esophagus, heart, lung, stomach, small intestine, large intestine, rectum, anal canal, hair, and skin. In some embodiments, the engineered bacterium is administered to specifically target a lesion in the subject. In some embodiments, the engineered bacterium senses changes in the local gut environment that indicates a disease or disorder.

[00180] In some embodiments, disclosed herein are methods for detecting the distribution of a biofilm produced by an engineered bacterium disclosed herein in a subject. In some embodiments, the biofilm comprises a protein having a nonstandard amino acid that is secreted by the engineered bacterium. In some embodiments, the biofilm comprises an engineered bacterium disclosed herein. In some embodiments, the biofilm comprises a protein having a nonstandard amino acid produced by the engineered bacterium disclosed herein (*e.g.*, CsgA). In some embodiments, the method comprises administering to a subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group, and administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid and detecting the detectable compound in the subject, thereby determining the distribution of the biofilm produced by the engineered bacterium in the subject. In some embodiments, the biofilm is present in the gastrointestinal tract of the subject. In some embodiments, the biofilm is present in the respiratory tract of the subject. In some embodiments, the biofilm is present in the circulatory system of the subject. In some embodiments, the biofilm is present in an organ or

tissue selected from the group consisting of mouth, esophagus, lung, heart, stomach, small intestine, large intestine, rectum, anal canal, hair, and skin.

[00181] In some embodiments, disclosed herein are methods for delivering a drug to a subject. In some embodiments, the method comprises administering to a subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group, and administering to the subject a drug comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the drug to the nonstandard amino acid, thereby delivering the drug to the subject. Any drug comprising a bioorthogonal reactive functional group that reacts with a bioorthogonal reactive functional group of the nonstandard amino acid may be used. In some embodiments, the drug comprises a drug delivery vehicle selected from the group consisting of a nanocarrier (*e.g.*, a polymer), a nanoparticle (*e.g.*, a metal gold, silver, or iron nanoparticle), a liposome, a dendrimer, a carbon nanotube, a micelle, and a protein. Thus, the bioorthogonal reactive functional group may be present on the drug delivery vehicle.

[00182] In some embodiments, the drug comprises a bioorthogonal reactive functional group selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a hydrazine moiety, a tetrazine moiety, a norbornene moiety and a hydroxylamine moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a ketone moiety, and the bioorthogonal reactive functional group of the drug comprises a hydrazine moiety or a hydroxylamine moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises an azide moiety, and the bioorthogonal reactive functional group of the drug comprises an alkyne moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises an alkyne moiety, and the bioorthogonal reactive functional group of the drug comprises an azide moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a tetrazine moiety, and the bioorthogonal reactive functional group of the drug comprises a norbornene moiety. In some embodiments, the bioorthogonal reactive functional group of the nonstandard amino acid comprises a norbornene moiety, and the bioorthogonal reactive functional group of the drug comprises a tetrazine moiety.

[00183] In some embodiments, the drug is selected from the group consisting of a non-steroidal anti-inflammatory drug (NSAID), a steroidal anti-inflammatory drugs, an anti-fungal drugs, a detoxifying drug, an analgesics, a bronchodilator, an antibiotic, a monoclonal antibody, a diuretic, an immunosuppressant, a lymphokine, a hormone and a calcium channel blocker.

[00184] In some embodiments, disclosed herein are methods for diagnosing a disease or a disorder in a subject. In some embodiments, the method comprises administering to a subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group, and administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid and detecting the detectable compound in the subject, thereby diagnosing the disease or disorder in the subject.

[00185] In some embodiments, the disease or disorder is a gastrointestinal disease or disorder. In some embodiments, the disease or disorder is a respiratory disease or disorder. In some embodiments, the disease or disorder is an autoimmune disease or disorder. In some embodiments, the disease is an infectious disease. In some embodiments, the disease or disorder is a proliferative disease or disorder. In some embodiments, the disease or disorder is cancer. In some embodiments, the disease or disorder is a precancerous lesion. In some embodiments, the disease or disorder is an inflammatory disease or disorder. In some embodiments, the disease or disorder is selected from the group consisting of cystic fibrosis, congestive heart failure, benign prostatic hyperplasia, a gastrointestinal motility disorder, irritable bowel syndrome, chronic constipation, gastroesophageal reflux disease, functional heartburn, dyspepsia, functional dyspepsia, non-ulcer dyspepsia, gastroparesis, chronic intestinal pseudo-obstruction, colonic pseudo-obstruction, Crohn's disease, ulcerative colitis, and inflammatory bowel disease.

[00186] In some embodiments, the subject is an animal. In some embodiments, the subject is a mammal. In some embodiments, the subject is a domesticated animal. In some embodiments, the subject is a ruminant. In some embodiments, the subject is a non-human mammal. In some embodiments, the subject is a human subject.

[00187] The method may comprise preparing a pharmaceutical composition with at least one genetically engineered species, strain, or subtype of bacteria described herein, and

administering the pharmaceutical composition to a subject in a therapeutically effective amount. In some embodiments, the engineered bacteria disclosed herein are administered orally, *e.g.,* in a liquid suspension. In some embodiments, the engineered bacteria are lyophilized in a gel cap and administered orally. In some embodiments, the engineered bacteria are administered via a feeding tube or gastric shunt. In some embodiments, the engineered bacteria are administered rectally, *e.g.,* by enema. In some embodiments, the engineered bacteria are administered topically, intraintestinally, intrajejunally, intraduodenally, intraileally, and/or intracolically.

[00188] The methods disclosed herein may comprise administration of a composition alone or in combination with one or more additional therapies, *e.g.,* chemotherapy. The pharmaceutical composition may be administered alone or in combination with one or more additional therapeutic agents. An important consideration in the selection of the one or more additional therapeutic agents is that the agent(s) should be compatible with the bacteria, *e.g.,* the agent(s) must not interfere with or kill the bacteria.

[00189] In some embodiments, the engineered bacterium described herein, or pharmaceutical compositions comprising the engineered bacterium described herein may be administered to a subject, and a period of time is allowed to lapse in order to allow the bacterium to engraft and/or colonize in the subject (*e.g.*, in the subject's gastrointestinal tract, skin and/or respiratory tract). Thereafter, any intervention using the methods described herein may be used.

[00190] In some embodiments, it may be necessary to administer a nonstandard amino acid to the subject that can be used by the orthogonal translation systems described herein to incorporate the nonstandard amino acid into the protein expressed by the engineered bacterium. In some embodiments, the nonstandard amino acid is administered in a pharmaceutical composition. In some embodiments, the nonstandard amino acid is administered to the subject as part of a nutritional product. In some embodiment, the nonstandard amino acid is administered orally, intravenously, intradermally or rectally. In some embodiments, the nonstandard amino acid is administered to the subject at least one, two, three, four, five, six, eight, twelve, sixteen, twenty, twenty-four, thirty-six or forty-eight hours prior to administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group. In some embodiments, the nonstandard amino acid is administered to the subject at least one, two, three, four, five, six, or seven days prior to

administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group.

[00191] In some embodiments, it may be necessary to administer an agent (*i.e.*, an inducer) that regulates one or more inducible promoter that regulate the expression of one or more genes being expressed by the engineered bacterium described herein. The inducer will activate the expression of one or more of the orthogonal translation system and the heterologous gene encoding the protein of interest. In some embodiments, the inducer is administered in a pharmaceutical composition. In some embodiments, the inducer is administered to the subject as part of a nutritional product. In some embodiment, the inducer is administered orally, intravenously, intradermally or rectally. In some embodiments, the inducer is administered to the subject at least one, two, three, four, five, six, eight, twelve, sixteen, twenty, twenty-four, thirty-six or forty-eight hours prior to administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group. In some embodiments, the inducer is administered to the subject at least one, two, three, four, five, six, or seven days prior to administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group.

[00192] In some embodiments, both an inducer and a nonstandard amino acid are administered to the subject. In some embodiments, the inducer is administered to the subject concurrently with the nonstandard amino acid. In some embodiments, the inducer is administered the subject after the nonstandard amino acid is administered to the subject. In some embodiments, the inducer is administered before the nonstandard amino acid is administered to the subject. In some embodiments, the inducer and the nonstandard amino acid are administered in the same dosage form. In some embodiments, the inducer and the nonstandard amino acid are administered in separate dosage forms. In some embodiments, the inducer and the nonstandard are administered concurrently to the subject at least one, two, three, four, five, six, eight, twelve, sixteen, twenty, twenty-four, thirty-six or forty-eight hours prior to administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group. In some embodiments, the inducer and the nonstandard amino acid are administered concurrently to the subject at least one, two, three, four, five, six, or seven days prior to administering a detectable compound or drug to the subject comprising a bioorthogonal reactive functional group.

[00193] In some embodiments, an inducer, a nonstandard amino acid and a detectable agent are administered to the subject. In some embodiments, the inducer is administered to

the subject concurrently with the non standard amino acid, followed by administration of the detectable agent to the subject. In some embodiments, the inducer is administered to the subject concurrently with the detectable agent, followed by administration of the nonstandard amino acid. In some embodiments, the nonstandard amino acid is administered concurrently with the detectable agent, followed by administration of the inducer. In some embodiments, the inducer, the nonstandard amino acid and the detectable agent are administered separately. In some embodiments, two or more of the inducer, the nonstandard amino acid and the detectable agent are administered in the same dosage form. In some embodiments, the inducer, the nonstandard amino acid and the detectable agent are administered in separate dosage forms.

[00194] Definitions of common terms in cell biology and molecular biology can be found in The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); Benjamin Lewin (2009) Genes X, published by Jones & Bartlett Publishing (ISBN-10: 0763766321); Kendrew *et al.* (eds.) (1995) Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc. (ISBN 1-56081-569-8); and Coligan *et al.* (eds) (2009) Current Protocols in Protein Sciences Wiley Intersciences.

[00195] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00196] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

EXAMPLES

[00197] The following examples are set forth as being representative of the present disclosure. These examples are not to be construed as limiting the scope of the present disclosure as these and other equivalent embodiments will be apparent in view of the present disclosure, figures and accompanying claims.

[00198] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1995); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmel Eds., Academic Press Inc., San Diego, USA (1987); and Current Protocols in Protein Science (CPPS) (John E. Coligan *et al.*, ed., John Wiley and Sons, Inc.), which are all incorporated by reference herein in their entireties.

Example 1: Generation of engineered bacteria expressing proteins comprising the nonstandard amino acid *p*-AzF

[00199] Engineered heterologous nucleic acids expressing proteins comprising InaV, Trat, CsgA (as part of a synthetic curli *csgBACEFG* operon, which excludes the gene encoding the regulatory protein csgD), and MBP were cloned in pBbB8k plasmids, under the control of arabinose-inducible promoters. The proteins were cloned to incorporate a poly-histidine tag that allowed for affinity purification. Plasmid maps of engineered proteins are shown in **Figures 1A** and **1B**. Table 2 lists the DNA sequences of engineered proteins.

Table 2. Sequences of engineered proteins containing the nonstandard amino acid *p*-AzF. All proteins contain a poly-His tag (a 12 amino acid flexible linker followed by a 6X His tag). *p*-AzF inserted in a linker are placed just after the 6 histidine tag, and before a 36 or 48 amino acid flexible linker and a myc Tag. The position of *p*-AzF amino acid residue(s) is indicated with a "*".

Protein	Protein sequence	Total molecular weight (Da)
MBP	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGI KVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRF GGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYN GKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPAL DKELKAKGKSALMFNLQEPYFTWPLIAADGGYA FKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKN KHMNADTDYSIAEAAFNKGETAMTINGPWAWS NIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGIN AASPNKELAKEFLENYLLTDEGLEAVNKDKPLG AVALKSYEEELAKDPRIAATMENAQKGEIMPNIP QMSAFWYAVRTAVINAASGRQTVDEALKDAQT NSSSGSGGSGGSGGSGHHHHHH (SEQ ID NO: 12)	42342.80
MBP with *p*-AzF at position 67	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGI KVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDR* GGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYN GKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPAL DKELKAKGKSALMFNLQEPYFTWPLIAADGGYA FKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKN KHMNADTDYSIAEAAFNKGETAMTINGPWAWS NIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGIN AASPNKELAKEFLENYLLTDEGLEAVNKDKPLG AVALKSYEEELAKDPRIAATMENAQKGEIMPNIP QMSAFWYAVRTAVINAASGRQTVDEALKDAQT NSSSGSGGSGGSGGSGHHHHHH (SEQ ID NO: 13)	42383.81
MBP with *p*-AzF in a linker	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGI KVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRF GGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYN GKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPAL DKELKAKGKSALMFNLQEPYFTWPLIAADGGYA FKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKN KHMNADTDYSIAEAAFNKGETAMTINGPWAWS NIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGIN AASPNKELAKEFLENYLLTDEGLEAVNKDKPLG AVALKSYEEELAKDPRIAATMENAQKGEIMPNIP QMSAFWYAVRTAVINAASGRQTVDEALKDAQT NSSSGSGGSGGSGGSGHHHHHH*GGSGSSGSGGS GGGSGSSGSGGSGGGSGSSGSGGSGEQKLISEED L (SEQ ID NO: 14)	46238.56

CsgA	MKLLKVAAIAAIVFSGSALAGVVPQYGGGGNHG GGGNNSGPNSELNIYQYGGGNSALALQTDARNS DLTITQHGGGNGADVGQGSDDSSIDLTQRGFGNS ATLDQWNGKNSEMTVKQFGGGNGAAVDQTASN SSVNVTQVGFGNNATAHQYGSGGSGGSGGSGH HHHHH (SEQ ID NO: 15)	16676.73
CsgA with *p*-AzF at position 89	MKLLKVAAIAAIVFSGSALAGVVPQYGGGGNHG GGGNNSGPNSELNIYQYGGGNSALALQTDARNS DLTITQHGGGNGADVGQGSDDS*IDLTQRGFGNS ATLDQWNGKNSEMTVKQFGGGNGAAVDQTASN SSVNVTQVGFGNNATAHQYGSGGSGGSGGSGH HHHHH (SEQ ID NO: 16)	16717.74
CsgA with *p*-AzF in a linker	MKLLKVAAIAAIVFSGSALAGVVPQYGGGGNHG GGGNNSGPNSELNIYQYGGGNSALALQTDARNS DLTITQHGGGNGADVGQGSDDSSIDLTQRGFGNS ATLDQWNGKNSEMTVKQFGGGNGAAVDQTASN SSVNVTQVGFGNNATAHQYGSGGSGGSGGSGH HHHHH*GGSGSSGSGGSGGGSGSSGSGGSGGGS GSSGSGGSGEQKLISEEDL (SEQ ID NO: 17)	20572.49
InaV	MNIDKALVLRTCANNMADHCGLIWPASGTVESK YWQSTRRHENGLVGLLWGAGTSAFLSVHADAR WKVCEVAVADIIGLEEPGMVKFPRAEVVHVGDR ISASHFISARQADPASTPTPTPTPMATPTPAAANIA LPVVEQPSHEVFDVALVSAAAPSVNTLPVTTPQN LQTATYGSTLSGDNNSRLIAGYGSNETAGNHSDL IAGYGSTGTAGSDSSLVFRLWDGKRYRQLVART GENGVEADIPYYVNEDDDIVDKPDEDDDWIEVE GSGGSGGSGGSGHHHHHH (SEQ ID NO: 18)	30246.32
InaV with *p*-AzF at position 174	MNIDKALVLRTCANNMADHCGLIWPASGTVESK YWQSTRRHENGLVGLLWGAGTSAFLSVHADAR WKVCEVAVADIIGLEEPGMVKFPRAEVVHVGDR ISASHFISARQADPASTPTPTPTPMATPTPAAANIA LPVVEQPSHEVFDVALVSAAAPSVNTLPVTTPQN LQTAT*GSTLSGDNNSRLIAGYGSNETAGNHSDLI AGYGSTGTAGSDSSLVFRLWDGKRYRQLVARTG ENGVEADIPYYVNEDDDIVDKPDEDDDWIEVEGS GGSGGSGGSGHHHHHH (SEQ ID NO: 19)	30287.33
InaV with *p*-AzF in a linker	MNIDKALVLRTCANNMADHCGLIWPASGTVESK YWQSTRRHENGLVGLLWGAGTSAFLSVHADAR WKVCEVAVADIIGLEEPGMVKFPRAEVVHVGDR ISASHFISARQADPASTPTPTPTPMATPTPAAANIA LPVVEQPSHEVFDVALVSAAAPSVNTLPVTTPQN LQTATYGSTLSGDNNSRLIAGYGSNETAGNHSDL IAGYGSTGTAGSDSSLVFRLWDGKRYRQLVART GENGVEADIPYYVNEDDDIVDKPDEDDDWIEVE GSGGSGGSGGSGHHHHHH*GGGSGGGSGGGSG GGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSG GGSEQKLISEEDL (SEQ ID NO: 20)	34736.62

Trat	MMKTKKLMMVALVSSTLALSGCGAMSTAIKKR NLEVKTQMSETIWLEPASERTVFLQIKNTSDKDM SGLQGKIADAVKAKGYQVVTSPDKAYYWIQAN VLKADKMDLRESQGWLNRGYEGAAVGAALGA GITGYNSNSAGATLGVGLAAGLVGMAADAMVE DVNYTMITDVQIAERTKATVTTDNVAALRQGTS GAKIQTSTETGNQHKYQTRVVSNANKVNLKFEE AKPVLEDQLAKSIANILGSGGSGGSGGSGHHHHH H (SEQ ID NO: 21)	27659.42
Trat with *p*-AzF at position 200	MMKTKKLMMVALVSSTLALSGCGAMSTAIKKR NLEVKTQMSETIWLEPASERTVFLQIKNTSDKDM SGLQGKIADAVKAKGYQVVTSPDKAYYWIQAN VLKADKMDLRESQGWLNRGYEGAAVGAALGA GITGYNSNSAGATLGVGLAAGLVGMAADAMVE DVNYTMITDVQIAERTKATVTTDNVAALRQGTS GAKIQ*TSTETGNQHKYQTRVVSNANKVNLKFE EAKPVLEDQLAKSIANILGSGGSGGSGGSGHHHH HH (SEQ ID NO: 22)	27700.43
Trat with *p*-AzF in a linker	MMKTKKLMMVALVSSTLALSGCGAMSTAIKKR NLEVKTQMSETIWLEPASERTVFLQIKNTSDKDM SGLQGKIADAVKAKGYQVVTSPDKAYYWIQAN VLKADKMDLRESQGWLNRGYEGAAVGAALGA GITGYNSNSAGATLGVGLAAGLVGMAADAMVE DVNYTMITDVQIAERTKATVTTDNVAALRQGTS GAKIQTSTETGNQHKYQTRVVSNANKVNLKFEE AKPVLEDQLAKSIANILGSGGSGGSGGSGHHHHH H*GGGSGGGSGGGSGGGSGGGSGGGSGGGSGG GSGGGSGGGSGGGSGGGSEQKLISEEDL (SEQ ID NO: 23)	32149.72

[00200] An *E. coli* Nissle (ECN) 1917 strain comprising a knockout of the *csgBAC* and *csgDEFG* operons was developed using the lambda red system. For expressing curli fibers in vitro, pBbB8k plasmids containing the whole synthetic curli operon (*csgBACEFG* operon) were used. The knockout of the native curli operons allows for clear assessment of recombinant curli fibers production. The developed curli operons knockout strain was used for all experiments described in **Examples 2 – 8** below.

[00201] Bacterial cells were transformed with a pBbB8k plasmid encoding the engineered proteins above by inserting a TAG codon at the corresponding nucleotide location in the open reading frame of the protein, and with the plasmid pEVOL-pAzF (a gift from Peter Schultz (Addgene plasmid # 31186)) which encodes an orthogonal translation system that incorporates *p*-AzF into proteins encoded by nucleic acids having the TAG codon. Two control bacterial strains were also developed: bacteria transformed with pBbB8k plasmid encoding engineered proteins without *p*-AzF and co-transformed with pEVOL-pAzF to

observe background incorporation of *p*-AzF into other *E. coli* proteins; and bacteria transformed with pBbB8k plasmids encoding engineered proteins without *p*-AzF alone.

[00202] Engineered ECN were grown in 3 to 5 mL of Luria broth (LB) with the appropriate antibiotics (kanamycin for pBbB8k plasmids only, or kanamycin and spectinomycin for pBbB8k and pEVOL-pAzF plasmids) overnight at 37°C. Fresh cultures were inoculated by diluting the overnight culture 100 times in fresh LB with the appropriate antibiotic, and the cultures were grown at 37° C to an optical density at 600 nm (OD600) of 0.6 to 0.7. The cultures were then induced with 0.01 to 0.2 % arabinose (inducer) overnight. Along with the inducer, *p*-AzF was added to the cultures to a final concentration ranging between 0.1 to 5 mM. The cells were then pelleted, resuspended in lysis buffer and sonicated to complete the lysis. After centrifugation, the supernatant was incubated with Ni NTA beads (as instructed by the manufacturer) to purify the proteins. Protein expression and purity was assessed via SDS-PAGE and Western blotting using an anti-His tag antibody. Protein integrity was assessed using Western blot with an anti-myc tag antibody for engineered proteins with *p*-AzF inserted in a linker.

Example 2: Maltose-binding protein (MBP) comprising the nonstandard amino acid *p*-AzF was expressed and purified from *E. coli* Nissle 1917

[00203] The purpose of this experiment was to determine if *E. coli* Nissle could successfully used to express a protein comprising the non-standard amino acid *p*-AzF without having to recode the bacterial genome to remove or reduce the amount of the labeling codon TAG. As described in Example 1, three different bacterial cells were used: cells transformed with a single plasmid to express wild-type MBP (without a nonstandard amino acid), where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type MBP (without a nonstandard amino acid) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS"). 0.05% arabinose was added as an inducer to induce the production of the orthogonal translation system and of MBP. *p*-AzF was not added to the culture media of the bacteria of the first group (*i.e.*, No mutation/No OTS and UAG mutation/With OTS). However, *p*-AzF was added at a final concentration of 1 mM to the culture media of the bacteria of the remaining two groups (*i.e.*, No mutation/With OTS and UAG mutation/With

OTS) at the time of induction. As shown in **Figure 3**, E. *coli* Nissle comprising an orthogonal translation system encoded by the plasmid pEVOL-pAzF, and a heterologous nucleic acid encoding MBP protein comprising a nonstandard amino acid can be successfully expressed in and purified from the bacterial cells without needing to recode the bacterial genome to remove the used labeling codon (TAG).

Example 3: MBP comprising the nonstandard amino acid *p*-AzF purified from in *E. coli* Nissle was labeled with DBCO-Cy5

[00204] The purpose of this experiment was to determine if MBP comprising the nonstandard amino acid *p*-AzF purified from in *E. coli* Nissle could successfully be reacted with a detectable moiety (DBCO-Cy5). MBP expression and purification was performed as described in Examples 1 and 2. For labeling of proteins with DBCO-Cy5, the purified protein eluates were incubated for 1 hour at room temperature with DBCO-Cy5 dye at molar ratios ranging between 1:10 to 10:1 MBP:DBCO-Cy5. Labeling reactions were run on SDS-PAGE gels and Cy5 fluorescence was detected using a FluorChem M System. As shown in **Figure 4**, MBP comprising *p*-AzF can be successfully reacted with DBCO-Cy5 to label the protein. Further, very little background incorporation of *p*-AzF into endogenous *E. coli* proteins appears to have occurred, as determined by analyzing the background fluorescence in cells transformed with a plasmid to express wild-type MBP (without a nonstandard amino acid) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS").

[00205] To further confirm whether non-specific binding of the DBCO-Cy5 to endogenous proteins and/or low specific incorporation of *p*-AzF into endogenous *E. coli* proteins was occurring, cell pellets from the three transformation conditions were reacted with DBCO-Cy5, and residual fluorescence was analyzed. As shown in **Figure 7**, low non-specific binding was observed in cells expressing wild-type MBP.

[00206] To determine whether the number of functional groups from p-AzF that are attached to a detectable moiety can be tittered, purified MBP was reacted with DBCO-Cy5 at various DBCO-Cy6:MBP molar rations including 2, 2.5, 1, 0.75, 0.5 and 0.25. As shown in **Figure 12**, the number of functional groups from *p*-AzF that are attached to a detectable moiety can be tittered.

Example 4: CsgA comprising the nonstandard amino acid *p*-AzF was expressed and purified from *E. coli* Nissle 1917

[00207] The purpose of this experiment was to determine if *E. coli* Nissle could successfully used to express an operon comprising a heterologous gene encoding CsgA comprising the non-standard amino acid *p*-AzF. As described in Example 1, three different bacterial cells were used: cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon), where the bacteria lacked an orthogonal translation system ("No mutation/No OTS"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("No mutation/With OTS"); and cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("UAG mutation/With OTS"). 0.05% arabinose was added as an inducer to induce the production of the orthogonal translation system and of the synthetic curli operon proteins. *p*-AzF was not added to the culture media of the bacteria of the first group (*i.e.*, No mutation/No OTS and UAG mutation/With OTS). However, *p*-AzF was added at a final concentration of 1 mM to the culture media of the bacteria of the remaining two groups (*i.e.*, No mutation/With OTS and UAG mutation/With OTS) at the time of induction. As shown in **Figure 5**, *E. coli* Nissle comprising an orthogonal translation system encoded by the plasmid pEVOL-pAzF, and a heterologous nucleic acid encoding the synthetic curli operon, which encodes the CsgA protein comprising a nonstandard amino acid can be successfully expressed in and purified from the bacterial cells. Without wishing to be bound by any particular theory, it appeared that the larger burden of expressing a whole operon instead of a single protein resulted in lower CsgA expression, as compared to MBP (see Example 2).

Example 5: CsgA comprising the nonstandard amino acid *p*-AzF purified from in *E. coli* Nissle was labeled with DBCO-Cy5

[00208] The purpose of this experiment was to determine if CsgA comprising the nonstandard amino acid *p*-AzF purified from in *E. coli* Nissle could successfully be reacted with a detectable moiety (DBCO-Cy5). CsgA expression and purification was performed as described in Examples 1 and 2. For labeling of proteins with DBCO-Cy5, concentrated 500 mM eluates of the purified protein were incubated for 1 hour at room temperature with DBCO-Cy5 dye at a molar excess of DBCO-Cy5 to MBP. Labeling reactions were run on

SDS-PAGE gels and Cy5 fluorescence was detected using a FluorChem M System. As shown in **Figure 6**, CsgA comprising *p*-AzF can be successfully reacted with DBCO-Cy5 to label the protein. Further, the apparent higher background staining observed could be due to the low CsgA concentration loaded onto the SDS-PAGE gel.

Example 6: CsgA comprising the nonstandard amino acid *p*-AzF expressed in *E. coli* Nissle 1917 forms curli fibers

[00209] The purpose of this experiment was to determine the optimal concentration of the inducer, arabinose, used to induce the expression of the orthogonal translation system and of the curli operon proteins necessary for optimal expression CsgA comprising the nonstandard amino acid *p*-AzF in *E. coli* Nissle. Congo Red dye binding to curli fiber (CsgA) was determined using spectrophotometry at OD490/OD600, a measure of bound Congo Red dye over cell density. Increased Congo Red binding signifies increased curli fiber (CsgA) production. For this experiment, cells expressing MBP were used as control. As shown in **Figure 8**, optimal expression of CsgA comprising *p*-AzF was observed at an arabinose concentration of 0.05%. The background signal observed for cells expressing MBP might be attributable to Congo Red binding to other extracellular appendages of E. coli Nissle (for example, cellulose).

