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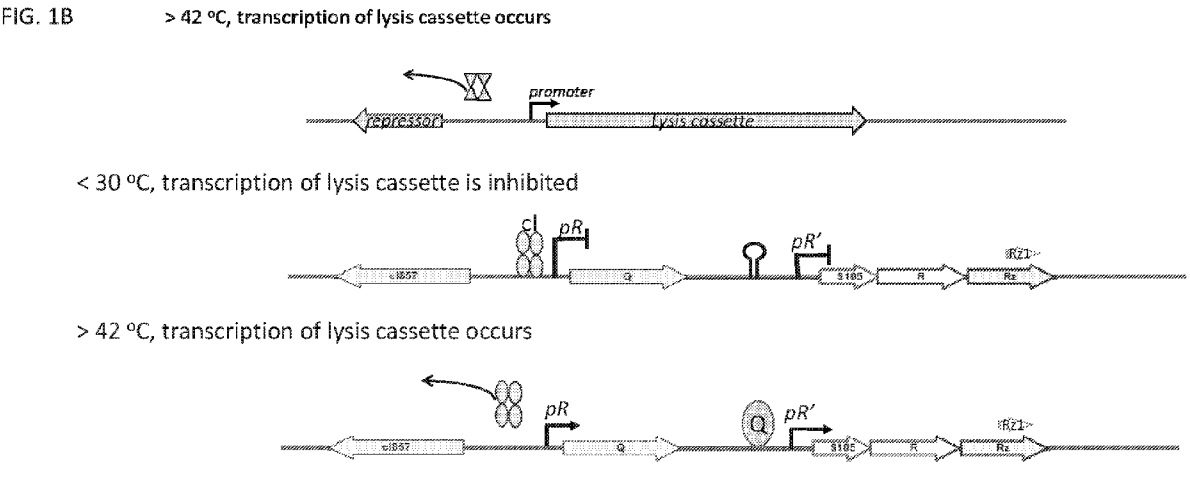
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(54) Title: STIMULUS-CONTROLLED CELL LYSIS

FIG. 1B



(57) Abstract: Stimulus-controlled cell lysis compositions and methods of their use are described.

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STIMULUS-CONTROLLED CELL LYSIS

[0001] This application claims the benefit under 35 USC 119(e) of U.S. Provisional Application No. 62/565,695, filed on September 29, 2017, which is incorporated herein by reference in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL IN ASCII TEXT FILE

[0002] This application incorporates by reference the Sequence Listing contained in the following ASCII text file:

File name: 0352_0039WO1_SL.txt; created on September 28, 2018, 24,633 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Disruption of cells is required to release their intracellular products for further use. While physical and chemical approaches can enable physical cell disruption, often this is non-optimal, particularly in the context of a high-throughput assay or for a sensitive enzyme.

SUMMARY OF THE INVENTION

[0004] A novel approach to disrupting cells/cellular membranes (i.e., lysing cell to release intracellular contents/components such as proteins) is described herein. More specifically, cells of interest can be genetically engineered/ programmed to lyse in response to a controlled, external stimulus, such as exposure to heat or light. The methods described herein are biologically sensitive, for example, lysis of the cells with minimal effect on the biological activity of enzymes or other proteins of interest released from the lysed cell.

[0005] The present invention encompasses methods of genetically engineering/modifying one, or more, transcription factors that regulate the expression of genes associated with cell death or lysis. As used herein, the term “transcription factor” or “transcription regulator” defines a sequence-specific DNA-binding protein that controls the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence. Transcription regulators have functionality/activity to promote or block the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific genes. For example, the regulator can act as promoter or activator, (i.e., a protein that binds to an enhancer, or activator binding

region of the DNA strand and enhances transcription from nearby promoter), or a repressor or blocker (i.e., a DNA binding protein that binds to the DNA strand and blocks the attachment of RNA polymerase to the promoter, thus preventing transcription of the genes). (See for example, Roeder, R.G. (September 1996). "The role of general initiation factors in transcription by RNA polymerase II". Trends in Biochemical Sciences. 21 (9): 327–35).

[0006] Specifically encompassed by the present invention is a cell lysis gene cassette comprising one, or more, cell lysis genes and one, or more, genetically modified transcriptional regulator genes associated with the expression of the cell lysis genes, wherein the transcriptional regulator encoded by the regulator gene(s) is stimulus-controlled. As used herein, the term "associated with" means that the expression of the cell lysis genes is regulated by, or otherwise dependent on, the biological activity or functionality of the transcriptional regulator protein. The function/activity of the transcriptional regulator protein is, in turn, controlled by (directed by) the stimulus (i.e., upon exposure to the stimulus). The transcriptional regulator gene can encode a repressor protein or an activator protein. The cell lysis gene encodes a cell-disrupting protein (a lysis protein), or a biologically active fragment of the lysis protein such as a biologically active, lytic peptide. Such proteins are known to those of skill in the art and can include, for example, members of the holin, pinholin, spanin, or endolysin families of proteins, or other homologous proteins with the biological activity/functionality of disrupting cell membrane structure (i.e., permeabilizing the cell membrane).

[0007] In one embodiment of the present invention, the transcriptional regulator gene encodes a genetically modified protein that, once expressed, undergoes a conformational change in response to the stimulus, whereby the activity/function of the regulator is altered (e.g., the conformational change in the protein results in the expression of one, or more, cell lysis genes, or the repression of one, or more cell lysis genes, resulting in cell lysis/death. Such stimulus can be, for example, changing the temperature during cell growth conditions or during analytical conditions. As described herein, the repressor protein AlpR has been modified (AlpR-A61T) to produce a thermally labile repressor protein.

[0008] In another embodiment of the present invention, the transcriptional regulator gene encodes a transcriptional regulator protein that is genetically modified to respond to

an external stimulus such as light. For example, the transcriptional regulator gene can encode a transcriptional regulator protein that changes the conformation of, or activity of, the regulator protein upon exposure to a specific wave length of light, for example, via an azobenzene photo switch.

[0009] Alternatively, particles (nanoparticles) or agents can be introduced into the medium surrounding the cells of interest, into the cells, or onto the cellular surface, that are stimulus-controlled, e.g., react to changes in heat or light exposure. For example, gold nano particles can be introduced into the cells that heat upon exposure to a specific energy source. The heat generated internally within the cell can trigger a conformational change of a regulator protein, resulting in the expression of the cell lysis genes and cell lysis.

