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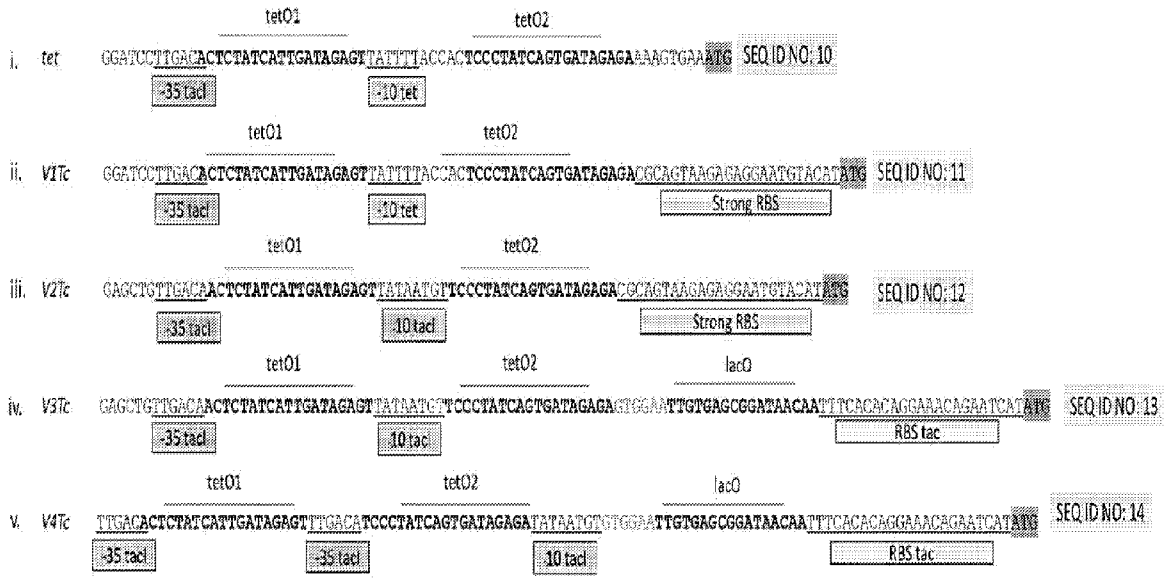

FIG. 2B

(57) **Abstract:** Provided herein are nucleic acid constructs that comprise an inducible promoter. Dual expression systems are provided comprising two nucleic acid constructs or a single nucleic acid construct with two inducible promoters. Also provided are methods of expressing transcripts by transforming a nucleic acid construct described herein into a prokaryotic cell and contacting the prokaryotic cell with an inducer. Methods of producing a carotenoid are also disclosed herein.

[Continued on next page]

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INDUCIBLE PROMOTERS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and priority to, U.S. Provisional Patent Application No. 63/332,507 filed April 19, 2022, and U.S. Utility Patent Application No. 18/136,660 filed April 19, 2023, the entirety of each of which is incorporated herein by reference.

BACKGROUND

[0002] Inducible promoters are ubiquitous biotechnology tools for manufacturing proteins, providing molecular models of biosynthesis pathways, and as synthetic switches for a variety of environmental, physiological, and cellular tools. Inducible promoters have a consistent architecture including two key elements: the operator region recognized by transcriptional regulatory proteins and consensus sequences that recruit the sigma (σ) subunits of RNA polymerase to initiate transcription of the inducible gene. Despite their widespread use, leaky transcription in the "OFF" state remains a challenge for inducible promoters. Therefore, improved inducible promoters, cellular systems, and methods of generating proteins are needed to enable advances in protein production and various biotechnology applications.

BRIEF SUMMARY

[0003] Provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TXTXXTGT (SEQ ID NO: 45) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TXGXCX (SEQ ID NO: 46) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence, wherein X is any nucleobase. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TATAAT (SEQ ID NO: 1) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. In some embodiments, the nucleic acid construct further comprises a transgene. In some embodiments, the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the

nucleic acid construct is inserted into a prokaryotic cell in the presence of an inducer; and wherein: (i) the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct; and (ii) the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. In some embodiments, the nucleic acid construct further comprises a transgene. In some embodiments, the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a prokaryotic cell in the presence of an inducer; and wherein: (i) the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct; and (ii) the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct. In some embodiments, a strong promoter increases the amount of expression of a transgene provided herein relative to a comparable inducible promoter that does not comprise SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46. In some embodiments, the strong promoter increases the amount of expression of a transgene provided herein by at least 10% relative to the expression of a transgene expressed by a comparable inducible promoter that does not comprise SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46.

[0004] Provided herein are methods of expressing a transgene in a cell, the methods comprising: (a) transforming a nucleic acid construct into the cell, wherein the nucleic acid construct comprises a modified inducible promoter, wherein the modified inducible promoter comprises: (i) a TATAAT (SEQ ID NO: 1) sequence or a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; (iii) a nucleic acid sequence encoding a bacterial ribosome binding sequence; and (iv) a transgene; and (b) contacting the cell

with an inducer, thereby expressing the transgene. In some embodiments, when cloned into a comparable nucleic acid construct that comprises a strong promoter, the transgene inhibits growth of the cell prior to the contacting step (b) with the inducer, thereby preventing expression of the transgene via the comparable nucleic acid. In some embodiments, the cell is a prokaryotic cell. In some embodiments, the cell is a eukaryotic cell.

[0005] Provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: (a) a first modified inducible promoter sequence, wherein the first modified inducible promoter comprises: (i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the first modified inducible promoter sequence; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the first modified inducible promoter sequence; and (iii) a nucleic acid sequence that encodes a first bacterial ribosome binding sequence; and (b) a second modified inducible promoter sequence, wherein the second modified inducible promoter sequence comprises: (i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the second modified inducible promoter sequence; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the second modified inducible promoter sequence; and (iii) a nucleic acid sequence that encodes a second bacterial ribosome binding sequence. In some embodiments, the nucleic acid constructs further comprise a transgene. In some embodiments, wherein the first modified inducible promoter sequence, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is in the presence of an inducer.

[0006] A composition comprising two or more of a nucleic acid construct provided herein.

[0007] Provided herein are isolated prokaryotic cells, wherein the isolated prokaryotic cells comprise a nucleic acid construct provided herein. Provided herein are isolated eukaryotic cells, wherein the isolated eukaryotic cells comprise a nucleic acid construct provided herein. Provided herein are cell-free systems, wherein the cell-free systems comprise a nucleic acid construct provided herein.

[0008] Provided herein are non-naturally occurring organisms, wherein the non-naturally occurring organisms comprise: a nucleic acid construct comprising: (a) a first inducible promotor sequence comprising:(i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (iii) a nucleic acid sequence that encodes a bacterial ribosome binding sequence; (b) one or more of a biosynthesis pathway transgene; and (c) a second modified inducible promotor sequence comprising: (i) a TATAATGT

(SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the second modified inducible promotor sequence; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the second modified inducible promotor sequence; and (iii) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. In some embodiments, the non-naturally occurring organisms comprise prokaryotic organisms. In some embodiments, the prokaryotic organisms comprise a population of bacteria. In some embodiments, the biosynthesis pathway transgene comprises a carotenoid synthesis gene.

[0009] Provided herein are compositions comprising a non-naturally occurring organism provided herein and an inducer.

[0010] Further provided herein are methods of expressing a transgene in a cell that does not comprise a T7 RNA polymerase, wherein the methods comprise: (a) transforming a nucleic acid construct into the cell, wherein the nucleic acid construct comprises a modified inducible promoter, wherein the modified inducible promoter comprises: (i) a TATAAT (SEQ ID NO: 1) sequence or a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (iii) a bacterial ribosome binding sequence; and a transgene: and (b) contacting the cell with an inducer, thereby expressing a protein encoded by the transgene. In some embodiments, the expression of the protein in the absence of the inducer is less than an amount of expression of the protein in the absence of the inducer of the transgene operatively coupled to a T7 promoter in a comparable nucleic acid construct. In some embodiments, the expression of the protein encoded by the transgene in the presence of the inducer is greater than an amount of expression of the protein in the presence of the inducer of the transgene operatively coupled to the T7 promoter in the comparable nucleic acid construct. In some embodiments, the transgene is toxic to a prokaryotic cell that does not express a modified inducible promoter. In some embodiments, the cell is a prokaryotic cell. In some embodiments, the cell is a eukaryotic cell.

[0011] Further provided herein are methods of expressing a transgene in a cell-free system, wherein the methods comprise: (a) transforming a nucleic acid construct into the cell-free system, wherein the nucleic acid construct comprises a modified inducible promoter, wherein the modified inducible promoter comprises: (i) a TATAAT (SEQ ID NO: 1) sequence or a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (iii) a bacterial ribosome binding sequence; and a transgene: and (b) contacting the cell-free system with an inducer, thereby expressing a protein encoded by the transgene. In some

embodiments, the cell-free system further comprises: an RNA polymerase, ribonucleotides, and/or a buffer.

[0012] Provided herein are methods for producing a protein, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the protein. Further provided herein are methods for producing a carotenoid, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the protein. Provided herein are methods for producing an organic compound, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the organic compound. Further provided herein are methods for producing a carotenoid, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the carotenoid. In some embodiments, the organic compound or the protein comprises a polyketide, a terpene, a non-ribosomal peptide, or an enzyme protein. Provided herein are methods for producing benzoic acid, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the benzoic acid. Further provided herein is a composition comprising benzoic acid made by a method provided herein. Further provided herein is a composition comprising a carotenoid made by a method provided herein.

[0013] Provided herein are kits, wherein the kits comprise a nucleic acid construct provided herein, a cell provided herein, a cell-free system provided herein, or a non-naturally occurring organism provided herein, packaging and materials, therefore.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Novel features of exemplary embodiments are set forth with particularity in the appended claims. A better understanding of the features and advantages will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosed systems and methods are utilized, and the accompanying drawings of which:

[0015] FIGS. 1A-1B show schematic diagrams of exemplary plasmids produced in accordance with the teachings of the present disclosure. **FIG. 1A** shows a schematic of a constitutive version of the synthetic *lac* and *tet* expression systems. **FIG. 1B** shows a schematic of inducible versions of the synthetic *lac* and *tet* expression systems in pColE1 backbone. Plasmids

contain the Kanamycin selection marker and the attP sites specific fox Bxb1 integrase. # indicates the version of the promoter (*e.g.* V1Tc, V2lac, etc).

[0016] FIGS. 2A-2C show the architecture of exemplary synthetic lac and tet promoters. The sequences of the operators lacO and tetO are overlined, in bold text. The -35 and -10 sequences of the promoters are underlined. Boxes represent the native -35 and -10 sequences, and consensus σ70 -35 and -10 sequences. Conserved regions of the original lac promoter and the original tacI promoter are shown. **FIG. 2A** shows the original lac (i) and synthetic lac (ii-vii) promoters. SEQ ID NOS: 3-9 are shown. **FIG. 2B** shows the original tet (i) and synthetic tet (ii-v) promoters. SEQ ID NOS: 10-14 are shown. **FIG. 2C** shows schematic representation of the function of synthetic lac and tet promoters. Each transcriptional unit is insulated by terminators. LacI/TetR regulators bind to the operators lacO/tetO. Addition of the inducers (yellow ovals) IPTG or anhydrotetracycline (aTc) remove the repressor allowing transcription from the promoters.

[0017] FIGS. 3A-3D show a graphical comparison of sfGFP and mCardinal emissions in Gram-negative bacteria. FIG. 3A shows absolute values of fluorescence measured in arbitrary units with far red wavelengths (excitation 604, emission 659, left panel), and green wavelengths (excitation 485, emission 510) *E. coli* DH10B, *P. putida* and *V. natriegens*. Note that these strains are wild type and do not contain any fluorescent protein genes. N=3. Error bars +/- SD. **FIGS. 3B-3D** show graphs of the fluorescence signal-to-background ratio of recombinant strains expressing sfGFP and mCardinal from the constitutive tacI promoter (these represent "signal") vs. wild type strains of (**FIG. 3B**) *E. coli* (**FIG. 3C**) *P. putida* & (**FIG. 3D**) *V. natriegens* (these wild type strains represent "background"). Signal-to-background measurement ratios are then plotted over time (left panels) The fluorescence values (in arbitrary units, AU) measured to create the signal-to-background ratios are shown in the middle (sfGFP) and right (mCardinal) panels. N=3. Error bars +/- SD.

[0018] FIGS. 4A-4C show graphs of the time course of mCardinal production in *E. coli* DH10B with synthetic lac and tet promoters. FIG. 4A shows the synthetic lac promoters, and **FIG. 4B** shows the synthetic tet promoters, with and without the transcriptional regulators tetR and lacI and induced after 3 hours with aTc or IPTG, respectively. **FIG. 4C** shows direct comparison of each promoter under evaluation against the strong constitutive promoter tacI. The recombinant DH10B carrying tacI-mCardinal was normalized to 100% mCardinal production and the wild type DH10B strain normalized to 0% mCardinal production. For all samples, the fluorescence mean of mCardinal signal (excitation 605, emission 659) was normalized by the cell density (OD600). N=4. Error bars +/- SD.

[0019] FIGS. 5A-5B show an SDS-Page gel and graph of the expression profile of CocE in *E. coli*. **FIG. 5A** shows gel electrophoresis of total crude extracts (top), the insoluble fraction analysis (middle panel), and the soluble fraction (bottom). The expected size of CocE is 63 kDa. *E. coli* expressing the pET and V2TcR expression systems, uninduced and induced (induced samples indicated with an asterisk). **FIG. 5B** shows a graph of benzoic acid production by CocE present in the soluble fraction, using cocaine as substrate. N=3. Error bars +/- SD. Key for X axis Groups: I: DH10B $p\sigma^{70}$ V2TcR-CocE; II: DH10B $p\sigma^{70}$ V2TcR-CocE aTc; III: DH10B $p\sigma^{70}$ V2TcR19-CocE; IV: DH10B $p\sigma^{70}$ V2TcR19-CocE aTc; V: BL21 pET21-cocE; VI: BL21 pET21-cocE IPTG.

[0020] FIGS. 6A-6C show graphs of the time course of mCardinal production in *P. putida*. Synthetic lac (**FIG. 6A**) and tet (**FIG. 6B**) promoters in their constitutive, repressed and induced states. All constructs were integrated in a single copy into the same genomic locus. **FIG. 6C** shows direct comparison of each promoter under evaluation against the constitutive promoter tacI in *P. putida*. *P. putida* with a single-copy integration of tacI-mCardinal was normalized to 100% mCardinal production and the wild type *P. putida* strain normalized to 0% mCardinal production. For all samples, the fluorescence mean of mCardinal signal (excitation 605, emission 659) was normalized by the cell density (OD600). N=4. Error bars +/- SD.