[00210] To determine whether the expressed CsgA comprising *p*-AzF could form curli fibers in the cells expressing the protein. The production of curli fiber comprising CsgA having *p*-AzF was determined using scanning electron microscopy (SEM). SEM samples were prepared by filtering bacterial cultures on Nucleopore filter membranes (0.22 μm pores) under vacuum. The samples were rinsed with 0.1 M sodium cacodylate buffer, and fixed with 2 % (m/v) glutaraldehyde and 2 % (m/v) paraformaldehyde for 2 hours at room temperature. The membranes were then washed in water, and the solvent was gradually exchanged to ethanol with an increasing ethanol 15 minute incubation step gradient (25 %, 50 %, 75 % and 100 % (v/v) ethanol). The membranes were dried in a critical point dryer and sputtered until they were coated in a 5 nm layer of Pt/Pd. Imaging was performed using a Zeiss Ultra 55 Field Emission SEM. As shown in **Figure 9**, no curli fiber production was observed in cells expressing MBP as expected, since the strain of E. coli Nissle has a deletion of the endogenous curli operons. However, when an exogenous synthetic curli operon was expressed in the cells, which included CsgA comprising *p*-AzF, curli fibers were observed near the bacterial cells.

[00211] To determine whether the amount of labeled fibers after incubation with DBCO-Cy5 could be varied by administering less *p*-AzF to the cultured cells. As shown in **Figure 13**, when cells were grown in media containing varying concentrations of the nonstandard amino acid, lower concentrations of *p*-AzF resulted in a smaller fraction of labeled fibers after incubation with DBCO-Cy5.

Example 7: Growth of engineered *E. coli* Nissle to express proteins having a nonstandard amino acid is not significantly affected

[00212] The purpose of this experiment was to determine whether genetic manipulation of *E. coli* Nissle to express proteins having a nonstandard amino acids affected cellular growth. Cells were grown in a deep-well 96 well plate, and arabinose, *p*-AzF, or arabinose and *p*-AzF were added to the culture media at an OD600 of ~ 0.2. Growth curves of *E. coli* Nissle cells either: lacking plasmid ("No plasmid"); comprising an orthogonal translation system (encoded by the pEVOL-pAzF plasmid) ("OTS"); cells transformed with a single plasmid to express wild-type MBP (without a nonstandard amino acid), where the bacteria lacked an orthogonal translation system ("MBP"); cells transformed with a plasmid to express wild-type MBP (without a nonstandard amino acid) expressed and a plasmid comprising an orthogonal translation system ("MBP + OTS"); and cells transformed with a plasmid to express MBP comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("MBP (mutation) + OTS"); cells transformed with a single plasmid to express wild-type CsgA (as part of the synthetic curli operon), where the bacteria lacked an orthogonal translation system ("Curli operon"); cells transformed with a plasmid to express wild-type CsgA (as part of the synthetic curli operon) expressed and a plasmid comprising an orthogonal translation system ("Curli operon + OTS"); or cells transformed with a plasmid to express CsgA (as part of the synthetic curli operon) comprising the nonstandard amino acid *p*-AzF and a plasmid comprising an orthogonal translation system ("Curli operon (mutation) + OTS"), were determined. Growth curves are shown in **Figures 10** and **11**. The growth of *E. coli* Nissle transformed with the OTS (pEVOL-pAzF) was not significantly affected, as compared to the non-transformed *E. coli* Nissle growth curve. The growth of cells expressing MBP with or without *p*-AzF, and with or without the presence of the orthogonal translation system, also was not significantly affected. As compared to the growth of cells expressing MBP, the growth of cells expressing curli fibers (including CsgA) was slower, but no significant differences between wild-type curli fibers, curli fibers

expressed in the presence of an orthogonal translation system, and curli fibers containing *p*-AzF expressed in the presence of the orthogonal translation system. Thus, the results show that recombinant proteins containing a nonstandard amino acid can be expressed in *E. coli* Nissle without significantly affecting bacterial growth.

Example 8: TraT was expressed and purified from *E. coli* Nissle 1917

[00213] The purpose of this experiment was to determine if *E. coli* Nissle could successfully used to express TraT. 0.05% arabinose was added as an inducer to induce the production of TraT. As shown in **Figure 14**, TraT protein can be successfully expressed in and purified from the *E. coli* Nissle bacterial cells. Three different lysate buffers (as indicated) were used to lyse the cells.

Example 9: *In vivo* labeling of engineered bacteria

[00214] Based on expression of MBP and CsgA containing *p*-AzF (described above), the number of recombinant proteins produced per cell is in the order of 10^6 proteins/cells (approximately the same number of available bioorthogonal reactive functional groups (*i.e.*, azide moieties) per cell). With an approximate number of cells colonizing the gastrointestinal tract of mice after 3 days of 10^4 to 10^8 cells, the concentration of the bioorthogonal reactive functional group azide in the gut of a mouse could range between tens of nM to approximately 10 μM. Assuming that the click reaction with the bioorthogonal functional group of a detectable compound proceeds to completion, this concentration should be sufficient to allow for signal detection for optical or MRI imaging techniques.

[00215] *In vivo* studies may be performed using engineered bacteria comprising the native curli operons, or lacking the native curli operons. Expression of native curli fibers may be important for colonization of the bacteria in the gut, especially when other engineered surface proteins are expressed (without co-expression of recombinant curli fibers).

[00216] For *in vivo* studies, 6-8 week old female mice are administered selection marker antibiotics (*e.g.*, kanamycin and spectinomycin; 1 g/L in drinking water), to maintain the plasmids of the engineered bacteria, continuously during the course of the experiment beginning 24 hours prior to administration of the engineered bacteria. The mice are divided into four separate groups: one experimental group and three control groups. The mice of control group 1 are administered engineered bacteria (as described, for example in Example 1 above) and a detectable compound (*e.g.*, DBCO-Cy5), but will not receive inducer (*e.g.*,

arabinose, to induce the expression of the protein comprising *p*-AzF and the expression of the orthogonal translation system) nor the nonstandard amino acid (*e.g.*, *p*-AzF). The mice of control group 2 are administered detectable compound, but will not be administered engineered bacteria, inducer, nor nonstandard amino acid. The mice of control group 3 do not receive engineered bacteria, inducer, nonstandard amino acid, nor detectable compound. At day 0, the mice the experimental group and of control group 1 are administered a single dose of engineered bacteria by oral gavage (10^9–10^{10} colony forming units (CFUs)). At days 1, 3, 5, 7, 9 and 11, mice of the experimental group and control group 1 are administered inducer (*e.g.*, arabinose at 10 g/L in drinking water) and nonstandard amino acid (*e.g.*, *p*-AzF at 0.25 g/L in drinking water). At days 2, 4, 6, 8, 10 and 12, the mice of the experimental group, control group 1 and control group 2 are administered detectable compound (*e.g.*, DBCO-Cy5, 100 μL at 0.08 mM) by oral gavage. Body weight, plasma samples, and fecal samples are taken throughout the duration of the study. *In vivo* imaging of the mice using the IVIS Spectrum *In Vivo* Imaging System (PerkinElmer) is performed throughout the study to determine labeling of the engineered bacteria *in vivo*. Fecal samples are also analyzed by fluorescence microscopy to determine bacterial labeling. Upon conclusion of the study at day 13, the mice can be euthanized, and internal organs (liver, spleen, intestines) collected and assayed.

Example 10: *In vivo* labeling and detection of engineered bacteria comprising mutant CsgA or mutant TraT having the non-standard amino acid pAzF

Protein Expression

[00217] Plasmids expressing either wild-type (wt) TraT or a mutant TraT having a labeling codon were transformed into PBP8 cells with or without the pEVOL-pAzF plasmid, which encodes an orthogonal translation system (OTS), and expression was induced. Proteins were purified after cells lysis and detected using Western Blot with either anti-myc or anti-his antibodies. Both wt and mutant TraT proteins have an expected molecular weight of about 27 kDa, each variants having a his-tag before either the stop-codon or the amber-codon (*i.e.*, labeling codon), respectively (**FIG. 15A**). A second band having a molecular weight of about 34 kDa was observed in Western Blots of cells expressing the mutant TraT protein using either an anti-His antibody (**FIG. 15A**) or an anti-myc antibody (**FIG. 15B**). The 34 kDa band is the expected size of the full length mutant TraT. As shown in **Fig. 15C**, in cells

lacking the OTS and not grown in the presence of NSAA, about 5% of the protein expressed was the 34 kDa variant. This read-through might be a result of an Amber suppression mutation. However, about 35% of TraT protein observed in the cells expressing both the mutant TraT, the OTS, and cultured in the presence of NSAA, was full length. Premature termination of translation appears to have occurred at the amber codon, perhaps due to inefficiencies of the OTS.

NSAA incorporation

[00218] Mutant TraT comprising pAzF, mutant CsgA comprising pAzF, wild-type CsgA, and wild-type TraT were purified, incubated with DBCO-Cy5 and analyzed through SDS PAGE. Two bands, each representing TraT or CsgA, were visible using red fluorescence imaging (**FIG. 16**), and solely observed in cells comprising both the plasmid expressing the mutant TraT or mutant CsgA and the plasmid comprising the OTS, when the cells were grown in the presence of the NSAA pAzF. Since no protein bands were observed in the negative controls (*i.e.*, bacterial cells expressing wt CsgA or wt TraT in the presence or absence of both the OTS and NSAA pAzF), both the mutation and OTS are necessary for the incorporation of the NSAA.

Cell Labeling

[00219] To determine the optimal conditions for labeling of cells expressing the mutant TraT comprising pAzF labeling experiments were performed using different incubation conditions. *E. coli* PBP8 cells expressing the mutant TraT comprising pAzF and the OTS were grown in the presence of 5 mM pAzF. Although higher concentrations of pAzF were tested, adverse effects on cell grown were observed when cells were grown in the presence of greater that 10 mM pAzF. Complete cell growth inhibition was observed when cells were grown in the presence of 20 mM pAzF.

[00220] First, either non-transformed PBP8 cells, or PBP8 cells transformed to express mutant TraT and the OTS were incubated with varying concentrations of DBCO-Cy5 for 1 hour at 25 °C. Fluorescence intensity was measured after cell pellets were deposited onto nitrocellulose. As shown in **FIG. 17A**, the intensity of the sham control increased linearly with the concentration of DBCO-Cy5. However, in cells expressing the mutant TraT increased fluorescence intensity, as compared to the control cells, was observed at low concentrations of DBCO-Cy5, with saturation observed at about 500 μM DBCO-Cy5.

[00221] To determine the optimal incubation time for labeling with DBCO-Cy5, either non-transformed PBP8 cells (control), or PBP8 cells transformed to express mutant TraT and the OTS were incubated with 200 μM DBCO-Cy5 at 37°C for 1-4 hours. As shown in **FIG. 17B**, fluorescence intensity increased linearly over time in the control cells. However, fluorescence intensity saturation was observed after 2 hours in cells transformed to express mutant TraT and the OTS.

[00222] To determine the optimal incubation temperature for labeling with DBCO-Cy5, either non-transformed PBP8 cells (control), or PBP8 cells transformed to express mutant TraT and the OTS were incubated with either 100 μM or 200 μM DBCO-Cy5 at either 25°C or 37°C. As shown in **FIG. 17C**, labeling performed using 200 μM DBCO-Cy5 at 25°C resulted in comparable fluorescence intensity as labeling performed using 100 μM DBCO-Cy5 at 37°C.

[00223] Additional labeling experiments were performed using PBP8 cells expressing either mutant CsgA comprising pAzF or mutant TraT comprising pAzF and the OTS to determine optimal labeling conditions. As shown in **FIGs. 18A** and **18B**, cells expressing either mutant CsgA or mutant TraT incubated at 37°C for 2 hours with increasing concentrations of DBCO-Cy5 for labeling demonstrated that fluorescence intensity increased proportionally with higher concentration of DBCO-Cy5, with saturation observed at 300 μM DBCO-Cy5. Further, as shown in **FIGs. 18C** and **18D**, cells expressing either mutant CsgA or mutant TraT incubated with 200 μM DBCO-Cy5 for labeling between 1-4 hours, showed maximum labeling after 2 hours. Fluorescence intensity decrease after 2 hours, which may be due to protein degradation.

[00224] To study the distribution of DBCO-Cy5 uptake and labeling efficiency in a population of bacterial cells, PBP8 cells expressing either mutant CsgA comprising pAzF and the OTS or mutant TraT comprising pAzF and the OTS were cultured in the presence of pAzF, and the cultures were induced overnight with arabinose, and incubated with Cy5-DBCO as described above. The labeled populations of cells were analyzed using flow cytometry and confocal microscopy. As shown in **FIGs. 21A, 21B, 21C** and **21D,** Cy5-DBCO labeling was detected in both PBP8 cells expressing either mutant CsgA comprising pAzF or mutant TraT comprising pAzF and the OTS. Further, as shown in **FIGs. 19** and **20**, a bimodal distribution was observed when flow cytometry was performed on cell populations, with minimal fluorescence observed in control untransformed PBP8 cells and

non-induced PBP8 cells transformed to express either mutant CsgA comprising pAzF or mutant TraT comprising pAzF and the OTS.

In Vivo Detection of Cy5-DBCO-labeled Bacterial Cells

[00225] To determine whether Cy5-DBCO-labelled could be detected *in vivo*, the following study was performed. PBP8 cells expressing either mutant CsgA comprising pAzF and the OTS were cultured, protein expression induced, and the cells were labeled with Cy5-DBCO *in vitro* as described above. Mice were either administered no bacteria (control), 1 x 10^6 cells, or 1 x 10^9 cells by oral gavage, and Cy5 fluorescence detected *in vivo* using the IVIS Lumina II system (PERKIN ELMER). Fluorescence was monitored over time. As shown in FIG. 22, the bacteria were readily detected *in vivo*. Some background fluorescence was detected in mice which may be due to their feed (non alfalfa-free feed), which may result in background fluorescence interference. In later experiments, regular food was replaced with alfalfa-free food to minimize any fluorescence background.

[00226] To determine whether cells could be labelled with Cy5-DBCO *in vivo*, the following experiments were performed. To determine the optimal concentration of Cy5-DBCO for detection using the VIS Lumina II system (PERKIN ELMER), mice were administered either 5 μM, 50 μM, 100 μM, or 200 μM of Cy5-DBCO. Maximum fluorescence detection without detector saturation was observed using 100 μM Cy5-DBCO (see **FIG. 23**). Therefore, 100 μM Cy5-DBCO was the concentration used for the labelling experiments described below.

[00227] Having demonstrated that labeled bacteria expressing mutant CsgA comprising pAzF labelled with Cy5-DBCO were detectable after passing the gastrointestinal tract, *in vivo* experiments were designed to label the bacteria *in vivo*. Briefly, four groups of mice were treated with either (a) Cy5-DBCO dye alone (Group 1); (b) bacteria expressing wild-type CsgA (Group 2); (c) bacteria expressing mutant CsgA comprising pAzF in the absence of arabinose inducer (Group 3); or (d) bacteria expressing mutant CsgA comprising pAzF in the presence of arabinose inducer (Group 4). For the groups of mice receiving bacteria (Groups 2-4), unlabeled bacteria were administered by oral gavage at day 0. Mice were continuously administered pAzF in water. Mice from Groups 2 and 4 were also administered the arabinose inducer. Subsequently (day 1), mice in all groups were administered Cy5-DBCO by gavage and no longer administered pAzF and arabinose (as applicable) 2 hours prior to administration of Cy5-DBCO. Mice were imaged using the VIS

Lumina II system (PERKIN ELMER) to detect the clearance rate of the dye alone as well as the labeled bacteria. As shown in **FIG. 24**, a difference in the residence time was observed between mice administed the Cy5-DBCO dye alone (Group 1) and mice administered both Cy5-DBCO and bacteria expressing mutant CsgA comprising pAzF in the presence of arabinose inducer (Group 4). The Cy5-DBCO dye was no longer detectable after about 24 hours in the mice of Group 1, while fluorescence was detectable for an extended period of time ($\geq$ 35 hours) in the majority of mice of Group 4, indicative of labeling of the the engineered bacteria. The differences in fluorescence residence time was statistically significant (see **FIG. 25**).

[00228] To determine whether pH changes and other biological factors affected the ability to detect the labelled bacteria *in vivo*, the following experiments were performed to determine whether the bacteria remained viable *in vivo*, and whether the bacteria lost either of the plasmids expressing the OTS or the mutant CsgA. Fecal samples were obtained from the mice three days after bacteria were administered to the mice, and fecal pellets were plated onto agar plates containing 50 μg/mL chloramphenicol, kanamycin and spectinomycin to culture the bacteria in the sample. Bacterial colonies were selected, grown in the presence and absence of pAzF. Cell lysates were analyzed by SDS-PAGE. SDS-PAGE gels were dyed with either Coomasie Blue or Cy5-DBCO in order to detect total protein, or mutant CsgA comprising pAzF, respectively. As shown in **FIG. 26A** and **26B** mutant CsgA comprising pAzF was detectable in some of the isolated bacteria. Not all of the bacteria retained the ability to express mutant CsgA comprising pAzF which may be due to the loss of the plasmid encoding the mutant CsgA protein, and/or the plasmid encoding the OTS.

[00229] In order to detect the distribution of the bacteria in the gastrointestinal tract of the mice *ex vivo*, the mice were sacrificed and the gastrointestinal tract was imaged using the IVIS Lumina II system (PERKIN ELMER). As shown in **FIGs. 27A** and **27B**, a significant difference was observed in the level of fluorescence detected in mice receiving the control untransformed PBP8 bacteria and mice receiving bacteria expressing the mutant CsgA comprising pAzF. Non-specific binding of the Cy5-DBCO dye was detected in the gut. However, as shown in **FIG. 27C**, when the total radiance efficiency was determined after stomach fluorescence was subtracted, the differences in radiance efficiency at sites distal to the gut were statistically significant. While no fluorescence was observed in the cecum of mice receiving the control, fluorescence was detected in mice receiving bacteria expressing

the mutant CsgA comprising pAzF. At 24 hours, the fluorescence detected in the cecum of the mice receiving bacteria expressing the mutant CsgA comprising pAzF decreased.

[00230] To further confirm that the bacteria could be labeled in the gastrointestinal tract of the mice, the following experiment was performed. Mice were administered PBP8 cells expressing either mutant CsgA comprising pAzF and the OTS, arabinose inducer, and pAzF. As control, a group of mice were not treated with bacteria, nor administered pAzF and arabinose. The gastrointestinal tract tissue was collected from the mice and imaged using the IVIS Lumina II system (PERKIN ELMER) (see **FIGs. 28** and **29**). The cecal tissue was isolated and homogenized. Following cell lysis, the pellet and soluble fractions of the ceca were analyzed to determine whether labeled mutant CsgA comprising pAzF (*i.e.*, bound to Cy5-DBCO) could be detected in either fraction (see **FIG. 30**). To extract all of the CsgA protein that was potentially present in the pellet fraction, samples were treated with HFIP:TFA to disassemble aggregated curli fibers. As shown in **FIGs. 31A, 31B, 32A,** and **32B**, Cy5-DBCO-labelled mutant CsgA was detected in the soluble fractions of untreated cecum homogenate of every mouse to which bacteria expressing mutant CsgA comprising pAzF was administered, but not in the cecum homogenate from mice not administered bacteria.

Materials and Methods

Cell strains and plasmids

[00231] The divergent curli operon regions consisting of *csgBAC* and *csgEFG* were PCR isolated from *E. coli* K12 substr. W3110 and cloned by overlap extension into the pBbB8k plasmid, to create a single operon, *csgBACEFG*, under the control of the araBp promoter. A six-histidine tag (HisTag) was added to the C-terminus of CsgA to allow for immunodetection (pBbB8k-WT). A control plasmid was constructed by cloning the *malE* gene encoding for the maltose binding protein (MBP) from the W3110 genome into the pBbB8k plasmid under the control of the araBp promoter (pBbB8k-MBP). To allow for the incorporation of NSAA, a mutation encoding for the amber stop codon (TAG) was inserted within the open-reading frame of CsgA (pBbB8k-Mut) or MBP (pBbB8k-MBPMut) using site-directed mutagenesis. The pEVOL-pAzF plasmid was used as orthogonal translation system (OTS).

[00232] Protein expression was performed in a curli operon deletion mutant, PBP8, an *E. coli* strain derived from *E. coli Nissle 1917* (ECN). PBP8 strain was constructed using the

lambda red recombineering method as previously described (*see* Datsenko and Wanner (2000) *Proc. Nat'l. Acad. Sci. USA* 97(12): 6640-5). Briefly, the chloramphenicol acetyltransferase (CAT) cassette was constructed with 500 bp homology arms upstream and downstream of the curli operon using SOEing PCR. The CAT cassette was transformed into electrocompetent EcN with pre-induced lambda red genes from pKD46 plasmid using 0.5% arabinose during the growth step of electrocompetent cell preparation. Transformants were expanded using SOC media at 30°C shaking incubator for 3 hours to promote the genomic replacement of the curli operon with the CAT cassette. Transformants were then plated onto LB agar plates containing 25 μg/mL chloramphenicol and incubated overnight at 37 °C. Colonies that were resistant to chloramphenicol were selected and verified for knockout of the curli operon by colony PCR and genomic DNA sequencing as compared to wild-type EcN.

In vitro expression of curli fiber

[00233] PBP8 cells transformed with a pBbB8k plasmid were streaked onto lysogeny broth (LB) agar plates containing 50 μg/mL kanamycin. PBP8 cells transformed both with a pBbB8k plasmid and with the OTS plasmid were streaked onto Luria Broth (LB) agar plates containing both 50 μg/mL kanamycin and 50 μg/mL spectinomycin. Colonies were picked from the plates and 5 mL cultures were inoculated (in LB containing the appropriate antibiotics). Cultures were grown overnight at 37°C. The overnight cultures were diluted 100-fold in fresh LB medium containing the appropriate antibiotics, and cultured at 37°C until they reached an optical density (OD) at 600 nm of 0.6 to 0.8. Arabinose was added to the bacterial cultures at a final concentration of 0.05 % to induce protein expression. If NSAA incorporation was desired, the NSAA 4-azido-L-phenylalanine (pAzF) (Toronto Research Chemicals) was also added to the cultures to a final concentration ranging between 1 mM and 5 mM. Higher concentrations of NSAA affected cell growth. Protein expression was allowed to occur at 37°C overnight.

Bacterial growth curves and doubling times

[00234] Bacterial cells were grown in LB overnight, diluted 1:100 in fresh LB, and grown in 1 mL-well 96 wells plate at 37 °C and shaken at 900 rpm. Once the culture reached an optical density (OD) of 0.6, measured at 600 nm (optimal for protein expression), the cultures were again diluted 1:100 in fresh LB in a 250 mL 96 well plate. Half of each cell

culture was induced with 0.05% arabinose and incubated with 5 mM NSAA. The other half of the cell culture was used as negative control and were not induced with arabinose nor exposed to NSAA. Bacterial cells expressing either the curli operon, or MBP as control, were used. Bacterial growth was assessed using a Biotek Kinetics Machine, which measured OD every 5 minutes over a period of 24 hours. The data was then plotted and the linear slope during exponential growth was used to calculate doubling times. Because curli-expression cells derivatives had more irregular exponential growths than MBP-expressing cells, the first exponential growth "hump" of curli variants was used to determine doubling times.

Electron microscopy

[00235] Cells expressing curli fiber (with and without NSAA) were imaged by scanning electron microscopy (SEM) to assess the formation of fibers. 100 µl of cell cultures were filtered onto Nucleopore filter membranes (0.22 um pore size, GE Healthcare Bio-Sciences) under vacuum. The samples were fixed with 2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer for 2 hrs. at room temperature. The membranes were then gently washed with water, and the solvent was gradually exchanged to ethanol using an increasing gradient ethanol 15-minute incubation step (25 %, 50 %, 75 % and 100 % (v/v) ethanol). Samples were dried in a critical point dryer, placed onto SEM sample holders using silver adhesive (Electron Microscopy Sciences), and sputtered until they were coated in a 5 nm layer of Pt/Pd. Imaging was performed using a Zeiss Ultra 55 Field Emission SEM.

In vitro labeling and detection of bacterial cells

[00236] Cells expressing curli fibers were pelleted at 4,000 x g for 10 min. The cells were resuspended in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). The centrifugation and resuspension process was repeated 3 times to remove excess free NSAA present in the culture medium. For labelling, 100 µL of cell culture at OD_{600} of 10 were incubated with Cy5-DBCO dye in PBS containing 1% (w/v) BSA for 2 hours at 37 °C with agitation. The labeled cells were pelleted again, and resuspended in PBS containing 1% (w/v) BSA. This wash step was repeated 3 times to remove any unbound dye. Labeling was assessed by spotting 10 µl of labeled cells at OD 1 on a nitrocellulose membrane and detecting the fluorescence of Cy5 using a FluorChem™ M system (Protein

Simple). Flow cytometry was used to determine the fraction of cells that were labeled. Samples were analyzed with a BD LSRFortessa Flow Cytometer System (BD Biosciences).

[00237] For confocal microscopy, bacterial cells were also stained with 5 μM Hoechst 33342 (THERMO SCIENTIFIC) in PBS for 1 hour and rinsed 3 times using 1 mL of PBS. Subsequently, the bacterial cells were fixed in 2% paraformaldehyde for 10 minutes at 25 °C and washed 3 times with 1 mL of PBS. 10 μl of cells were deposited on a glass slide for imaging using a Leica SP5 X MP Inverted Confocal Microscope (Leica Microsystems).

Purification and identification of proteins labeled in vitro

[00238] Pre-labeled bacterial cells were pelleted at 4,000 x g for 20 min, and resuspended in lysis buffer (50 mM phosphate buffer, 100 mM NaCl, 7 M guanidinium hydrochloride). A protease inhibitor cocktail was added, and the suspension was stored at –20 °C overnight. The suspension was thawed on ice, and sonicated to fully lyse the cells (40% amplitude, 3 x 25 seconds ON, 35 seconds OFF). After centrifugation at 10,000 x g for 30 minutes, the supernatant was applied to a NiNTA column and incubated 2 hours with agitation at room temperature in the presence of 10 mM imidazole. His-tag purification was performed by washing the column 3 times with 40 mM imidazole, and eluting His-tagged CsgA proteins with 500 mM imidazole (both in 50 mM phosphate buffer with 100 mM NaCl). Eluates were concentrated using 3 kDa Amicon ultracentrifugal filters and analyzed by SDS-PAGE using a 4-20% polyacrylamide gel. Prior to staining with Coomassie Blue, Cy5 fluorescence within the SDS-PAGE gel was also detected. Mass spectrometry analysis was used to confirm the presence of CsgA in the Cy5-labeled bands.