[0010] Also encompassed by the present invention are compositions comprising a cell of interest, (e.g., an isolated cell) and the cell lysis gene cassette described above. The cell can be a prokaryotic cell or a eukaryotic cell, and in particular is a bacterial cell. More specifically, the present invention comprises a transformed bacterial cell comprising the cell lysis gene cassette described herein. The transformed bacterial cell can be an *E. coli*, or, more specifically, an *E. coli* strain BL2l, and the cell lysis gene cassette can comprise one or both plasmid vectors pLysis or pINT_HK. In particular, the thermally labile variant of *E. coli* BL2l comprising recombinant plasmids pLysis and pINT_HK is encompassed by the present invention. Also encompassed by the present invention is a vector such as an expression vector comprising the cell lysis cassette (e.g., pLysis and/or pINT_HK) that is suitable for use in the methods of stimulus-controlled cell lysis described herein, and the cells described herein. Suitable vectors can be designed by methods known to those of skill in the art. Primer sequences as shown in FIG. 4C used to construct the plasmids are also encompassed by the present invention.

[0011] Methods encompassed herein include any method using the cell lysis cassette or vector described above to result in cell lysis or death. In particular, a method of stimulus controlled cell lysis is encompassed by the present invention. For example, the method comprises the steps of introducing the cell lysis cassette or vector as described herein into the cell of interest. Such methods of transforming or transfecting cells are known to those of skill in the art. After introducing the cassette into the cell(s), the cells are maintained under suitable conditions for growth or analysis. When cell lysis is to be initiated, the cells are exposed to the appropriate external stimulus, under conditions sufficient to alter the

activity/function of the transcriptional regulator proteins encoded by the transcriptional regulator genes of the cassette, resulting in the expression of the cell lysis genes and cell lysis.

[0012] A further method encompassed herein is a method of lysing cells wherein the cells are encapsulated within a droplet such as in a microfluidic device suitable and wherein the cells comprise a gene cassette as described herein. Such a microfluidic device can be used for analysis of molecular interactions of proteins within cells of interest, or for protein synthesis. Applications of droplet microfluidics can be found for example, in "Recent Advances in Applications of Droplet Microfluidics" Micromachines 2015, 6, 1249-1271. More particularly, the method can comprise specifically targeting and selectively lysing one, or more, cells in a droplet while the other cells in the droplet are not lysed and therefore remain viable for e.g., further molecular interactions for molecular pathway elucidation, signal detection or diagnostic purposes.

[0013] Also encompassed by the present invention is a method of producing a protein, peptide or other biomolecule in a cell. For example, the method can comprise introducing a cell lysis gene cassette into a target cell and further substantially simultaneously, or subsequently, introducing a protein expression vector comprising a gene encoding a protein or biomolecule of interest into the target cell. More specifically, a cell such as a bacterial cell can be transformed with an expression vector comprising one, or more genes encoding one, or more, proteins/peptides/biomolecules of interest and co-transformed (or subsequently transformed) with a plasmid vector comprising the cell lysis gene cassette as described herein. Alternatively the protein expression vector can further comprise the cell lysis gene cassette. The cell is incubated under conditions suitable for the expression of the protein of interest. After sufficient incubation, the cell lysis gene cassette is stimulated (e.g., by thermal stimulation of increasing the incubation temperature) and the targeted cell specifically lyses and releases the protein of interest into a cell lysate. The cell lysate can be recovered by standard means and made available for further analysis or reactions. For example, the method can comprise recovering the cell lysate comprising the proteins/biomolecules of interest (i.e., cell lysate components) from the lysed cells, contacting the components of the cell lysate (e.g., proteins) with additional reagents or a second population of cells, and monitoring or detecting interactions of the cell lysate

components with the reagents or cells. Reagents can be, for example, chemicals or molecules that detect a specific protein such as an enzyme or fluorescent detection reagent.

[0014] Alternatively, target cells can be selectively lysed using the methods and cell lysis gene cassettes described herein. For example, a first population of targeted cells can be lysed and the cell lysate components can be maintained in contact with a second population of intact cells that, for example, express a protein or biomolecule that specifically interacts with the proteins in the cell lysate. Such specific interactions can be, for example, a specific binding reaction such as between an antibody and antigen, or a cell surface receptor and ligand. These specific interactions can be detected by means known to those of skill in the art.

[0015] A further method encompassed by the present invention is a method of using gold nanoparticles to transduce an optical stimulus to a genetically controlled thermal stimulus for cell lysis. In particular, the gold nanoparticles can be in solution, or selectively attached to the surface of the cell, or wherein the gold nanoparticles are encapsulated in the cells.

[0016] The above and other features of the invention including various novel details of construction and combinations of parts, and other advantages, will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will be understood that the particular method and device embodying the invention are shown by way of illustration and not as a limitation of the invention. The principles and features of this invention may be employed in various and numerous embodiments without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] In the accompanying drawings, reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale; emphasis has instead been placed upon illustrating the principles of the invention. . The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings(s) will be provided by the Office upon request and payment of the necessary fee. Of the drawings:

[0018] FIG 1A. A generalized he cell lysis cassette. FIG 1B the cell lysis cassette constructed for example 1 < 30 °C, repressor cI is expressed, repressing the transcription

from pR promoter. Lysis genes are not transcripted either, due to the terminator sequence present upstream of pR' promoter. At > 42 °C, cI becomes inactive, anti-terminator Q is thus expressed. Q binds to the terminator sequence, unwinds it, so that lysis genes can be transcribed.

[0019] FIG 2A and 2B. Amino acid sequences of the thermally labile repressor AlpR (SEQ ID NO. 1) and modified AlpR, AlpR-A61T (SEQ ID NO. 2).

[0020] FIG 3A and 3B. Plasmid maps of the two plasmids, pLysis (SEQ ID NO. 3) and PINT_HK (SEQ ID NO. 4) used to produce a thermally labile *E. coli* BL21

[0021] FIG 4A-C. FIG 4A shows a list of E. coli strains. FIG 4B shows a list of plasmids. FIG 4C is a list of primer sequences (SEQ ID NOS: 5-20) used in the assembly of the thermally labile *E. coli* BL21.

[0022] FIG 5 depicts different schemes for interacting gold nanoparticles (depicted as black circles) with *E. coli* to transduce optical signals to thermal signals to stimulate cell lysis.

[0023] FIG 6 is a schematic showing microfluidic work flow for encapsulation of cells in droplets and the selective thermal lysis of a subset of cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0024] The invention now will be described more fully hereinafter with reference to the accompanying drawings, in which illustrative embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0025] As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. Further, the singular forms and the articles "a", "an" and "the" are intended to include the plural forms as well, unless expressly stated otherwise. It will be further understood that the terms: includes, comprises, including and/or comprising, when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. Further, it will be understood that when an element,

including component or subsystem, is referred to and/or shown as being connected or coupled to another element, it can be directly connected or coupled to the other element or intervening elements may be present.