[0021] FIGS. 7A-7C show graphs of the time courses of mCardinal production in *V. natriegens*. Synthetic lac (**FIG. 7A**) and tet (**FIG. 7B**) promoters in their constitutive, repressed and induced states are represented. **FIG. 7C** shows direct comparison of each promoter under evaluation against the constitutive promoter tacI in *V. natriegens*. *V. natriegens* carrying tacI-mCardinal was normalized to 100% mCardinal production and the wild type *V. natriegens* strain normalized to 0% mCardinal production. For all samples, the fluorescence mean of mCardinal signal (excitation 605, emission 659) was normalized by the cell density (OD600). N=4. Error bars +/- SD.

[0022] FIG. 8 shows a graphical comparison of the pET21a expression system in *E. coli* BL21 versus the synthetic *V2Lac/lacI* and *V2Tc/tetR* promoters in *E. coli* DH10B. Cultures were induced after 3 hours of cultivation and mCardinal mean was normalized by the cell density (OD_{600}). N=4. Error bars +/- SD.

[0023] FIGS. 9A-9F shows a schematic representation of an exemplary dual expression system. **FIG. 9A** shows the design of the V2TcR-V2(3)LacI system. **FIG. 9B** shows a dual expression system controlling expression of mCardinal. **FIG. 9C** shows a dual expression system controlling expression of sfGFP and mCardinal. **FIG. 9D** shows a V2TcR expression system controlling the LYC operon. **FIG. 9E** shows a V2TcR expression system controlling expression of crtEBIY. **FIG.**

9F shows a dual expression system controlling expression of crtEBI and crtY. Exemplary sequences tested include SEQ ID NOS: 69-73 and 76-79.

[0024] FIGS. 10A-10C show graphs of the time course of mCardinal production in *E. coli* (**FIG. 10A**), *P. putida* (**FIG. 10B**) & *V. natriegens* (**FIG. 10C**). Left graphs show the recombinant strains containing the V2TcR-mCardinal (SEQ ID NO: 56) and V2(3)LacI-mCardinal expression systems and the right graphs show the recombinant strains containing the dual V2TcR-mCardinal/V2(3)LacI-mCardinal expression system. For all samples, the fluorescence mean of mCardinal signal (excitation 605, emission 659) was normalized by the cell density (OD600). N=3. Error bars +/- SD.

[0025] FIGS. 11A-11C shows graphs of the time course of the reporter systems mCardinal and sfGFP in *E. coli* (**FIG. 11A**), *P. putida* (**FIG. 11B**), and *V. natriegens* (**FIG. 11C**) containing the dual expression system V2TcR-sfGFP/V2(3)LacI-mCardinal. Left graphs measure mCardinal production and right graphs measure sfGFP. For all samples, the fluorescence mean of mCardinal signal (excitation 605, emission 659) and sfGFP signal (excitation 485, emission 510) was normalized by the cell density (OD600). N=3. Error bars +/- SD.

[0026] FIGS. 12A-12B shows graphs of the production of lycopene and β-carotene with the V2TcR expression system in *E. coli*, *P. putida,* and *V. natriegens*. Cultures were induced with aTc at OD600. 0.7 and cultured for 4 hours. **FIG. 12A** shows Lycopene levels that were extracted and measured by UHPLC. **FIG. 12B** shows B-carotene levels that were extracted and measured by UHPLC. N=3. Error bars +/- SD.

[0027] FIGS. 13A-13B shows graphs of the production of lycopene and beta (β)-carotene by the dual expression system in *E. coli*, *P. putida,* and *V. natriegens*. Cultures were induced with aTc, IPTG or both inducers at OD600. 0.7 and cultured for 4 hours. **FIG. 13A** shows lycopene levels that were extracted and measured by UHPLC. **FIG. 13B** shows β-carotene levels that were extracted and measured by UHPLC. N=3. Error bars +/- SD.

[0028] FIG. 14 shows a schematic of an exemplary dual expression system. An exemplary sequences include SEQ ID NO: 54 and SEQ ID NO: 55.

DETAILED DESCRIPTION

Overview

[0029] Disclosed herein are nucleic acid constructs that comprise a modified inducible promoter. Such constructs can be utilized to express a transgene operatively coupled to the promoter upon transformation into a cell or a cell-free system. As described herein, the modified inducible

promoter does not substantially express the transgene in the absence of an inducer and in the presence of a specific transcriptional regulator. In some cases, a transgene is toxic to the cell when expressed utilizing the modified inducible promoter described herein. In some cases, a transgene is not toxic to the cell when expressed utilizing the modified inducible promoter described herein. Expression of toxic genes is one of many advantages to the modified inducible promoter systems provided herein. In addition, non-toxic gene product yields (e.g., proteins or organic compounds) are also higher as compared to inducible promoters that are not modified.

[0030] In contrast, many inducible promoters produce significant expression of the transgene even in the absence of an inducer. Such promoters, known as "leaky" promoters, cannot be used to express transgenes that are toxic to a cell, as the leaky expression of the transgene inhibits growth of the cell culture and thus prevents overexpression or avoids formation of inclusion bodies.

[0031] Further, nucleic acid constructs provided herein comprising a modified inducible promoter that generates the expression of a protein encoded by a transgene in the presence of an inducer that is comparable or to a greater extent than the expression of a protein encoded by the transgene when expressed using a different promoter (*e.g.*, a T7 promoter such as a pET vector). In some embodiments, expression of the transgene can be carried out in various prokaryotic cells, eukaryotic cells, or cell-free systems without the need for T7 lysogenization.

Definitions

[0032] The terminology used herein is for the purpose of describing particular cases only and is not intended to be limiting. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising".

[0033] The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, such as plus or minus 10%. Where ranges and/or subranges of values are provided, the ranges and/or subranges include the endpoints of the ranges and/or subranges.

[0034] The term "substantially" as used herein refers to a value approaching 100% of a given value. For example, an expression system described herein that does not "substantially" express a transgene in the absence of an inducer can indicate that less than 10% of the transgene (*e.g.* less

than 5%, less than 1%, less than 0.1%, or less than 0.01%) is expressed, relative to an amount of transgene expressed in the presence of the inducer.

[0035] As used herein, the term "operably linked" indicates that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence it regulates to control transcriptional initiation and/or expression of that sequence.

Modified Inducible Promoters

[0036] Inducible promoters often have a consistent architecture including two elements: (1) the operator region recognized by the transcriptional regulator proteins (*e.g.*, lacI and tetR, and the -10) and -35 consensus sequences that recruits the sigma (σ) subunits of RNA polymerase to initiate transcription. Improvements to the lac promoter can be made to increase its strength and improve its regulation. For example, the -10 and -35 boxes of the original *lac* promoter (FIG. 1Ai) can be updated to generate improved variants such as the *lacUV5* (FIG. 1Aii) and *tacI* (FIG 1Aiii) promoters. In the *lacUV5* promoter, the -10 box of the original *lac* promoter can be replaced by the Pribnow box (*e.g.*, TATAAT, SEQ ID NO: 1) or any one of SEQ ID NOS: 44, 47 to 51. The *lacUV5* and *trp* promoters shown in the working examples can be combined to create the *tacI* promoter, which increased transcription 11-fold compared to its predecessor with the incorporation of the -35 consensus box TTGACA. The *tet* promoter (FIG. 1Bi) shares the highly conserved -35 hexamer with *tac* promoter, but does not contain the Pribnow box.

[0037] Despite their ubiquitous use in biology, there remain problems with the current *lac*-based inducible expression systems. Leaky transcription in the OFF state remains a consistent challenge. Tight transcriptional control is indispensable to produce high yields of challenging recombinant proteins, including toxic genes and proteins that are impossible for a heterologous host to process or fold. For the pET system, target proteins are driven indirectly by controlling expression of the T7 polymerase under the *lacUV5* promoter, and then driving the target gene transcription by the T7 promoter. Still, low level T7 transcription in the uninduced state leads to leaky transcription of the target gene. To combat this problem further, *E. coli* strains such as pLysS and pLysE with integration of the T7 lysozyme that inhibits low level T7 activity are used to obtain tighter transcriptional control. However, a host strain with T7 RNA polymerase under control of the *lacUV5* promoter, and integration of T7 lysozyme, is required to tightly regulate target gene expression. Expression with the *tet* system has historically provided tighter transcriptional control than *lac* derived promoters, does not required specialized strains (such as the BL21), and fully induction is achieved by anhydrotetracycline (aTc) at concentrations that do not cause growth

defect due the high affinity of aTc to TetR, and its imperceptible antibiotic activity. Remarkably, at the uninduced state just one mRNA molecule per three cells is produced, and up to 5000-fold induction has been reported, however, the yields of recombinant protein obtained by the tet expression system remains low compared to the pET expression system when using *E. coli* as heterologous host.

[0038] The repertoire of organisms used both in academic and industrial settings is rapidly expanding. To address challenges related to complex protein expression in *E. coli*, other chassis organisms such as *Pseudomonas putida* and *Vibrio natriegens* have been employed to produce challenging proteins (*e.g.*, carotenoids) in a variety of biotechnological processes. Both *lac* and *tet* expressions systems can be adapted to *P. putida* and *V. natriegens*, though in some instances with lower total protein yield than achieved in pET. However, previous systems for inducible expression were not configured for use across various cell types (*e.g.*, prokaryotic cells). Accordingly, disclosed herein are universal expression systems than can be directly ported between different gram-negative species, yield high quantities of recombinant protein comparable to expression via a pET in *E. coli*, *and* maintain tight transcriptional repression in the uninduced state. The expression system of the current disclosure is a significant improvement over the pET system in providing tight OFF state control while achieving similar yields of recombinant protein, and with the advantage of direct portability to alternative host species. Further, additional modifications can be performed to enable the expression of the transgene to be turned OFF after induction, thus allowing for reversible control of expression.

[0039] A modified, inducible promoter of the current disclosure comprises modified architecture of the *lac* and *tet* expression systems to improve their strength, control, and portability. In some embodiments, the genetic architecture of the *lac* and *tet* expression systems were modified in three ways: (1) addition of the consensus -10 and -35 sequence boxes to be strongly targeted by σ^{70}, (2) incorporation of a nucleic acid sequence that encodes a strong ribosome binding site recognized by a broad spectrum of gram-negative bacteria, and (3) independent control of the transcriptional regulators by appropriately-tuned constitutive promoters.

[0040] The nucleic acid constructs provided herein can comprise DNA, RNA, an artificial nucleic acid analog, or any combination thereof. In some embodiments, the nucleic acid constructs provided herein comprise, a nucleic acid modification (*e.g.*, a chemical modification), a nucleobase substitution, or a nucleotide substitution. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises one or more nucleobase substitutions. In some embodiments, the sequence at position -10, relative to the

transcriptional start site of the promoter comprises a sequence of TXTXXT (SEQ ID NO: 47), where X is any nucleobase. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises a sequence of TXTXXTGT (SEQ ID NO: 45), where X is any nucleobase. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises a sequence of TATXXTGT (SEQ ID NO: 48), where X is any nucleobase. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises a sequence of TATAXTGT (SEQ ID NO: 49), where X is any nucleobase. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises a sequence of TATAATGT (SEQ ID NO: 50), where X is any nucleobase. In some embodiments, the sequence at position -10, relative to the transcriptional start site of the promoter comprises a sequence of TATAATGT (SEQ ID NO: 51), where X is any nucleobase.

[0041] In some embodiments, the sequence at position -35, relative to the transcriptional start site of the promoter comprises one or more nucleobase substitutions. In some embodiments, the sequence at position -35, relative to the transcriptional start site of the promoter comprises a sequence of TXGXCX (SEQ ID NO: 46), where X is any nucleobase. In some embodiments, the sequence at position -35, relative to the transcriptional start site of the promoter comprises a sequence of TTGXCX (SEQ ID NO: 52), where X is any nucleobase. In some embodiments, the sequence at position -35, relative to the transcriptional start site of the promoter comprises a sequence of TTGACX (SEQ ID NO: 53), where X is any nucleobase. In some embodiments, the sequence at position -35, relative to the transcriptional start site of the promoter comprises a sequence of TTGACA (SEQ ID NO: 2), where X is any nucleobase.

[0042] In some embodiments, a nucleic acid construct provided herein comprises a nucleotide analogue. Nucleotide analogues include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil, and the like; and 2´-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2´-OH is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2, or CN. shRNAs also can comprise non-natural elements such as non-natural bases, *e.g.*, inosine and xanthine, sugars, e.g., 2´-methoxy ribose, or non-natural phosphodiester linkages, *e.g.*, methylphosphonates, phosphorothioates and peptides.

[0043] The nucleic acid construct provided herein can comprise a sequence encoding a ribosome binding site. Ribosome binding sites (RBSs) are nucleic acid sequences that promote efficient and

accurate translation of mRNAs for protein synthesis, and are also provided for use in the inducible promoters provided herein to permit modulation of the efficiency and rates of synthesis of the proteins encoded by the system. An RBS affects the translation rate of an open reading frame in two main ways—i) the rate at which ribosomes are recruited to the mRNA and initiate translation is dependent on the sequence of the RBS, and ii) the RBS can also affect the stability of the mRNA, thereby affecting the number of proteins made over the lifetime of the mRNA. Accordingly, one or more nucleic acid sequence encoding a ribosome binding site (RBS) or an RBS mRNA can be added to the nucleic acid constructs described herein to control expression of proteins.

[0044] In some embodiments, a nucleic acid construct provided herein further comprises a terminator sequence. Terminators are sequences that usually occur at the end of a gene or operon and cause transcription to stop, and are also provided for use in the modules and engineered systems described herein to regulate transcription and prevent transcription from occurring in an unregulated fashion, i.e., a terminator sequence prevents activation of downstream modules by upstream promoters. A terminator or termination signal can include the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a terminator that ends the production of an RNA transcript is contemplated. A terminator can be necessary for use *in vivo* to achieve desirable message levels.

[0045] The most commonly used type of terminator is a forward terminator. When placed downstream of a nucleic acid sequence that is usually transcribed, a forward transcriptional terminator will cause transcription to abort. In some embodiments, bidirectional transcriptional terminators are provided. Such terminators will usually cause transcription to terminate on both the forward and reverse strand. Finally, in some embodiments, reverse transcriptional terminators can be used to terminate transcription on the reverse strand only.

[0046] In some embodiments, a nucleic acid construct provided herein comprises additional regulatory elements that increase or decrease transgene expression in a cell or cell-free system depending on the absence or presence of a particular inducer or set of inducers. In some embodiments, the regulatory element is an enhancer. Additional non-limiting examples of regulatory elements include: lasR activator (*e.g.,* from *P. aeruginosa*), cinR activator, toxicity-gene activator (*e.g.*, ToxR, from *Vibrio cholerae*), lacI (*e.g.*, a wild-type, derivative, or variant thereof), lacI repressor, tetracycline repressor (*e.g.*, TetR from transposon Tn10), mnt repressor, TP901, heat shock proteins, and any derivative or variant thereof.