***In vivo* tracking of click microbes**

[00239] Prior to the experiments, mice were given a non-fluorescent food (alfalfa-free) for at least 5 days to minimize background autofluorescence. At six hours prior to oral gavage with bacteria, mice were fasted and given water containing antibiotics (1 g/L kanamycin (RPI), 1 g/L spectinomycin (RPI)), inducer (10 g/L L-(+)-Arabinose (Sigma)) and NSAA (5 mM) based on their assigned experimental conditions. The starting culture of PBP8 transformed with pBbB8k-WT was expanded 1:100 dilution in LB containing 50 μg/mL kanamycin, while that of PBP8 transformed with pBbB8k-Mut and pEVOL-pAzF was expanded 1:100 dilution in LB containing 50 μg/mL kanamycin and spectinomycin to OD of 0.5. Log-phase cultures were centrifuged at 4,000 x g for 15 minutes at 4°C and resuspended

in 20% sucrose (OmniPur) in PBS to an OD of 10. Once the mice were fasted for six hours, 100 μL of the prepared log-phase bacteria were administered to the mice via oral gavage.

[00240] The alfalfa-free food and water were administered again to the mice to allow for proliferation and protein induction of the engineered bacteria *in vivo*. Meanwhile, the abdominal hair of the mice was removed to allow for imaging. 40 hours after gavage with the bacteria, pAzF and arabinose-containing water was switched for antibiotic-only-containing water, and the food was briefly remove to prepare the mice for dye administration.

[00241] Two hours after the water was switched and the food withheld, 100 μL of 100 μM of Cy5-DBCO in 20% sucrose in PBS were administered to the mice by oral gavage. Then, mice were imaged under anesthesia using IVIS Lumina II (PerkinElmer). The IVIS instrument was equipped with 10 narrow-band excitation filters (30 nm bandwidth) and 4 broadband emission filters (60- and 75-nm bandwidth). At the time point, the mice were imaged using excitation filter at 675 nm, and 500 nm for background subtraction, and emission filter at 695-770 nm, with field of view (FOV) = D (12.5 cm), fstop = 2 and medium binning. Living Image software version 4.3.1/4.4 was used for image analysis.

Whole GI tract *ex vivo* fluorescence imaging

[00242] The mouse experiments were performed in the same manner as the *in vivo* tracking experiment described above, except that 36 hours after administration of the Cy5-DBCO dye. the mice were euthanized to harvest the whole gastrointestinal (GI) tract for *ex vivo* fluorescence imaging. The GI tract was placed on the lid of 150x25 mm polystyrene tissue culture plate (Falcon) and imaged using IVIS Lumina II with the same setting as the *in vivo* imaging. After imaging, the GI tract was kept at -20°C for protein extraction.

Whole cecum fluorescence and protein analysis

[00243] The GI tract specimens used in the *ex vivo* fluorescence imaging were further analyzed for curli and Cy5-DBCO interaction. The mouse GI tracts were removed from -20°C. The cecum from each sample was excised, transferred to a 15-ml falcon tube, and crushed to homogenize. 3 mL of PBS was added to each sample, supplemented with 4 μL of benzonase nuclease (NOVAGEN) and 8 μL of 1 M magnesium sulfate ($MgSO_4$) (SIGMA). The samples were incubated at 37°C overnight.

[00244] Each sample was transferred to 2-ml microtube and centrifuged at 14,000 rpm for 10 minutes to extract the supernatant. The supernatant samples were transferred to another

set of tubes, and concentrated using 10-kDa Amicon Ultra 0.5 mL centrifugal filters (Millipore) at 10,000 rpm centifugation for 15 minutes twice. The pellet samples were resuspended with 200 μL of PBS. Both supernatant and pellet samples were diluted five times, mixed with 2X dyeless laemli buffer in 1:1 ratio, incubated at 95°C for 5 minutes and run on 12% SDS-PAGE gel (Bio-Rad) at 200 Volts for 30 minutes. After the gel electrophoresis, the gels were rinsed with water and imaged to detect the fluorescence of Cy5 using a FluorChem™ M system (Protein Simple). Then, the gels were stained with Coomassie Brilliant Blue to detect the presence of proteins and compared the size against standard protein ladder (Bio-Rad).

Re-labeling of click microbes from fecal pellets

[00245] During the *in vivo* tracking experiment, fecal pellets were collected from each mouse in a sterile manner at 24-hour time point after dye administration. The pellets were homogenized using bead mill homogenizer TissueLyser LT (Qiagen) and 5-mm stainless steel beads (Qiagen) with the frequency of 30 Hz for 2 minutes. The fecal homogenates were centrifuged briefly at 1,000 rpm for 1 minute to loosely pellet the large particulates. The supernatant was serially diluted and plated on appropriate antibiotic-selection plates. The colonies were grown, induced, and undergone similar *in vitro* labeling experiment and analysis mentioned previously.

References

Each of the following references is hereby incorporated by reference in its entirety.

1. Chin *et al.* (2002) *J. Am. Chem. Soc.* 124(31): 9026-7.
2. Lang and Chin (2014) *Chem. Rev.* 114: 4764-4806.
3. Datta *et al.* (2002) *J. Am. Chem. Soc.* 124(20): 5652-3.
4. Young *et al.* (2010) *J. Mol. Biol.* 395: 361-74.

CLAIMS

1. An engineered bacterium comprising:

a. an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and

b. a heterologous nucleic acid comprising a labeling codon, wherein the heterologous nucleic acid comprises a heterologous gene encoding a protein selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein;

wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with a nonstandard amino acid; and

wherein the orthogonal tRNA incorporates the nonstandard amino acid at the residue corresponding to the labeling codon during translation.

2. The engineered bacterium of claim 1, wherein the heterologous nucleic acid further encodes a polypeptide linker sequence.

3. The engineered bacterium of claim 2, wherein the polypeptide linker sequence is flexible.

4. The engineered bacterium of any one of claims 1-3, wherein the heterologous nucleic acid further encodes a polypeptide tag.

5. The engineered bacterium of any one of claims 1-4, wherein the labeling codon is located at a nucleic acid position within the heterologous gene encoding the protein.

6. The engineered bacterium of any one of claims 2-4, wherein the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide linker sequence.

7. The engineered bacterium of any one of claims 2-4, wherein the labeling codon is located at a nucleic acid position within the heterologous nucleic acid encoding the polypeptide tag.

8. The engineered bacterium of any one of claims 1-7, wherein the heterologous nucleic acid comprises 2, 3, 4, 5, 6, 7, or 8 labeling codons.

9. An engineered bacterium comprising:

a. an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and

b. a heterologous nucleic acid encoding a protein, wherein the protein comprises a nonstandard amino acid, and wherein the protein is selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein;

wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid; and

wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation.

10. The engineered bacterium of claim 9, wherein the protein further comprises a polypeptide linker.

11. The engineered bacterium of claim 10, wherein the polypeptide linker is flexible.

12. The engineered bacterium of any one of claims 9-11, wherein the protein further comprises a polypeptide tag.

13. The engineered bacterium of claim 4 or claim 12, wherein the polypeptide tag is selected from the group consisting of a poly-histidine tag, a myc tag, a FLAG tag, a hemagglutinin (HA) tag, and a V5 tag.

14. The engineered bacterium of any one of claims 9-13, wherein the protein comprises 2, 3, 4, 5, 6, 7, or 8 nonstandard amino acids.

15. The engineered bacterium of any one of claims 1-14, wherein the bacterial extracellular matrix protein is a fiber protein, a flagellar protein, or a pilin protein.

16. The engineered bacterium of any one of claims 1-14, wherein the protein is selected from the group consisting of InaV, Trat, and CsgA.

17. The engineered bacterium of claim 16, wherein the protein is CsgA.

18. The engineered bacterium of any one of claims 9-17, wherein the protein is CsgA and the nonstandard amino acid is located at an amino acid residue corresponding to residue 89 of wild-type CsgA.

19. The engineered bacterium of any one of claims 9-16, wherein the protein is InaV and the nonstandard amino acid is located at an amino acid residue corresponding to residue 174 of wild-type InaV.

20. The engineered bacterium of any one of claims 9-16, wherein the protein is Trat and the nonstandard amino acid is located at an amino acid residue corresponding to residue 200 of wild-type Trat.

21. The engineered bacterium of any one of claims 1-8, wherein the labeling codon is TAG.

22. The engineered bacterium of claim 1 or claim 9, wherein the orthogonal tRNA comprises an anticodon loop that specifically recognizes the codon UAG.

23. The engineered bacterium of any one of claims 1-22, wherein the nonstandard amino acid comprises a bioorthogonal reactive functional group.

24. The engineered bacterium of claim 23, wherein the bioorthogonal reactive functional group is selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a tetrazine moiety, or a norbornene moiety.

25. The engineered bacterium of any one of claims 1-24, wherein the nonstandard amino acid is selected from the group consisting of *p*-azidophenylalanine (*p*-AzF), azidohomoalanine (Aha), azidolysine, homopropargylglycine (Hpg), homoallylglycine (Hag), oxonorvaline (Onv), *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine (*p*-EtF), *para*-acetylphenylalanine (*p*-AcF), azidonorleucine (Anl), *trans*-crotylglycine (Tcg), selenomethionine (Se-Met), 2-aminooctynoic acid (Aoa), and propargylglycine (Pra).

26. The engineered bacterium of claim 25, wherein the nonstandard amino acid is *p*-AzF.

27. The engineered bacterium of any one of claims 1-26, wherein the engineered bacterium is non-pathogenic.

28. The engineered bacterium of any one of claims 1-27, wherein the engineered bacterium is of the species *Escherichia coli*.

29. The engineered bacterium of any one of claims 1-28, wherein the engineered bacterium is of an *Escherichia coli* strain selected from the group consisting of C321, C321.ΔA and C321.ΔA.exp.

30. The engineered bacterium of any one of claims 1-28, wherein the engineered bacterium is of the *Escherichia coli* Nissle 1917 strain.

31. The engineered bacterium of any one of claims 1-30, wherein the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a codon.

32. The engineered bacterium of claim 31, wherein the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a TAG codon.

33. The engineered bacterium of any one of claims 1-30, wherein the engineered bacterium comprises a genome that has not been recoded to reduce or eliminate a codon.

34. The engineered bacterium of any one of claims 1-33, wherein the engineered bacterium further comprises a *prfA* gene deletion.

35. The engineered bacterium of any one of claims 1-34, wherein the orthogonal aminoacyl tRNA synthetase is an Archea aminoacyl tRNA synthetase or a bacterial aminoacyl tRNA synthetase.

36. The engineered bacterium of any one of claims 1-35, wherein the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina*, *Desufitobacterium*, *Pyrococcus* or *Methanocaldococcus* aminoacyl tRNA synthetase gene.

37. The engineered bacterium of any one of claims 1-36, wherein the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina mazei*, *Methanosarcina barkeri*, *Desulfitobacterium hafniense, Pyrococcus horikoshii*, or *Methanocaldococcus jannaschii* aminoacyl tRNA synthetase gene.

38. The engineered bacterium of any one of claims 1-37, wherein the orthogonal translation system is located in the bacterial genome.

39. The engineered bacterium of any one of claims 1-37, wherein the orthogonal translation system is located in a plasmid.

40. The engineered bacterium of claim 39, wherein the plasmid is pEVOL-pAzF.

41. The engineered bacterium of any one of claims 1-40, wherein the expression of the heterologous nucleic acid is under the control of a constitutive promoter.

42. The engineered bacterium of any of claims 1-40, wherein the expression of the heterologous nucleic acid is under the control of an inducible promoter.

43. The engineered bacterium of claim 42, wherein the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

44. The engineered bacterium of any one of claims 1-43, wherein the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the heterologous gene encoding the orthogonal tRNA is under the control of a constitutive promoter.

45. The engineered bacterium of any one of claims 1-43, wherein the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the heterologous gene encoding the orthogonal tRNA is under the control of an inducible promoter.

46. The engineered bacterium of claim 45, wherein the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

47. A biofilm comprising the engineered bacterium of any one of claims 1-46.

48. A pharmaceutical composition comprising an engineered bacterium of any one of claims 1-46 and a pharmaceutically-acceptable excipient.

49. The pharmaceutical composition of claim 48, wherein the composition is formulated for oral administration to a subject.

50. The pharmaceutical composition of claim 48, wherein the composition is formulated for rectal administration to a subject.

51. The pharmaceutical composition of claim 49 or claim 50, wherein the subject is a mammalian subject.

52. The pharmaceutical composition of claim 51, wherein the mammalian subject is a human subject.

53. The pharmaceutical composition of any one of claims 48-52, wherein the pharmaceutical composition is formulated as a pill, a capsule, a lozenge, or a suppository.

54. A method for labeling of an engineered bacterium in a subject *in vivo*, the method comprising:

(a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group; and

(b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group,

wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid of the protein,

thereby labeling the engineered bacterium in the subject *in vivo*.

55. A method for detecting the distribution of an engineered bacterium in a subject *in vivo*, the method comprising:

(a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group;

(b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid; and

(c) detecting the detectable compound in the subject,

thereby detecting the distribution of the engineered bacterium in the subject *in vivo*.

56. A method for diagnosing a gastrointestinal disease in a subject, the method comprising

(a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group;

(b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the detectable compound to the nonstandard amino acid; and

(c) detecting the distribution of the detectable compound in the gastrointestinal tract of the subject, thereby diagnosing a gastrointestinal disease in the subject.

57. A method for detecting the distribution of a biofilm produced by an engineered bacterium in a subject, the method comprising:

(a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid to a subject, and wherein the nonstandard amino acid comprises a first bioorthogonal reactive functional group;

(b) administering to the subject a detectable compound comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo,* thereby attaching the detectable compound to the nonstandard amino acid; and

(c) detecting the detectable compound in the subject,

thereby determining the distribution of the biofilm produced by the engineered bacterium in the subject.

58. A method for delivering a drug to a subject in need thereof, the method comprising:

(a) administering to the subject an engineered bacterium comprising a protein having at least one nonstandard amino acid, wherein said nonstandard amino acid comprises a first bioorthogonal reactive functional group;

(b) administering to the subject a drug comprising a second bioorthogonal reactive functional group, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group *in vivo*, thereby attaching the drug to the nonstandard amino acid,

thereby delivering the drug to the subject in need thereof.

59. The method of any one of claims 54-58, wherein the engineered bacterium comprises:

(a) an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and

(b) a heterologous nucleic acid encoding the protein having the at least one nonstandard amino acid;

wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid; and

wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation.

60. The method of any one of claims 54-58, wherein step (a) is performed concurrently with step (b).

61. The method of any one of claims 54-58, wherein step (a) is performed before step (b).

62. The method of any one of claims 54-60, further comprising administering a nonstandard amino acid to the subject.

63. The method of claim 62, wherein the nonstandard amino acid is administered to the subject prior to administering the detectable compound or the drug to the subject.

64. The method of any one of claims 54-63, wherein the first bioorthogonal reactive functional group is selected from the group consisting of an azide moiety, a ketone moiety, an alkyne moiety, an alkene moiety, a tetrazine moiety, and a norbornene moiety.

65. The method of any one of claims 54-64, wherein the second bioorthogonal reactive functional group is selected from the group consisting of a hydrazine moiety, a hydroxylamine moiety, an alkyne moiety, an azide moiety, a tetrazine moiety, and a norbornene moiety.

66. The method of any one of claims 54-65, wherein the first bioorthogonal reactive functional group is a ketone moiety, and the second bioorthogonal reactive functional group is either a hydrazine moiety or a hydroxylamine moiety.

67. The method of any one of claims 54-65, wherein the first bioorthogonal reactive functional group is an azide moiety, and the second bioorthogonal reactive functional group is an alkyne moiety.

68. The method of any one of claims 54-65, wherein the first bioorthogonal reactive functional group is an alkyne moiety, and the second bioorthogonal reactive functional group is an azide moiety.

69. The method of any one of claims 54-68, wherein the first bioorthogonal reactive functional group reacts with the second bioorthogonal reactive functional group via a click chemistry reaction.

70. The method of any one of claims 54-69, wherein the reaction between the first bioorthogonal reactive functional group and the second bioorthogonal reactive functional group requires a catalytic agent.

71. The method of any one of claims 54-69, wherein the reaction between the first bioorthogonal reactive functional group and the second bioorthogonal reactive functional group does not require a catalytic agent.

72. The method of any one of claims 54-70 further comprising administering a catalytic agent to the subject.

73. The method of claim 70 or claims 72, wherein the catalytic agent is copper or nickel.

74. The method of any one of claims 54-73, wherein the detectable compound comprises a fluorescent moiety, a radioactive moiety, a colorimetric dye, a fluorescent dye, a luminescent dye, a magnetic resonance imaging (MRI) contrast agent, a CT contrast agent, a PET contrast agent, or an ultrasound contrast agent.

75. The method of any one of claims 54-74, wherein the protein further comprises a polypeptide linker.

76. The method of claim 75, wherein the polypeptide linker is flexible.

77. The method of any one of claims 54-76, wherein the protein further comprises a polypeptide tag.

78. The method of claim 77, wherein the polypeptide tag is selected from the group consisting of a poly-histidine tag, a myc tag, a FLAG tag, a hemagglutinin (HA) tag, and a V5 tag.

79. The method of any one of claims 54-78, wherein the protein is selected from the group consisting of a secreted protein, a membrane protein, or a bacterial extracellular matrix protein.

80. The method of claim 79, wherein the bacterial extracellular matrix protein is a curli fiber protein, a flagellar protein, or a pilin protein.

81. The method of any one of claims 54-79, wherein the protein is selected from the group consisting of InaV, Trat, and CsgA.

82. The method of claim 81, wherein the protein is CsgA.

83. The method of any one of claims 54-82, wherein the protein is CsgA and the nonstandard amino acid is located at an amino acid residue corresponding to residue 89 of wild-type CsgA.

84. The method of any one of claims 54-81, wherein the protein is InaV and the nonstandard amino acid is located at an amino acid residue corresponding to residue 174 of wild-type InaV.

85. The method of any one of claims 54-81, wherein the protein is Trat and the nonstandard amino acid is located at an amino acid residue corresponding to residue 200 of wild-type Trat.

86. The method of any one of claims 54-85, wherein the nonstandard amino acid is selected from the group consisting of *p*-azidophenylalanine (*p*-AzF), azidohomoalanine (Aha), azidolysine, homopropargylglycine (Hpg), homoallylglycine (Hag), oxonorvaline (Onv), *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine

(*p*-EtF), *para*-acetylphenylalanine (*p*-AcF), azidonorleucine (Anl), trans-crotylglycine (Tcg), selenomethionine (Se-Met), 2-aminooctynoic acid (Aoa), and propargylglycine (Pra).

87. The method of claim 86, wherein the nonstandard amino acid is *p*-AzF.

88. The method of claim 87, wherein the detectable compound is DBCO-Cy5.

89. The method of any one of claims 54-88, wherein the engineered bacterium is non-pathogenic.

90. The method of any one of claims 54-89, wherein the engineered bacterium is of the species *Escherichia coli*.

91. The method of any one of claims 54-90, wherein the engineered bacterium is of an *Escherichia coli* strain selected from the group consisting of C321, C321.ΔA and C321.ΔA.exp.

92. The method of any one of claims 54-90, wherein the engineered bacterium is of the *Escherichia coli* Nissle 1917 strain.

93. The method of any one of claims 54-92, wherein the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a codon.

94. The method of any one of claims 54-93, wherein the engineered bacterium comprises a genome that has been recoded to reduce or eliminate a TAG codon.

95. The method of any one of claims 54-92, wherein the engineered bacterium comprises a genome that has not been recoded to reduce or eliminate a codon.

96. The method of any one of claims 54-95, wherein the engineered bacterium further comprises a *prfA* gene deletion.

97. The method of any one of claims 54-96, wherein the orthogonal aminoacyl tRNA synthetase is an Archea aminoacyl tRNA synthetase or a bacterial aminoacyl tRNA synthetase.

98. The method of any one of claims 54-97, wherein the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina, Desufitobacterium, Pyrococcus* or *Methanocaldococcus* aminoacyl tRNA synthetase gene.

99. The method of any one of claims 54-98, wherein the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase is a *Methanosarcina mazei, Methanosarcina barkeri, Desulfitobacterium hafniense, Pyrococcus horikoshii,* or *Methanocaldococcus jannaschii* aminoacyl tRNA synthetase gene.

100. The method of any one of claims 54-99, wherein the orthogonal translation system is located in the bacterial genome.

101. The method of any one of claims 54-99, wherein the orthogonal translation system is located in a plasmid.

102. The method of claim 101, wherein the plasmid is pEVOL-pAzF.

103. The method of any one of claims 54-102, wherein the expression of the heterologous nucleic acid is under the control of a constitutive promoter.

104. The method of any one of claims 54-102, wherein the expression of the heterologous nucleic acid is under the control of an inducible promoter.

105. The method of claim 104, wherein the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

106. The method of any one of claims 54-105, wherein the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the orthogonal tRNA is under the control of a constitutive promoter.

107. The method of any one of claims 54-105, wherein the expression of the heterologous gene encoding the orthogonal aminoacyl tRNA synthetase and/or the orthogonal tRNA is under the control of an inducible promoter.

108. The method of claim 107, wherein the inducible promoter is responsive to an inducer selected from the group consisting of IPTG, arabinose, and tetracycline.

109. The method of claim 104, further comprising administering to the subject an inducer that regulates the inducible promoter.

110. The method of claim 107, further comprising administering to the subject an inducer that regulates the inducible promoter.

111. The method of claim 109 or claim 110, wherein the inducer is administered to the subject prior to administering the detectable compound or the drug to the subject.

112. The method of any one of claims 54-111, wherein the engineered bacterium is administered to the subject orally or rectally.

113. The method of any one of claims 54-112, wherein the engineered bacterium is comprised in a pharmaceutical composition.

114. The method of any one of claims 54-112, wherein the detectable compound or the drug is administered to the subject orally, subcutaneously, intravenously, rectally, or transdermally.

115. The method of any one of claims 54-112, wherein the detectable compound or the drug is comprised in a pharmaceutical composition.

116. The method of any one of claims 109-115, wherein the inducer is comprised in a pharmaceutical composition.

117. The method of any one of claims 54-116, wherein the detectable compound or the drug further comprises a drug delivery vehicle selected from the group consisting of a nanocarrier, a nanoparticle, a liposome, a dendrimer, a carbon nanotube, a micelle, and a protein.

118. The method of claim 58, wherein the drug is delivered to the gastrointestinal tract of the subject.

119. The method of claim 58, wherein the drug is delivered to the mouth, esophagus, stomach, large intestine, small intestine, rectum, colon, or anal canal of the subject..

FIG. 1A

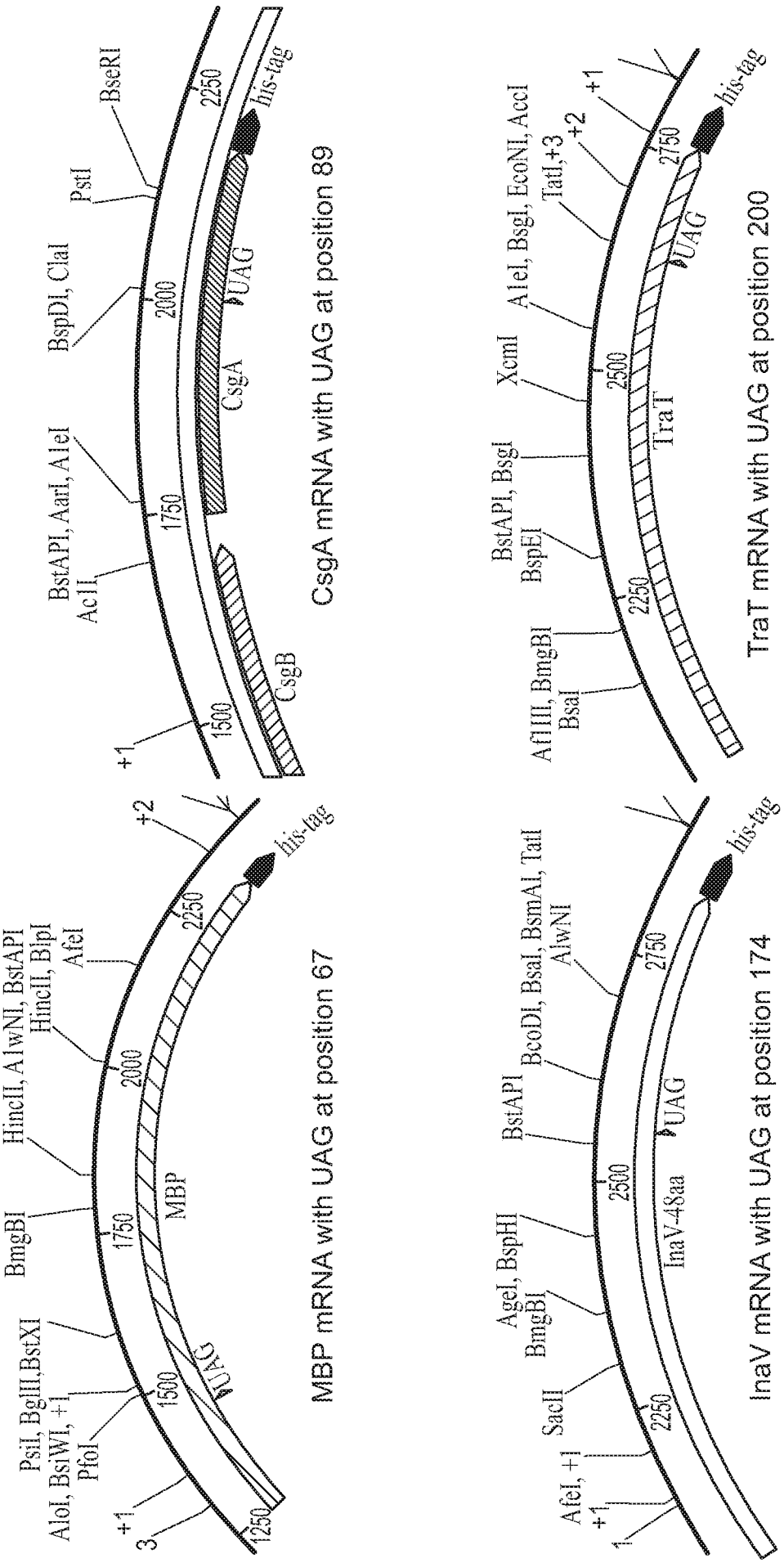
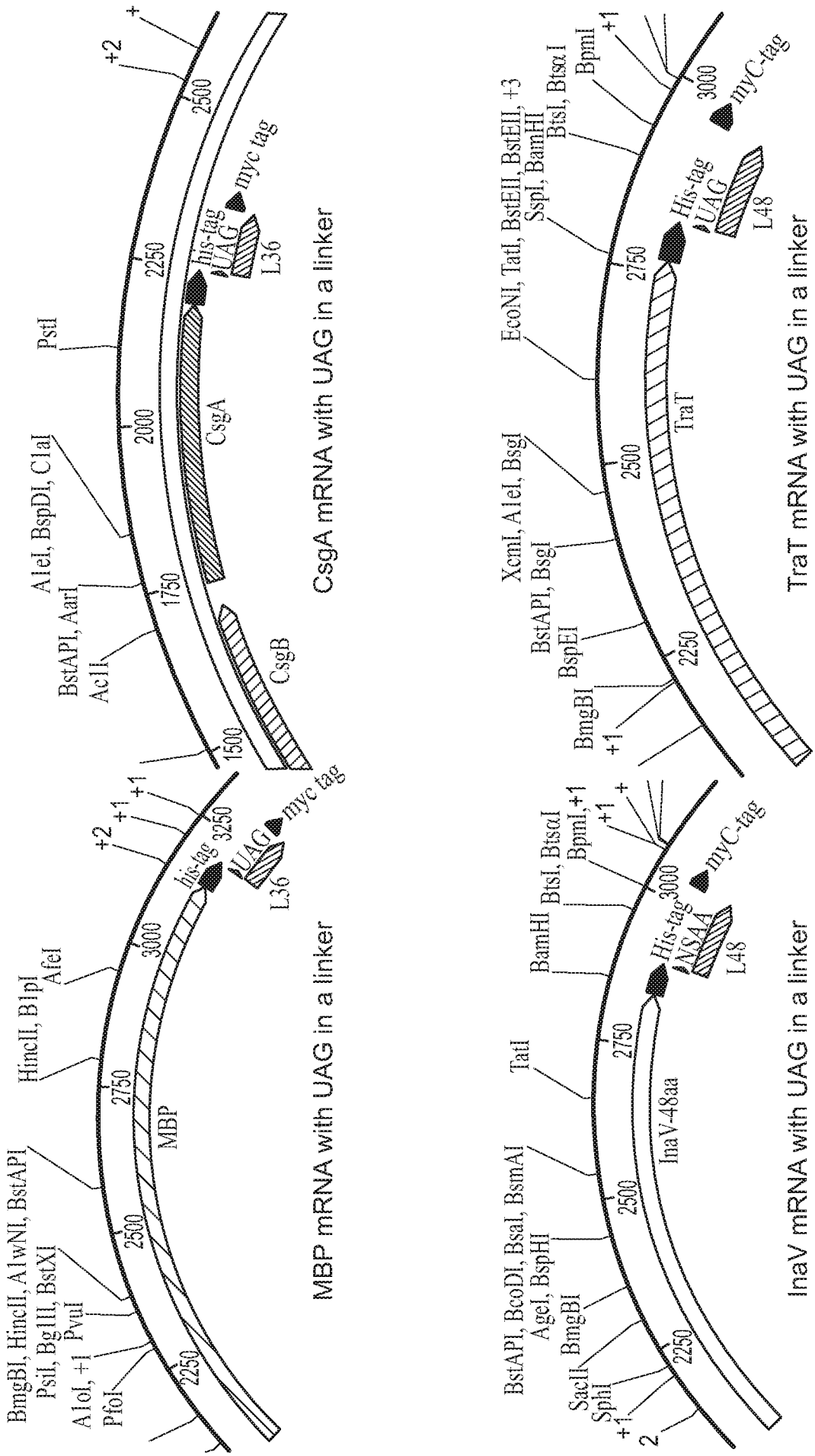
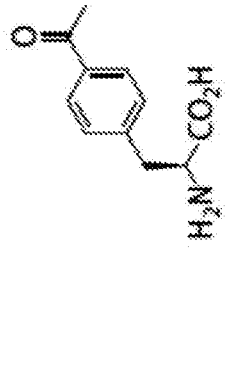
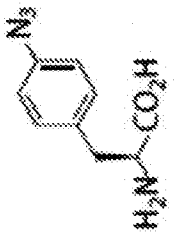