[0026] As described herein, cells can be genetically manipulated to lyse in response to a controlled external stimulus using the molecular/genetic machinery (transcriptional and translational processes) of the host cell (e.g., cell of interest) by which the cell turns on/off the transcription of, or expression of, proteins essential to cell membrane integrity, resulting in cell lysis. For example, lysis can result from the initiation of a programmed cell death pathway such as apoptosis by inducing, promoting or repressing the transcription of certain genes associated with apoptosis. Lysis can also result from the accumulation of cell-death associated proteins expressed at high levels within the cell, such as the accumulation of holins in bacteriophage-infected cells.

[0027] As described herein, a genetic circuit (see for example, "Principles of genetic circuit design", J. A. N. Brophy & C. A. Voigt; Nature Methods 11, 508–520 (2014) can be engineered into cells to enable cell lysis in response to a controlled stimulus. In particular, the cells of interest are bacterial cells, but the methods described herein can be adapted to any cell, such as yeast or mammalian cells. An example of the methods described herein is to engineer such a circuit based on the machinery of phages. This machinery includes the genes responsible for cell lysis and the transcriptional regulators that control this lysis. The transcriptional regulator could be placed under the control of an inducible promoter or the regulator can be stimulus labile (thermal, optical, chemical). In one particular case, the transcriptional regulator is a DNA binding protein that binds its cognate site as a dimer, and does not function except as at least a dimer. Therefore, anything that disrupts dimerization will also cause the cell lysis genes to be expressed. Another method encompassed herein is to activate expression of cell lysis genes by placing them directly under the control of an inducible promoter, or activator or repressor protein.

Stimulus-induced lysis of bacterial cells

The variant λ bacteriophage holin S105 containing the A52G substitution is of particular interest, as its lysis occurs more rapidly after induction than the native S105 holin. (Johnson-Boaz R, Chang CY, Young R. A dominant mutation in the bacteriophage lambda S gene causes premature lysis and an absolute defective plating phenotype. Mol Microbiol. 1994 Aug;13(3):495-504.)

Thermally-induced lysis

[0028] A gene cassette/genetic circuit can be designed that comprises one, or more, lysis genes and a thermal-labile repressor as described herein. In one example, a genetic lysis circuit can be produced by placing the λSRRzRZ1 lysis cassette downstream from the pR' promoter, which is under repression by the thermally labile repressor, λCI857, expression of genes in the lysis cassette can be thermally induced. Another example consists of the repressor AlpR, that could be rendered thermally labile by the A61T substitution, whose removal would allow expression of the lysis machinery AlpB and AlpC. The sequence for ALP-R is shown in FIG. 2A and the modified sequence is shown in 2B. The transcription factor AlpR represses expression of the AlpB-AlpC lysis proteins, which are native to Pseudomonas aeruginosa PAO1. The genes encoding these proteins are introduced to E. coli, or another susceptible host, by introduction on a plasmid or integration in the host chromosome. After thermal upshift, the AlpR-A61T protein is no longer capable of maintaining repression of the lysis proteins, which causes the cells to lyse. The expression of the lysis proteins may also be placed under control of other stimuli, for example, a chemical agent.

[0029] The cells are grown at < 37° C. Cell lysis will be thermally induced by incubation at 42° for ~15 min and resuming growth at < 37° C for 30 minutes, whereupon the cells containing the particular genetic circuit, as described herein, would lyse. Soluble proteins can be isolated from the cell lysate by centrifugation, or other suitable techniques known to those of skill in the art.

[0030] In particular, as described herein, engineering the genetic circuit into a protein expression strain such as the *E. coli* BL21 can control cell lysis and enable the release of other expressed protein products.

[0031] **Example 1: A thermally labile *E. coli* for protein expression**

[0032] To construct the thermally labile variant of the *E. coli,* BL21, two plasmids, one termed pLysis (FIG. 3A) carrying the cell lysis machinery, and one termed pINT_HK (FIG. 3B) are used to integrate the lysis machinery into *E. coli* chromosome were designed. The pINT_HK plasmid was constructed by two-way isothermal assembly. Fragment 1 containing lacI gene and Ptac promoter was amplified from plasmid pAH55, using primers F_lacI-Ptac (SEQ ID NO: 5) and R_lacI-Ptac (SEQ ID NO: 6). Fragment 2 containing the integrase from phage HK022 was amplified from plasmid pAH69, by primers F_pAH69

(SEQ ID NO: 7) and R_pAH69 (SEQ ID NO: 8). The plasmid was transformed into NEB® 10-beta Competent E. coli cells, and selected on LB plates containing 100 µg/ml ampicillin at 30 oC. The correct colony was named TL_coli_1.

[0033] The pLysis plasmid was constructed by four-way isothermal assembly. Fragment 1 containing the attP site of phage HK022 was amplified from pAH144 using primers F_pAH144 (SEQ ID NO: 9) and R_pAH144 (SEQ ID NO: 10). Fragment 2 containing the cI gene and pR promoter was amplified from plasmid pAH69 using primers F_cIpR (SEQ ID NO: 11) and R_cIpR (SEQ ID NO: 12). Fragment 3 containing anti-terminator Q was amplified from lambda genomic DNA using primers F_Q (SEQ ID NO: 13) and R_Q (SEQ ID NO: 14). Fragment 3 containing the lysis cassette was amplified from plasmid pS105 using primers F_pS105 (SEQ ID NO: 15) and R_pS105 (SEQ ID NO: 16). The plasmid was transformed into E.coli strain BW23473 and selected on LB plates containing 100 µg/ml spectinomycin at 30 °C. The correct colony was named TL_coli_2.

[0034] The pLysis plasmid was then transformed into TL_coli_1 competent cells, which was made by inducing the cells with 1mM IPTG for 30 minutes to allow the phage HK022 integrase to express. The transformants were selected on LB plates containing 35 µg/ml spectinomycin at 30 oC. Correct single integration event was confirmed by PCR amplification using primers CRIM_HK_P1 (SEQ ID NO: 17), CRIM_P2 (SEQ ID NO: 18), CRIM_P3 (SEQ ID NO: 19), and CRIM_HK_P4 (SEQ ID NO: 20). The E.coli strain with pLysis integrated into the chromosome at HK022 attachment site (attB) was then named TL_coli_3.

[0035] To introduce pLysis into the protein expression E.coli strain BL21(DE3), phage P1vir was used to infect strain TL_coli_3. The phage lysate was then used to infect BL21(DE3) by the standard transduction protocol. The transductants were selected on LB plates containing 20mM Sodium Citrate and 35 ug/ml spectinomycin at 30 oC. Correct transductants were confirmed by PCR amplification using primers CRIM_HK_P1, CRIM_P2, CRIM_P3, and CRIM_HK_P4, and renamed TL_coli_4.

[0036] The thermal induced cell lysis event of strains TL_coli_3 and TL_coli_4 were confirmed by growing the cells at 30 °C until turbid, and shifting to 42 °C for 15 minutes, followed by shifting to 37 °C until cell culture became transparent.