[0047] Thus, in some embodiments, a nucleic acid construct provided herein can be part of a synthetic gene network. Synthetic gene networks can include an engineered composition that

comprises at least one nucleic acid construct provided herein and can perform a function including, but not limited to, sensing the presence or absence of an analyte or inducer, a logic function, or a regulatory function. In some embodiments of a synthetic gene network comprising at least two nucleic acid constructs, the nucleic acid constructs can interact with each other directly or indirectly. A synthetic gene network can comprise a nucleic acid encoding a transgene operably linked to a modified inducible promoter provided herein.

[0048] The nucleic acid constructs provided herein can be used to visualize chemical, analyte, or protein production in the presence and absence of an inducer provided herein. In some embodiments, a nucleic acid construct provided herein further comprises a reporter gene. In some embodiments, the reporter gene is mCardinal or a green fluorescent protein (*e.g.*, superfolder GFP or sfGFP). A reporter gene encoding any fluorescent protein can be applicable to the nucleic acid constructs and methods of use provided herein. Additional examples of genes encoding fluorescent proteins that can be used in accordance with the compositions and methods described herein include, without limitation, enhance yellow fluorescent protein (EYFP), engineered cyan fluorescent protein (ECFP), mOrange, mCherry, Venus YFP, Cerulean, mBanana, orange fluorescent protein (OFP), derivatives, or variants thereof.

[0049] In some embodiments, the reporter gene encodes for a colorimetric protein enzyme. Colorimetric enzymes can cleave a substrate (*e.g.*, a chemical) to yield a color-changing product. In some embodiments, the protein tag is chitinase (which cleaves colorless 4-Nitrophenyl N,N´-diacetyl-beta-D-chitobioside substrate to yield a yellow p-nitrophenol product). In some embodiments, the reporter gene is *LacZ* (which encodes beta-galactosidase) or a fragment thereof. When *LacZ* is expressed, the enzyme cleaves the yellow chlorophenol Red-β-D-galactopyranoside (CPRG) substrate to produce the purple chlorophenol red product.

[0050] In some embodiments, the reporter gene comprises a catalytic nucleic acid. Examples of catalytic nucleic acids include, but are not limited to, a ribozyme, an RNA-cleaving deoxyribozyme, a group I ribozyme, RNase P, a Hepatitis delta ribozyme, and DNA-zymes.

[0051] In some embodiments, the reporter gene comprises an antigen for which a specific antibody or antibody fragment is available. In some embodiments, a reporter gene comprises an antibody, which when expressed, binds to a complementary antigen.

Methods of Expression

[0052] Provided herein are methods for expressing a transgene or a protein encoded by a transgene sequence using a nucleic acid construct provided herein having a modified inducible promoter as

described herein. In some embodiments, a transgene can be expressed in a host cell that is toxic to the host cell or a non-naturally occurring organism (*e.g.*, a bacterium). Such transgenes can be difficult to express utilizing a pET or other vector containing a leaky promoter, because the leaky expression upon transformation can inhibit growth of the host cell and thereby either prevent expression or express the transgene as an inclusion body. Indeed, tight OFF state control can allow the host cell to reach mid-log phase growth prior to induction, thus allowing for improved expression of the toxic transgene in the prokaryotic cell relative to expression via a vector having a leaky promoter.

[0053] The nucleic acid constructs provided herein can comprise a transgene encoding a protein or a fragment thereof. In some embodiments, the transgene or a protein encoded by the transgene is not toxic to the host cell. A protein and/or peptide or fragment thereof can be any protein of interest, for example, but not limited to: biological proteins; mutated proteins; therapeutic proteins; truncated proteins, and the like. Proteins can also be selected from a group comprising: mutated proteins, genetically engineered proteins, peptides, synthetic peptides, recombinant proteins, chimeric proteins, antibodies, midibodies, tribodies, humanized proteins, humanized antibodies, chimeric antibodies, modified proteins and fragments thereof. A transgene provided herein can comprise a gene that is, for example, part of a biosynthesis pathway for the production of a protein, an organic compound, or a molecule of interest. The cells, systems, and organisms provided herein provide for a facile method of manufacturing such proteins.

[0054] The modified inducible promoters provided herein can be introduced to any cell type or any system that can transcribe and translate the protein encoded by the transgene downstream of the promoter, In some embodiments, a cell provided herein is a prokaryotic cell or a eukaryotic cell. In some embodiments, the system is a cell-free system that can be used to produce the protein or organic compound of interest. A cell-free system is a composition comprising a set of reagents capable of providing for or supporting a biosynthetic reaction (*e.g.*, transcription reaction, translation reaction, or both) *in vitro* in the absence of cells. For example, to provide for a transcription reaction, a cell-free system comprises promoter-containing DNA, RNA polymerase, ribonucleotides, and a buffer system. Cell-free systems can be prepared using enzymes, coenzymes, and other subcellular components either isolated or purified from eukaryotic or prokaryotic cells, including recombinant cells, or prepared as extracts or fractions of such cells. A cell-free system can be derived from a variety of sources, including, but not limited to, eukaryotic and prokaryotic cells, such as bacteria including, but not limited to, *E. coli*, *P. putida*, *V. natriegens*, thermophilic bacteria and the like, wheat germ, rabbit reticulocytes, mouse L cells, Ehrlich's ascitic cancer cells,

HeLa cells, CHO cells, or budding yeast. In some embodiments, the cell-free system comprises an RNA polymerase. In some embodiments, the cell-free system comprises components sufficient for the translation reaction. In some embodiments, the cell-free system comprise ribosomes, aminoacyl transfer RNAs, translation factors, and a buffer system. The components can also comprise amino acids or amino acids and aminoacyl tRNA synthetases. Components of translation factors are disclosed, for example, in Shimizu and Ueda, "Pure Technology," Cell-Free Protein Production: Methods and Protocols, Methods in Molecular Biology, Endo et al. (Eds), Humana 2010, the contents of which is incorporated herein by reference in its entirety. Exemplary translation factors include, but are not limited to, factors responsible for protein biosynthesis are initiation factors (IF1, IF2, and IF3), elongation factors (EF-G, EF-Tu, and EF-Ts), and release factors (RF1, RF2, and RF3), as well as RRF for termination.

[0055] Prior to induction, the modified inducible promoter provided herein does not substantially express the transgene in the absence of an inducer (*e.g.* isopropyl β- d-1-thiogalactopyranoside (IPTG), anhydrotetracycline, and the like). In some embodiments, the amount of expressed transgene produced in the absence of inducer is less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.9%, less than 0.8%, less than 0.7%, less than 0.6%, less than 0.5%, less than 0.4%, less than 0.3%, less than 0.2%, less than 0.1%, less than 0.09%, less than 0.08%, less than 0.07%, less than 0.06%, less than 0.05%, less than 0.04%, less than 0.03%, less than 0.02%, or less than 0.01% of an amount of expressed transgene produced in the presence of the inducer. In some embodiments, the amount of expressed transgene produced in the absence of inducer is less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.9%, less than 0.8%, less than 0.7%, less than 0.6%, less than 0.5%, less than 0.4%, less than 0.3%, less than 0.2%, less than 0.1%, less than 0.09%, less than 0.08%, less than 0.07%, less than 0.06%, less than 0.05%, less than 0.04%, less than 0.03%, less than 0.02%, or less than 0.01% of an amount of expressed transgene produced by a comparable nucleic acid having a weak promoter (*e.g.* lacI) promoter in the absence of the inducer.

[0056] Expression of the transgene is performed by contacting the host cell or cell-free system provided herein with an inducer (*e.g.* isopropyl β- d-1-thiogalactopyranoside (IPTG), anhydrotetracycline, and the like). Non-limiting examples of inducers include: a chemical, a compound, and organic compound, a protein, an analyte, tetracycline and derivatives thereof, metallothionine, ecdysone, cocaine, hormones, steroids, and antibiotics (*e.g.*, rapamycin, kanamycin). Exemplary environmental inducers include exposure to heat (*i.e.*, thermal pulses or

constant heat exposure), light (*e.g.*, photoirradiation within the defined range of wavelengths), various steroidal compounds, divalent cations (including Cu^{2+} and Zn^{2+}), galactose, tetracycline, IPTG (isopropyl-β-D thiogalactoside), as well as other naturally occurring and synthetic inducing agents and gratuitous inducers.

[0057] In some embodiments, the amount of expressed transgene produced in the presence of inducer is at least equal to the amount of expressed transgene produced by a comparable nucleic acid having a lacUV5 promoter and a T7 polymerase in the presence of the inducer. In some embodiments, the amount of protein expressed in the presence of the inducer is at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 100%, at least 105%, at least 110%, at least 115%, at least 120%, at least 125%, at least 130%, at least 135%, at least 140%, at least 145%, at least 150%, at least 155%, at least 160%, at least 165%, at least 170%, at least 175%, at least 180%, at least 185%, at least 190%, at least 195%, at least 200%, at least 300%, at least 400%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or at least 1000% an amount of expressed transgene produced by a comparable nucleic acid having a strong (*e.g.*, T7) promoter in the presence of the inducer.

[0058] Such expression can be carried in a variety of prokaryotic host cells. Indeed, while most expression is carried out in T7 lysogenized *E. coli* DE3 strains, the nucleic acid constructs of the present disclosure having a modified inducible promoter can be utilized in prokaryotic cells in the absence of T7 lysogenization.

[0059] The host cell for expression of a nucleic acid provided herein (*e.g.*, a transgene) can comprise a prokaryotic cell or a eukaryotic cell. In some embodiments, the prokaryotic cell is a bacterial cell. In some embodiments, a prokaryotic cell can include any Gram-positive strain bacterial cell. In some embodiments, a prokaryotic cell can include any Gram-negative strain bacterial cell. Examples of bacterium that can be used include, but are not limited to: non-T7 lysogenized *E. coli* strains such as DH5α, DH10β, or W3110, *P. putida, P. aeruginosa, H. influenzae, C. trachomatis, P. mirabilis, P. vulgaris, C. pneumoniaea, K. pneumoniaea, N. gonorrhoeae, H. pylori, A. cholera, S. aureus, S. enterica, C. jejuni, B. fragilis, L. pneumophila, V. parahaemolyticus,* and *V. natriegens*.

[0060] The host cell for expression can be a eukaryotic cells, e.g., a mammalian cell, an insect cell, a yeast cell, a fungal cell, and the like. A nucleic acid construct provided herein can be regulated

in a cell-specific or tissue-specific manner such that it is only active in transcribing the associated coding region of a given transgene in a specific tissue type(s).

Exemplary biosynthesis pathways for producing gene products

[0061] Provided herein are methods of producing a protein, an organic compound, or a molecule using the inducible promoters provided herein, a cell expressing an inducible promoter provided herein, a non-naturally occurring organism provided herein, or kits provided herein. In some embodiments, the non-naturally occurring organism provided herein comprises a nucleic acid construct comprising one or more inducible promoters provided herein and one or more sequence encoding a transgene. In some embodiments, the one or more transgene comprises a sequence encoding a protein in a biosynthesis pathway. In some embodiments, the nucleic acid construct provided herein comprise a biosynthesis pathway transgene. In some embodiments, the biosynthesis pathway is a polyketide synthesis pathway, a terpene synthesis pathway, a non-ribosomal peptide biosynthesis pathway, or a carotenoid biosynthesis pathway. In some embodiments, the cell expressing an inducible promoter provided herein or the a non-naturally occurring organism provided herein are cultured for a period of time. In some embodiments, the period of time is for at least 4 hours, 6 hours, 10 hours, 12 hours, or more. In some embodiments, the period of time is for at least 24 hours. In some embodiments, the period of time is for at least 96 hours.

[0062] In some embodiments, the biosynthesis pathway is a carotenoid synthesis pathway. Industrially useful carotenoids are generally produced by chemical synthesis processes for which possibility of undesired actions such as contamination of synthesis auxiliary materials is a major concern for the quality of the product. In addition, tastes of consumers tend to lean toward naturally-occurring carotenoids. However, there is a limit to extraction of carotenoids from plants and natural products, and an effective industrial process is not entirely established. As a production method of naturally-occurring carotenoids, microbial fermentation methods have been used. However, none of such cases enable production of carotenoids in an amount which is enough for economical industrial production. In many cases, through classical mutation and breeding, wild-type of carotenoid producing microorganisms do not generate enough carotenoid product for large-scale manufacturing. carotenoid biosynthesis pathway is made up of various enzymes, and genes encoding such enzymes have been analyzed by many researches. In a typical pathway, for example, carotenoid is synthesized in its early stage by an isoprenoid biosynthesis pathway which is shared by steroid and terpenoid, starting from mevalonic acid which is a basic metabolite. Farnesyl pyrophosphate having 15 carbons (C15) generating through the isoprenoid basic synthesis system

is condensed with isopentenyl diphosphate (IPP) (C5), to give geranylgeranyl diphosphate (GGPP) (C20). Then through condensation of two molecules of GGPP, colorless phytoene which is the first carotenoid is synthesized. The phytoene is then converted into lycopene through a series of unsaturation reactions, and then the lycopene is converted into β-carotene through a cyclization reaction. Then, a hydroxyl group and a keto group are introduced into the β-carotene, which leads synthesis of various xanthophylls represented by astaxanthin.

[0063] Provided herein are methods for producing a carotenoid, the method comprises culturing the non-naturally occurring organism provided herein or a plurality of non-naturally occurring organisms under conditions and for a sufficient period of time and contacting the non-naturally occurring organism with an inducer, thereby producing the carotenoid. In some embodiments, the cells or non-naturally occurring organisms provided herein are cultured in a cell culture medium (*e.g.*, a lysogeny broth, also called LB broth or Luria Broth). In some embodiments, the cell or non-naturally occurring organisms are cultured in a bioreactor, a spinning flask, or a vessel suitable for cell growth and survival.