MBP mRNA with UAG at position 67
PsiI, BglII,BstXI
AloI, BsiWI, +1
PfoI
BmgBI
HincII, A1wNI, BstAPI
HincII, BlpI
AfeI
MBP
UAG
his-tag
+1
3
1250
1500
1750
2000
2250
+2
CsgA mRNA with UAG at position 89
BstAPI, AarI, A1eI
AclI
BspDI, ClaI
PstI
BseRI
CsgA
CsgB
UAG
his-tag
+1
1500
1750
2000
2250
TraT mRNA with UAG at position 200
BstAPI, BsgI
BspEI
AflIII, BmgBI
BsaI
XcmI
A1eI, BsgI, EcoNI, AccI
TatI,+3
+2
+1
TraT
UAG
his-tag
2250
2500
2750
InaV mRNA with UAG at position 174
AfeI, +1
+1
1
SacII
AgeI, BspHI
BmgBI
BstAPI
BcoDI, BsaI, BsmAI, TatI
A1wNI
InaV-48aa
UAG
his-tag
2250
2500
2750

FIG. 1B


BmgBI, HincII, A1wNI, BstAPI
PsiI, Bg1II, BstXI
A1oI, +1
PfoI
PvuI
2250
2500
2750
HincII, B1pI
AfeI
MBP
3000
his-tag
UAG
myc tag
L36
MBP mRNA with UAG in a linker
PstI
A1eI, BspDI, C1aI
BstAPI, AarI
AclI
1500
1750
2000
2250
2500
CsgA
CsgB
his-tag
UAG
myc tag
L36
CsgA mRNA with UAG in a linker
BstAPI, BcoDI, BsaI, BsmAI
AgeI, BspHI
SacII
BmgBI
SphI
2250
2500
TatI
2750
BamHI
BtsI, BtsaI
BpmI, +1
InaV-48aa
His-tag
WSAA
3000
myC-tag
L48
InaV mRNA with UAG in a linker
XcmI, A1eI, BsgI
BstAPI, BsgI
BspEI
BmgBI
2250
2500
EcoNI, TatI, BstEII, BstEII, +3
SspI, BamHI
BtsI, BtsaI
BpmI
2750
TraT
His-tag
UAG
3000
myC-tag
L48
TraT mRNA with UAG in a linker



Acetylphenylalanine (p-AcF)



Azidophenylalanine (p-AzF)

FIG. 2A

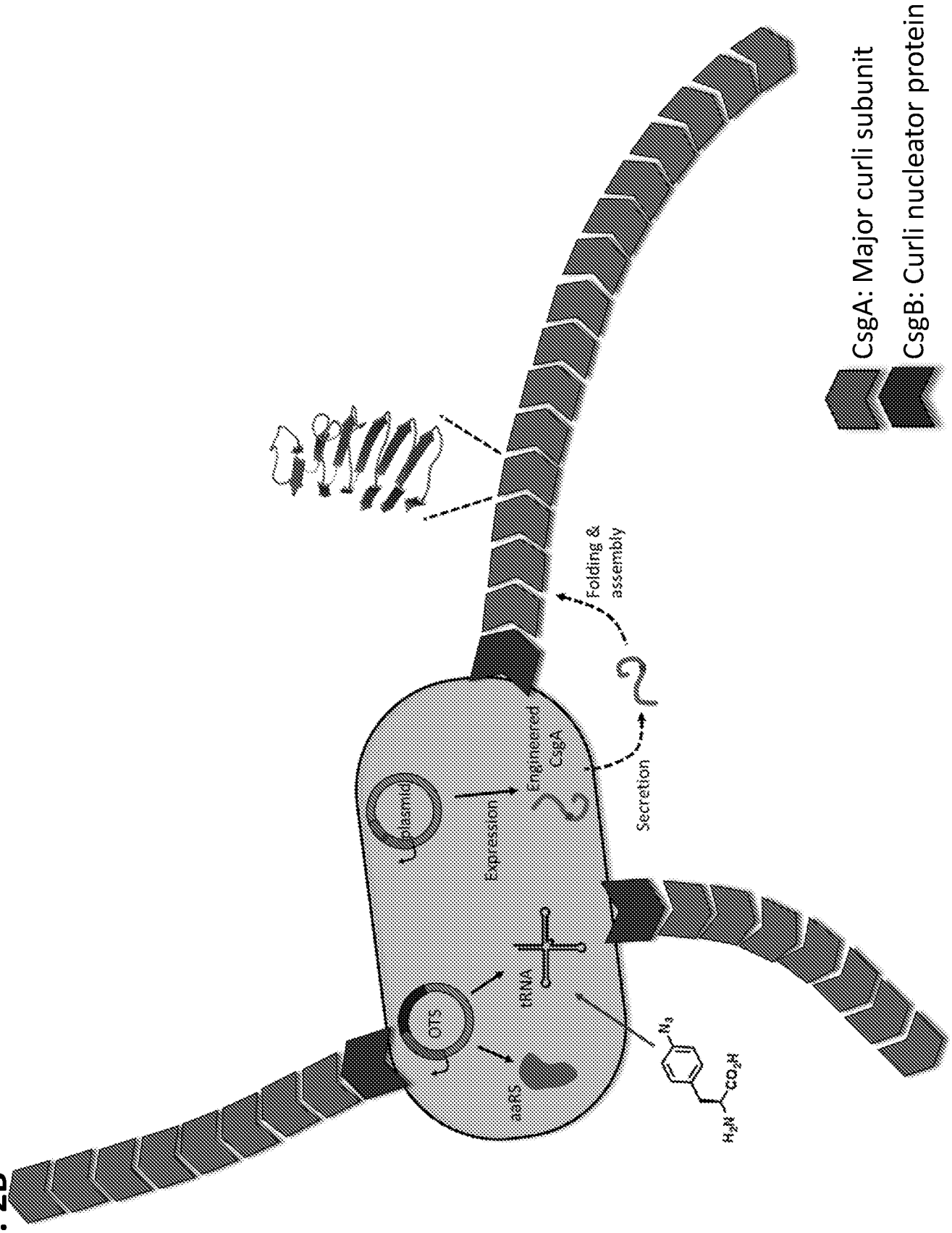

CsgA: Major curli subunit
CsgB: Curli nucleator protein
Folding & assembly
Secretion
Engineered CsgA
Expression
Plasmid
tRNA
OTS
aaRS
N_3
CO_2H
H_2N

FIG. 2B

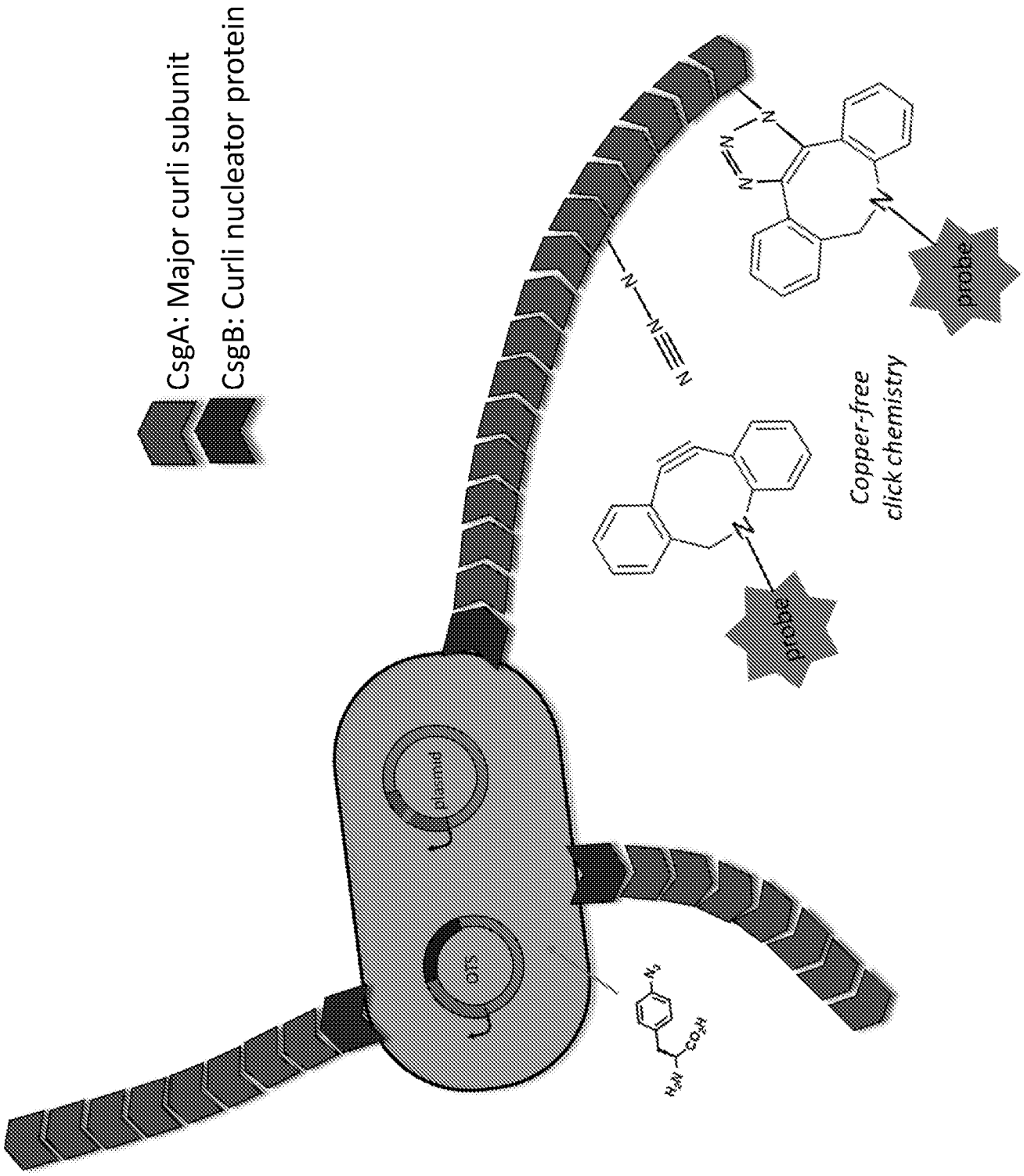

CsgA: Major curli subunit
CsgB: Curli nucleator protein
Signal
SUC
probe
probe
Copper-free
click chemistry

FIG. 2C

FIG. 3

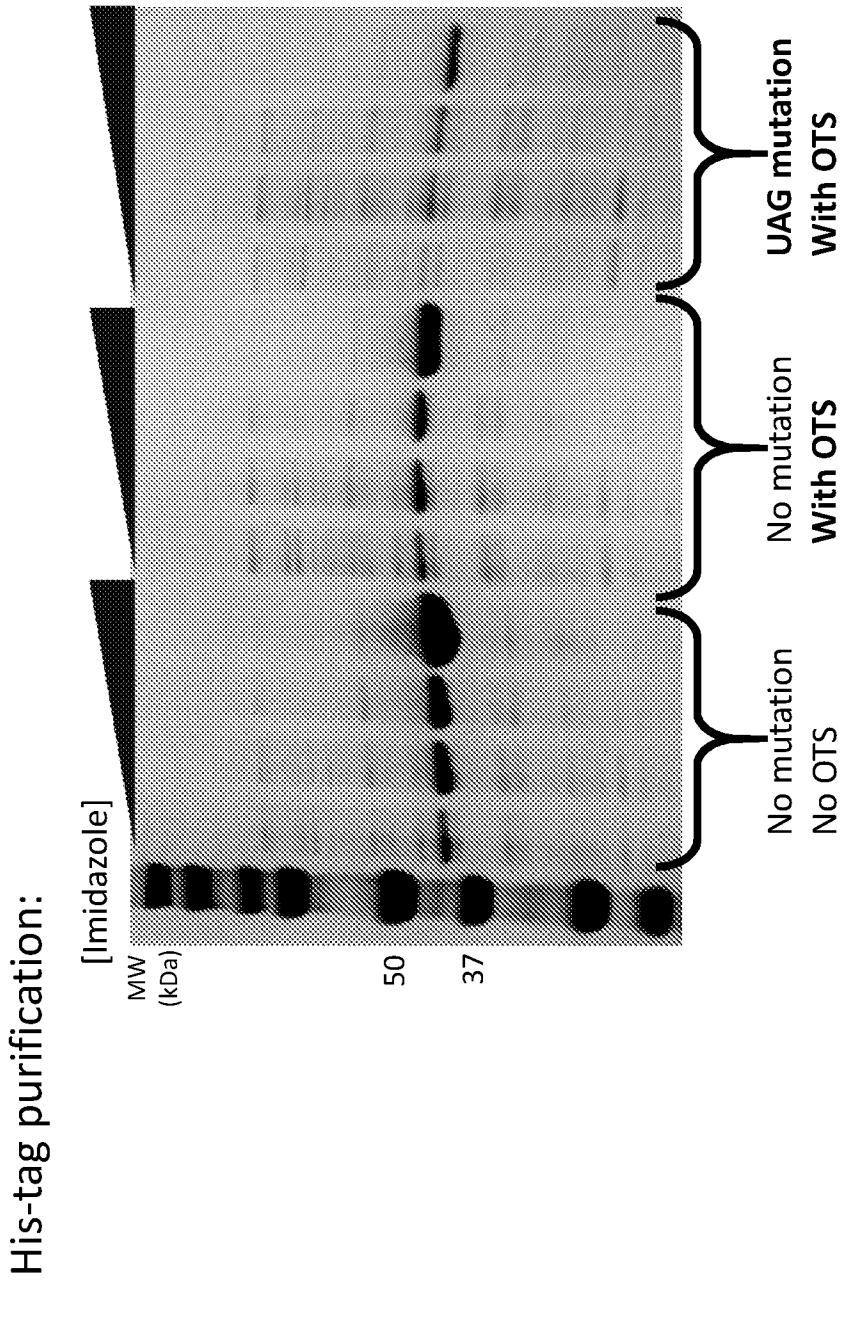

His-tag purification:
[Imidazole]
MW (kDa)
50
37
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS
Imidazole gradient:
10, 20, 40, 500 mM

FIG. 4A

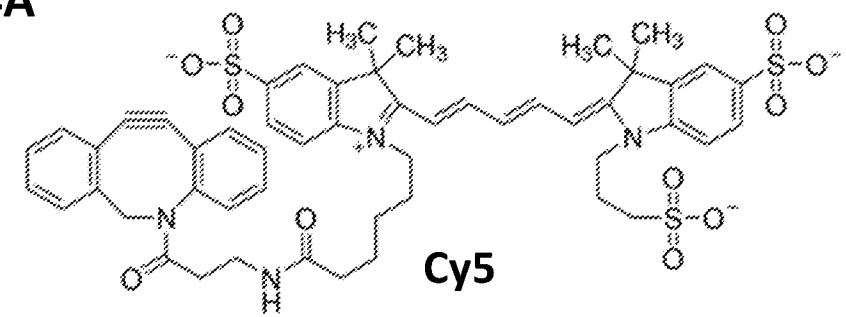


FIG. 4B

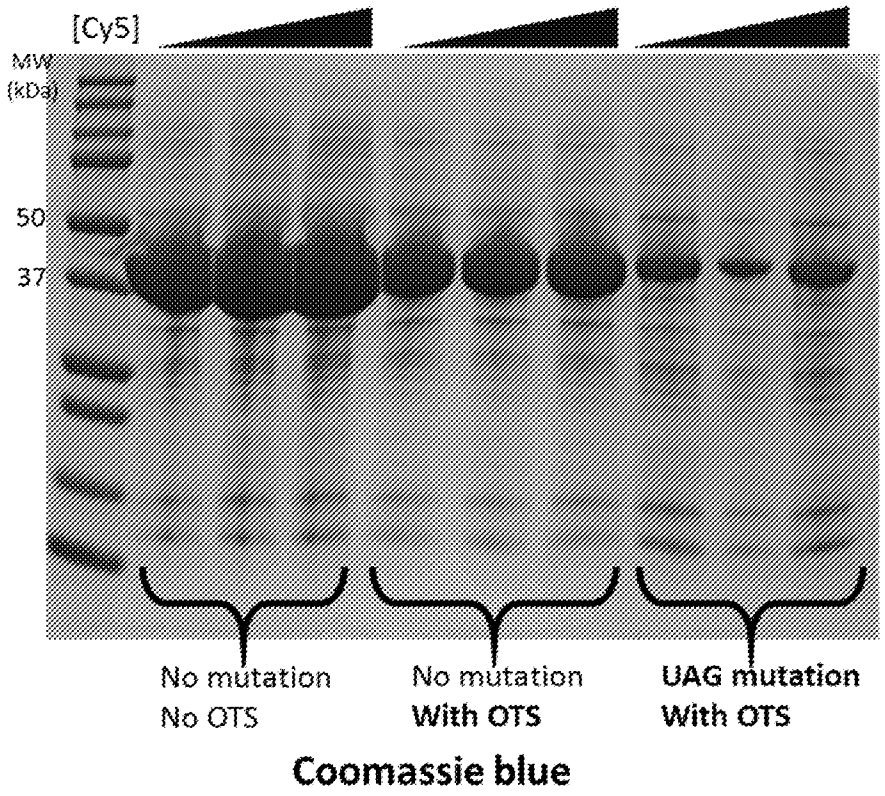


FIG. 4C

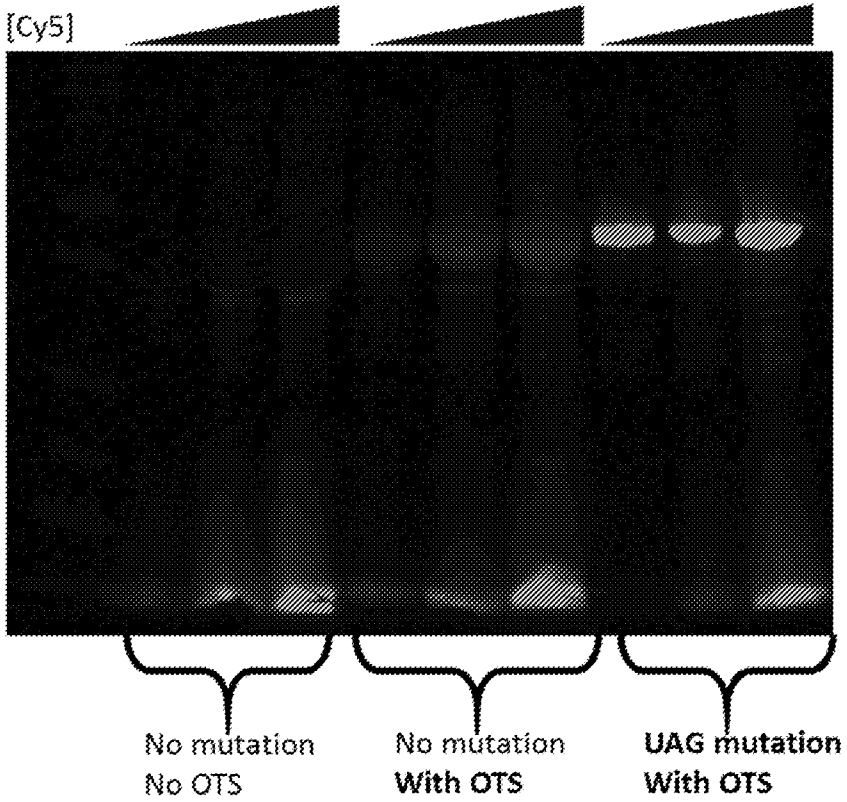


FIG. 5

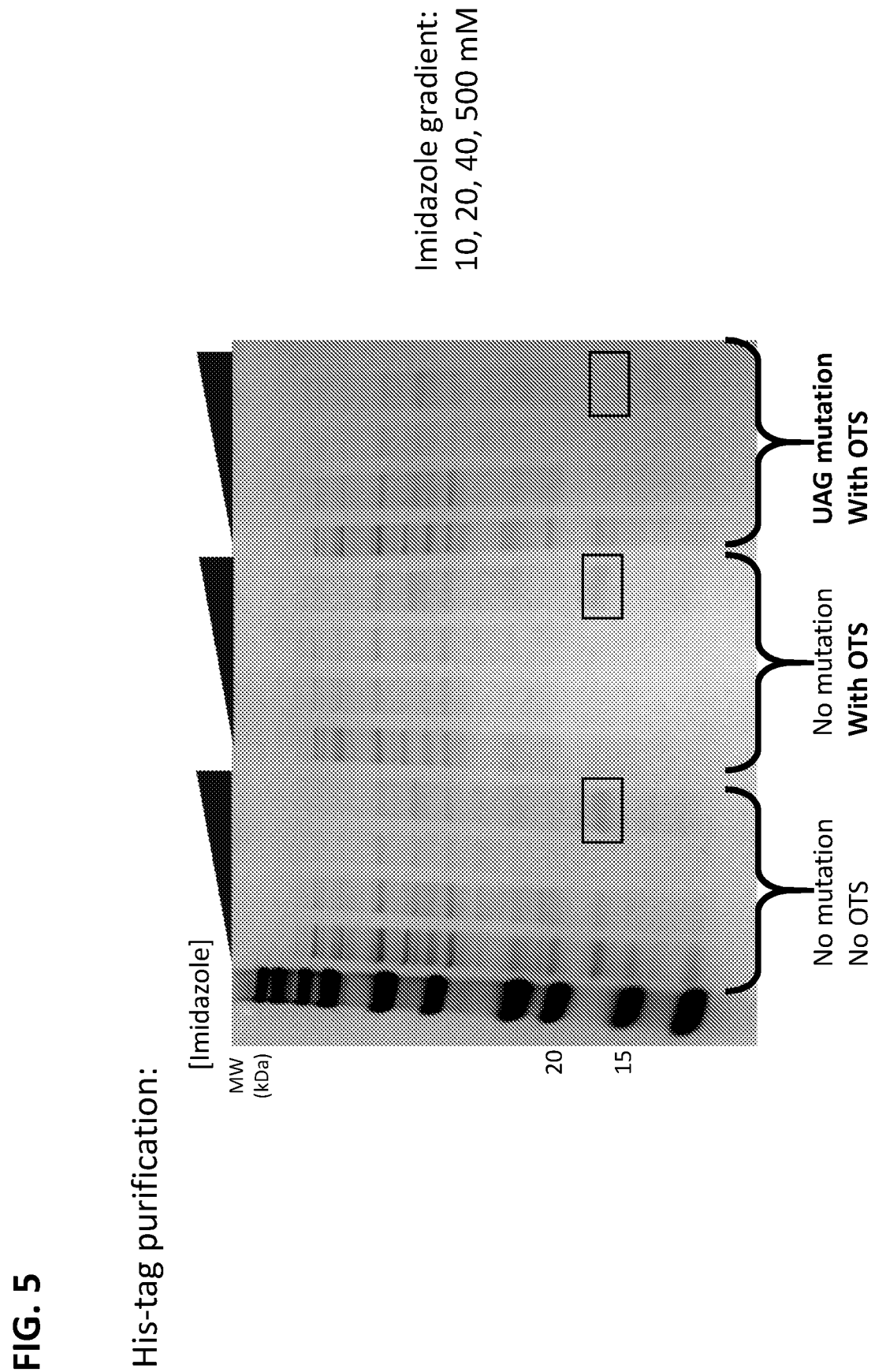

His-tag purification:
[Imidazole]
MW (kDa)
20
15
Imidazole gradient:
10, 20, 40, 500 mM
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS

FIG. 6A

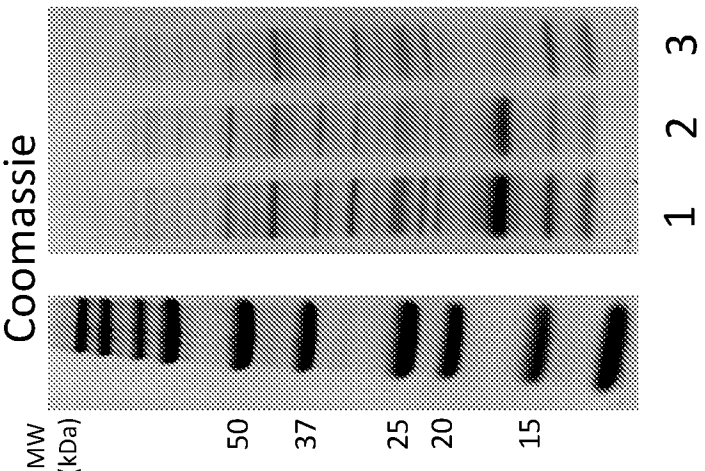

Coomassie
MW (kDa)
50
37
25
20
15
1 No mutation No OTS
2 No mutation With OTS
3 UAG mutation With OTS
1 2 3