[0037] A thermally labile ***Staphylococcus aureus***

[0038] Gram positive bacteria such as *S. pyogenes,* and *S. aureus* are known to be difficult to lyse by traditional means. Phage proteins of bacteria can be used to facilitate lysis of these organisms. More rapid, genetically-controlled lysis may facilitate the release and harvest of some bacterial products, such as RNA species that are sensitive to certain stimuli that are currently used to facilitate lysis by standard means.

[0039] Example of phage lytic proteins are the *S. aureus* bacteriophage vB_SauS-philPLA88 derived endolysin LysH5 and fusion proteins between lysostaphin and a virion-associated peptidoglycan hydrolase HydH5, described in "The Phage Lytic Proteins from the Staphylococcus aureus," PLOS ONE, 2013 May, 8:5, e64671. Additional description of *S. aureus* phage lytic proteins is described in the review article, "Are Phage Lytic Proteins the Secret Weapon To Kill Staphylococcus aureus?," mBio, 2018 Jan/Feb, 9:1, e01923.

[0040] The genes that produce these lytic proteins can be placed under the control of known elements that respond to certain stimuli, such as the thermally controlled repressors λCI857 or AlpR described above, or novel elements that may be derived from new sequences based on sequence homology.

Strain list:

Name	Relevant genotype and features	Source
NEB® 10-beta	*E.coli* K12 strain *Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14-ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	NEB®
BW23473	*Δ(lacIZYA argF)U169 rph-1 rpoS396(Am) robA1 creC510 hsdR514 ΔendA9 uidA(ΔMluI)::pir(wt)recA1*	Haldimann et al., CGSC (Coli Genetic Stock Center)
TL_coli_1	NEB® 10-beta (pINT_HK)	This work
TL_coli_2	BW23473 (pLysis)	This work
TL_coli_3	NEB® 10-beta *attB_HK022*::pLysis	This work
BL21 (DE3)	E.coli B strain for protein expression *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS* *λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	NEB®
TL_coli_4	BL21 (DE3) *attB_HK022*::pLysis	This work

Plasmid list:

Name	Relevant features	Source
pAH55	Plasmid harboring *lacI^q, Ptac,* kanR	Haldimann et al., CGSC
pAH69	Plasmid having expression of Int_{HK022} controlled by cI857, *pR* promoter, AmpR	Haldimann et al., CGSC
pAH144	Integration plasmid having *attP_HK022* site, SpecR	Haldimann et al., CGSC
pS105	Plasmid containing part of lambda genome from part of *Q* to the lysis cassette (*S, R, Rz, Rz1*)	Smith et al.
pINT_HK	Plasmid expressing Int_{HK022} under the control of *lacI^q, Ptac*	This work
pLysis	Integration plasmid containing cI857, *pR* controlled *Q, lysis cassette*	This work

Primer list:

Name	Sequence (SEQ ID NOS: 5-20)	Source
F_lacI-Ptac	CTGCATCGATCTGCAGCATACACTCCGCTATC	This work
R_lacI-Ptac	TTCTTCCCATATGTATACCTCCTTCTTACAAGCTTGG	This work
F_pAH69	AGGTATACATATGGGAAGAAGGCGAAGTCATG	This work
R_pAH69	TATGCTGCAGATCGATGCAGGTGGCACTTTTCG	This work
F_pAH144	GTGCAGATAGGAATTCTCATGTTTGACAGCTTATCAC	This work
R_pAH144	CTTCATCGATGGATCCTCTAGAGTCGACC	This work
F_cIpR	TAGAGGATCCATCGATGAAGATTCTTGCTCAATTG	This work
R_cIpR	CGAGTCTCATTTCTGATCCTCTTCAAAAGGC	This work
F_Q	AGGATCAGAAATGAGACTCGAAAGCGTAGC	This work
R_Q	GCAATCATGCTGCTAACGTGTGACCGCATTC	This work
F_pS105	CACACGTTAGCAGCATGATTGCCACGGATG	This work
R_pS105	ATGAGAATTCCTATCTGCACTGCTCATTAATATACTTC	This work
CRIM_HK_P1	GGAATCAATGCCTGAGTG	Haldimann et al.,
CRIM_P2	ACTTAACGGCTGACATGG	Haldimann et al.,
CRIM_P3	ACGAGTATCGAGATGGCA	Haldimann et al.,
CRIM_HK_P4	GGCATCAACAGCACATTC	Haldimann et al.,

Example 2: Direct Optically-induced lysis

[0041] The cell lysis gene(s) can be placed under control of light-controlled transcriptional regulator. Such a photoactivatable regulator can also be referred to as a "photo caged", or "caged" regulator which is "uncaged" as a result of being exposed to a specific energy source/wavelength of light, and the caged transcriptional regulator is inhibited from activating gene expression (see, e.g., US Patent 7,541,193). One method to control cell lysis is to engineer a transcriptional regulator as a DNA binding protein dimer for which dimerization is disrupted in response to an optical stimulus. This can potentially be achieved by the coupling of a photo switch molecule such as an azobenzene or a light responsive moiety to the dimer. A regulator protein coupled to a photo switch can be produced using post translational modification methods known to those of skill in the art. (See e.g., Binder, et al., Interg. Biol. 2014, 6, 755; Mart, R.J. and Allerman, R. K., "Azobenzene photocontrol of peptides and proteins" Chem. Commun. 2016, 52, 12262).

[0042] **Example 3: Optically-induced thermal lysis**

[0043] An alternative approach to use light to stimulate cell lysis is to use plasmonic nanoparticles that will heat upon illumination. When illuminated, these particles will heat locally. The temperature can be controlled through the density of the gold nanoparticles, the laser temperature, and spectral detuning of the illumination of the illumination source from the plasmon resonance of the gold nanoparticles. ("Light-Induced Heating of Gold Nanoparticles in Colloidal Solution: Dependence on Detuning from Surface Plasmon Resonance", Plasmonics **11**, 345-350 (2016)).

[0044] Gold nanoparticles can be used extracellularly to raise the temperature of the surrounding cell media (FIG 5), can be functionalized to selectively interact with the cell surface to provide more localized temperature control, or can be utilized intracellularly. To introduce gold nanoparticles into *E. coli* intracellulary. The *E. coli* can be engineered to produce caveolin enabling endocytosis ("Constitutive formation of caveolae in a Bacterium," Cell, 2012 Aug 17, 150, 752-75) thereby allowing uptake of gold nanoparticles.

[0045] In the presence of the thermally controlled cell lysis gene this illumination can locally raise the temperature stimulate cell lysis. (See, e.g., "Plasmonic photothermal therapy (PPTT) using gold nanoparticles", Lasers Med Sci. 2008 Jul;23(3):217-28; Huang, X. and El-Sayed, M. A., "Plasmonic photo-thermal therapy (PPTT) Alexandria J. Med.