[0064] In some embodiments, the transgene encodes a polypeptide having such an enzymatic activity that converts a methylene group at 4 position in β-ionone ring into a keto group. In some embodiments the transgene comprises a crtW gene. In some embodiments, the transgene encodes a polypeptide having such an enzymatic activity that adds one hydroxyl group to a carbon at 3-position of 4-keto-β-ionone ring and/or at 3-position of β-ionone ring. In some embodiments, the transgene comprises a crtZ gene sequence. In some embodiments, the transgene encodes for a polypeptide having such an enzymatic activity that converts lycopene into β-carotene. In some embodiments, the transgene comprises a crtY gene sequence. In some embodiments, the transgene encodes for a polypeptide having such an enzymatic activity that converts phytoene into lycopene. In some embodiments, the transgene comprises a crtI gene sequence. In some embodiments, the transgene encodes for a polypeptide having prephytoene synthase activity. In some embodiments, the transgene comprises a crtB gene sequence. In some embodiments, the transgene encodes for a polypeptide having geranylgeranyl diphosphate synthase activity. In some embodiments, the transgene comprises a crtE gene sequence. In some embodiments, the transgene encodes for a polypeptide having a lycopene elongase/hydratase activity. In some embodiments, the transgene comprises a crtEB gene sequence. In some embodiments, the transgene comprises a crtEBI gene sequence. In some embodiments, the transgene comprises a crtEBIY gene sequence. In some embodiments, the transgene comprises a crtEBIYZ gene sequence. In some embodiments, the transgene comprises a crtEBI-YZW gene sequence. In some embodiments, the transgene

comprises an ABA1 gene sequence. In some embodiments, the transgene comprises an ABA2 gene sequence. In some embodiments, the transgene comprises a sequence that is at least 85% identical to any one of SEQ ID NOS: 69-73, 76-79. In some embodiments, the comprises a sequence that is at least 90% identical to any one of SEQ ID NOS: 69-73, 76-79. In some embodiments, the comprises a sequence that is at least 95% identical to any one of SEQ ID NOS: 69-73, 76-79. In some embodiments, the comprises a sequence that is at least 99% identical to any one of SEQ ID NOS: 69-73, 76-79. In some embodiments, the comprises any one of SEQ ID NOS: 69-73, 76-79.

[0065] Further provided herein are methods of producing benzoic acid. In some embodiments, the biosynthetic pathway gene is a CocE gene. In some embodiments, the transgene comprises a sequence that is at least 85% identical to SEQ ID NO: 74. In some embodiments, the transgene comprises a sequence that is at least 90% identical to SEQ ID NO: 74. In some embodiments, the transgene comprises a sequence that is at least 95% identical to SEQ ID NO: 74. In some embodiments, the transgene comprises a sequence that is at least 99% identical to SEQ ID NO: 74. In some embodiments, the transgene comprises SEQ ID NO: 74.

Exemplary embodiments:

[0066] Provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TXTXXTGT (SEQ ID NO: 45) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TXGXCX (SEQ ID NO: 46) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence, wherein X is any nucleobase. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TATAAT (SEQ ID NO: 1) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. Further provide herein are nucleic acid constructs, wherein the nucleic acid constructs further comprises a transgene. Further provided herein are nucleic acid constructs, wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a prokaryotic cell in the presence of an inducer; and wherein: (i) the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable

nucleic acid construct; and (ii) the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise: a modified inducible promoter, wherein the modified inducible promoter comprises: (a) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (b) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise a transgene. Further provided herein are nucleic acid constructs, wherein the transgene is selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, an ABA1 gene, an ABA2 gene, and a CocE gene. Further provided herein are nucleic acid constructs, wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a cell in the presence of an inducer. Further provided herein are nucleic acid constructs, wherein the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein a strong promoter increases the amount of expression of a transgene provided herein relative to a comparable inducible promoter that does not comprise SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46. Further provided herein are nucleic acid constructs, wherein the strong promoter increases the amount of expression of a transgene provided herein by at least 10% relative to the expression of a transgene expressed by a comparable inducible promoter that does not comprise SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 44, SEQ ID NO: 45, or SEQ ID NO: 46. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise a sequence encoding a reporter gene. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise one or more regulatory element. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise a terminator sequence.

[0067] Provided herein are nucleic acid constructs comprising: a sequence that is at least 85% identical to a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise a sequence that is at least 90% identical to a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise a sequence that is at least 95% identical to a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise a sequence that is at least 99% identical to a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs comprise a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73.

[0068] Provided herein are nucleic acid constructs comprising: (a) a first modified inducible promoter sequence provided herein; and (b) a second modified inducible promoter sequence, wherein the second modified inducible promoter sequence comprises: (i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the second modified inducible promoter sequence; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the second modified inducible promoter sequence; and (iii) a nucleic acid sequence that encodes a second bacterial ribosome binding sequence. Further provided herein are nucleic acid constructs, wherein the nucleic acid construct further comprises a transgene. Further provided herein are nucleic acid constructs, wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is in the presence of an inducer. Further provided herein are nucleic acid constructs, wherein the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise a terminator sequence. Further provided herein are nucleic acid constructs, wherein the nucleic acid constructs further comprise one or more regulatory elements. Further provided herein are nucleic acid constructs, wherein the transgene comprises a carotenoid biosynthesis pathway gene. Further provided herein are nucleic acid constructs, wherein the transgene is selected from the group consisting of: a crtW gene, a crtE

gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, an ABA1 gene, an ABA2 gene, and a CocE gene.

[0069] Provided herein are compositions, wherein the compositions comprise two or more nucleic acid constructs provided herein.

[0070] Provided herein are cells comprising a nucleic acid construct or a composition provided herein. Further provided herein are cells, wherein the cells are prokaryotic cells. Further provided herein are cells, wherein the cells are bacterial cells. Further provided herein are cells, wherein the cells are Gram-negative bacterial cells. Further provided herein are cells, wherein the cells are *P. putida* bacterial cells or *V. natriegens* bacterial cells. Further provided herein are cells, wherein the cells are eukaryotic cells. Further provided herein are cells, wherein the cells are mammalian cells.

[0071] Provided herein are cell-free systems comprising a nucleic acid construct provided herein, a composition provided herein and/or an RNA polymerase. Further provided herein are cell-free systems, wherein the cell-free systems further comprise ribosomes, aminoacyl transfer RNAs, translation factors, and a buffer.

[0072] Provided herein are methods of expressing a protein encoded by a transgene in a cell, the methods comprising: (a) transforming a cell with a nucleic acid construct, wherein the nucleic acid construct comprises a modified inducible promoter that comprises: (i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; (iii) a nucleic acid that encodes a bacterial ribosome binding sequence; and a transgene; and then (b) contacting the cell with an inducer, thereby expressing the protein encoded by the transgene. Further provided herein are methods, wherein the transgene when cloned into a comparable nucleic acid construct that comprises a strong promoter inhibits growth of the cell prior to the contacting with the inducer, thereby preventing expression of the transgene via the comparable nucleic acid. Further provided herein are methods, wherein the cell is a prokaryotic cell. Further provided herein are methods, wherein the prokaryotic cell is a bacterium. Further provided herein are methods, wherein the cell does not comprise a T7 promoter or a T7 polymerase. Further provided herein are methods, wherein when in the absence of the inducer, the expression of the protein encoded by the transgene is lower relative to the expression of the protein in a comparable cell comprising the transgene operably linked to a T7 promoter. Further provided herein are methods, wherein when in the presence of the inducer the expression of the protein encoded by the transgene is greater relative to the expression of the protein in a comparable cell comprising the transgene operably linked to a T7 promoter.

[0073] Provided herein are non-naturally occurring organisms comprising a nucleic acid construct comprising: (a) a first inducible promotor sequence comprising: (i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (iii) a nucleic acid sequence that encodes a bacterial ribosome binding sequence; (b) one or more of a biosynthesis pathway transgene; and (c) a second modified inducible promotor sequence comprising: (i) a TATAATGT (SEQ ID NO: 44) consensus sequence at position -10, relative to a transcriptional start site of the promoter; (ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and (iii) a nucleic acid sequence that encodes a bacterial ribosome binding sequence. Further provided herein are non-naturally occurring organisms wherein the one or more biosynthesis pathway transgene is selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, and a CocE gene. Further provided herein are non-naturally occurring organisms, wherein the non-naturally occurring organism comprises a bacterium. Further provided herein are non-naturally occurring organisms wherein the bacterium is a Gram-negative bacterium. Further provided herein are non-naturally occurring organisms, wherein the Gram-negative bacterium is a P. putida bacterium or a V. natriegens bacterium. Further provided herein are non-naturally occurring organisms, wherein the bacterium is a Gram-positive bacterium. Further provided herein are non-naturally occurring organisms, wherein the nucleic acid construct comprises a terminator sequence. Further provided herein are non-naturally occurring organisms, wherein the nucleic acid construct comprises one or more regulatory element. Further provided herein are non-naturally occurring organisms, wherein the nucleic acid construct comprises a reporter gene.

[0074] Provided herein are compositions, wherein the compositions comprise: the non- naturally occurring organism provided herein; and an inducer. Further provided herein are compositions, wherein the inducer comprises: anhydrotetracycline (aTc), isopropyl β- d-1-thiogalactopyranoside, cocaine, metallothionine, ecdysone, an antibiotic agent, galactose, a steroid, or a divalent cation.

[0075] Provided herein are methods for producing a carotenoid, the methods comprising: culturing the non-naturally occurring organism provided herein; and contacting the non-naturally occurring organism with an inducer, thereby producing the carotenoid. Further provided herein are methods, wherein the non-naturally occurring organism comprises a nucleic acid comprising a gene selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, and a crtEBI-YZW gene.

[0076] Provided herein is a carotenoid produced by the methods provided herein, wherein the carotenoid is lycopene, beta-carotene, zeaxanthin, canthaxanthin, or astaxanthine.

[0077] Provided herein are methods for producing benzoic acid, the methods comprising: culturing the cell provided herein; or the non-naturally occurring organism of provided herein; and contacting the cell or the non-naturally occurring organism with an inducer, thereby producing benzoic acid. Further provided herein are methods, wherein the cell or the non-naturally occurring organism comprises a nucleic acid comprising a CocE gene.

[0078] Provided herein are compositions comprising benzoic acid, wherein the benzoic acid is produced by the methods provided herein.

[0079] Provided herein are kits comprising the nucleic acid construct provided herein, packaging, buffers, and materials therefor.

[0080] Provided herein are kits comprising the cell provided herein, packaging, culture medium, buffers, and materials therefor.

[0081] Provided herein are kits comprising the non-naturally occurring organism provided herein, packaging, culture medium, buffers, and materials therefor.

[0082] Provided herein are nucleic acid constructs comprising a modified inducible promoter, wherein the modified inducible promoter comprises: a TATAAT consensus sequence at position -10, relative to a transcriptional start site of the promoter; a TTGACA sequence at the -35 position, relative to a transcriptional start site of the promoter; a bacterial ribosome binding sequence; wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a cell in the presence of an inducer; and wherein: the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct; and the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct.

[0083] Provided herein are isolated prokaryotic cells comprising a nucleic acid construct provided herein.

[0084] Provided herein are methods of expressing a transgene toxic to a prokaryotic cell, the methods comprising: transforming a nucleic acid construct into the prokaryotic cell, wherein the nucleic acid construct comprises a modified inducible promoter comprises: a TATAAT consensus sequence at position -10, relative to a transcriptional start site of the promoter; a TTGACA

sequence at the -35 position, relative to a transcriptional start site of the promoter; a bacterial ribosome binding sequence; and contacting the prokaryotic cell with an inducer, thereby expressing the transgene; wherein the transgene when cloned into a comparable nucleic acid construct that comprises a strong promoter inhibits growth of the prokaryotic cell prior to the contacting with the inducer, thereby preventing expression of the transgene via the comparable nucleic acid.

[0085] Provided herein are methods of expressing a transgene in a prokaryotic cell that does not comprise a T7 RNA polymerase, the methods comprising: transforming a nucleic acid construct into the prokaryotic cell, wherein the nucleic acid construct comprises a modified inducible promoter comprises: a TATAAT consensus sequence at position -10, relative to a transcriptional start site of the promoter; a TTGACA sequence at the -35 position, relative to a transcriptional start site of the promoter; a bacterial ribosome binding sequence; and contacting the prokaryotic cell with an inducer, thereby expressing the transgene; wherein the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a T7 promoter in a comparable nucleic acid construct; and the expression of the transgene in the presence of the inducer is greater than an amount of expression in the presence of the inducer of the transgene operatively coupled to the T7 promoter in the comparable nucleic acid construct.

[0086] Provided herein are nucleic acids, nucleic acid constructs, compositions, cells, non-naturally occurring organisms, cell-free systems, kit, or method provided herein.

[0087] For a better understanding of the present disclosure and of its many advantages, the following examples are given by way of illustration and without limiting the scope of this disclosure.

EXAMPLES

EXAMPLE 1: INDUCIBLE PROMOTOR ARCHITECTURE.

[0088] In exemplary embodiments, the -10 and -35 boxes of the original lac promoter (FIG. 1Ai), which are targeted by sigma 70 (σ^{70}), were modified to generate improved variants such as the lacUV5 (FIG. 1Aii) and tacI (FIG. 1Aiii) promoters. In the lacUV5 promoter, the -10 box of the original lac promoter was replaced by the Pribnow box (TATAAT). Later the lacUV5 and trp promoters were combined to create the tacI promoter, which increased transcription 11-fold compared to its predecessor with the incorporation of the -35 consensus box TTGACA. The tet

promoter (FIG. 1Bi) shares the highly conserved -35 hexamer with tac promoter, however it does not contain the Pribnow box.

[0089] Provided herein are exemplary lac and tet expression systems with improved strength, control, and portability.

[0090] The genetic architecture of the lac and tet expression systems were modified in three ways:

(1) addition of the consensus -10 and -35 sequence boxes to be strongly targeted by σ70;

(2) incorporation of a strong ribosome binding site recognized by a broad spectrum of Gram-negative bacteria; and

(3) independent control of the transcriptional regulators by appropriately-tuned constitutive promoters.

[0091] The results were validated with the reporter protein mCardinal, which significantly improves the dynamic range of promoter measurements over more commonly used green fluorescent proteins. The improvement seen in the mCardinal dynamic range is due to intrinsic fluorescence of many bacterial species that interferes with measurements in the green wavelengths, and the bacteria provided herein have reduced autofluorescence in the far red spectrum. Additionally, the inducible promoters provided herein were compared with the pET system with the production of the cocaine esterase CocE, a thermosensitive enzyme capable of metabolizing cocaine into benzoic acid. CocE, which is prone to form inclusion bodies in leaky *E. coli* expression systems, is expressed as an inducible and soluble protein using the promoters provided herein. The results and assays provided in Example 2 further support that the expression system provided herein is a significant improvement over available expression systems in providing tight OFF state control while achieving high yields of recombinant protein, and with the advantage of direct portability to alternative host species.

EXAMPLE 2: GENERATION OF INDUCIBLE PROMOTORS IN GRAM NEGATIVE BACTERIA.