FIG. 6B

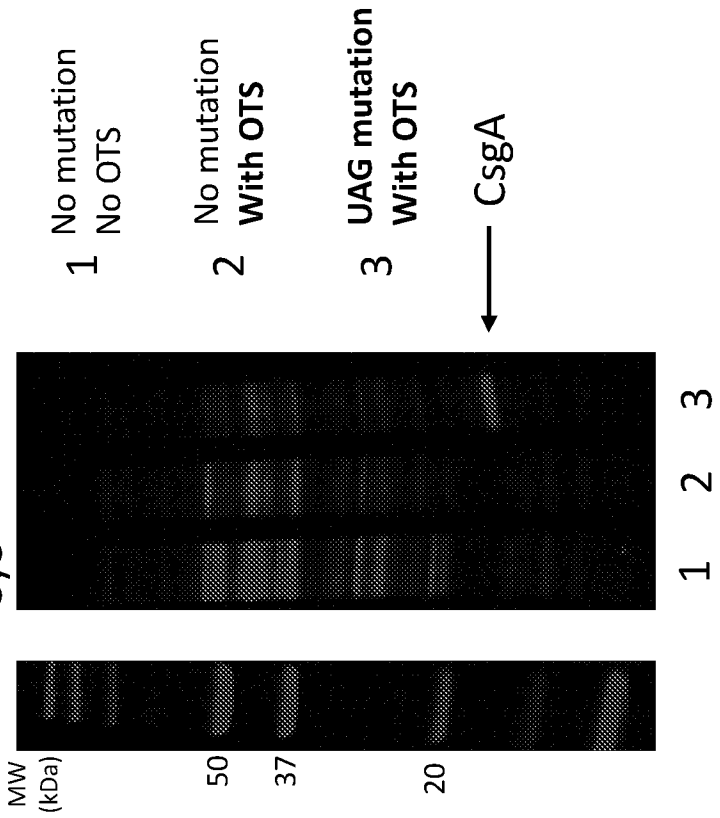
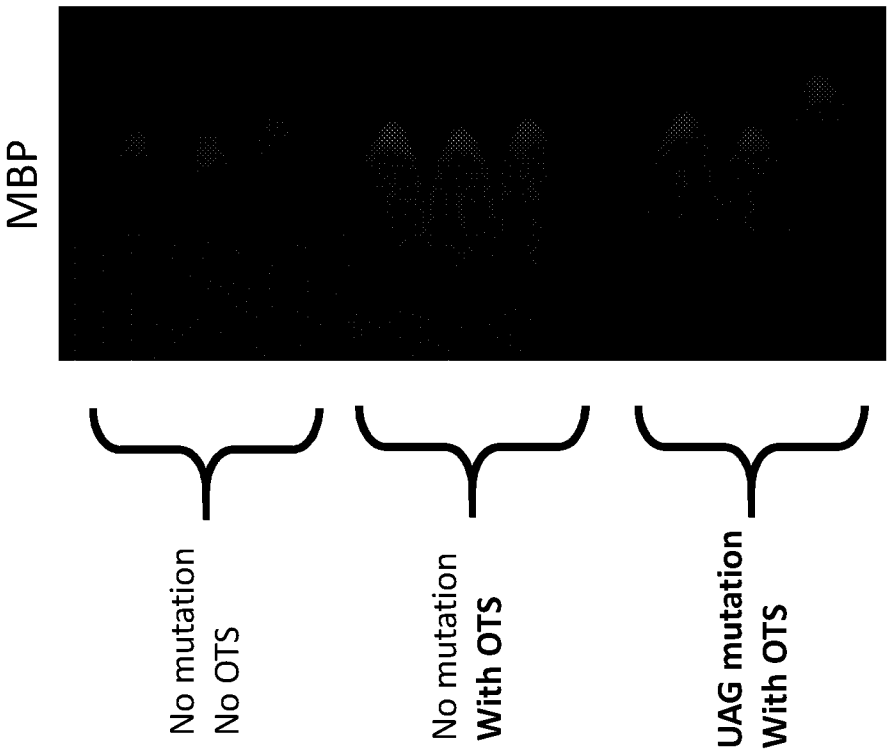

Cy5
MW (kDa)
50
37
20
1 No mutation No OTS
2 No mutation With OTS
3 UAG mutation With OTS
CsgA
1 2 3

FIG. 7


MBP
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS

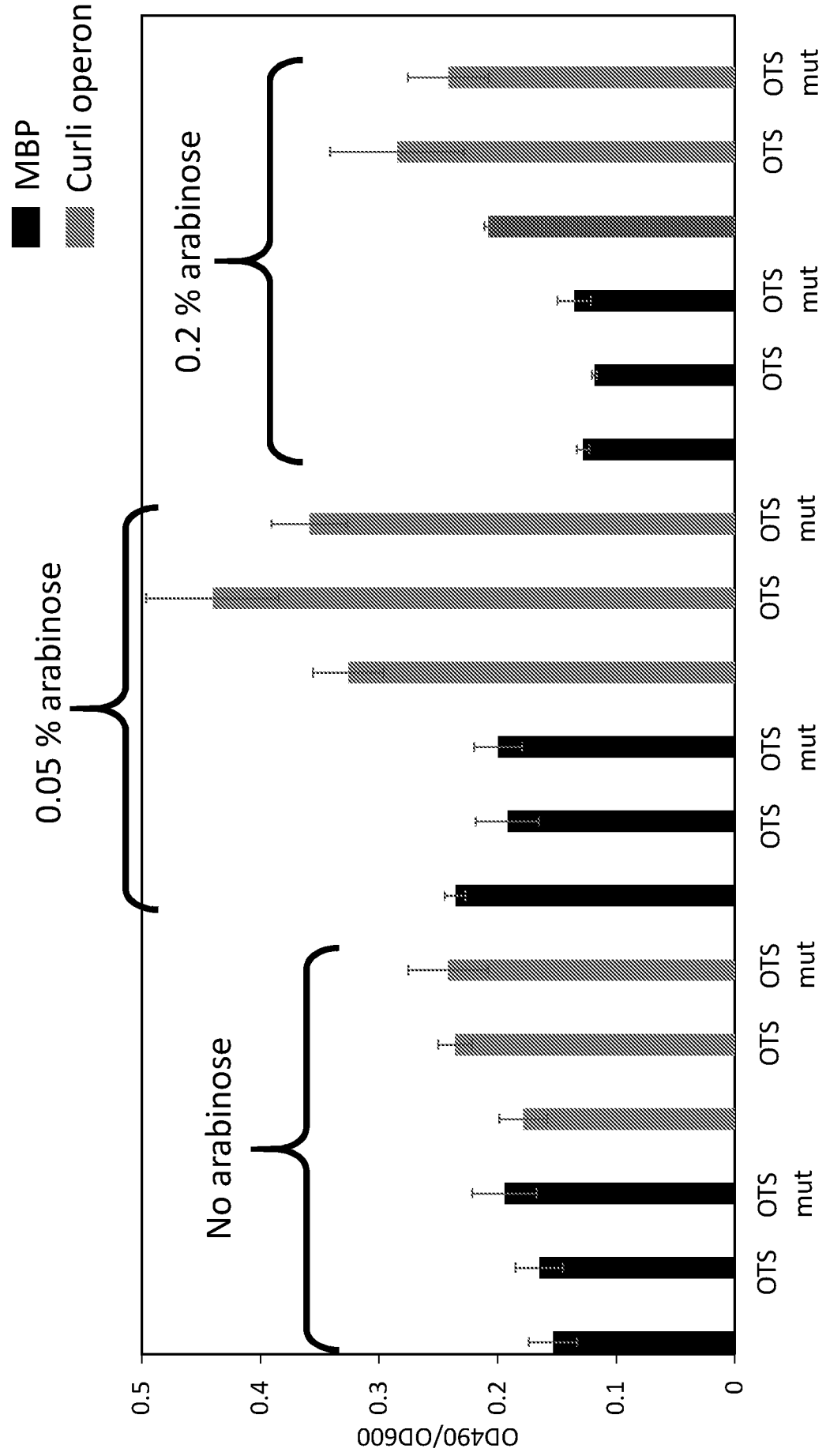

MBP
Curli operon
0.05 % arabinose
0.2 % arabinose
No arabinose
OTS
OTS mut
OD490/OD600
0
0.1
0.2
0.3
0.4
0.5

FIG. 8

FIG. 9

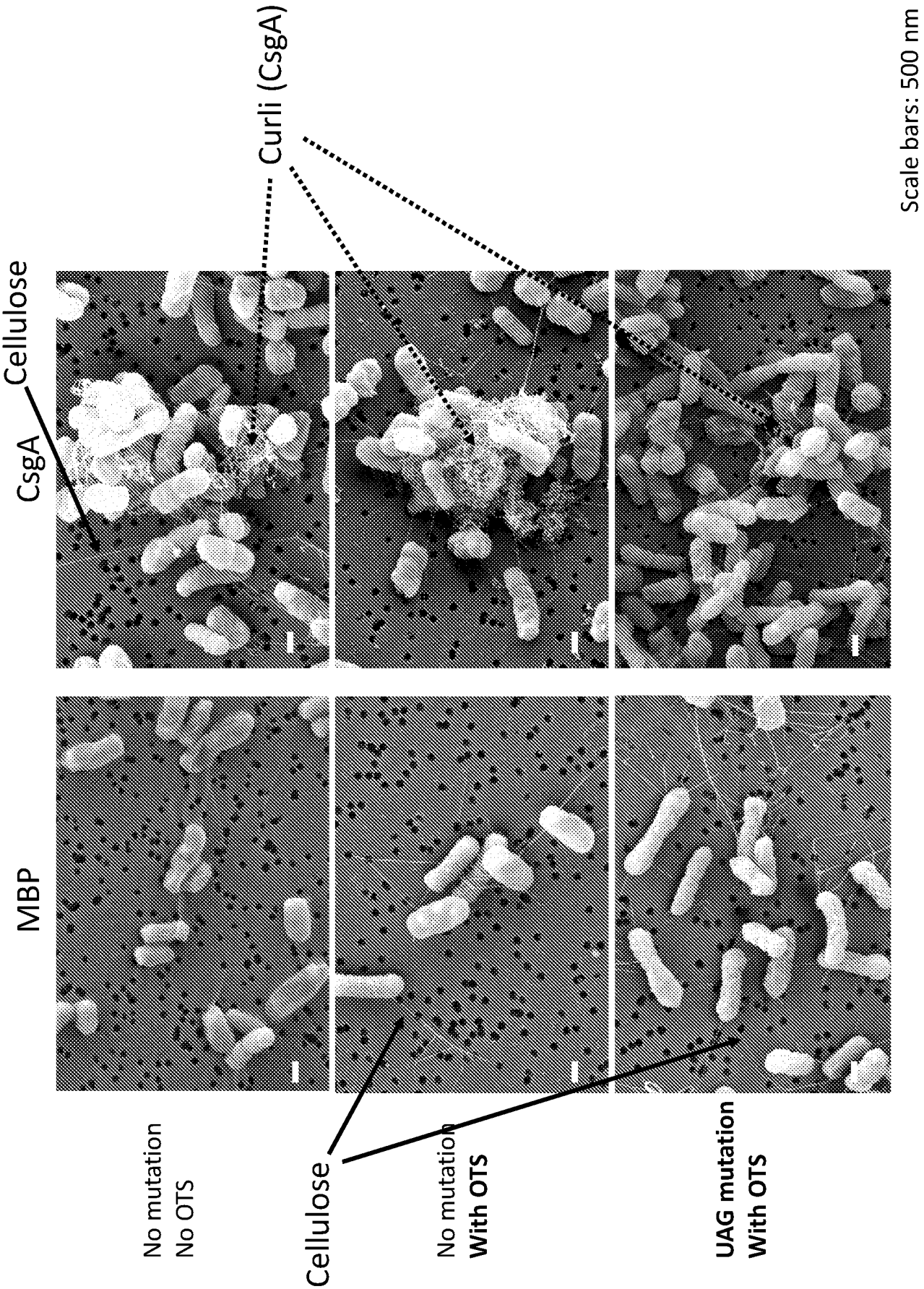

MBP
CsgA
Cellulose
Curli (CsgA)
Cellulose
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS
Scale bars: 500 nm

FIG. 10A

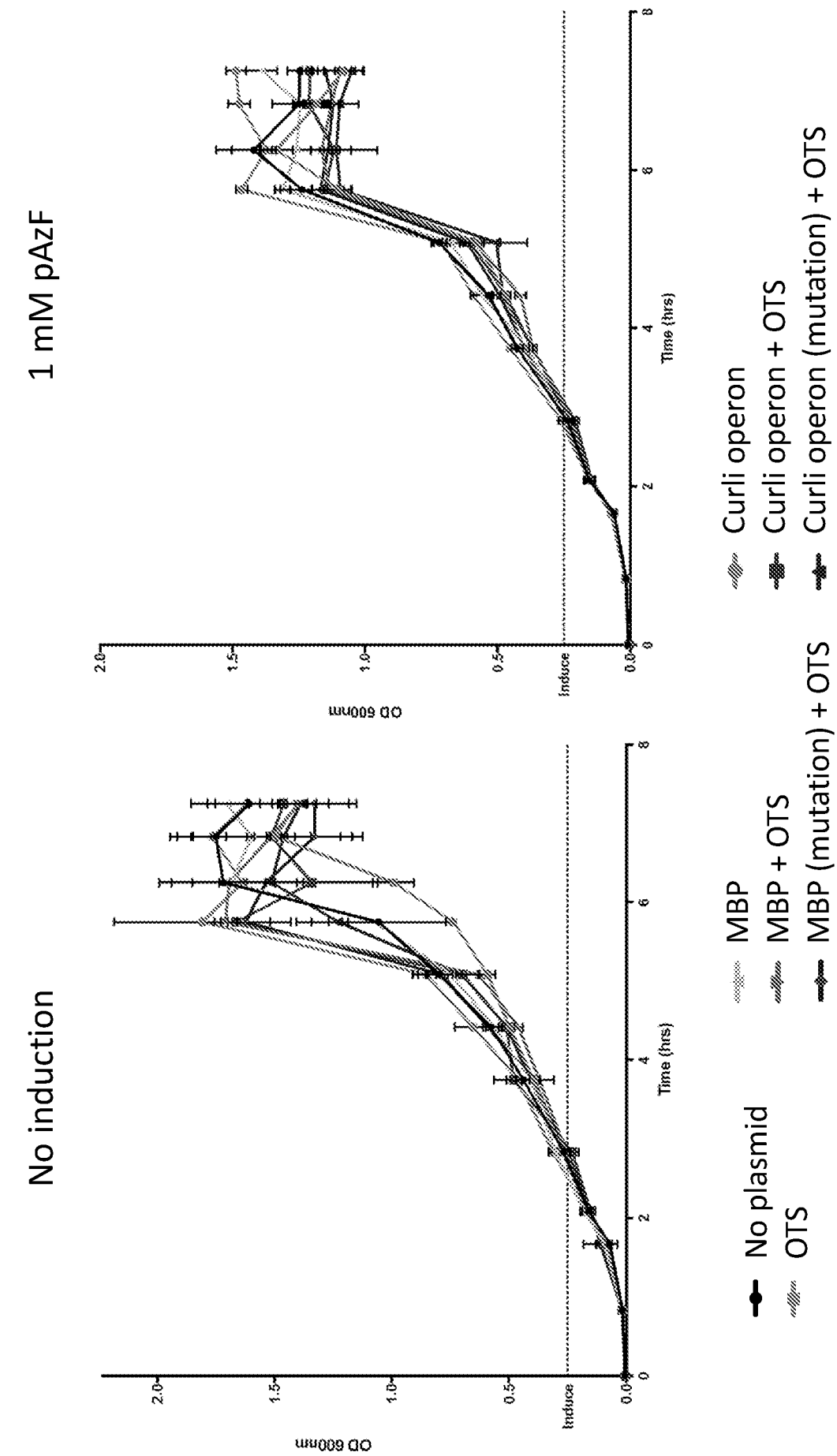


FIG. 10B

FIG. 11A

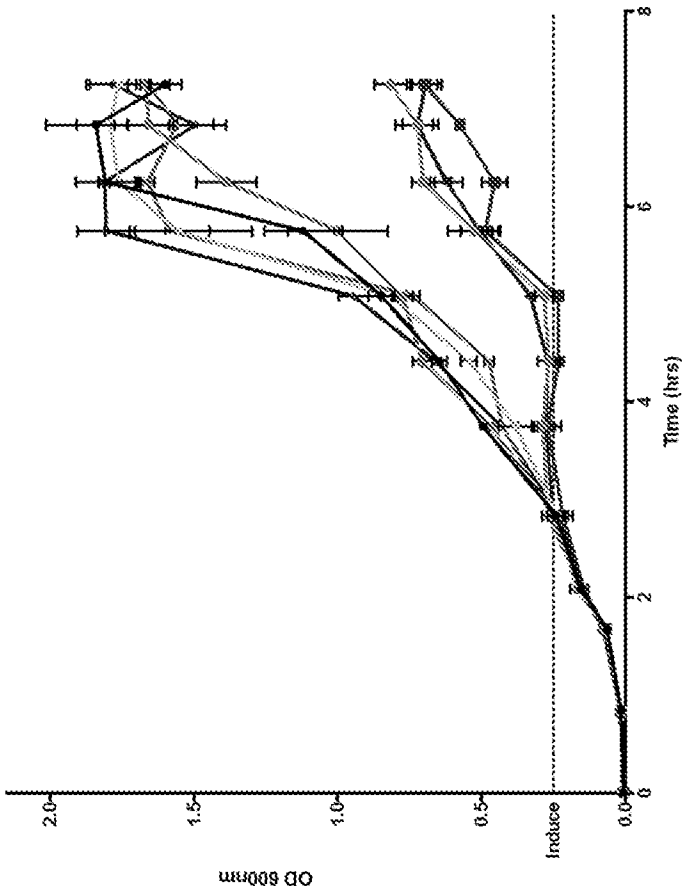


FIG. 11B

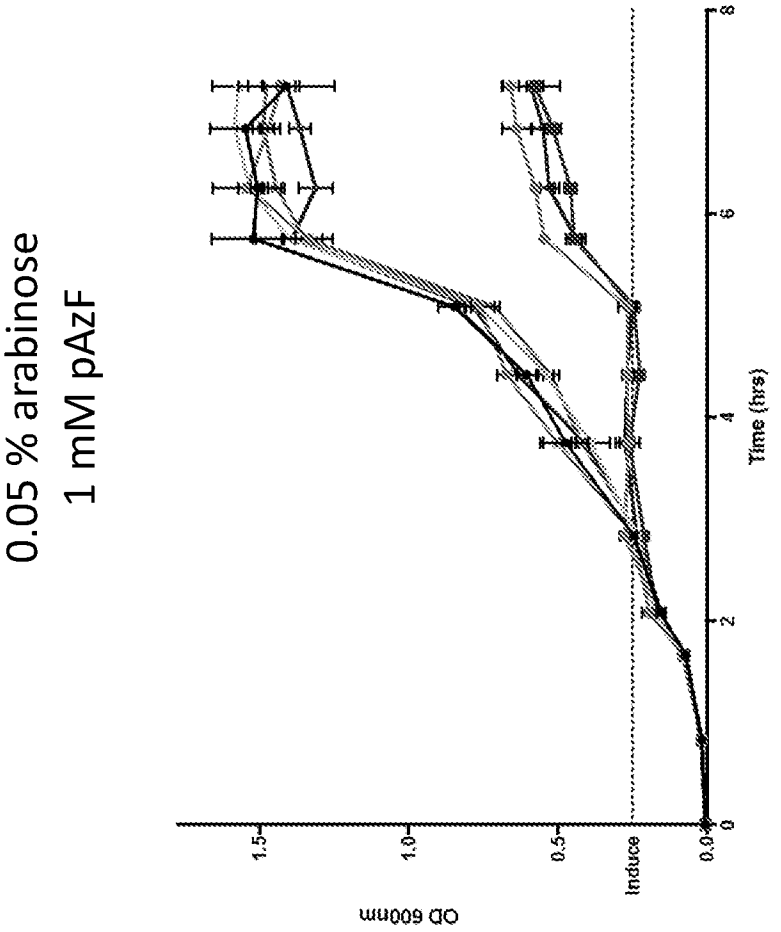
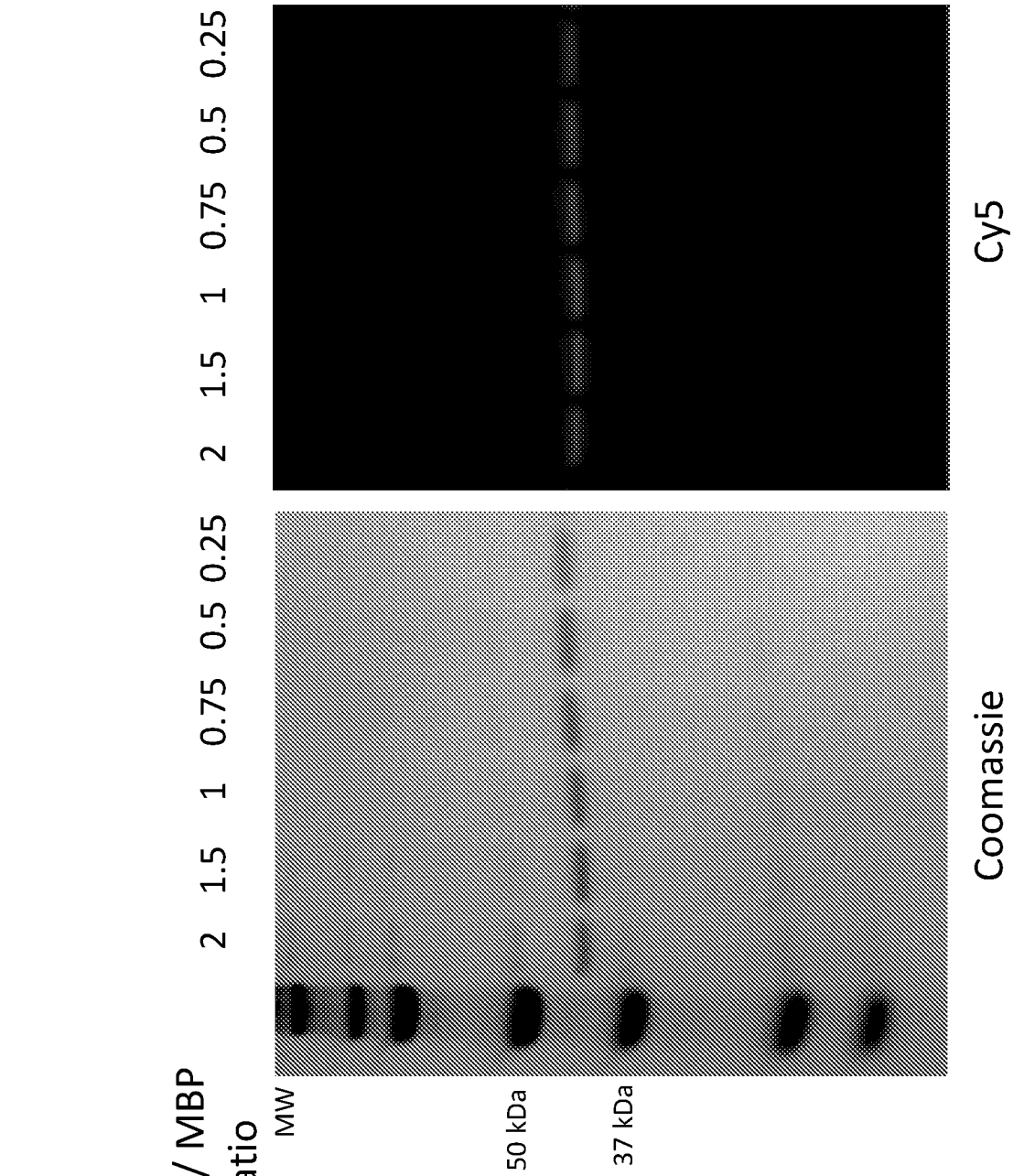


FIG. 12


DBCO-Cy5 / MBP
molar ratio
MW
2 1.5 1 0.75 0.5 0.25
50 kDa
37 kDa
Coomassie
2 1.5 1 0.75 0.5 0.25
Cy5

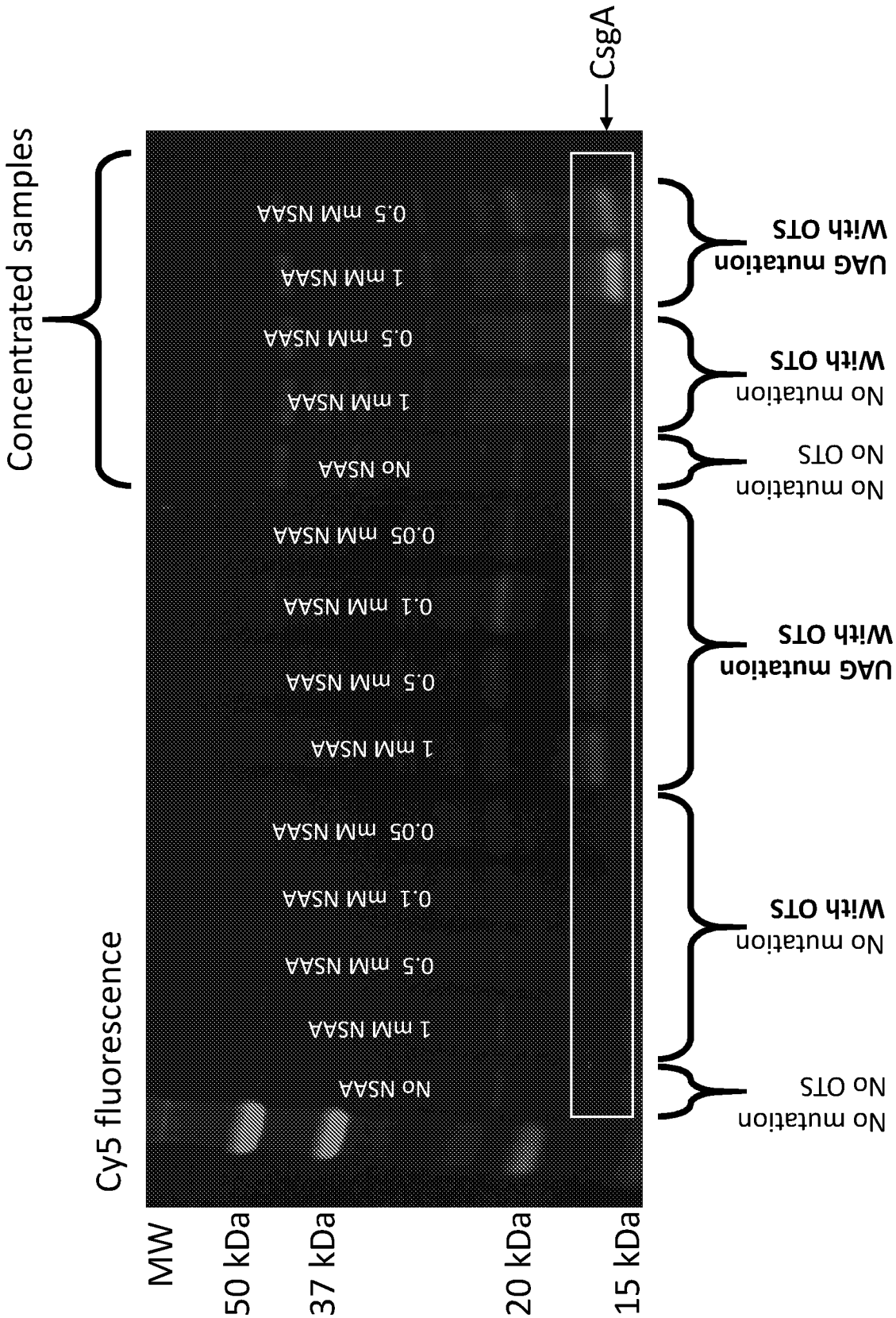

MW
50 kDa
37 kDa
20 kDa
15 kDa
Cy5 fluorescence
Concentrated samples
No NSAA
1 mM NSAA
0.5 mM NSAA
0.1 mM NSAA
0.05 mM NSAA
1 mM NSAA
0.5 mM NSAA
0.1 mM NSAA
0.05 mM NSAA
No NSAA
1 mM NSAA
0.5 mM NSAA
CsgA
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS
No mutation
No OTS
No mutation
With OTS
UAG mutation
With OTS