(2011) 47, 1-9). Cell lysis is possible directly using the heating from gold nanoparticles by heating to more extreme temperatures, however high temperatures can damage proteins or other biological components produced within the cells or damage/kill other neighboring cells that are not targeted for lysis.

[0046] **Example 4: Use of genetically controlled lysis in droplets**

[0047] Lysis of cells inside droplets can be important to enable the analysis of biomolecules that are contained within the cell. Current approaches involve either the fusion of lysis reagents, electrical lysis, or the freezing and thawing of the droplets, all of which can disrupt the droplet, be denaturing, or otherwise interfere with this analysis. The ability to control the lysis genetically in response to a particular signal will enable the selection of a broad range of stimuli. In particular, the use of a thermal or optical stimulus, as described above, will be non-invasive to the droplet.

[0048] A mixed population of cells can be contained within a droplet and some portion of this population could contain genetic circuits enabling them to be selectively lysed. An example application of this is to have a biomolecule-producing bacterium that is co-encapsulated in the same droplet as a different type of cell. The selective lysis of the biomolecule-producing cell would enable interactions of the biomolecule with the second cell type, which could then be assayed, e.g., in high-throughput screening analysis.

[0049] Microfluidic encapsulation of thermally labile single cells

Commercially available systems such as the μEncapsulator System (Dolomite Bio) can enable the encapsulation of individual cells in pL aqueous droplets dispersed in an oil phase. Cells containing the machinery for thermal cell lysis and that produce a protein, antibody or other compound of interest are prepared in one set of droplets and a second set of cells that do not lyse thermally are prepared in a second set of droplets. A microfluidic chip is used to merge droplet set 1 with droplet set 2, creating droplets that contains a cell that will lyse under a thermal stimulus and cells that will not lyse under a thermal stimulus. The droplets are heated to 42 °C causing the thermally responsive cells to lyse (FIG. 6) The cell lysate containing any protein or other products will interact with the cells from droplet set 2 and this response can be monitored. FADS (fluorescent activated droplet sorting) can be used to sort cells that exhibit a particular response to the cell lysate from those that do not. In an alternative version of this example, the cells that thermally lyse can also contain

gold nanoparticles and a light source can be used to selectively lyse cells rather than a heat source.

References (all references are herein incorporated by reference in their entirety)

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[0061] "Plasmonic photo-thermal therapy (PPTT) Alexandria J. Med. (2011) 47, 1-9).

[0062] While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A cell lysis gene cassette comprising one, or more cell lysis genes and one, or more, genetically modified transcriptional regulator genes associated with the expression of the cell lysis genes, wherein the transcriptional regulator protein encoded by the transcriptional regulator gene(s) is stimulus-controlled.

2. The cell lysis cassette of claim 1, wherein the transcriptional regulator gene encodes a repressor protein.

3. The repressor protein of claim 2, wherein the protein is a thermally labile repressor protein.

4. The repressor protein of claim 3, wherein the protein is AlpR-A61T.

5. The cell lysis cassette of claim 1, wherein the transcriptional regulator gene encodes an activator protein.

6. The cell lysis cassette of claim 1, wherein the transcriptional regulator encoded by the transcriptional regulator gene undergoes a conformational change in response to the stimulus whereby the activity/function of the regulator protein is altered.

7. The cell lysis cassette of claim 6, wherein the alteration of the transcriptional regulator protein function results in the expression of one, or more cell lysis genes.

8. The cell lysis cassette of claim 7, wherein the cell lysis gene encodes a membrane disruptive protein.

9. The membrane disruptive protein of claim 8, wherein the protein is selected from the group of proteins consisting of holins, endolysins, or spanins.

10. The cell lysis cassette of claim 1, wherein the transcriptional regulator is genetically modified to encode a transcriptional regulator protein that

responds to a stimulus selected from the group consisting of thermal stimulus, optical stimulus or chemical stimulus.

11. A composition comprising an isolated cell comprising the cell lysis cassette of claim 1.

12. The composition of claim 11, wherein the cell is a prokaryotic cell or a eukaryotic cell.

13. A recombinant plasmid vector selected from the group consisting of pLysis or pINT_HK.

14. A transformed bacterial cell comprising one, or both, of the plasmids of claim 13.

15. A thermally labile variant of *E.coli* strain BL2l.

16. The thermally labile variant *E. coli* of claim 15, wherein the *E. coli* comprises the plasmids pLysis and pINT_HK.

17. A primer sequence selected from the group consisting of SEQ ID NOS: 5-16.

18. A method of stimulus controlled cell lysis, the method comprising the steps of:

a) introducing the cell lysis cassette of claim 1 into the cell of interest;

b) maintaining the cell under conditions suitable for the expression of the genes of the cell lysis cassette; and

c) exposing the cell to the appropriate stimulus, under conditions sufficient to alter the activity/function of the transcriptional regulator protein encoded by the cell lysis gene cassette, resulting in the expression of the cell lysis genes and cell lysis.

19. The method of claim 18, wherein the transcriptional regulator is genetically modified to encode a transcriptional regulator protein that responds to a

stimulus selected from the group consisting of thermal stimulus, optical stimulus or chemical stimulus.

20. The method of claim 18, wherein the transcriptional regulator is AlpR-A61T.

21. The method of either claim 18 or 19, wherein the cell is encapsulated in a droplet.

22. The method of either claim 18 or 19, wherein the lysis is a selective lysis comprising selectively lysing one population of cells in a droplet while the second population of cells is not lysed.

23. A method of producing a protein or a biomolecule in a cell, the method comprising:

a) introducing into the cell a cell lysis gene cassette of claim 1;

b) introducing into the cell an expression vector encoding a protein or biomolecule of interest;

c) incubating the cell under conditions suitable for the expression of the protein of interest within the cell; and

d) exposing the cell to the appropriate stimulus, under conditions sufficient to alter the activity/function of the transcriptional regulator protein encoded by the cell lysis gene cassette, resulting in the expression of the cell lysis genes and lysis of the cell, thereby releasing the proteins from the cell.

24. The method of claim 23, wherein the transcriptional regulator is genetically modified to encode a transcriptional regulator protein that responds to a stimulus selected from the group consisting of thermal stimulus, optical stimulus or chemical stimulus.

25. The method of claim 24, wherein the transcriptional regulator is AlpR-A61T.

26. The method of either claim 23 or 24, wherein the cell is encapsulated in a droplet.

27. The method of either claim 23 or 24, wherein the lysis is a selective lysis comprising selectively lysing a first population of cells in a droplet while the second population of cells are not lysed, thereby releasing the components of the lysed cells into a cell lysate.

28. The method of either claim 23 or 24, wherein the components of the cell lysate are maintained in contact with the second population of cells under conditions suitable for specific interactions with the cells and monitoring or detecting the specific interactions of the components of the cell lysate with the cells of the second population.