Bacterial strains

[0092] *E. coli* DH10B, *P. putida* JE90 derivative of KT2440 with BxB1int-attB, and *V. natriegens* Vmax X2 (Codex DNA, Inc.) were used to evaluate the synthetic lac and tet promoters. The plasmid pET21a-mCardinal was evaluated in *E. coli* BL21 strain. Selective markers kanamycin (50 μg/mL for *E. coli* and *P. putida*, and 400 μg/mL for *V. natriegens*), ampicillin (100 μg/mL for *E. coli*) and spectinomycin (60 μg/mL for *E. coli* and *P. putida*, and 250 μg/mL for *V. natriegens*) were supplemented to LB medium when required. *E. coli* strains were transformed by

electroporation and *V. natriegens* by chemical transformation. Integration of plasmids into *P. putida* chromosome was performed by electroporation following the protocol as described, for example, in Elmore *et al. Metab Eng Commun*, 5: 1-8 (2017), the contents of which is incorporated herein by reference in its entirety.

[0093] Plasmid information is listed in Table 1 below. Any plasmid containing any version of the σ^{70}-based promoters as shown in FIG. 2A and FIG. 2B has been labeled "pσ^{70}" followed by information about the construct and the specific promoter. "Lac" and "Tc" constructs are constitutive and do not express a repressor protein; "LacI" and "TcR" constructs are inducible and include expression of LacI or TetR, respectively. Numbers in parentheses indicate a vector backbone other than pJH0204 ((13) for pJH0228 with the pCloDF13 origin of replication, and (19) for pUC19).

Table 1. List of Plasmids and Vectors.

Name	Backbone	Origin of replication	Selective marker	Construct
pJH0204	pJH0204	pColE1	Kanamycin	
pJH0228	pJH0228	pCloDF13	Spectinomycin/Streptomycin	
pJH0204F	pJH0204	pColE1	Kanamycin	
pFCtacI-GFP	pJH0204	pColE1	Kanamycin	*tacI*-sfGFP
pFCtacI-cardi	pJH0204	pColE1	Kanamycin	*tacI*-mCardinal
pσ^{70} V1Lac-cardi	pJH0204	pColE1	Kanamycin	*V1lac*-mCardinal
pσ^{70} V2Lac-cardi13	pJH0228	pCloDF13	Spectinomycin/Streptomycin	*V2lac*-mCardinal
pσ^{70} V3Lac-cardi	pJH0204	pColE1	Kanamycin	*V3lac*-mCardinal
pσ^{70} V4Lac-cardi	pJH0204	pColE1	Kanamycin	*V4lac*-mCardinal
pσ^{70} V1LacI-cardi	pJH0204	pColE1	Kanamycin	*V1lac/lacI*-mCardinal
pσ^{70} V2LacI-cardi	pJH0204	pColE1	Kanamycin	*V2lac/lacI*-mCardinal
pσ^{70} V2Lac-cardi13	pJH0204	pCloDF13	Spectinomycin/Streptomycin	*V2lac/lacI*-mCardinal
pσ^{70} V3LacI-cardi	pJH0204	pColE1	Kanamycin	*V3lac/lacI*-mCardinal
pσ^{70} V4LacI-cardi	pJH0204	pColE1	Kanamycin	*V4lac/lacI*-mCardinal
pσ^{70} V1Tc-cardi	pJH0204	pColE1	Kanamycin	*V1Tc*-mCardinal
pσ^{70} V2Tc-cardi	pJH0204	pColE1	Kanamycin	*V2Tc*-mCardinal
pσ^{70} V3Tc-cardi	pJH0204	pColE1	Kanamycin	*V3Tc*-mCardinal
pσ^{70} V4Tc-cardi	pJH0204	pColE1	Kanamycin	*V4Tc*-mCardinal
pσ^{70} V1TcR-cardi	pJH0204	pColE1	Kanamycin	*V1Tc/tetR*-mCardinal
pσ^{70} V2TcR-cardi	pJH0204	pColE1	Kanamycin	*V2Tc/tetR*-mCardinal
pσ^{70} V3TcR-cardi	pJH0204	pColE1	Kanamycin	*V3Tc/tetR*-mCardinal
pσ^{70} V4TcR-cardi	pJH0204	pColE1	Kanamycin	*V4Tc/tetR*-mCardinal

pσ^{70} V1TcR-cardi19	pUC19	pMB1	Ampicillin	*V2Tc/tetR*-mCardinal
pET21a-cardi	pET21a	pET	Ampicillin	*pET*

Molecular cloning:

[0094] A schematic diagram of plasmid construction are shown in FIGS. 1A-1B. Polymerase chain reactions (PCR) were carried out with Phusion DNA polymerase (ThermoFisher Scientific, USA). Digestion of DNA was performed with fast digest restriction enzymes and DNA fragments were join by T4 DNA ligase or Gibson assembly kit (ThermoFisher Scientific, USA). Oligonucleotides and DNA synthesis were ordered to IDT (IDT, USA). DNA sequencing was performed (QuintaraBio, USA). Shuttle vectors pJH0204 and pJH0228 were used. The synthetic Vlac and VTc promoters were synthesized as gBLocks and incorporated into the MCS of pJH0204 using BamHI and XhoI. Further, sfGFP and mCardinal ORF were codon optimized for *P. putida* and inserted downstream the synthetic promoters using NdeI and HindIII. The transcriptional regulator tetR gene was synthesized with the PJ23119 promoter in a single gBlock and tetR was codon optimized for *P. putida* using the IDT DNA codon optimization tool (available on the world wide web at https:// www.idtdna.com/pages/tools/codon-optimization-tool) and inserted after mCardinal with EcoRI and XhoI. The lacI gene was PCR amplified from pET21a vector and placed under the control of PJ23119 using NcoI and XhoI, but this combination resulted toxic for *E. coli*, therefore lacI was PCR amplified together with its native promoter and ligated after mCardinal using EcoRI and XhoI. pJH0228-V2lac-mCardinal and pJH0228-V2lac/lacI-mCardinal vectors were produced using the same cloning strategy. mCardinal was inserted into pET21a using NdeI and XhoI. The transcriptional unit V2Tc/tetR-mCardinal was inserted into the pUC19 vector by Gibson removing the NdeI RE nucleotide sequence in the plasmid and incorporating terminators insulating the transcriptional unit. DNA sequence of CocE was codon optimized for *P. putida* using the IDT DNA codon optimization tool and cloned into the pET21a and V2Tc-tetR expression system using NdeI and HindIII. A complete list of plasmids generated in this study is shown in **Table 1**. Primers used in this study are listed in **Table 2**.

Table 2. List of Primers.

Primer name	SEQ ID NO:	Sequence 5' to 3'
pUC19 modified FW XhoI	15	actgcCTCGAGACTGATTTTTAAGGCGACTGATGAGTCGCCTTTTTTTTGTC TAGCTAACTCACATTAATTGCGTTGCGCT
pUC19 modified FW	16	gcaatGGATCCagtcaaaagcctccgaccggaggcttttgactAGTACAATC TGCTCTGATGCCGCATAGTT

XhoI		
204F1_fwd	**17**	Ggcgcgtactccaaaaggatctaggtgaagatc
204F1_rev	**18**	gagttcttctgattagacaaaaaaaaggcgac
204F2_fwd	**19**	tttttttgtctaatcagaagaactcgtcaagaagg
204F2_rev	**20**	gcccgacggcgaggatctcgtcgtgacgcatg
204F3_fwd	**21**	cacgacgagatcctcgccgtcgggcatccgc
204F3_rev	**22**	gtctagactgcaggaattcaagcttcatatgggatccggaccaaaacgAAA
204F4_fwd	**23**	aagcttgaattcctgcagtctagaccatggctcgaggacgaacaataaggc
204F4_rev	**24**	Cctagatccttttggagtacgcgcccgggga
placI upstream	**25**	TACTGGTTTCACATTCACCAC
lacI downstream	**26**	GTGGTGAATGTGAAACCAGTAA
lacI upstream	**27**	TTTCCAGTCGGGAAACCT
lacI downstream2	**28**	AGGTTTCCCGACTGGAAA
tetR upstream	**29**	CCAATACAACGTGGGTTGCT
FwplacI	**30**	AACCGGAATTCccccgtggccggg
LXhoI RV	**31**	AACCGCTCGAGTCACTGCCCGCTTTCC
Fw PstI-lacI	**32**	attgcCTGCAGTCACTGCCCGCTTTCCAGTCG
Rv XbaI-lacI	**33**	gcaatTCTAGAagtcaaaagcctccgaccggaggcttttgactCCCCGTGGC CGGGGGACTG
XbaI-V3Lac-NcoI_fwd	**34**	gcctccggtcggaggcttttgactt CTAGAGAGCTGTTGACACTTTATG
XbaI-V2Lac-NcoI_rev	**35**	taatgagctcctcacccttactcacCATGGGTACATTCCTCTCTTACTGC
XbaI-V3Lac-	**36**	taatgagctcctcacccttactcac
NcoI_rev	**37**	CATGGGATTCTGTTTCCTGTGTG
Rv cardi XhoI	**38**	TCAGTCTCGAGTTACTTGTCGTCATCATCCTTAT

[0095] Primers were used as sequencing primers (*e.g.*, placI upstream, tetR upstream). 204F primers were used to incorporate BamHI, NdeI, HindIII, KpnI, EcorI, PstI, XbaI, NcoI, XhoI RE into the MCS of pJH0204 and remove NcoI from kanamycin cassette. The pUC19 modified FW XhoI primers were used to incorporate terminators into pUC19 vector and BamHI/XhoI sites.

[0096] Sequences of *cocE*, *tetR*, and mCardinal, codon optimized for *P. putida*, and the PJ23119 promoter sequence, are provided below.

[0097] DNA sequence of mCardinal codon optimized for *P. putida:*

ATGGTGAGTAAGGGTGAGGAGCTCATTAAGGAGAACATGCACATGAAGCTGTATATGGAGGGCACCGTAAACAACCA
CCACTTCAAGTGTACCACCGAGGGTGAAGGTAAACCCTACGAGGGGACGCAGACCCAACGCATCAAGGTCGTGGAGG
GCGGCCCGCTGCCTTTCGCATTCGACATTCTGGCGACCTGTTTTATGTACGGCTCGAAGACCTTCATCAACCACACC
CAAGGCATCCCGGACTTCTTCAAGCAGAGCTTCCCTGAGGGCTTCACCTGGGAGCGCGTCACCACGTATGAAGACGG
TGGGGTGCTCACCGTGACCCAGGACACGAGCTTGCAGGATGGCTGCTTGATTTACAACGTCAAGCTGCGCGGGGTGA
ACTTCCCTAGCAACGGGCCAGTGATGCAGAAAAAGACGCTGGGTTGGGAGGCCACCACCGAGACCCTGTACCCGGCC
GACGGGGGGCTGGAAGGGCGGTGCGATATGGCCCTGAAATTGGTCGGCGGCGGTCATTTGCACTGCAATCTCAAGAC
CACGTACCGCTCCAAGAAACCCGCCAAAAACCTGAAGATGCCTGGTGTTTATTTTGTCGACCGGCGCCTGGAGCGCA
TCAAGGAAGCGGACAATGAGACGTACGTGGAACAGCACGAAGTGGCCGTGGCTCGTTATTGCGATCTGCCGTCGAAG
CTGGGTCACAAACTGAACGGCATGGATGAGCTGTACAAAGATTATAAGGATGATGACGACAAGTAA (**SEQ ID NO: 39**)

[0098] DNA sequence of sfGFP codon optimized for *P. putida:*

ATGTCCAAAGGTGAAGAGCTGTTTACCGGCGTCGTGCCCATTCTGGTGGAGCTGGATGGCGACGTCAACGGGCACAA
GTTTAGCGTCCGTGGCGAAGGTGAGGGCGACGCCACGAACGGTAAGCTGACGCTGAAATTCATTTGCACCACCGGCA
AATTGCCTGTACCCTGGCCCACCCTGGTGACCACGCTCACCTACGGCGTACAGTGCTTCAGCCGTTACCCGGACCAC
ATGAAGCGTCACGACTTCTTCAAAAGCGCCATGCCGGAGGGTTACGTGCAGGAGCGTACGATTAGTTTCAAGGACGA
CGGCACCTATAAGACCCGTGCCGAAGTGAAGTTCGAAGGCGATACGTTGGTGAACCGTATCGAGTTGAAGGGTATCG
ACTTTAAGGAAGACGGCAACATCCTGGGCCATAAGCTGGAGTACAATTTCAACAGCCATAACGTTTACATCACCGCC
GATAAACAGAAGAACGGCATTAAAGCCAACTTTAAGATCCGCCACAACGTCGAAGACGGCTCGGTGCAGCTGGCCGA
CCATTATCAGCAAAACACCCCCATCGGTGATGGGCCCGTGCTGCTGCCGGATAACCATTATCTGAGCACGCAGTCGG
TGCTCAGCAAGGACCCTAACGAAAAGCGCGATCACATGGTGCTGCTGGAGTTCGTCACGGCGGCGGGGATCACCCAT
GGGATGGACGAGCTCTACAAAGACTATAAAGATGACGATGACAAGTAAA (**SEQ ID NO: 40**)

[0099] DNA sequence of tetR codon optimized for *P. putida:*

ATGTCCCGCCTGGATAAATCGAAAGTGATTAACTCGGCCCTCGAATTGCTGAATGAAGTCGGTATCGAGGGGCTGAC
GACCCGTAAATTGGCACAAAAGTTGGGGGTGGAGCAACCCACGTTGTATTGGCACGTCAAAAATAAGCGGGCATTGC
TGGATGCCCTCGCTATTGAAATGTTGGATCGCCACCATACCCATTTCTGTCCACTGGAGGGCGAGTCCTGGCAGGAC
TTTCTCCGCAACAACGCGAAATCCTTTCGCTGTGCACTCTTGTCCCATCGGGACGGTGCTAAGGTGCACTTGGGCAC
CCGTCCCACCGAAAAACAATACGAAACCTTGGAAAATCAATTGGCGTTTTTGTGCCAGCAAGGGTTTAGCTTGGAGA
ATGCTCTCTATGCGCTCTCGGCTGTCGGGCACTTTACGTTGGGGTGCGTGTTGGAGGACCAGGAGCATCAAGTCGCA
AAAGAGGAGCGTGAAACCCCAACCACGGACTCGATGCCACCTCTGCTCCGCCAAGCTATCGAACTCTTCGATCATCA
GGGCGCGGAGCCAGCCTTCCTCTTTGGGCTGGAGCTGATTATCTGCGGTTTGGAAAAACAACTCAAGTGTGAAAGCG
GGTCCTAA (**SEQ ID NO: 41**)