FIG. 13

FIG. 14A

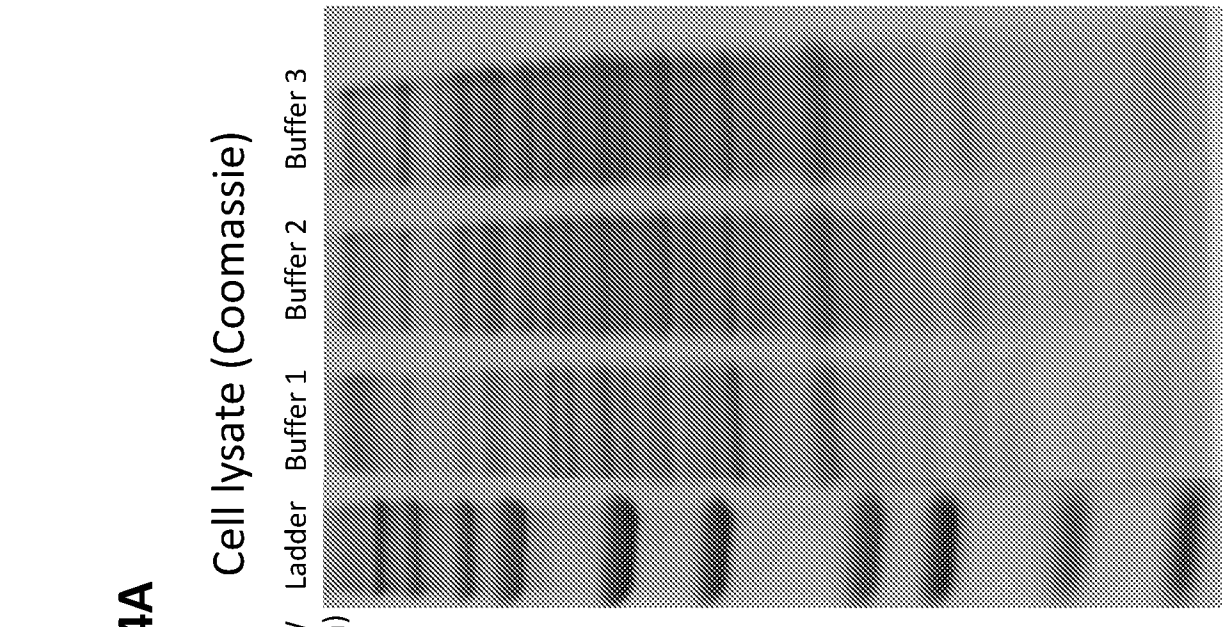


FIG. 14B

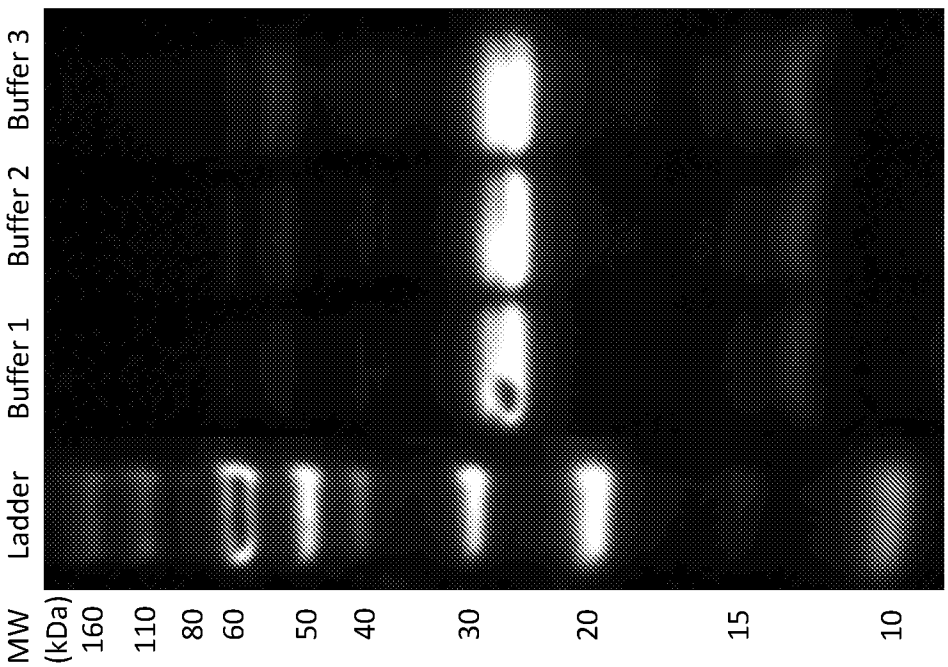


FIG. 15A

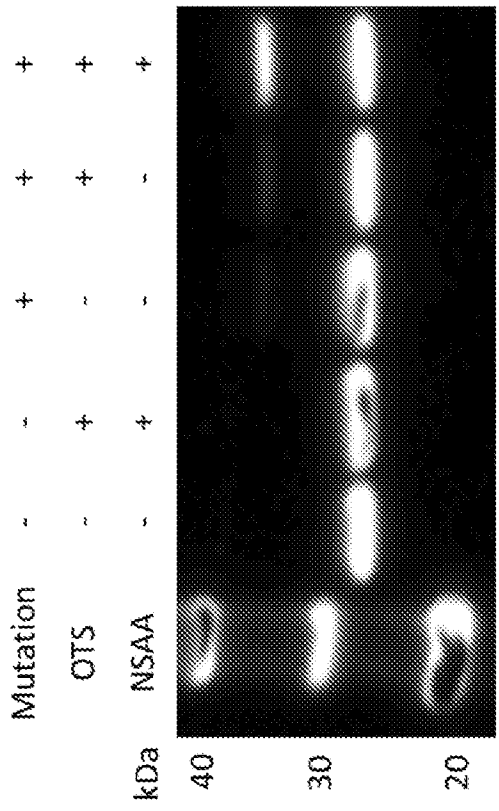


FIG. 15B

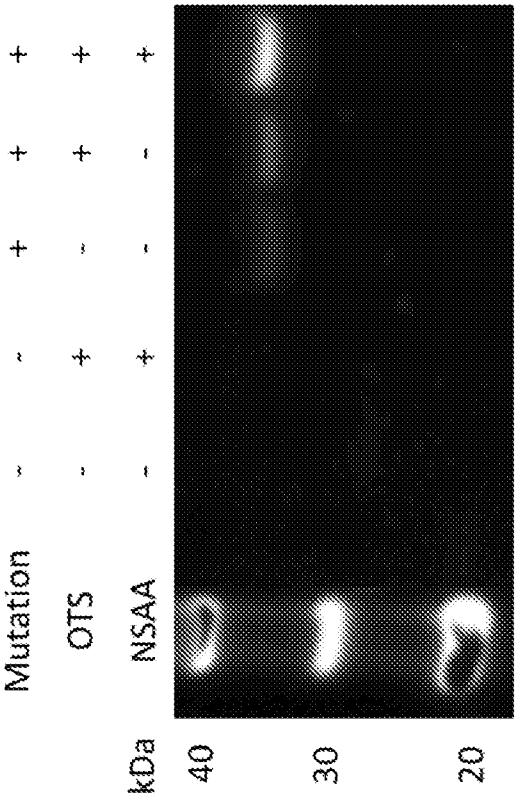


FIG. 15C

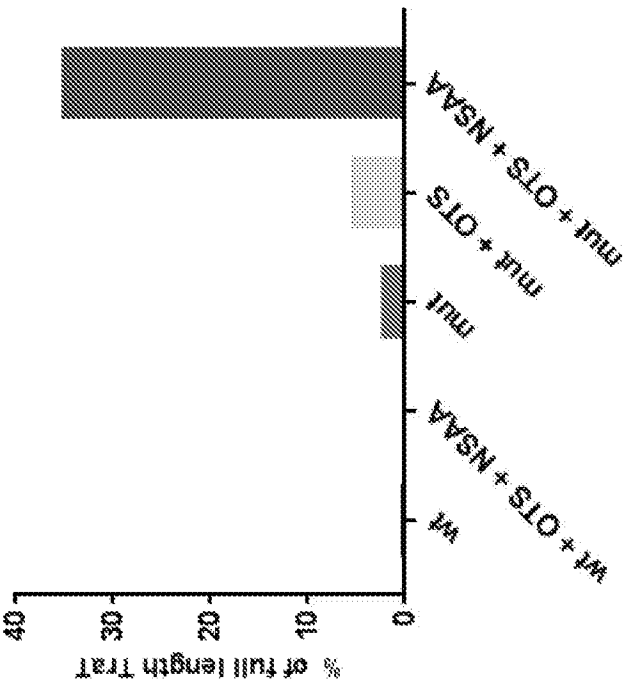
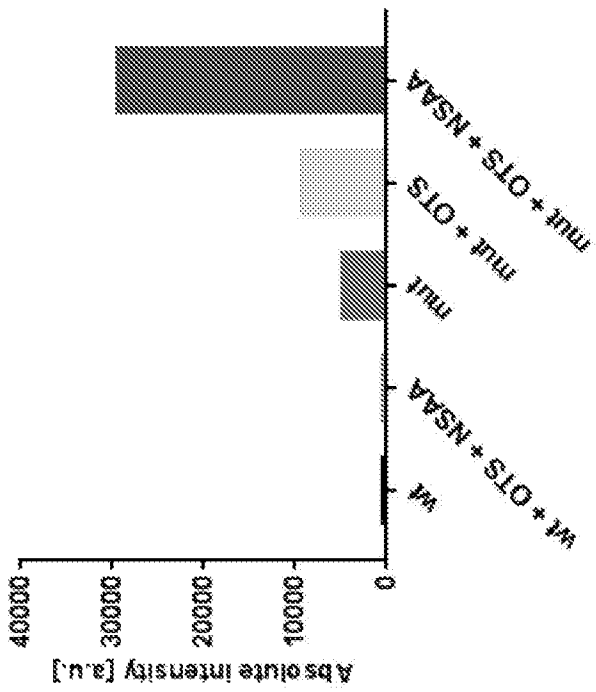


FIG. 15D



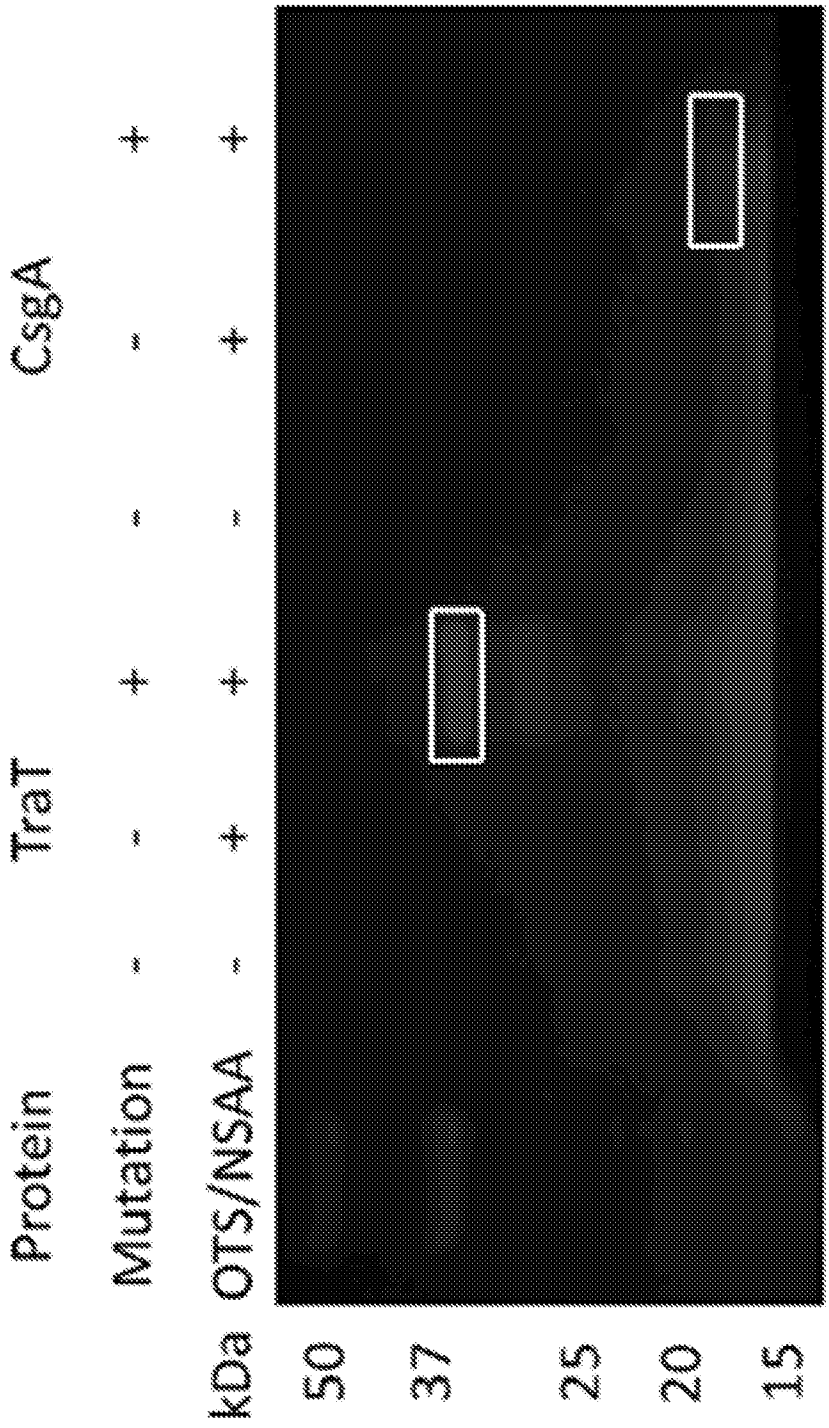


FIG. 16

FIG. 17A

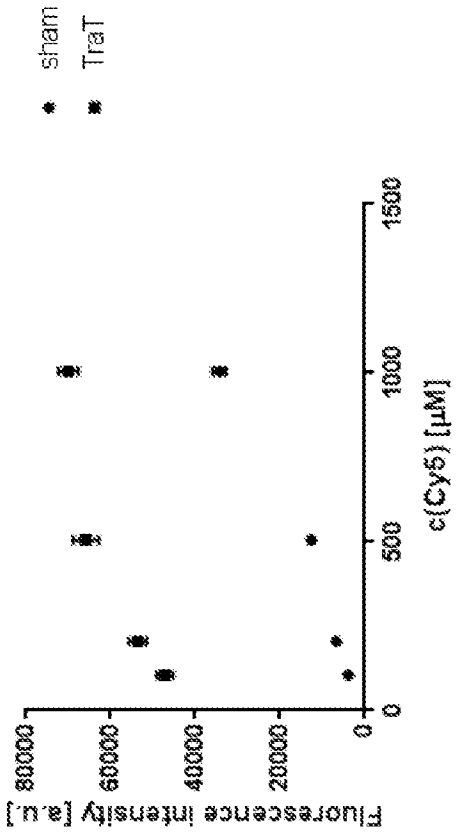


FIG. 17B

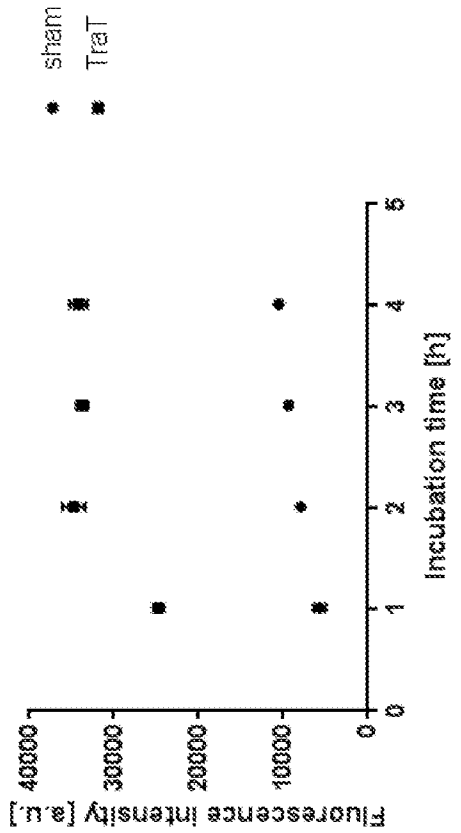


FIG. 17C

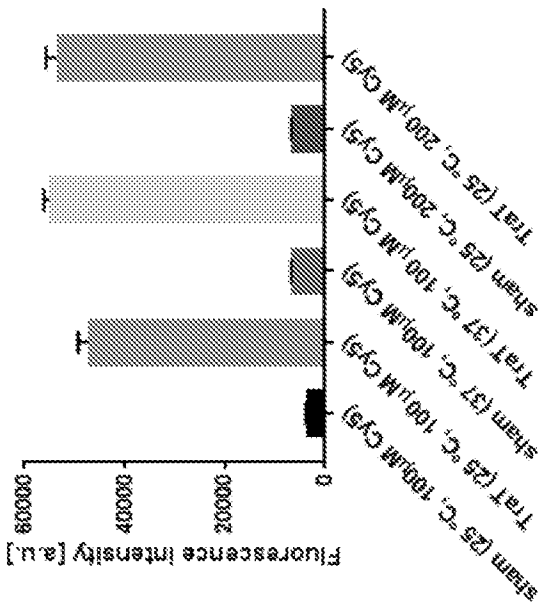


FIG. 18A

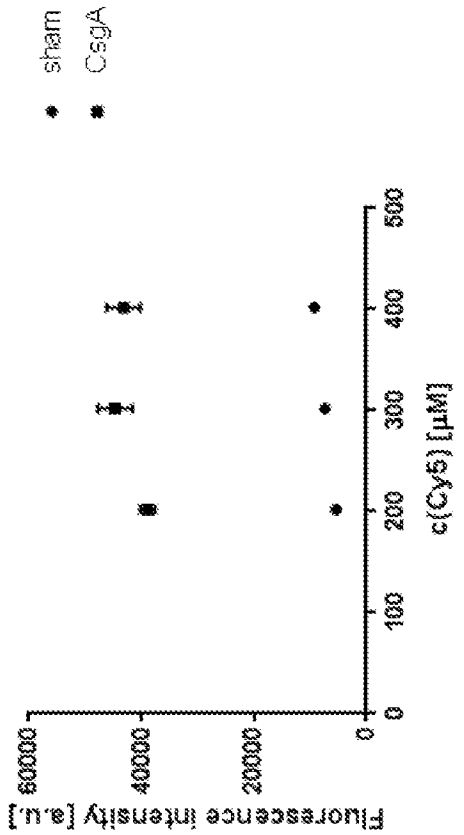


FIG. 18B

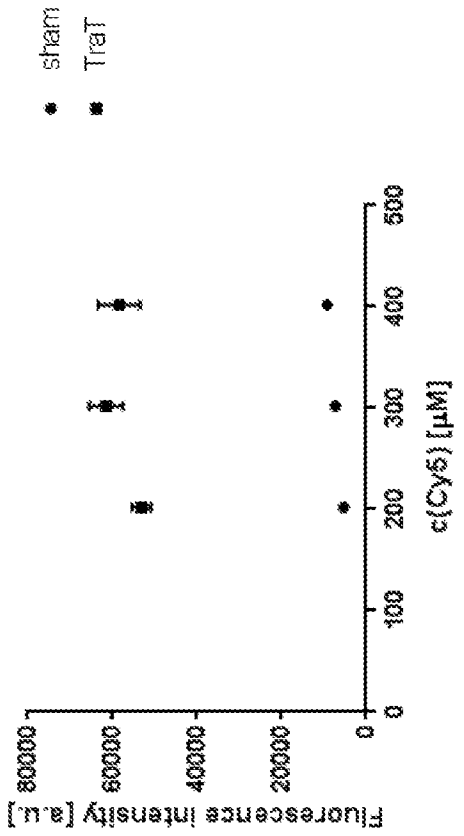


FIG. 18C

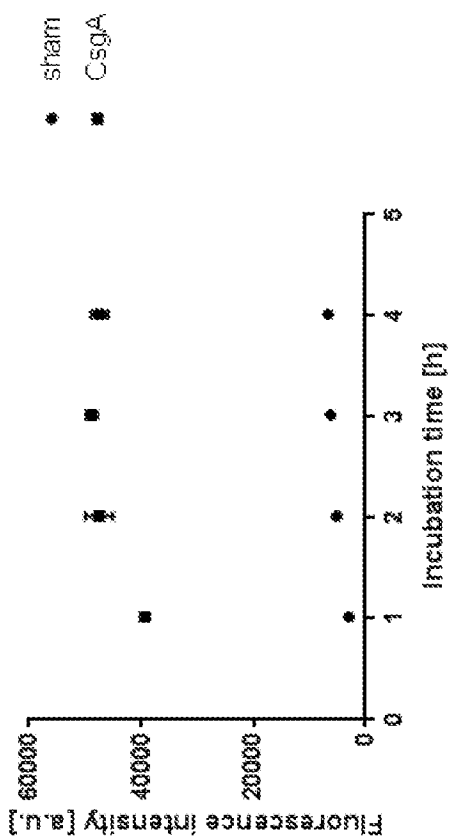
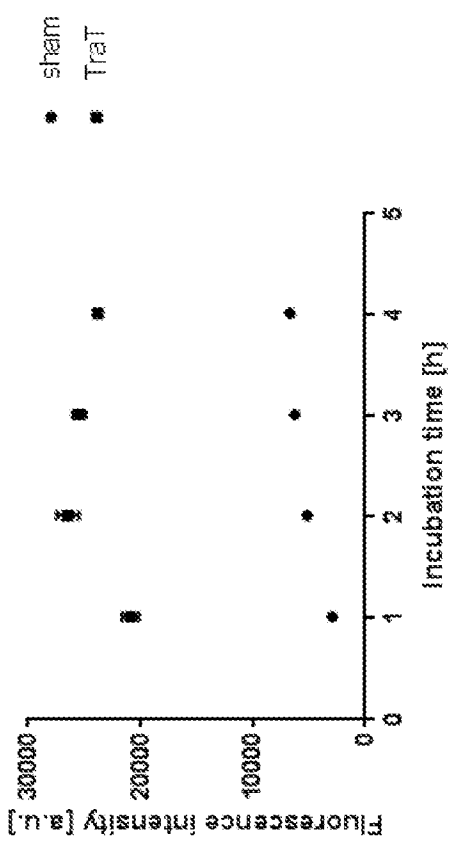


FIG. 18D



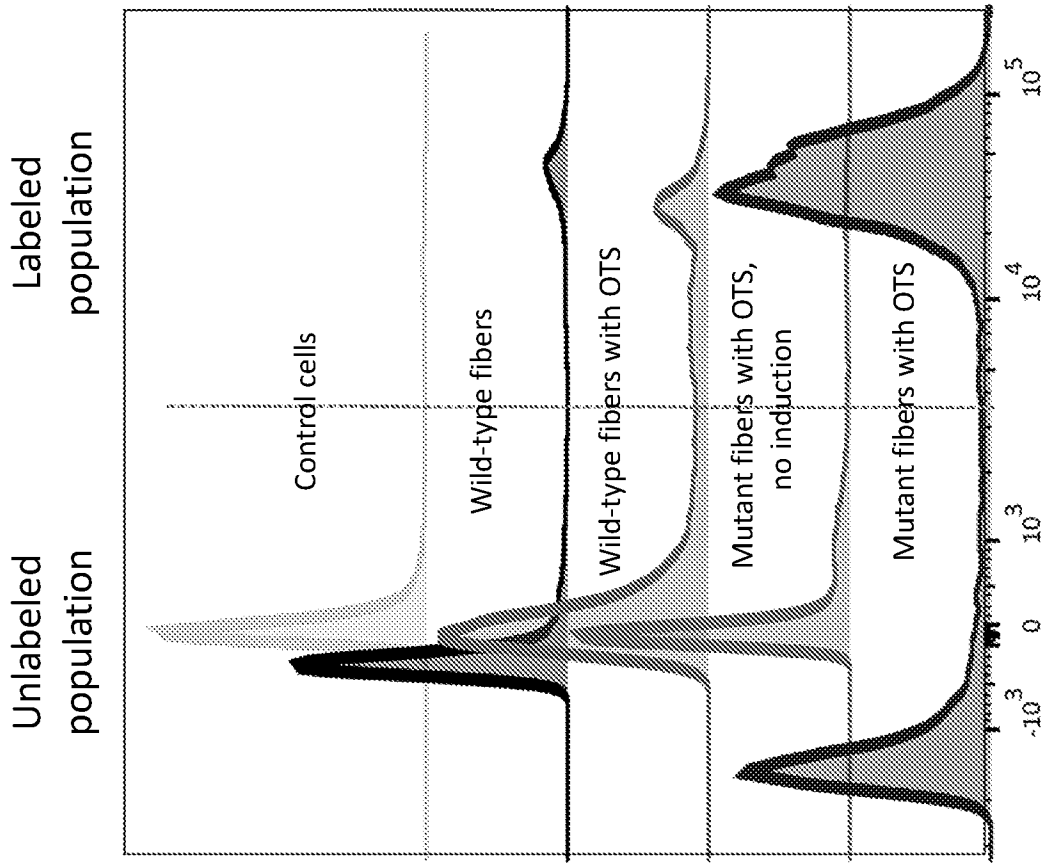

Unlabeled population
Labeled population
Control cells
Wild-type fibers
Wild-type fibers with OTS
Mutant fibers with OTS, no induction
Mutant fibers with OTS
-10^3
0
10^3
10^4
10^5