29. The method of any of claims 18, 19, 23 or 24, wherein thermal stimulus is used to lyse the cells comprising using gold nanoparticles to transduce an optical stimulus to a genetically controlled thermal stimulus for cell lysis.

30. The method of claim 29, wherein the gold nanoparticles are in solution surrounding the cells.

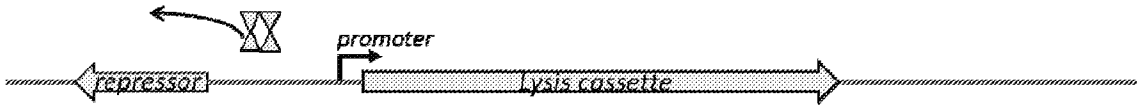
31. The method of claim 29, wherein the gold nanoparticles are selectively attached to the surface of the cells.

32. The method of claim 29, wherein the gold nanoparticles are encapsulated in the cells.

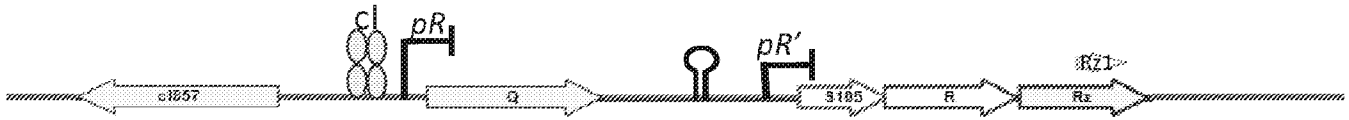
FIG. 1A **< 30 °C, transcription of lysis cassette is inhibited**



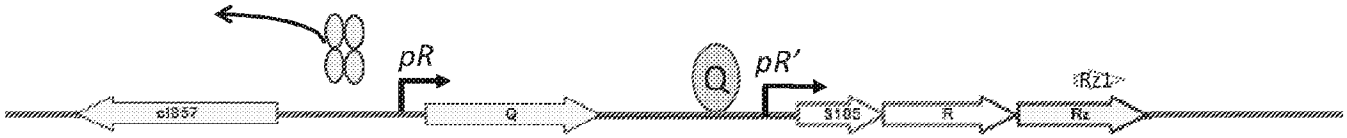
FIG. 1B **> 42 °C, transcription of lysis cassette occurs**



< 30 °C, transcription of lysis cassette is inhibited



> 42 °C, transcription of lysis cassette occurs



AlpR

MELKDRIKAARKHAHLSQVQL**AQAVGMTQTS**ISDLERGKSR**ATSFVAQIAG**ACGVNPLWL
AEGRGEMLAERGQANAGPNASWLGAVESWDDETPLDADEIELPFYKEIELSGGKGSTVILQ
TGGRKLRFGKYTLRKKNIDPASAACVTVSGNSMEPVLPDGSTVGVDTSARTIKDGDMYAFD
HDGQLRVKLLYRLPGGGLRIRSFNSDEHPDERYEPQEAAEHINVIGRVFWYSVLV

FIG. 2A

AlpR-A61T

MELKDRIKAARKHAHLSQVQL**AQAVGMTQTS**ISDLERGKSR**ATSFVAQIAG**ACGVNPLWL
TEGRGEMLAERGQANAGPNASWLGAVESWDDETPLDADEIELPFYKEIELSGGKGSTVILQ
TGGRKLRFGKYTLRKKNIDPASAACVTVSGNSMEPVLPDGSTVGVDTSARTIKDGDMYAFD
HDGQLRVKLLYRLPGGGLRIRSFNSDEHPDERYEPQEAAEHINVIGRVFWYSVLV