[00100] DNA sequence of cocE codon optimized for *P. putida:*

CATATGGTGGACGGTAATTATTCGGTAGCGTCCAACGTTATGGTGCCGATGCGCGACGGGGTGCGCTTGGCTGTAGA
TCTGTACCGCCCGGACGCAGATGGCCCTGTACCGGTCCTGCTGGTCCGCAACCCCTACGACAAATTCGACGTGTTCG
CTTGGAGTACGCAGAGCACGAACTGGCTGGAATTTGTGCGCGATGGGTACGCCGTCGTCATCCAAGACACCCGGGGC

CTCTTTGCATCCGAAGGTGAGTTCGTTCCACATGTTGATGACGAGGCGGATGCGGAAGACACGCTGAGCTGGATCTT
GGAACAAGCATGGTGCGACGGCAATGTGGGTATGTTCGGTGTAAGCTACCTGGGCGTTACGCAGTGGCAAGCTGCTG
TTAGCGGTGTGGGTGGTTTGAAGGCAATCGCCCCGAGCATGGCGAGCGCGGATCTGTACCGTGCCCCCTGGTACGGT
CCTGGCGGCGCCCTGAGCGTGGAAGCACTCCTGGGCTGGAGCGCATTGATCGGTACGGGCCTGATTACCAGCCGTAG
CGATGCCCGCCCGGAAGACGCAGCCGACTTCGTACAGCTGGCAGCCATCCTGAACGATGTGGCCGGTGCCGCAAGCG
TGACCCCTCTGGCCGAACAGCCCTTGTTGGGCCGCCTGATCCCTTGGGTGATCGACCAGGTGGTGGACCATCCAGAC
AACGACGAGTCGTGGCAGAGCATCTCGCTCTTTGAACGTTTGGGTGGGCTCGCTACCCCGGCCTTGATTACCGCCGG
GTGGTACGATGGCTTCGTGGGCGAGAGCCTCCGTACCTTCGTAGCTGTGAAGGACAACGCGGATGCGCGTCTGGTGG
TGGGGCCGTGGAGCCACAGCAATCTGACCGGCCGTAATGCCGACCGTAAGTTTGGGATCGCCGCGACCTACCCCATC
CAGGAGGCGACGACCATGCACAAGGCTTTTTTCGACCGGCACCTCCGTGGCGAGACCGATGCCCTGGCAGGGGTGCC
CAAGGTGCGCCTCTTCGTAATGGGTATCGATGAGTGGCGCGACGAGACCGACTGGCCATTGCCAGATACCGCTTACA
CGCCTTTTTACCTCGGGGGCTCCGGTGCGGCCAACACGAGCACGGGTGGTGGGACCCTGTCGACCTCGATCAGCGGC
ACGGAGTCGGCGGACACCTACCTGTATGATCCTGCCGACCCCGTGCCAAGTCTGGGCGGCACCCTCCTCTTCCATAA
TGGGGACAACGGTCCAGCTGACCAGCGCCCGATTCACGATCGCGACGACGTGCTGTGCTACTCCACCGAGGTGTTGA
CCGACCCCGTGGAAGTAACGGGGACGGTTTCGGCTCGCCTGTTCGTGTCCTCGTCGGCCGTGGATACCGATTTTACC
GCCAAGTTGGTCGACGTGTTCCCCGATGGTCGGGCAATCGCTCTCTGCGACGGCATCGTGCGTATGCGCTACCGGGA
GACCTTGGTAAATCCTACGCTCATTGAGGCCGGTGAGATTTACGAGGTGGCTATTGATATGCTGGCCACCAGCAACG
TGTTTTTGCCGGGCCACCGCATCATGGTGCAAGTTAGCAGCTCGAACTTCCCGAAGTACGACCGCAACTCCAACACC
GGCGGCGTCATCGCTCGCGAGCAACTGGAGGAAATGTGCACCGCCGTAAACCGCATTCACCGCGGCCCCGAACACCC
GTCCCATATCGTGCTGCCGATCATTAAGCGCGACTATAAGGACGACGACGATAAGTGAAAGCTT (**SEQ ID NO: 42**)

[00101] DNA sequence of pJ23119 promoter:

TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCGCAGTAAGAGAGGAATGTACAC (**SEQ ID NO: 43**)

SDS-PAGE

[00102] SDS-PAGE was carried out on a 4–12% Bis-tris Midi Protein Gel in an XCell4 SureLock Midi system (Invitrogen, USA). *E. coli*, *P. putida* and *V. natriegens* cell extracts were obtained from diluting an overnight culture 1:50 in 10 mL of fresh LB media and grown at 37°C for *E. coli* and 30°C for *P. putida* and *V. natriegens* at 220 rpm until the culture reached an optical density (OD) at 600 nm (OD_{600})of 0.7 measured spectrophotometrically, then induced with 0.2 mM IPTG or 0.1 µg/mL aTc accordingly. Induced cultures were grown for 5 hours, and 2 mL culture were spun down at 14.000 rpm and 4°C for 10 minutes and frozen at - 20°C for further analysis. Cells were lysed with Complete Bacterial Protein Extraction Reagent (ThermoFisher Scientific), and crude extracts were analyzed by SDS-PAGE. Total protein concentration was estimated with Thermo Scientific NanoDrop one and ~10 mg total protein of each cell extract was loaded into the SDS-gel.

Expression of CocE and measurement of benzoic acid production

[00103] Recombinant *E. coli* BL21 strain carrying the pET21a-cocE and DH10B containing either pσ^{70} V2TcR-cocE or pσ^{70} V2TcR-cocE19 plasmids were cultivated overnight at 30°C at 220 rpm and fresh cultures were started with 5% of the ON culture and induced at ~OD_{600} 0.7 with 0.2 mM IPTG or 0.1 µg/mL aTc accordingly. 1 mL samples were collected each hour for 6 hours by centrifugation at 4°C and 14000 rpm and stored at - 80°C. Cell pellets were disrupted using 150 µL of B-PE Complete Bacterial Protein Extraction Reagent (ThermoFisher Scientific) for 25 minutes at room temperature and soluble fractions were collected after centrifugation for 25 minutes at 4°C and 14000 rpm. ~ 3 mg/mL of soluble fractions were incubated with 0.015 mg of cocaine for 20 minutes at 28°C and benzoic acid production was estimated using the benzoic acid detection kit for feed (Attogene, EZ2013-03).

EXAMPLE 3: DESIGN OF SYNTHETIC LAC AND TET PROMOTERS.

[00104] A schematic diagram of plasmid construction is shown in **FIGS. 1A-1B**. All plasmids used in the assay are listed in **Table 1**. The original *lac* promoter (FIG. 2Ai) lacks the -35 conserved box recognized by the σ^{70} subunit of RNA polymerase. Original *lac* and *tet* (FIG. 2Bi) promoters lack the Pribnow (-10) box. Three synthetic variants of each promoter were constructed containing the highly conserved σ^{70} consensus hexamers at positions -10 and -35. Version 1 (*V1lac*, FIG. 2Aiv, and *V1Tc*, FIG. 2Bii) of each promoter resembles the original *lac* and *tet* promoters, with the incorporation of a strong RBS from *P. putida*. The version 2 (*V2lac*, FIG. 2Av, and *V2Tc*, FIG. 2Biii) replaces the -35 and -10 boxes of Version 1 for the highly conserved σ^{70} consensus hexamers TTGACA and TATAAT, respectively. Version 3 (*V3lac*, FIG. 2Avi, and *V3Tc*, FIG. 2Biv) incorporates an additional lacO operator and the RBS of the *tacI* promoter. Version 4 (*V4lac*, FIG. 2Avii, and *V4Tc*, FIG. 2Bv) displaces the location of the -10 and -35 boxes (*V4lac*) and incorporates an additional -35 box in *V4Tc*. The synthetic promoters were cloned into the MCS of the shuttle vector pJH0204 containing the origin of replication pColE1 with 25-30 copies per cell in *E. coli* . The design allowed the incorporation of the transcriptional regulators *lacI* and *tetR* after a terminator L3S2P21 and under the control of the constitutive promoter PJ23119 (FIG. 2C).

[00105] No aberrant phenotypes in the *E. coli* strains carrying the PJ23119-*tetR* construct were observed. However, *E. coli* failed to maintain the PJ23319-*lacI* construct. The plasmids containing the *lacI* repressor gene in this configuration produced slow-growing colonies and yielded plasmids with aberrant restriction patterns, indicating gross plasmid rearrangements. Therefore, the strong PJ23119 promoter was replace for the native *lacI* promoter and observed no further toxicity.

[00106] The reporter protein mCardinal was incorporated downstream of the synthetic promoters and the plasmids were transformed into *E. coli* DH10B, *P. putida* JE90 derivative KT2440, and *V. natriegens* Vmax *X2* strains. The *V2lac-mCardinal* constructs could not be maintained in *E. coli*, likely due the high strength of the *V2lac* promoter. To avoid this abnormal phenotype, the genetic circuit *V2lac-mCardinal* was produced in the pJH0228 vector containing the CloDF13 origin of replication at about 10 copies per cell, allowing stable maintenance of the *V2lac* promoter.

EXAMPLE 4: SELECTION OF THE FLUORESCENT REPORTER SYSTEM.

[00107] GFP and other green fluorescent protein derivatives are widely used as reporters in bacterial expression systems. However, the intrinsic green fluorescence produced by endogenous molecules, such as proteins containing aromatic amino acids, negatively impact the interpretation of the exogenous green fluorescence generated by the reporter system. The autofluorescence is exacerbated in *P. putida*, a member of the fluorescent *Pseudomonas* species. Under iron limited conditions *P. putida* secretes the siderophore pyoverdine, a soluble fluorescent yellow green pigment. Far-red fluorescent proteins were explored, specifically mCardinal, a monomeric far red shifted derivative of mKate, as wavelengths between 600 and 900 nm are not absorbed by cells and organic molecules, thus reducing the noise of endogenous autofluorescence.

[00108] To quantify the impact of autofluorescence on the selected Gram-negatives strains, the endogenous fluorescence of these strains cultivated in LB over time were measured. All three species emit fluorescence in the green spectrum, as expected (FIG. 3A, right panel). This fluorescence is more or less constant over time with *E. coli*, however *P. putida* and *V. natriegens* show variable production of molecules that absorb green light at different cell densities. In contrast, when measured in the far-red spectrum specific for mCardinal, there was approximately 400 times less detection of autofluorescence, in arbitrary units on the same instrument, in all strains as compared to the measurements in the green spectrum (FIG. 3A, left panel). Again, *E. coli* displayed rather consistent far red autofluorescence over time whereas *P. putida* and *V. natriegens* autofluorescence varies with cell density.

[00109] To further validate the benefit of using mCardinal instead of sfGFP, the inherent noise of each reporter system was quantified by measuring the fluorescence signal-to-background ratio of both reporter systems expressed under the control of the constitutive *tacI* promoter in the three species. After 16 hours of growth, the recombinant *E. coli, P. putida* and *V. natriegens* expressing sfGFP emitted 64, 11 and 5 times more green fluorescence than their respective wild type strains (not expressing a fluorescent protein). Meanwhile, mCardinal-expressing strains

displayed 294, 34 and 13-fold higher red-light emission in *E. coli, P. putida* and *V. natriegens* compared to their wild type controls (FIGS. 3B-3D). Overall, the use of mCardinal rather than sfGFP significantly improves the dynamic range and facilitates measurement of promoter strength, as there is little endogenous autofluorescence in the far-red spectrum.

EXAMPLE 5: STRENGTH AND REGULATION OF SYNTHETIC *LAC* PROMOTERS IN *ESCHERICHIA COLI.*

[00110] The *lac* promoter, and its derivates, are constitutively active in the absence of its transcriptional regulator *lacI*. The strength of the synthetic *lac* promoters were evaluated without *lacI* and compared these against the strong *tacI* promoter, which drives high levels of transcription and can result in recombinant protein production of up to 30% of total protein. The *V1Lac* promoter was 10-fold weaker than the *tacI* promoter (FIG. 4A), consistent with previous data observing an 11-fold difference between these two promoters. The *V3lac* promoter matched the *tacI* promoter strength, while the *V4lac* promoter showed 5 times less mCardinal than *tacI* (FIG. 4A). The *V2lac* promoter had to be tested in the low copy plasmid pCloDF13 because the medium copy pColE1 derived plasmid could not be maintained in *E. coli*. Even in the low copy configuration, the *V2Lac* promoter surpassed by ~1.4-fold the *tacI* promoter and was therefore the strongest constitutive promoter despite its expression in a low copy plasmid. The result suggests that maintenance of a promoter of this strength within in a medium copy plasmid exceeds the sustainable metabolic burden of *E. coli* (FIG. 4A). These results indicate that the incorporation of σ^{70} consensus sequences at positions -35 and -10 efficiently increase the strength of the *lac* promoter.

[00111] Next, the transcriptional regulator *lacI* was incorporated into these circuits to quantify the efficiency of the OFF state. The *V1Lac* and *V4lac* constructs containing the *lacI* repressor produced a red fluorescence signal comparable to wild type *E. coli* (FIG. 4A), indicating tight transcriptional repression. The *V3lac* promoter could not be totally repressed by *lacI*, showing a slight tendency to leak, even with the presence of two lacO operators. The *V2lac* promoter with *lacI* was stably maintained by *E. coli* in both pColE1 and CloDF13-derived vectors. However, the repressed state of the *V2lac* promoter in the low and medium copy plasmids showed rather a constitutive behavior. *LacI* repressed ~9-fold the *V2Lac* promoter in the CloDF13 derived plasmid as compared to its constitutive construct (FIG. 4A), while the medium copy version of the *V2lac-lacI* system (which was not sustainable as a constitutive circuit) exhibited a strong constitutive expression ~1.3-fold higher than the *tacI* promoter, thus showing that one lacO operator region is not sufficient to block transcription of *V2Lac* containing both σ^{70} consensus sequences by LacI,

but incorporation of a second lacO reduces the leakage as observed in the *V3Lac* promoter (FIG. 4A).

[00112] To verify the inducibility of the synthetic *lac* promoters, *E. coli* was exposed to IPTG at the beginning of exponential phase. The *V1lac* and *V4lac* promoters showed minimal induction of mCardinal (FIG. 4A). The *V3lac* promoter, which mimics the *V2lac* promoter with an additional lacO operator, demonstrated a dynamic range of ~17-fold versus the uninduced state, but could only produce 24% of its full constitutive potential (FIG. 4A). The *V2lac* promoter in the low copy plasmid produced ~2-fold more mCardinal upon induction; similar behavior was observed in the medium copy version, where production of mCardinal reached the maximum values of all promoters tested at ~2.6-fold stronger than the *tacI* promoter (FIG. 4A). Overall, the results demonstrate that incorporation of σ^{70} consensus sequences to the *lac* promoter improve its strength (*V2Lac*), producing more mCardinal than the strong *tacI* promoter in both, the constitutive LacI-less version in the low copy plasmid and the repressed and induced states in the medium copy plasmid. However, LacI could not repress the transcription of the synthetic *V2Lac* promoter. Thus, additional *lacO* operators are necessary to turn the *lac* promoter OFF containing the perfect σ^{70} boxes, as observed in *V3Lac*, though full induction then becomes impossible in this architecture.