FIG. 19

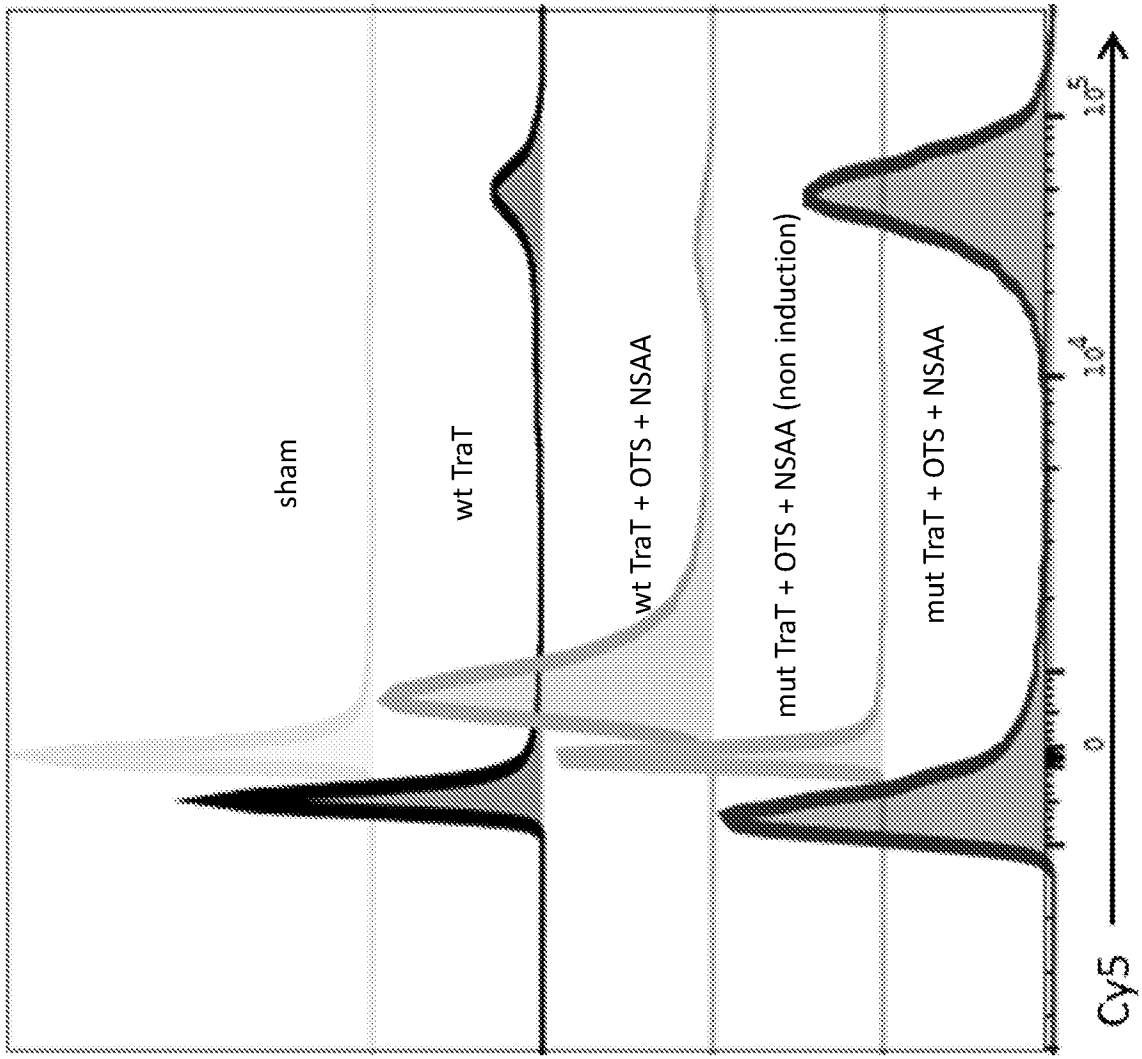

sham
wt TraT
wt TraT + OTS + NSAA
mut TraT + OTS + NSAA (non induction)
mut TraT + OTS + NSAA
0
10^4
10^5
Cy5

FIG. 20

FIG. 21A

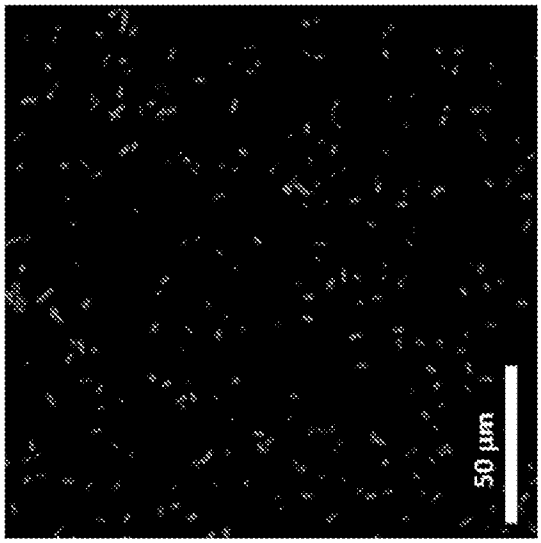


FIG. 21B

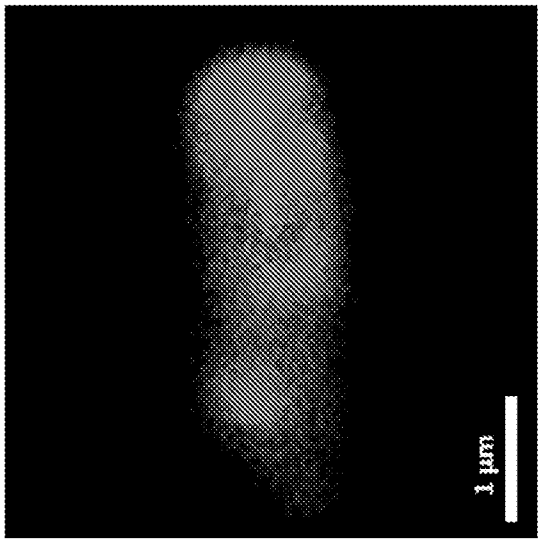


FIG. 21C

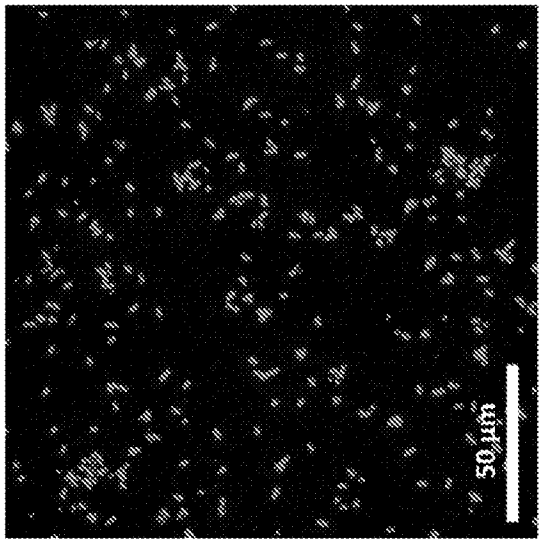


FIG. 21D

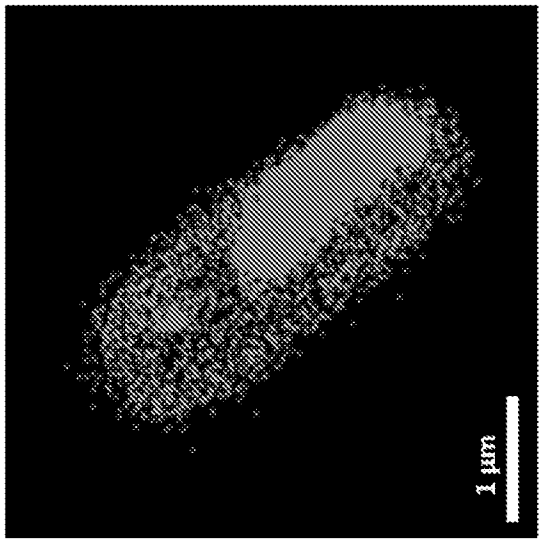
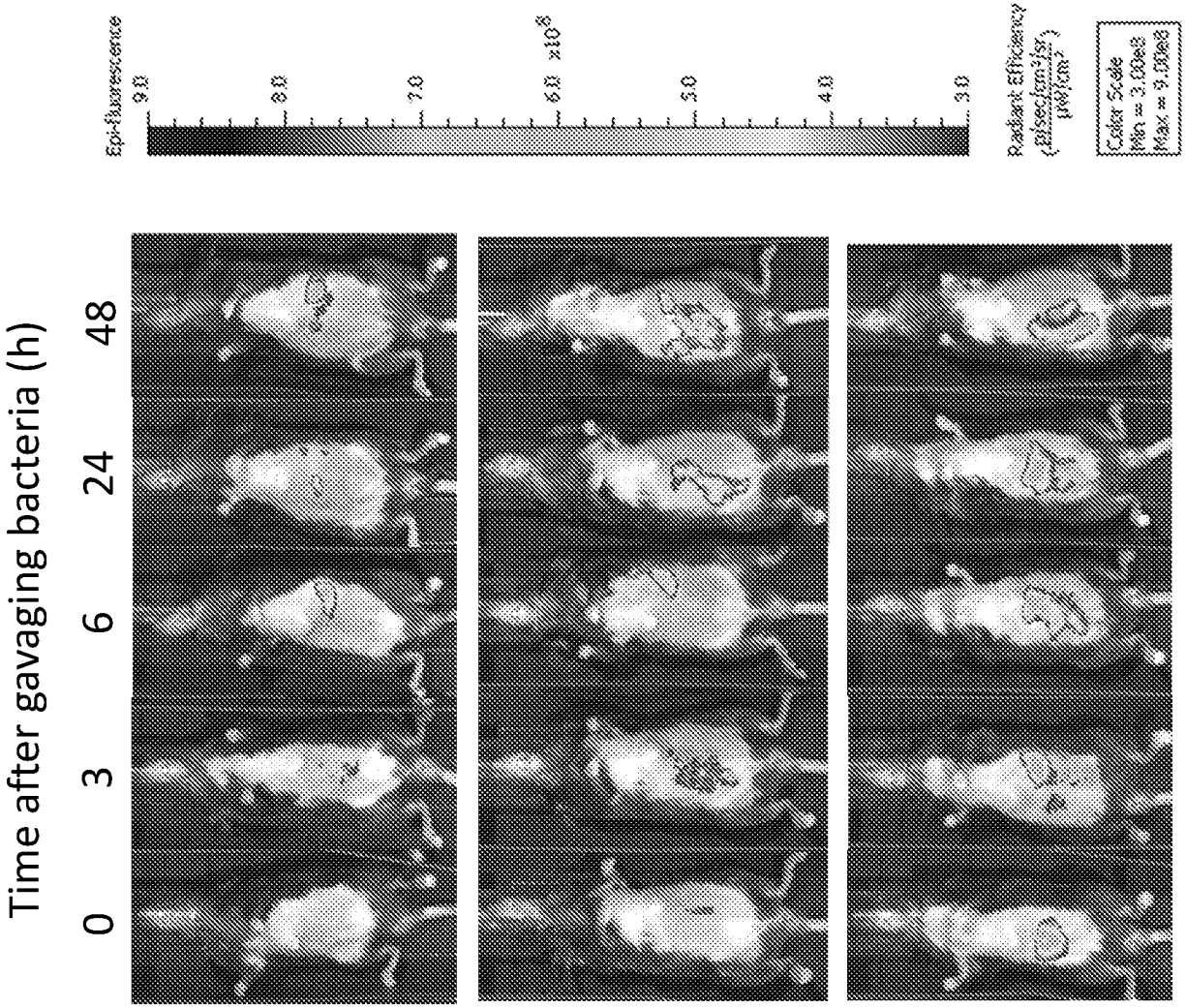


FIG. 22


Time after gavaging bacteria (h)
0
3
6
24
48
No bacteria
10^6 bacteria
10^9 bacteria
Epi-fluorescence
9.0
8.0
7.0
6.0 x10^8
5.0
4.0
3.0
Radiant Efficiency
(photons/cm²/sr)
μW/cm²
Color Scale
Min = 3.00e8
Max = 9.00e8

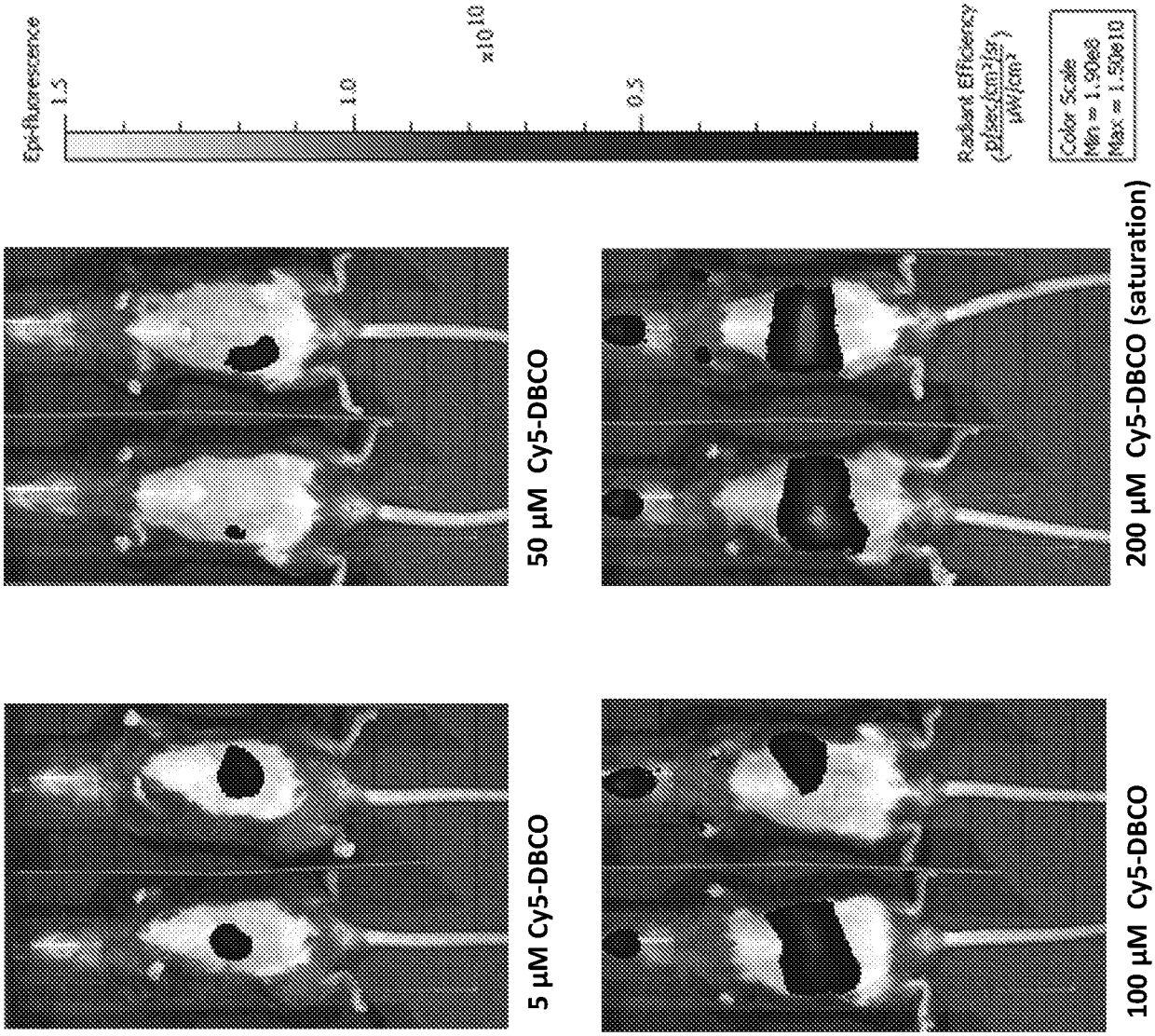
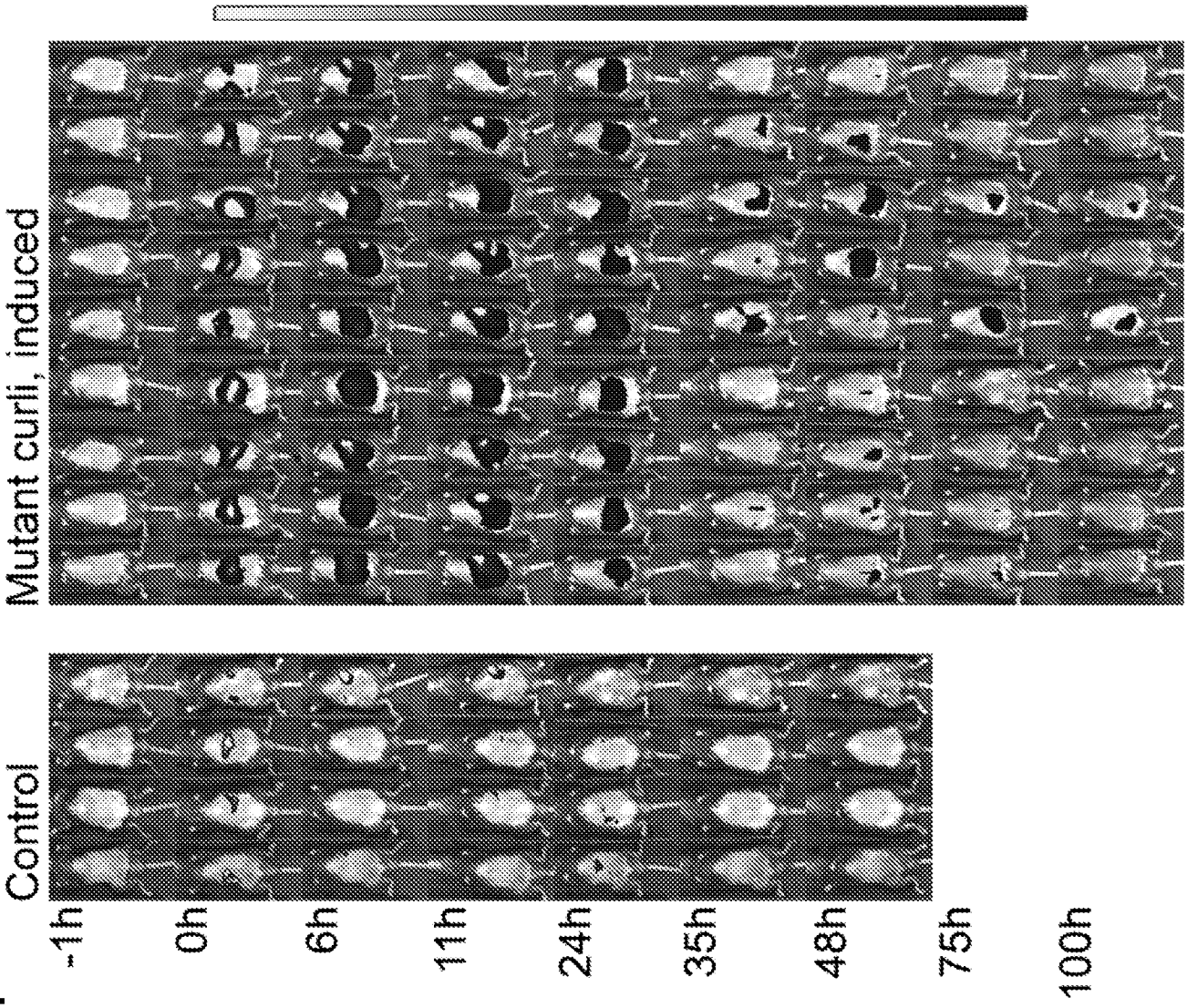

Epi-fluorescence
1.5
1.0
0.5
x10^10
Radiant Efficiency
(photons/sec/cm²/sr/
μW/cm²)
Color Scale
Min = 1.90e8
Max = 1.58e10
50 μM Cy5-DBCO
200 μM Cy5-DBCO (saturation)
5 μM Cy5-DBCO
100 μM Cy5-DBCO

FIG. 23

FIG. 24


Control
Mutant curli, induced
-1h
0h
6h
11h
24h
35h
48h
75h
100h

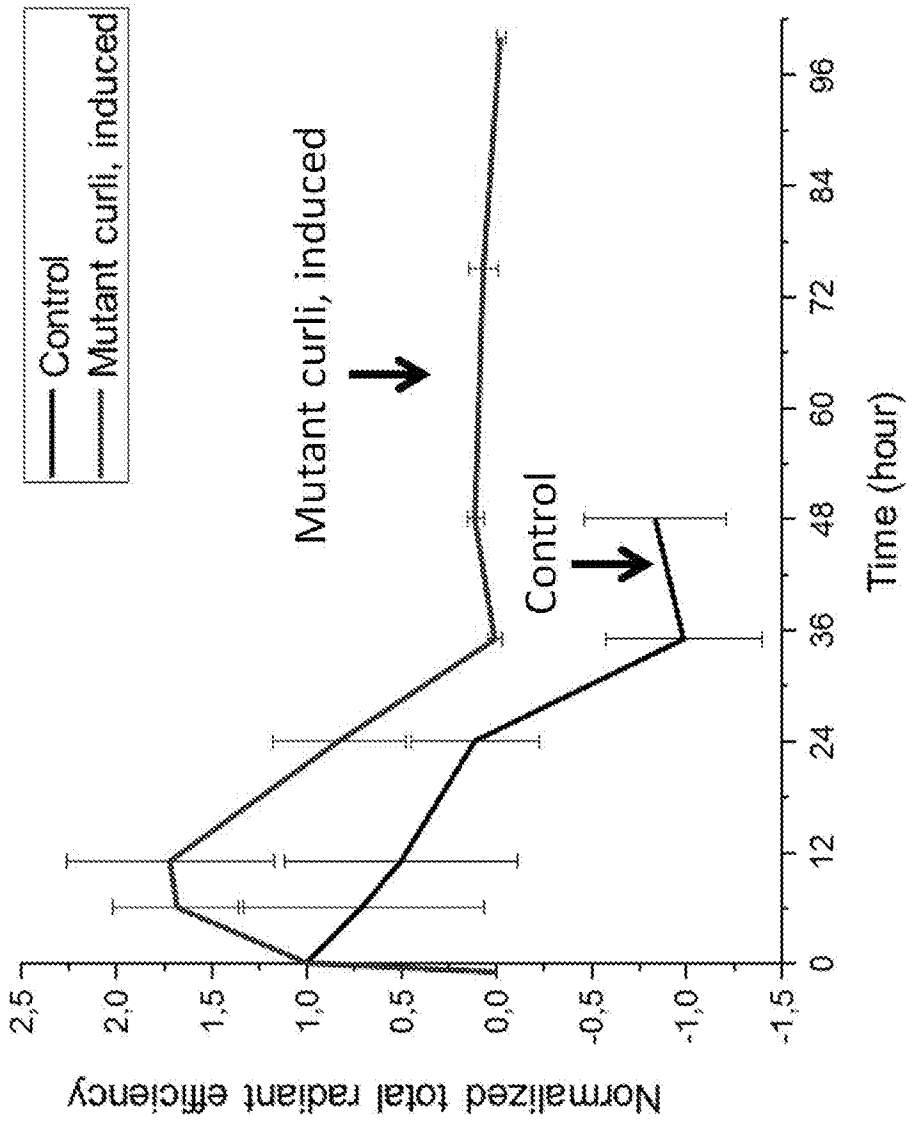

Control
Mutant curli, induced
Mutant curli, induced
Control
Normalized total radiant efficiency
Time (hour)
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
0
12
24
36
48
60
72
84
96

FIG. 25

FIG. 26A

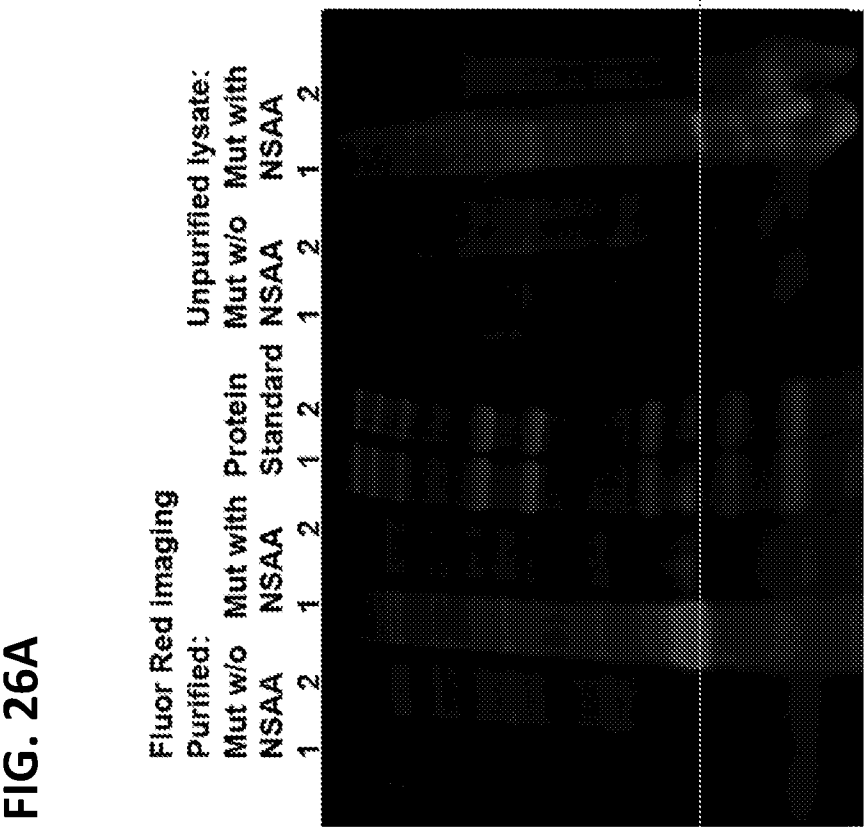
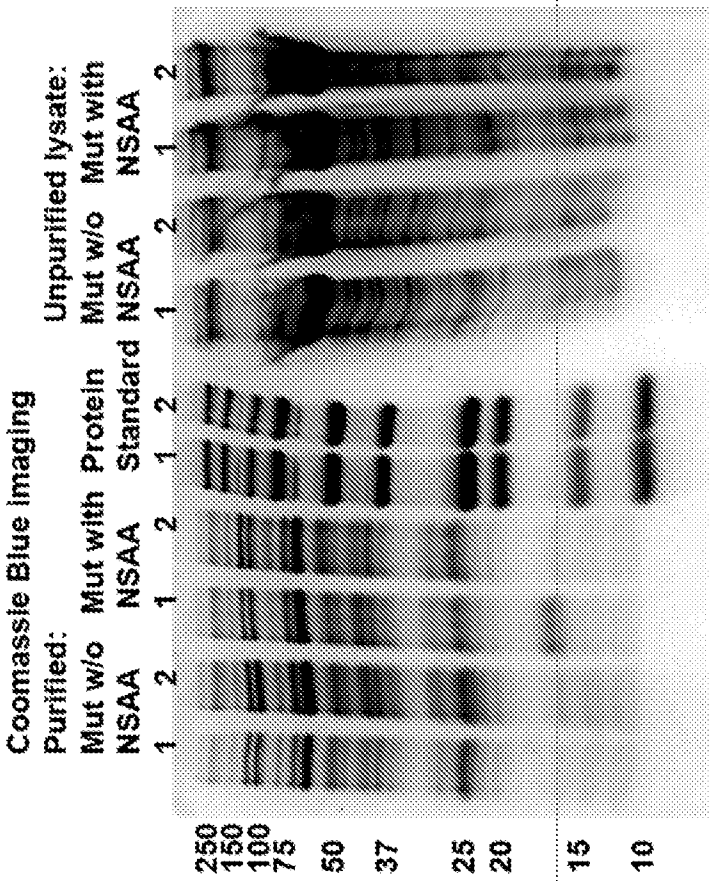