FIG. 2B

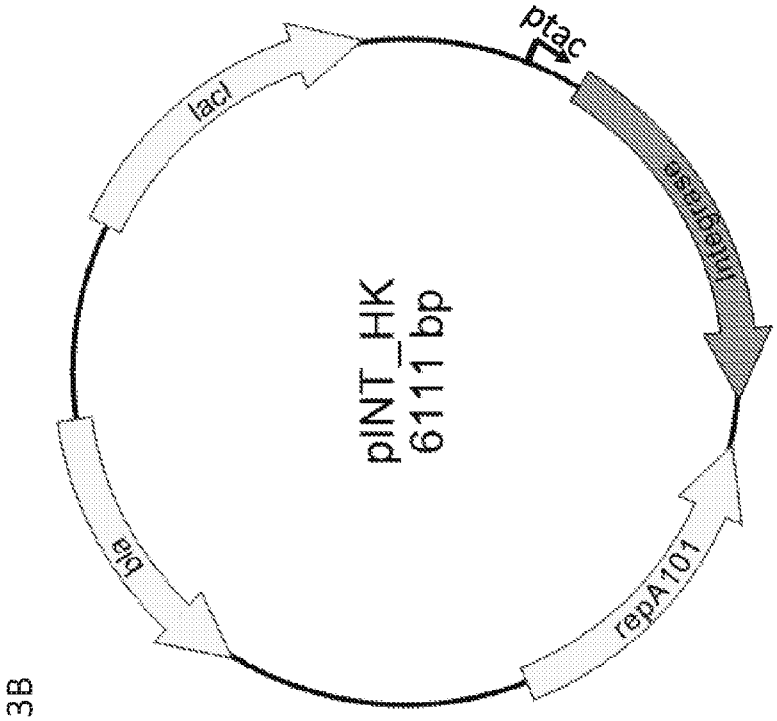

pINT_HK
6111 bp
ptac
lacI
Integrase
bla
repA101

FIG. 3B

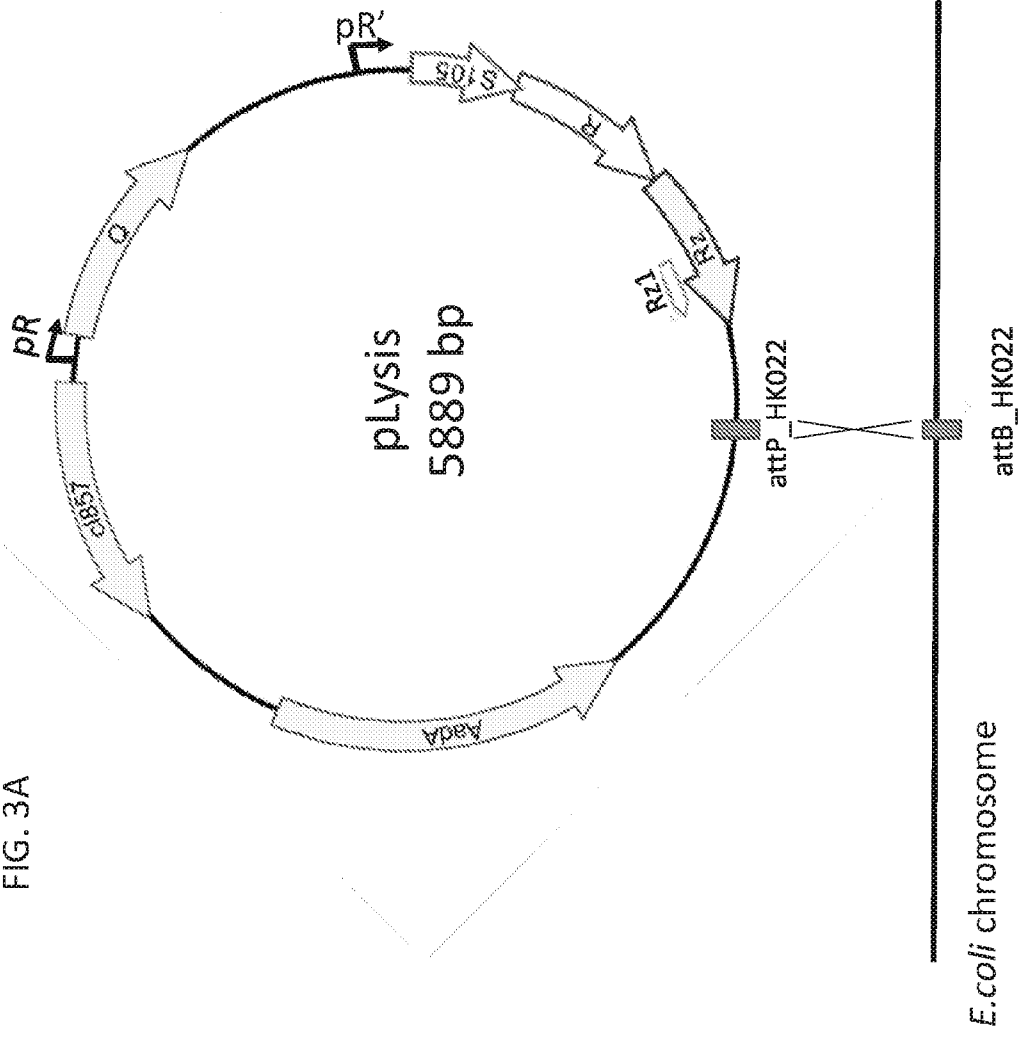

pLysis
5889 bp
pR'
S105
R
R2
R21
attP_HK022
attB_HK022
pR
Q
cI857
kanA
E.coli chromosome

FIG. 3A

FIG. 4A

Strain list:

Name	Relevant genotype and features	Source
NEB® 10-beta	*E.coli* K12 strain *Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14-ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	NEB®
BW23473	*Δ(lacIZYA argF)U169 rph-1 rpoS396(Am) robA1 creC510 hsdR514 ΔendA9 uidA(ΔMluI)::pir(wt)recA1*	Haldimann et al., CGSC (Coli Genetic Stock Center)
TL_coli_1	NEB® 10-beta (pINT_HK)	This work
TL_coli_2	BW23473 (pLysis)	This work
TL_coli_3	NEB® 10-beta *attB_HK022*::pLysis	This work
BL21 (DE3)	E.coli B strain for protein expression *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS* *λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	NEB®
TL_coli_4	BL21 (DE3) *attB_HK022*::pLysis	This work

Plasmid list:

Name	Relevant features	Source
pAH55	Plasmid harboring *lacI*^{q}, *Ptac*, kanR	Haldimann et al., CGSC
pAH69	Plasmid having expression of Int_{HK022} controlled by ci857, *pR* promoter, AmpR	Haldimann et al., CGSC
pAH144	Integration plasmid having *attP_HK022* site, SpecR	Haldimann et al., CGSC
pS105	Plasmid containing part of lambda genome from part of *Q* to the lysis cassette (*S, R, Rz, Rz1*)	Smith et al.
pINT_HK	Plasmid expressing Int_{HK022} under the control of *lacI*^{q}, *Ptac*	This work
pLysis	Integration plasmid containing ci857, *pR* controlled *Q, lysis cassette*	This work

FIG. 4B

FIG. 4C

Primer list:

Name	Sequence	Source
F_lacI-Ptac	CTGCATCGATCTGCAGCATACACTCCGCTATC	This work
R_lacI-Ptac	TTCTTCCCATATGTATACCTCCTTCTTACAAGCTTGG	This work
F_pAH69	AGGTATACATATGGGAAGAAGGCGAAGTCATG	This work
R_pAH69	TATGCTGCAGATCGATGCAGGTGGCACTTTTCG	This work
F_pAH144	GTGCAGATAGGAATTCTCATGTTTGACAGCTTATCAC	This work
R_pAH144	CTTCATCGATGGATCCTCTAGAGTCGACC	This work
F_clpR	TAGAGGATCCATCGATGAAGATTCTTGCTCAATTG	This work
R_clpR	CGAGTCTCATTTCTGATCCTCTTCAAAAGGC	This work
F_Q	AGGATCAGAAATGAGACTCGAAAGCGTAGC	This work
R_Q	GCAATCATGCTGCTAACGTGTGACCGCATTC	This work
F_pS105	CACACGTTAGCAGCATGATTGCCACGGATG	This work
R_pS105	ATGAGAATTCCTATCTGCACTGCTCATTAATATACTTC	This work
CRIM_HK_P1	GGAATCAATGCCTGAGTG	Haldimann et al.,
CRIM_P2	ACTTAACGGCTGACATGG	Haldimann et al.,
CRIM_P3	ACGAGTATCGAGATGGCA	Haldimann et al.,
CRIM_HK_P4	GGCATCAACAGCACATTC	Haldimann et al.,