EXAMPLE 6: STRENGTH AND REGULATION OF SYNTHETIC *TET* PROMOTERS IN *ESCHERICHIA COLI.*

[00113] The *tet* promoter also drives transcription constitutively without TetR. The expression profile of the constitutive synthetic *tet* promoters were evaluated and compared against the *tacI* promoter. As observed for the *lac* promoter, replacement of the −10 and −35 sequences with the σ^{70} consensus boxes in the *tet* promoter also increased the constitutive efficiency of the *V2Tc* promoter over the original *V1Tc*, in this case by 20%, and reached the yields obtained with *tacI* promoter (FIG. 4B). The *V4Tc* promoter showed similar results to the *V2Tc*, while the *V3Tc* lost any capability to initiate transcription (FIG. 4B). Further, incorporation of the *tetR* repressor to each of the four circuits under the control of the strong constitutive PJ23119 promoter completely silenced mCardinal production from all the synthetic *tet* promoters, even in the strong *V2Tc* promoter which harbors the optimal combination of consensus sequences recognized by the σ^{70} (FIG. 4B).

[00114] To confirm the induction of each promoter and determine its functional dynamic range, each circuit was induced with anhydrotetracycline (aTc). The original tet promoter (*V1Tc*) only achieved ~37% of its full constitutive potential, reaching only 30% of mCardinal compared to *tacI*

promoter confirming the middle level strength of the *tet* promoter (FIG. 4B). The *V2Tc* promoter achieved maximal induction reaching the full potential dynamic range and produced similar yields as the strong *tacI* promoter (FIG. 4B). Interestingly, the *V4Tc* promoter could not be de-repressed by aTc and showed a weak induction of only 5%, and as expected, the *V3Tc* promoter showed no activity (FIG. 4B). Together these results confirm that in *E. coli* the incorporation of the consensus boxes targeted by the σ^{70} is key to boost the expression profile of promoters recognized by this transcription initiation factor and achieve the maximal transcriptional capacity of these promoters upon induction.

EXAMPLE 7: DIRECT COMPARISON OF SYNTHETIC *LAC* AND *TET* EXPRESSION SYSTEMS TO PET IN *ESCHERICHIA COLI*.

[00115] In *E. coli*, the pET series of expression plasmids are the most popular systems for recombinant protein production. The efficiency of the synthetic *lac* and *tet* promoters were compared directly against pET21a. The pET expression system in the BL21 strain can produce more than 50% of the target gene as the total protein per cell, mCardinal production was measured in BL21. As expected, the pET system induced by IPTG produced ~1.5-fold more mCardinal than the strong constitutive *tacI* promoter, which is expected to accumulate up to 30% of total cell protein. Despite the massive production of mCardinal by the pET system, its OFF or uninduced state can be considered as a medium high constitutive expression system, yielding 30% of the constitutive *tacI* promoter as evidenced by mCardinal production (FIG. 4B, FIG. 4C). This transcriptional leakage in the pET system is well known in the scientific community.

[00116] Among the synthetic *lac* and *tet* promoters, all tested in *E. coli* DH10B, only the *V2Lac* promoter surpassed the pET system in the medium copy plasmid pColE1. The IPTG induced *V2Lac* expression system produced ~1.8 times more recombinant protein than the pET system, however, the leakage of *V2Lac* promoter equals the induced state of the pET system (FIG. 4A and FIG. 8), therefore cancelling the advantages of an inducible expression system. The *V3Lac* expression system showed a tighter control of the OFF state, therefore, to increase its strength the *V3Lac-lacI* construct containing mCardinal was inserted into the pUC19 vector, thus increasing the copy number from 30 to 250 copies per cell. *E. coli* failed to maintain the *V3lac-lacI* construct in the pUC19 vector, likely due to the toxicity of *LacI*. The *V2Tc-tetR* expression system was evaluated in the pUC19 vector. *E. coli* stably maintained the *V2Tc-tetR* construct and no phenotypic or genotypic abnormalities were observed as in pUC19-*V3Lac-lacI*. mCardinal production by pUC19-*V2Tc-tetR* matched the pET21a expression system, with one exceptional difference, the

pUC19-*V2Tc-tetR* maintained complete repression in the uninduced after 12 hours (FIG. 4B and FIG. 8). Thus, pUC19-*V2Tc-tetR* shows significant improvement in transcriptional control and dynamic range over pET21a (FIG. 4B, C). Further, equivalent protein production and tighter transcriptional control were achieved without the obligatory use of the BL21(DE3) strain, suggesting the system provided herein is readily portable to other strains and species.

EXAMPLE 8: EXPRESSION OF THE COCAINE ESTERASE COCE AND PRODUCTION OF BENZOIC ACID.

[00117] To further validate the advantage of the expression system provided herein over the pET expression system, the production of a functional protein product, the cocaine esterase CocE, was assayed. This enzyme hydrolyses cocaine into benzoic acid and could expand the use of the narcotic compound as raw source in the production of the carboxylic acid widely used as precursor and preservative in the food and pharmaceutical industries. Expression of CocE has previously been shown to be a difficult and laborious task with the pET expression system, because CocE forms inclusion bodies; consequently, long incubation methods at low temperatures are required to isolate sufficient yields of functional CocE. *E. coli* could not support CocE expression in the repressor-less variants of the synthetic promoters, thus confirming this is a toxic protein for *E. coli*. Next, CocE in the *V2Tc-tetR* expression system was assessed in both the medium and high copy plasmid backbones, pJH0204 and pUC19 respectively (pσ70 V2TcR-cocE and pσ70 V2TcR-cocE, see Table 1), and compared against the pET expression system for the production of benzoic acid. Benzoic acid production is an indication that CocE was correctly folded and properly hydrolyzing cocaine.

[00118] The soluble fraction recovered from the recombinant *E. coli* strain containing the pET21a-cocE expression system showed equivalent benzoic acid production in the presence of cocaine in both the uninduced and induced states (FIG. 5B), with a clear tendency to form inclusion bodies, as evidenced by the accumulation of protein in the insoluble fraction upon activation by IPTG (FIG. 5A). Thus, while induction of CocE by IPTG in the pET system does result in the additional production of protein, the additional protein made is insoluble and non-functional protein. Functionally speaking, the expression of CocE in pET21a is not inducible, but instead behaves constitutively, as there is no difference in cocaine hydrolysis in the uninduced and induced states (FIG. 5B).

[00119] The soluble fractions from the recombinant *E. coli V2Tc-tetR*-cocE expression system, in both the pJH0204 and pUC19 plasmids, showed no benzoic acid production in the absence of the

inducer. The addition of aTc triggered production of CocE with protein production and function roughly equivalent to the soluble pET21a-cocE plasmid (FIG. 5A, FIG. 5B). In both plasmids, benzoic acid production was observed after only one hour of aTc induction, and reached maximal production by 3 hours (FIG. 5B). These results confirmed the advantage of the incorporation of the σ^{70} boxes to the tetracycline promoter, keeping the toxic enzyme CocE OFF in the uninduced state and producing sufficient amounts of mature CocE after 3 hours of induction. This is significantly shorter than induction of 16 hours previously recorded (data not shown). Further, the expression system provided herein produced soluble and functional protein without evidence of massive toxicity or inclusion body formation. This method facilitates the scalability of this bioprocess which has potential as an alternative, environmentally friendly method to obtain benzoic by replacing petroleum-based starting materials.

EXAMPLE 9: STRENGTH AND REGULATION OF SYNTHETIC *LAC* AND *TET* PROMOTERS IN *PSEUDOMONAS PUTIDA*.

[00120] Genetic control in the soil-dwelling species *P. putida* is of great interest for biotechnological applications due to the ability of this microorganism to synthesize complex natural products and metabolize a variety of xenobiotic compounds. The constitutive strength, repression, and inducibility of synthetic promoters in *P. putida* were assayed. The lac-based promoter systems (which include the tacI promoter) are reported to have poor dynamic range and instability in high-copy plasmids when harbored in *P. putida* as replicative plasmids. To address this problem, the genetic circuits were integrated into the *P. putida* chromosome. The constitutive strength of V1lac, V2lac, V3lac and V4lac promoters were measured using mCardinal, and found out that tacI is stronger than all the synthetic lac variants by ~3, 1.5, 10 and 33-fold respectively (FIG. 6A, FIG. 6C). The incorporation of the transcriptional regulator lacI efficiently repressed the V1lac, V3lac and V4lac promoters, but as seen in *E. coli*, the V2lac showed a tendency to leak, though not as egregiously as in *E. coli* (FIG. 6A, FIG. 6C). Induction by IPTG was barely detected in the V1lac, V3lac and V4Lac promoters, thus indicating the weakness of the original lac promoter in *P. putida* (FIG. 6A, FIG. 6C). Interestingly and analogously to *E. coli*, the V2lac promoter showed ~27-fold increase in mCardinal production against the uninduced state and surpassed its theoretical dynamic range as compared to the constitutive version, ultimately achieving similar yields as the strong tacI promoter (FIG. 6A, FIG. 6C). These results confirm that the incorporation of the σ70 consensus sequences to the lac promoter (V2Lac) significantly improve the strength of this promoter in the *P. putida* host.

[00121] The tet promoters in *P. putida* were also evaluated. The original tet promoter with a strong RBS (V1Tc) was the strongest among all synthetic tet promoters in the absence of tetR, followed by V4Tc, V2Tc and V3Tc (FIG. 6B, FIG. 6C). Incorporation of the tetR repressor under the control of the PJ23119 promoter silenced all the synthetic tet promoters, as in *E. coli* (FIG. 6B, 6C). Addition of aTc activated the four tet promoters and remarkably, the induced V2Tc promoter showed the highest mCardinal induction exceeding its constitutive expression by ~10-fold and matching the tacI promoter. The V2Tc promoter was revealed to be ~2 and 3-fold more efficient than the V1Tc and V4Tc promoters after induction respectively, while the V3Tc proved to be inefficient in *P. putida* (FIG. 6B, FIG. 6C). These results highlight the importance of the consensus sequences targeted by σ70 to amplify the strength of inducible promoters also in *P. putida*.

EXAMPLE 10: STRENGTH AND REGULATION OF SYNTHETIC *LAC* AND *TET* PROMOTERS IN *VIBRIO NATRIEGENS*.

[00122] The circuits were next evaluated in the marine bacterium *V. natriegens* which has gained popularity for routine molecular biology applications due its ability to double in <10 min. In this host, previous studies indicated that the *tacI* promoter produced GFP upon activation with IPTG, while induction of the *tet* promoter resulted in low GFP yields. The constitutive and inducible versions of the synthetic *lac* and *tet* promoters were evaluated in the pColE1 derived plasmid with ~300 copies per cell, except for the constitutive *V2Lac*, which was evaluated in pCloDF13 derived vector with ~64 copies per cell in this strain. The constitutive *V2Lac* and *V4Tc* promoters outperformed *tacI* by ~16 and ~2-fold respectively, while *V3Lac*, *V1Tc* and *V2Tc* produced similar levels as *tacI* (FIGS. 7A-7C). *V1Lac* and *V3Lac* underperformed *tacI* by ~2.7-fold, and *V3Tc* showed no activity (FIGS. 7A-7C). Incorporation of the transcriptional regulators *lacI* and *tetR* turned OFF all the synthetic promoters except for *V2Lac*, which continued to show leakage, as in *E. coli* and *P. putida* (FIG. 7A). Induction of the synthetic *lac* promoters by IPTG revealed that only the *V2Lac* promoter was fully activated, surpassing its constitutive version by ~1.3-fold and showed a potential dynamic range of ~25-fold (FIG. 7A). The *V3Lac* reached 30% of its full potential, and the *V1Lac* and *V4Lac* showed no induction (FIG. 7A). While addition of aTc activated all four synthetic *tet* promoters, the *V3Tc* and *V4Tc* promoters rather showed weak mCardinal production, and *V1Tc* produced 2-fold more mCardinal than its constitutive variant (FIG. 7B). The *V2Tc* promoter, which harbors the 2 consensus σ^{70} boxes, demonstrated strong inducibility producing 11-fold more mCardinal than the *tacI* promoter and displaying full potential of its dynamic range producing 100-fold more mCardinal than its OFF version (FIG. 7B, C). These

results confirmed that the adaptation of the two consensus boxes recognized by the σ^{70} in the *V2lac* and *V2Tc* promoters improved the performance of the inducible *lac* and *tet* promoters in *V. natriegens*.

EXAMPLE 11: ADDITIONAL CONSIDERATIONS FOR INDUCIBLE PROMOTER DESIGN.

[00123] Production of recombinant proteins is and will continue to be one of the main tools scientists use to understand biological processes and transfer academic results to industrial applications. The pET system is well known to induce the formation of inclusion bodies, a major drawback in the production of soluble proteins. pET requires specific strains that carry the T7 RNA polymerase, it lacks real tuneability, and fails to keep the target gene OFF. Leakiness in the OFF state negates the main advantage of an inducible system, which is intended to permit time- or context-specific control of gene expression.

[00124] In contrast to pET, the results provided in Examples 2-9 show that inclusion of the σ^{70} consensus sequences into the *lac* and *tet* inducible promoters improve both repression and induction. The tight transcriptional control does not require any particular strain background, and permits rapid expression of soluble proteins, including toxic proteins such as CocE. The inducible promoters provided herein can be easily optimized to be used in a variety of Gram-negative hosts, including *P. putida* and *V. natriegens*, thus widening the applicability of these tools to a broad spectrum of bacteria. The inducible promoters provided herein offer a significant advance to the biotechnology industry in offering additional platforms for exogenous protein expression and purification.

[00125] The *V2lac* promoter expressed very highly across three Gram-negative species. Indeed, in *V. natriegens* the *V2lac* promoter is the strongest promoter yet described, as it surpasses the widely used *tacI* promoter by 16-20-fold (FIG. 7). Additional factors can affect promoter performance, for instance, the length and sequence of the spacer between the -10 and -35 elements can have dramatic effects on promoter strength. .

[00126] The results in FIG. 5 suggest that complete OFF state control is key to avoiding common problems with protein expression of challenging proteins, such as the formation of inclusion bodies. Future promoter configurations could be aided by the combination of additional mechanisms to achieve complete OFF state control, such as the additional of small RNA transcriptional and translational regulators. These approaches have increased appeal over the pET system with pLysE, for example, as they permit facile portability among strains and species. OFF state control of the

lac promoters was less of an issue in *P. putida* and *V. natriegens*, where better repression of the strong *V2lac* promoter was achieved (FIGS. 6A-7C). Without being bound by a particular theory, it is likely that species-specific differences in RNA polymerase binding and promoter clearance may affect the behavior of inducible promoters.