FIG. 26B



5 µM dye

FIG. 27A

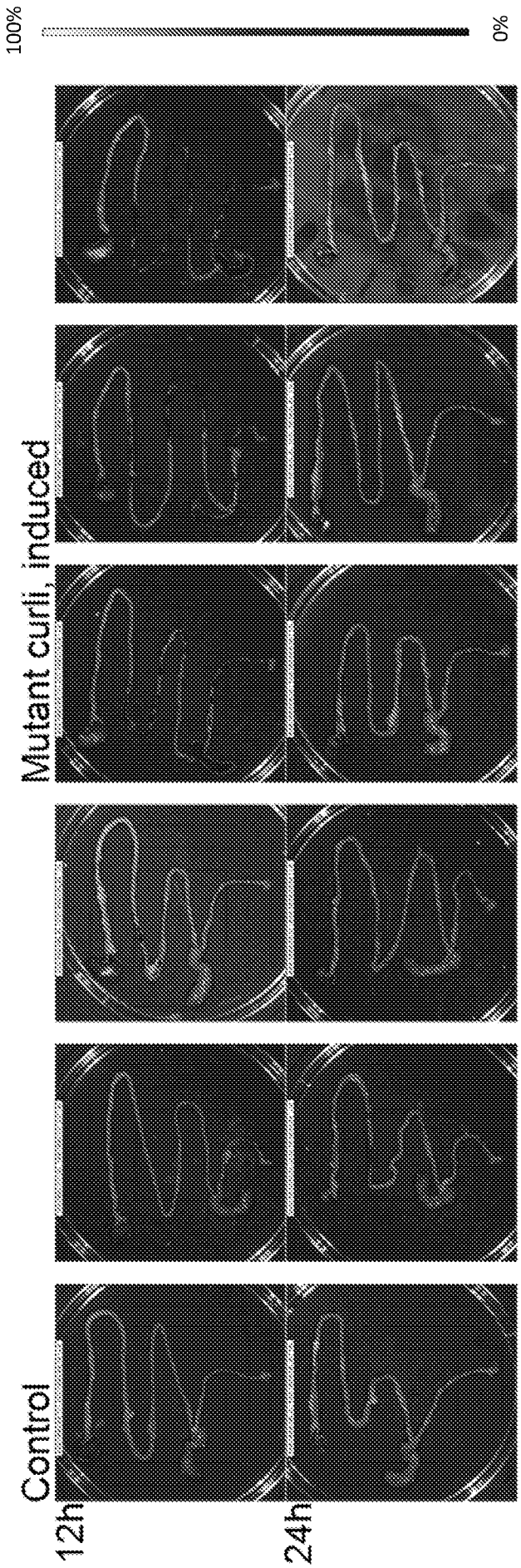


FIG. 27B

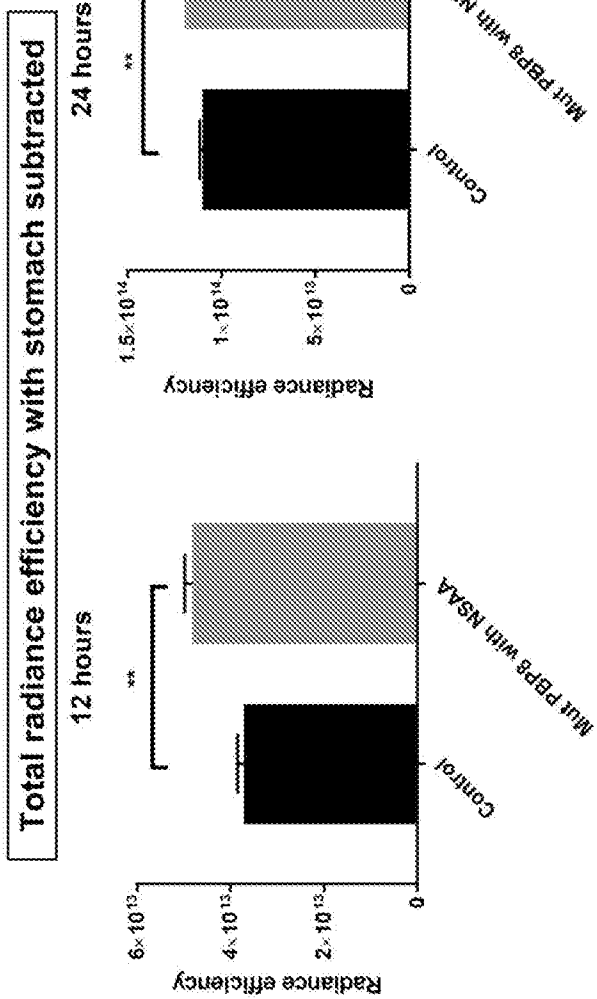


FIG. 27C

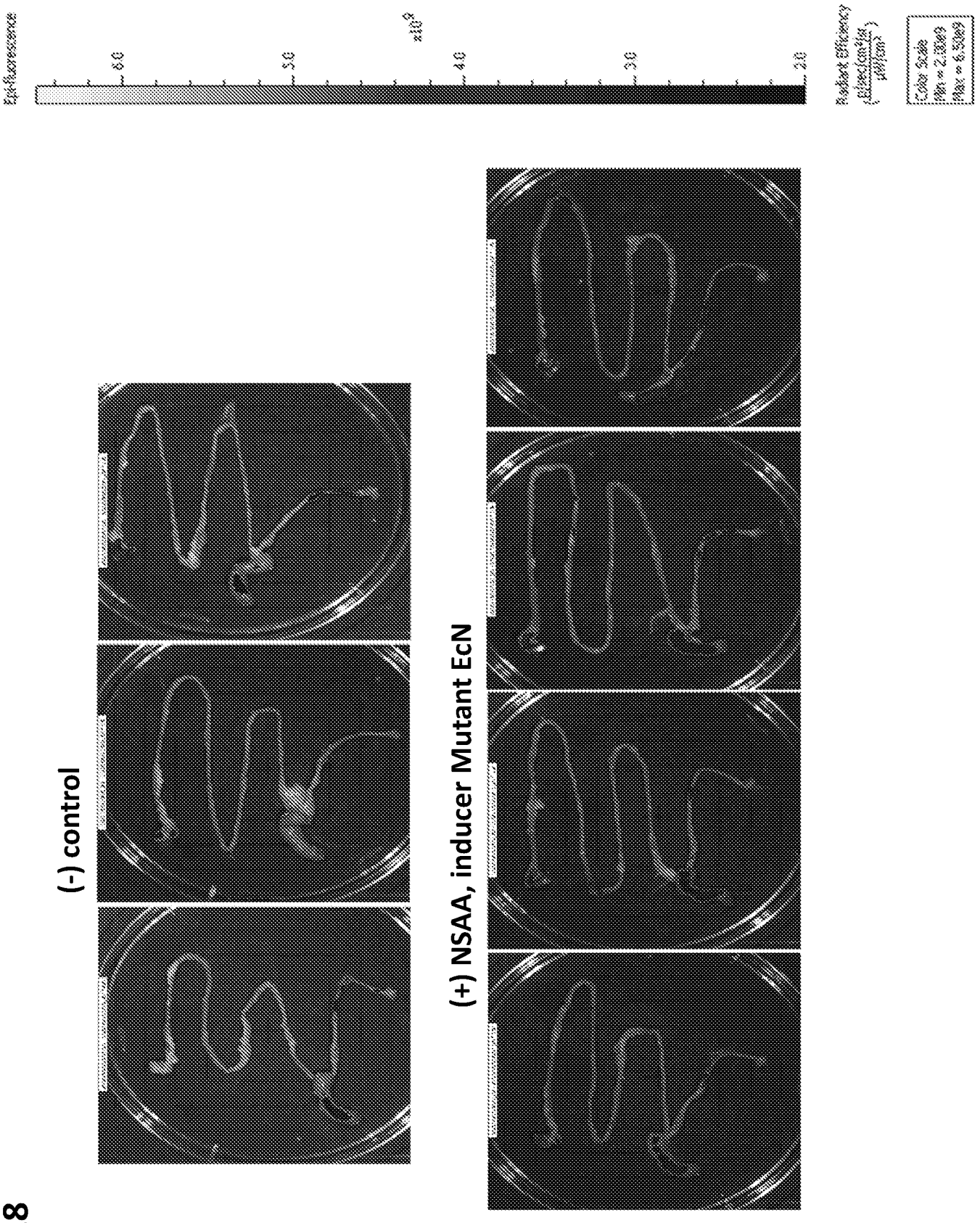

Epifluorescence
6.0
5.0
x10^9
4.0
3.0
2.0
Radiant Efficiency
(photons/s/cm²/sr/µW/cm²)
Color Scale
Min = 2.33e9
Max = 6.53e9
(-) control
(+) NSAA, inducer Mutant EcN

FIG. 28

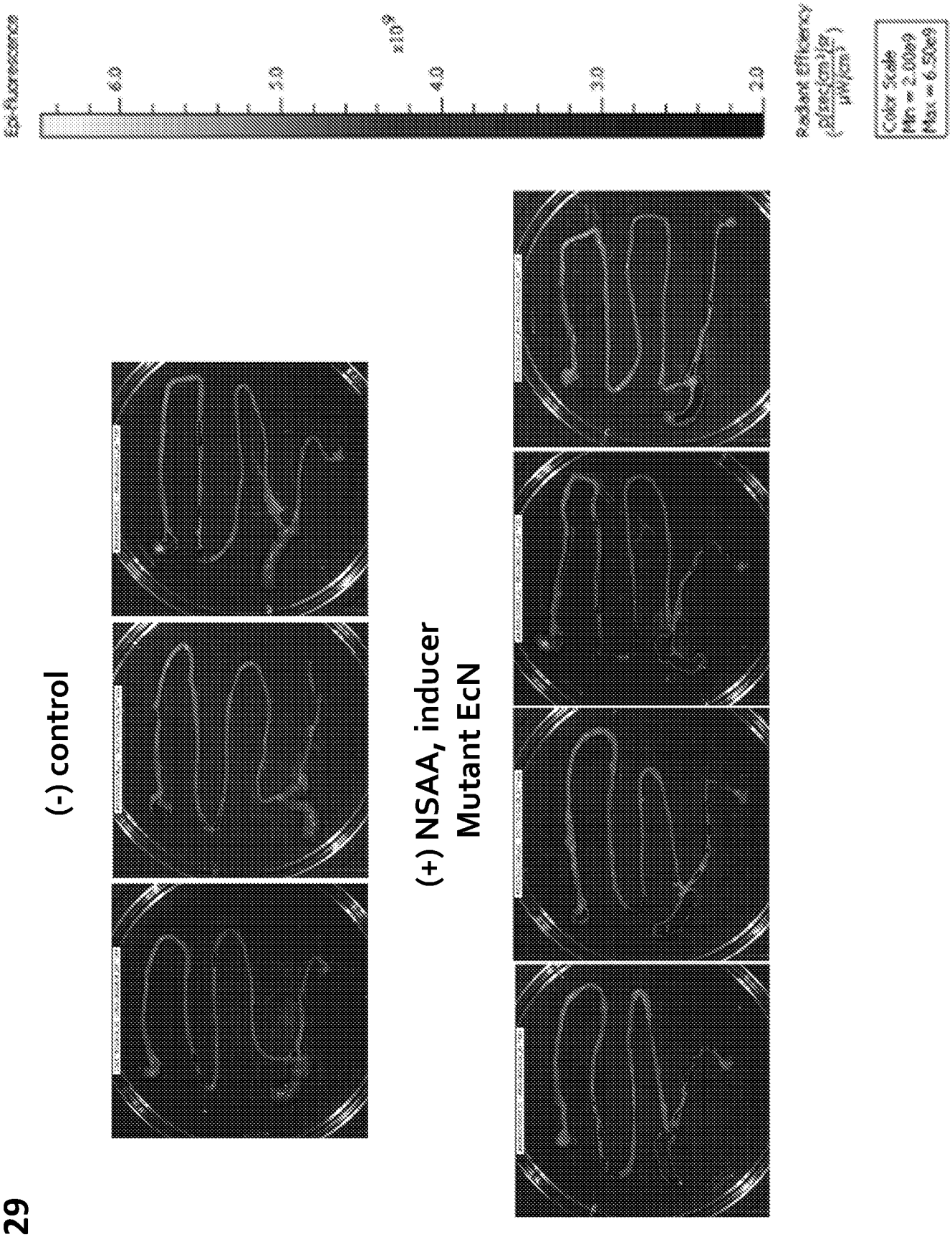

Epi-fluorescence
4.0
3.0
x10^9
4.0
3.0
2.0
Radiant Efficiency
(Photons/sec
/cm2/sr)
Color Scale
Min = 2.00e9
Max = 6.50e9
(-) control
(+) NSAA, inducer
Mutant EcN

FIG. 29

FIG. 30A

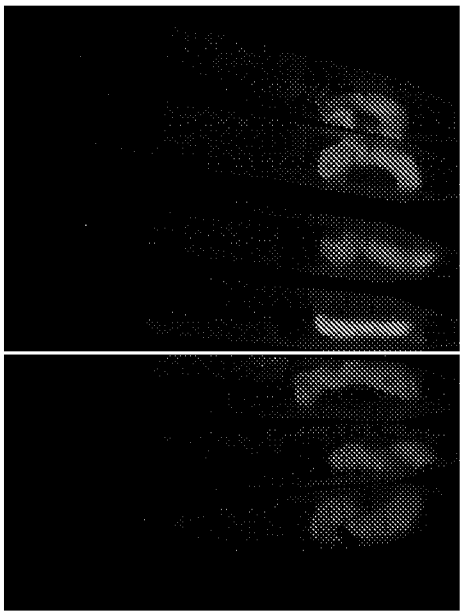


FIG. 30B

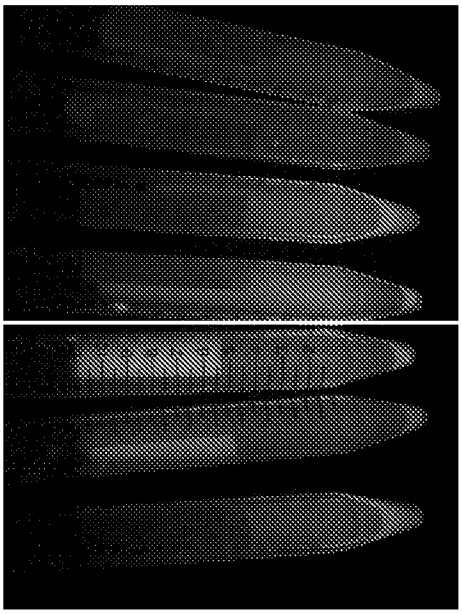


FIG. 30C

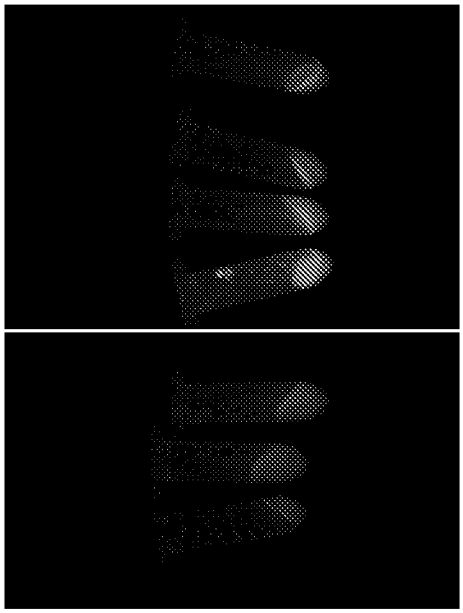


FIG. 31A

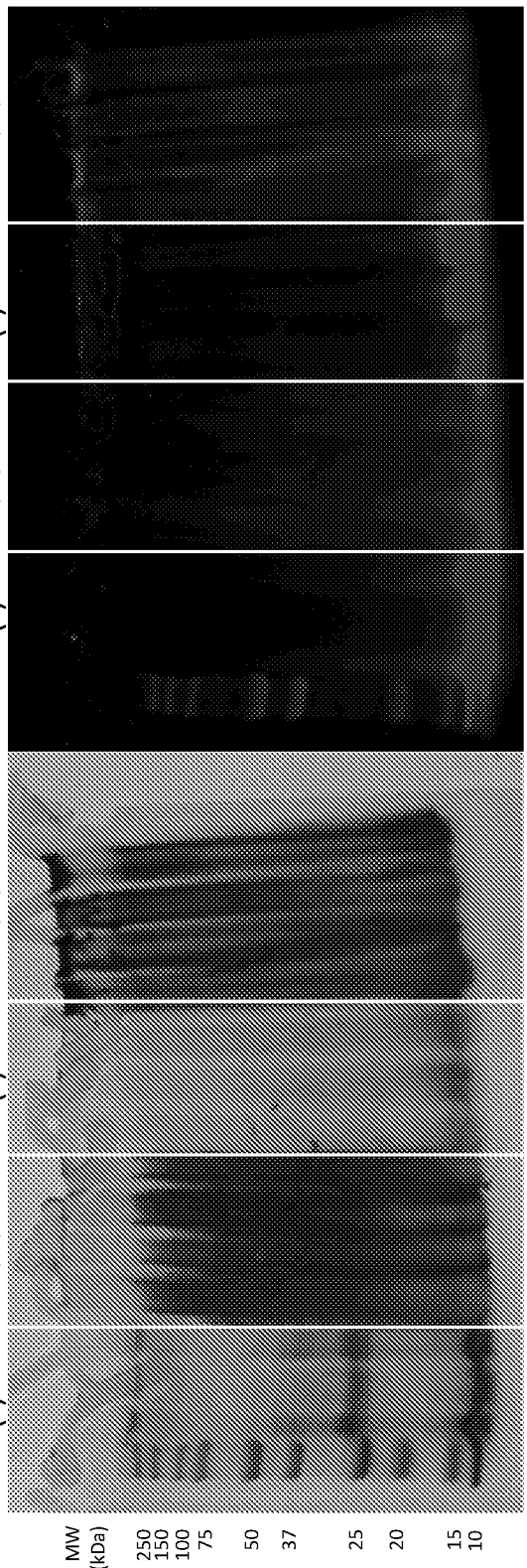


FIG. 31B

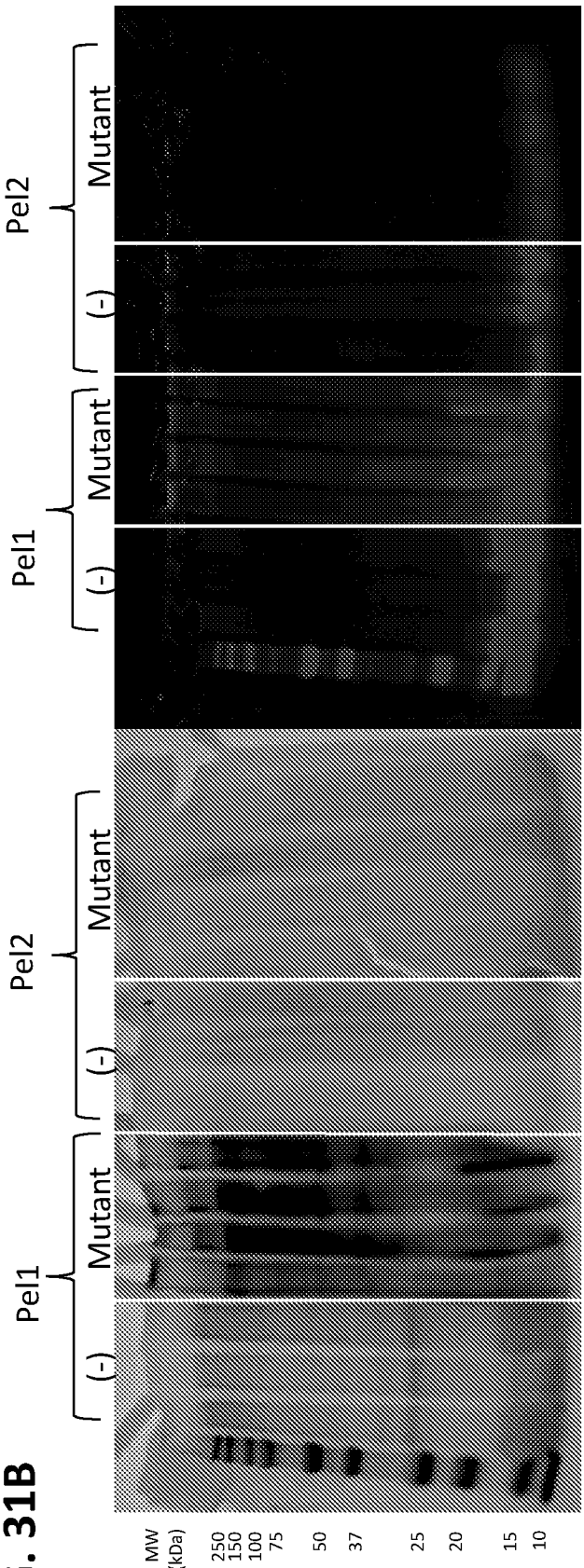


FIG. 32A

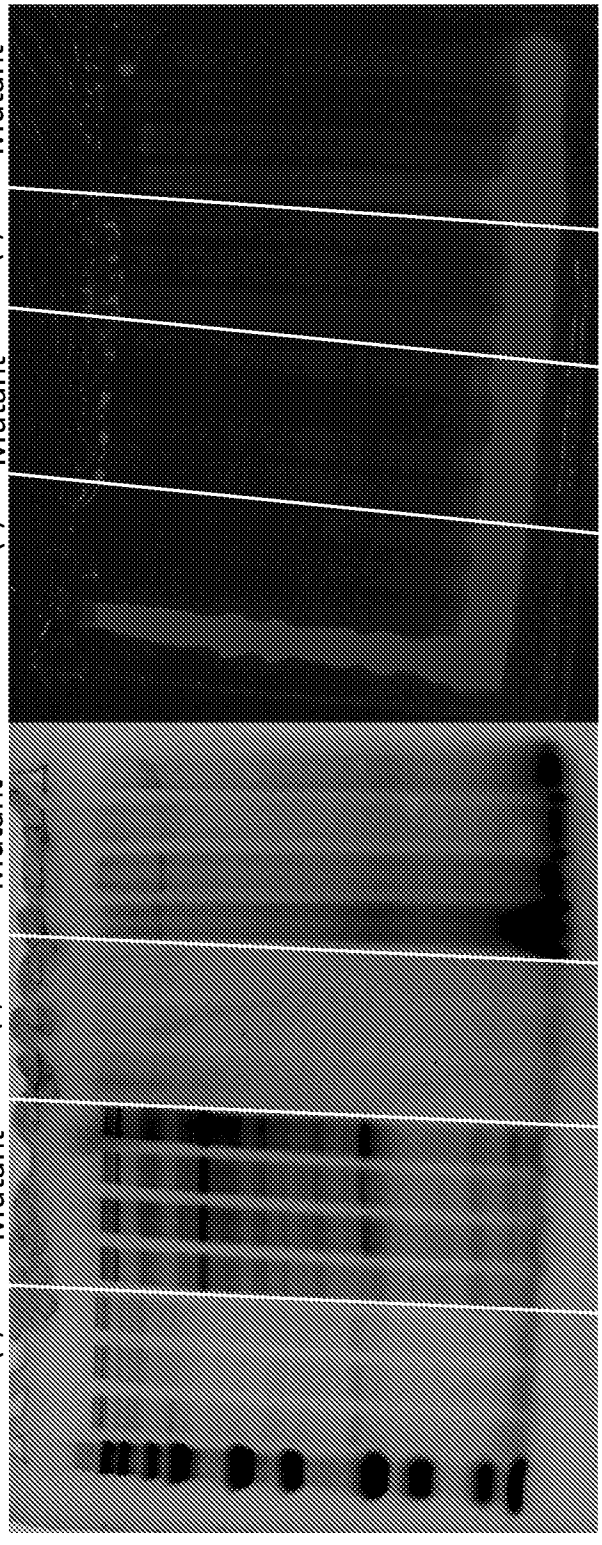


FIG. 32B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/45869

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/00, C12N 9/00 (2017.01)
CPC - C12N 15/09, C12P 21/02

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0244245 A1 (SCHULTZ et al.) 19 September 2013 (19.09.2013) abstract, para [0010], [0012], [0014], [0015], [0017], [0109], [0272], [0279], [0392]	1-4, 9-12, 22
A	US 2013/0323821 A1 (TIAN et al.) 5 December 2013 (05.12.2013) para [0013], [0015], [0259], [0261]	1-4, 9-12, 22
A	US 2009/0081669 A1 (SHIVA et al.) 26 March 2009 (26.03.2009) abstract, para [0017]	1-4, 9-12, 22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2017	21 DEC 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/45869

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-8, 13-21, 23-53 and 62-117
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

---------- see extra sheet ----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 9-12 and 22

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III, Observations where unity of invention is lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, 9-12 and 22, directed to an engineered bacterium composition comprising an orthogonal translation system, an orthogonal tRNA and a heterologous nucleic acid encoding a protein in which the tRNA is incorporated.

Group II: Claims 54-61 and 118-119 directed to a method of administering to a subject an engineered bacterium comprising an amino acid comprising a first bioorthogonal reactive functional group that reacts with a second bioorthogonal reactive functional group administered to said subject.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group II does not require an isolated engineered bacterium composition, as required by group I.

Group I does not require a method of labeling or detecting of an engineered bacterium, a method of diagnosising a disease, and a method of drug delivery, as required by group II

Common Technical Features

The common technical feature shared by Groups I and II is an engineered bacterium comprises:
(a) an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA; and
(b) a heterologous nucleic acid encoding a protein having at least one nonstandard amino acid;
wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid; and wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by US 2013/0244245 A1 to Schultz et al. (hereinafter 'Schultz'). Schultz discloses an engineered bacterium (abstract "The invention relates to orthogonal pairs of tRNAs and aminoacyl-tRNA synthetase that can incorporate unnatural amino acid into proteins produced in eubacterial host cells such as E. coli") comprising
(a) an orthogonal translation system comprising a heterologous gene encoding an orthogonal aminoacyl tRNA synthetase and a heterologous gene encoding an orthogonal tRNA (para [0010] "the invention provides translation systems. These systems comprise a first orthogonal aminoacyl-tRNA synthetase (O-RS), a first orthogonal tRNA (O-tRNA)", para [0017] "the providing step furnishes a polynucleotide encoding the O-tRNA" [i.e. a tRNA gene], para [0014] "the translation system resides in a host cell (and includes the host cell). The host cell ... can be a eubacterial cell, such as E. coli");
(b) a heterologous nucleic acid encoding a protein having at least one nonstandard amino acid (para [0012] "the translation system further comprises a nucleic acid encoding a protein of interest", para [0015] "the nucleic acid comprises at least one selector codon that is recognized by the first O-tRNA. The method then incorporates the unnatural amino acid at the selected position in the protein ... producing the protein comprising the unnatural amino acid at the selected position");
wherein the aminoacyl tRNA synthetase is capable of specifically aminoacylating the orthogonal tRNA with the nonstandard amino acid (para [0010] "These systems comprise a first orthogonal aminoacyl-tRNA synthetase (O-RS), a first orthogonal tRNA (O-tRNA), and an unnatural amino acid, where the first O-RS preferentially aminoacylates the first O-tRNA with the first unnatural amino acid."), and wherein the orthogonal tRNA incorporates the nonstandard amino acid into the protein during translation (para [0015] "The method then incorporates the unnatural amino acid at the selected position in the protein during translation of the protein in response to the selector codon").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.