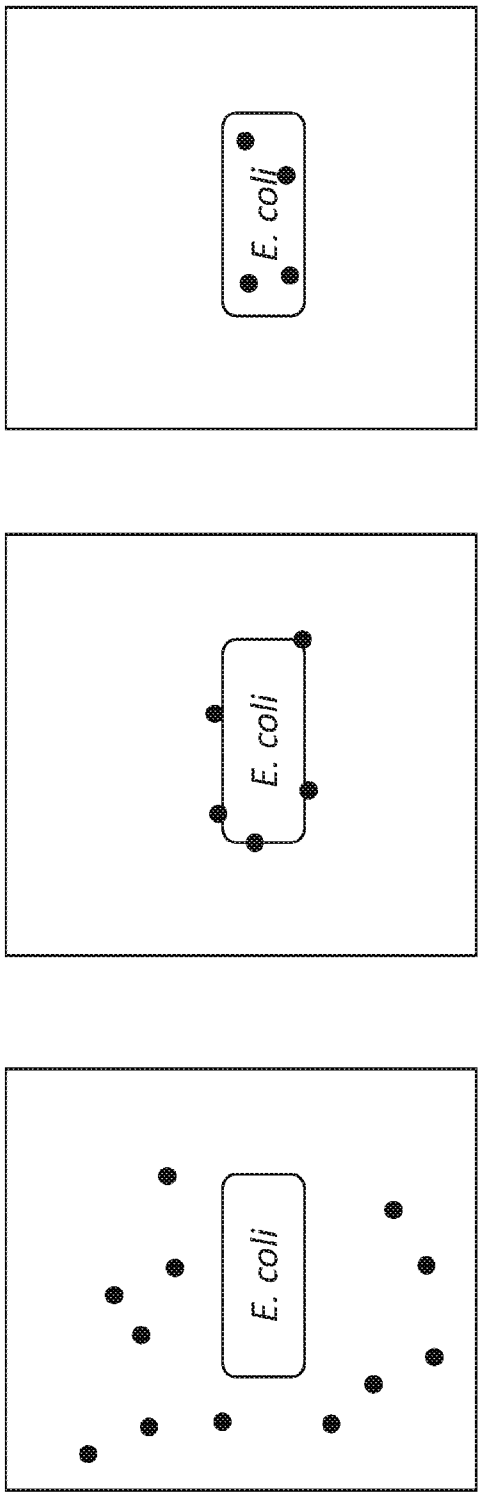


FIG. 5

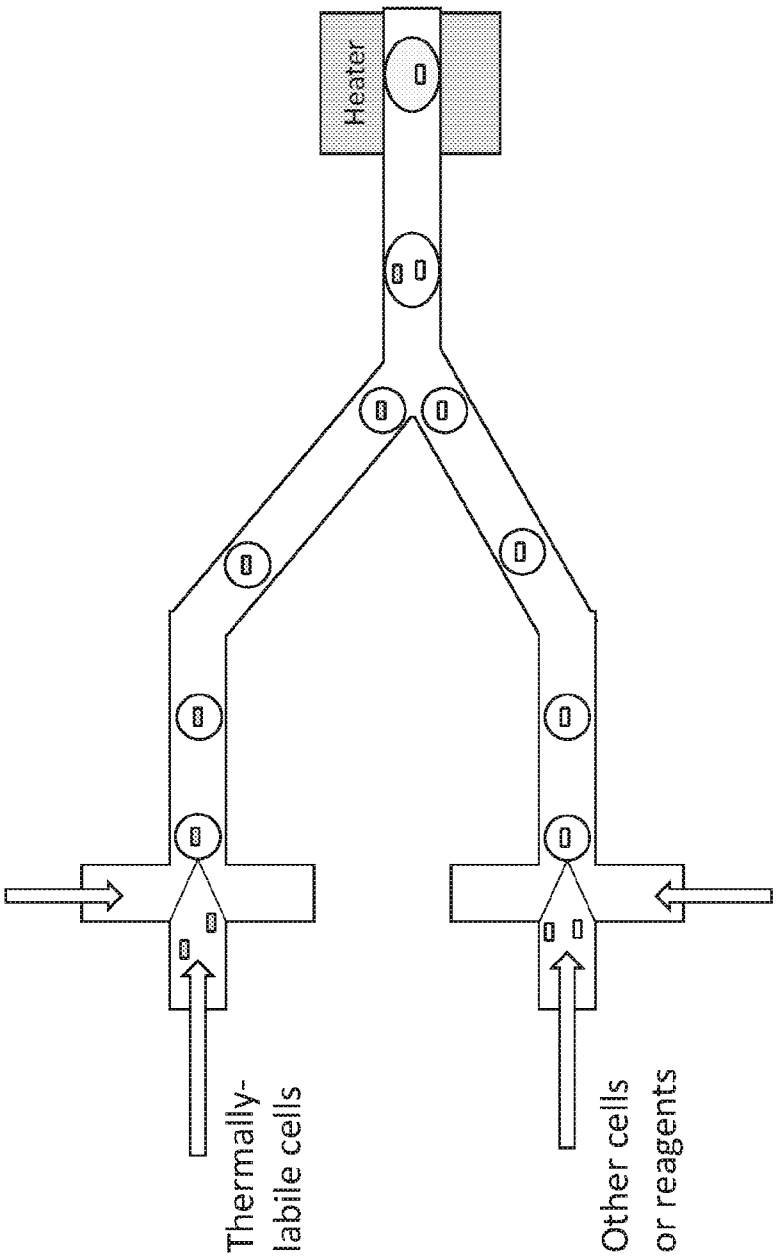


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/053582

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435 C12N15/09
ADD.

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)
C07K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIRSTY A. MCFARLAND ET AL: "A self-lysis pathway that enhances the virulence of a pathogenic bacterium", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 112, no. 27, 22 June 2015 (2015-06-22), pages 8433-8438, XP055534092, US ISSN: 0027-8424, DOI: 10.1073/pnas.1506299112 page 8433 - page 8436 ----- -/--	1-32

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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
18 December 2018	07/01/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gonçalves Mauger, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/053582

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOHNSON-BOAZ R ET AL: "A dominant mutation in the bacteriophage lambda S gene causes premature lysis and an absolute defective plating phenotype", MOLECULAR MICROBIOL, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 13, no. 3, 1 August 1994 (1994-08-01) , pages 495-504, XP009097863, ISSN: 0950-382X, DOI: 10.1111/J.1365-2958.1994.TB00444.X cited in the application abstract	1-32
A	K. A. MCFARLAND ET AL: "Autoregulated expression of the gene coding for the leucine-responsive protein, Lrp, a global regulator in Salmonella enterica serovar Typhimurium", JOURNAL OF GENERAL MICROBIOLOGY, vol. 154, no. 7, 1 July 2008 (2008-07-01), pages 2008-2016, XP055534150, ISSN: 1350-0872, DOI: 10.1099/mic.0.2008/018358-0 abstract	1-32
X	Michele Leroux: "Characterization of antagonistic interbacterial pathways at the single-cell level", Molecular and cellular biology, 29 September 2015 (2015-09-29), XP055535870, Retrieved from the Internet: URL:http://hdl.handle.net/1773/34066 [retrieved on 2018-12-18] chapter 4 and chapter 5; page 99 - page 137; figures 4.10, 4.11	1-32
X	US 2006/040393 A1 (JIA XIYU [US] ET AL) 23 February 2006 (2006-02-23) abstract; claims; figures	1-32
A	WO 02/34892 A1 (CISTEM BIOTECHNOLOGIES GMBH [AT]; BLAESI UDO [AT]) 2 May 2002 (2002-05-02) abstract; claims	1-32
X	EP 1 309 604 A2 (PHAGE BIOTECHNOLOGY CORP [US]) 14 May 2003 (2003-05-14) claims; examples	1-32
X	WO 00/71731 A1 (PHAGE BIOTECHNOLOGY CORP [US]) 30 November 2000 (2000-11-30) abstract; claims	1-32

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Information on patent family members

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