[00127] The challenges of leakiness were entirely eliminated by the use of *tet*-based promoters in the system provided herein. An often-cited drawback of *tet* promoters for protein expression was the limitation of protein production as compared to *lac*-based systems. Incorporation of the σ^{70} consensus sequence into the *tet* promoter (*V2Tc*) significantly increased expression above that of the original *tet* promoter (*V1Tc*) to the point where the promoter output equaled (and in the case of *V. natriegens*, exceeded) that of the *tacI* promoter. As a complementary approach to inducible promoter manufacturing and design, the sigma factor can be replaced with σ^{70} sequences to assess promoter performance. Promoter performance was quantified herein by using far-red reporters, which significantly decreased background fluorescence and increased dynamic range in the bacterial species assayed.

[00128] Different yields of recombinant protein can be achieved based on promoter and host selection. Overall, the incorporation of the consensus −35 sequence and Pribnow (-10) box unlocks the strength of the *lac* and *tet* promoters in Gram-negative bacteria, facilitating the production of any given target gene in different host with the same set of plasmids.

[00129] The *V2Tc* promoter provided herein offers an improvement over the pET-based protein expression systems that remain very widely used. *V2Tc* is tightly regulated, robustly inducible, and drives protein production comparable to or better than the *tacI* promoter in all three species that were examined. No specific strain backgrounds were necessary, as it does not rely on the presence of the T7 polymerase.

EXAMPLE 12: DEVELOPMENT OF THE DUAL EXPRESSION SYSTEM V2TCR/V2(3)LACI.

[00130] The σ^{70} adapted expression systems V2TcR and V3LacI have tight regulation and strong induction in *E. coli* in the presence of anhydrotetracycline (aTc) and IPTG respectively, while in *P. putida* and *V. natriegens* the V2TcR and V2LacI showed the best performance as inducible promoters in these host bacteria. A dual expression system was developed using as backbone the pJH0204 vector containing the origin of replication pColE1 and the attB sites specific for bxb1 recombinase. Each transcriptional unit of the dual expression system was insolated by terminators and the transcriptional regulators tetR and lacI were located in-between and in opposite direction

of the inducible promoters V2Tc and V2/3Lac to block undesired transcription of the open reading frames (ORF). Further, 2 different multicloning sites were located after the inducible promoters V2Tc and V2/3Lac to facilitate the incorporation of target genes (FIGS. 9A-9F, and FIG. 14).

[00131] The dual expression systems were tested in *E. coli*, *P. putida* and *V. natriegens* to evaluate i) synergy ii) expression of different recombinant proteins and iii) production of the biosynthetic gene cluster (BGC) leading to the biosynthesis of lycopene and B-carotene. Synergy was evaluated with the reporter system mCardinal which was accommodated in both inducible promoters of the dual expression system (FIG. 9B). Expression of different recombinant proteins was performed by adapting the sfGFP under the control of V2TcR and mCardinal controlled by V2/3LacI (FIG. 9C). Finally, production of the terpenoids lycopene and B-carotene by the dual expression system was achieved by the assembly of the crtEBIY genes from *Pantoea ananatis*. The crtEBIY genes were synthesized with the codon usage for *E. coli*. The crtEBI operon was assembled in the pUC19 vector via Gibson adapting the ribosome binding site (RBS) of J23119 9 to crtB and the RBS pJL1 to crtI, further the synthetic construct was transferred to the V2TcR expression system to evaluate lycopene production (FIG. 9D). B-carotene production was evaluated with the incorporation of the crtY gene with the J23119 RBS to V2TcR-crtEBI yielding V2TcR-crtEBIY (FIG. 9E). The dual control of the crtEBI operon together with the crtY gene to produce B-carotene was achieved by adapting the crtEBI to the V2TcR expression system and the crtY gene to V2(3)LacI expression system present in the dual vector (FIG. 9F). All measurements were performed using LB as growth medium as the scope of this work is not to optimize the production of terpenoids. After transforming the plasmids listed in **Table 3** in the three bacterial species no difference in the wild type strains was observed, thereby confirming the stability of the dual expression systems.

Table 3. List of Plasmids.

Name	Expression system 1	ORF1	Expression system 2	ORF2
$p\sigma^{70}$ V2TcR-mCardinal	V2TcR	mCardinal		
$p\sigma^{70}$ V2lacI-mCardinal	V2lacI	mCardinal		
$p\sigma^{70}$ V3LacI-mCardinal	V3LacI	mCardinal		
$p\sigma^{70}$ V2TcR-sfGFP	V2TcR	sfGFP		
$p\sigma^{70}$ V2lacI-sfGFP	V2LacI	sfGFP		
$p\sigma^{70}$ V3LacI-sfGFP	V3LacI	sfGFP		
pJH0204F				
$p\sigma^{70}$ V2TcR-mCardinal/V2LacI-	V2TcR	mCardinal	V2LacI	mCardinal

mCardinal				
$p\sigma^{70}$ V2TcR-mCardinal/V3LacI-mCardinal	V2TcR	mCardinal	V3LacI	mCardinal
$p\sigma^{70}$ V2TcR-sfGFP/V2LacI-mCardinal	V2TcR	mCardinal	V2LacI	sfGFP
$p\sigma^{70}$ V2TcR- sfGFP /V3LacI- mCardinal	V2TcR	mCardinal	V3LacI	sfGFP
pUC19-crtEBI	-	crtEBI		
$p\sigma^{70}$ V2TcR-crtEBI	V2TcR	crtEBI		
$p\sigma^{70}$ V2TcR-crtEBIY	V2TcR	crtEBIY		
$p\sigma^{70}$ V2TcR-crtEBI/V2LacI-crtY	V2TcR	crtEBI	V2LacI	crtY
$p\sigma^{70}$ V2TcR-crtEBI/V3LacI-crtY	V2TcR	crtEBI	V3LacI	crtY

Note: SEQ ID NOS: are listed in the SEQUENCES section of this paper.

EXAMPLE 13: SYNERGY OF THE DUAL EXPRESSION SYSTEM V2TCR/V2(3)LACI.

[00132] Increasing the copy number in *E. coli* efficiently boosted production of mCardinal by the pσ70V2TcR expression system but had a negative impact in the pσ70 V3LacI expression system due the toxicity of the transcriptional regulator LacI. Therefore, the synergistic effect of the duet expression system pσ70V2TcR /V3LacI both controlling mCardinal as different studies demonstrated that combined promoters enhance production of the target protein. The *E. coli* strain containing the duet expression system V2TcR/V3LacI produced 2-fold and 4-fold less red fluorescence than the strains containing the V2TcR and V3LacI when induced with aTc and IPGT respectively (FIG. 10A). Synergy was observed by the addition of IPTG and aTc to the V2TcR/V3LacI strain improving mCardinal production by 1.5-fold against the induction obtained with aTc alone (FIG. 10A). However, the synergy in mCardinal production in the duet system reached equal amounts of mCardinal as the V2TcR expression system (FIG. 10A). These results indicate that the tandem version of the synthetic promoters V2TcR/V3LacI offer an alternative to co-express different genes at high levels using different inducers.

[00133] In *P. putida* the V2TcR and V2lacI expression systems showed the best performance activating transcription of heterologous proteins. Consequently, we integrated both promoters controlling expression of mCardinal into the chromosome of this host. Single induction of the dual system by aTc resulted in induction of mCardinal equivalent to V2TcR system; single induction of the dual system with IPTG resulted in a 3-fold decrease relative to V2lacI alone (FIG. 10B). However, the addition of both inducers (aTc+IPTG) increased mCardinal by 1.1-fold over the

V2TcR and V2lacI expression system, thus showing a minimal synergistic effect of the dual promoters in *P. putida* (FIG. 10B).

[00134] The duet expression system V2TcR/V2LacI decreased substantially the efficiency of the synthetic promoters in *V. natriegens* compared to the single expression system counterparts by 12 and 2-fold for V2LacI and V2TcR after induction with IPTG and aTc, respectively (FIG. 10C). Co-induction with IPTG + aTc did not have a synergistic effect in mCardinal production, but reached similar yields as obtained by induction with aTc alone (FIG. 10C). Together, this information demonstrates that synergy cannot be achieved with the dual expression system V2TcR/V2(3)LacI in medium copy plasmids as is the case for *E. coli* and *V. natriegens*. On the contrary, the duet expression system diminishes the activity of each promoter probably due the constitutive expression of the transcriptional regulators TetR and LacI, and competition of the σ^{70} for both promoters. In *P. putida* the duet expression system was directly integrated into the chromosome and reduced the quantities of the transcriptional regulators TetR and LacI. Surprisingly, in this host the V2TcR expression system gained strength in the dual version, the V2lacI expression system lost efficiency, and synergy was observed, thus indicating that duet expression system gain performance as single copies in the chromosome of the host bacterium.

EXAMPLE 14: EXPRESSION OF DIFFERENT RECOMBINANT PROTEINS WITH THE DUAL EXPRESSION SYSTEM V2TCR/V2(3)LACI.

[00135] Prior to the expression systems provided herein there was not a universal dual expression system that allows differential expression of distinct set of genes via two independent inducible promoters that is portable among different Gram-negative species. The V2TcR/V2(3)LacI dual expression system demonstrated that it can control the co-expression of mCardinal, although synergy was only observed in *P. putida*. Two different reporter proteins were expressed to evaluate their production in *E. coli*, *P. putida* and *V. natriegens* using the V2TcR/V2(3)LacI expression system. The transcriptional unit V2TcR controlling the expression of sfGFP while the V2(3)LacI unit controlling mCardinal production.

[00136] The three bacterial species under study containing V2TcR-sfGFP/V2(3)LacI-mCardinal expression system showed no green fluorescence in the uninduced state, thus confirming the tight regulation of the V2TcR expression system, however, the V2(3)LacI-mCardinal is prone to leak and red fluorescence was observed in the absence of IPTG (FIGS. 11A-11C C). Addition of aTc induced the production of sfGFP, and similar result was observed with the addition of IPTG which triggered production of mCardinal. Interestingly, when the cultures were co-induced with aTc +

IPTG the green and red fluorescence reached similar levels compared with the cultures induced with only one inducer, thus indicating that both promoters (V2TcR/V2(3)LacI) are unaffected by the activation of each other reaching their maximum activity either in their sole activation or their independent combined activation (FIGS. 11A-11C).

EXAMPLE 15: PRODUCTION OF LYCOPENE AND B-CAROTENE IN *E. COLI*, *P. PUTIDA* AND *V. NATRIEGENS* WITH THE EXPRESSION SYSTEM V2TCR.

[00137] To decipher the capability of the dual-expression system V2TcR /V2(3)LacI to produce natural products we first had to test the ability of the V2TcR expression system to control the expression of multiple genes, we therefore decided to evaluate the production of lycopene and B-carotene in *E. coli*, *P. putida* and *V. natriegens*. Lycopene production has already been demonstrated in *E. coli* and *P. putida* via heterologous expression of the lycopene-producing operon (LYC) containing the crtEBI genes from Pantoea ananatis. Lycopene is the biosynthetic precursor of B-carotene, thus expression of the LYC operon together with crtY yields B-carotene production, which also has been demonstrated in *E. coli* and V. natriegens. The LYC and the crtEBIY operons were accommodated in the V2TcR expression system and lycopene and B-carotene production were measured by UHPLC after 4 hours of induction with aTc. In *E. coli* production of both, lycopene and B-carotene, was achieved but the induced culture produced only 1.7 and 1.2-fold more lycopene and B-carotene respectively than the uninduced culture, thus demonstrating that the tight control of the V2TcR can't totally repress the expression of the crtEBI and crtEBIY genes (FIGS. 12A-12B). Lycopene biosynthesis in *E. coli* using the pET expression system also demonstrated abundant leakage and production of 170 mg/L lycopene after 40 hours of induction, 1.1-fold more than the uninduced culture. Despite the V2TcR expression system produced 94 mg/L, this was achieved after 4 hours of induction, against the 40 hours reported with the pET expression system. This result confirms the advantage of the V2TcR expression system over the pET expression system as described in Examples 2-10. In *P. putida* and *V. natriegens* containing the V2TcR-crtEBI (SEQ ID NO: 70) and V2TcR-crtEBIY (SEQ ID NO: 71) transcriptional units no lycopene or B-carotene production was observed in the uninduced state, and activation of by aTc yielded production of the terpenoids (FIGS. 12A-12B). *P. putida* was reported to produce 1.22 ng/mL of lycopene after 24 hours of growth with the pSEVA421 vector, with the V2TcR expression system lycopene production reached 17 mg/L after 4 hours of cultivation, which is a substantial increment (13.000x), and B-carotene production by *P. putida* reached 840 mg/L, the highest among the three species under study (FIG. 12). *V. natriegens* was

reported to produce 2.93 mg/L of B-carotene, while the V2TcR expression system achieved 597 mg/L of B-carotene and 31 mg/L of lycopene. These results demonstrate that the V2TcR can be efficiently programmed for production of biosynthetic gene clusters in Gram-negative bacteria.

EXAMPLE 16: COORDINATED EXPRESSION OF THE LYCOPENE AND B-CAROTENE BIOSYNTHETIC GENE CLUSTERS (BGC) WITH THE DUAL EXPRESSION SYSTEM V2TCR/V2(3)LACI.

[00138] Natural products are encoded by BGC and ability to activate each gene at a different time point with a different inducer represent an advantage when the genes are toxic, or the metabolites produce by the BGC affect the cell growth of the heterologous host. As provided herein, the V2TcR expression system can activate the production of lycopene and B-carotene. In order to control the production of B-carotene with two different inducers, aTc and IPTG were used. The LYC operon was incorporated to the V2TcR expression system and the crtY gene was controlled by the V2(3)LacI expression system. The plasmid $p\sigma^{70}$ V2TcR-crtEBI/V3LacI-crtY (SEQ ID NO: 73) was transformed into *E. coli* and the plasmid $p\sigma^{70}$ V2TcR-crtEBI/V2lacI-crtY (SEQ ID NO: 72) was transformed into *P. putida* and *V. natriegens*. In the three bacteria species no lycopene production was observed despite the V2TcR expression system was controlling the crtEBI genes and activation by aTc should trigger production of lycopene (FIG. 13A). This result is explained in *E. coli* by the leakage of the crtEBIY genes under the control of the synthetic promoters V2TcR and V3LacI, demonstrating the high processivity of the B-carotene BGC to metabolize the endogenous IPP isopentenyl diphosphate directly into B-carotene. However, the V2TcR-crtEBI showed no leakage in *P. putida* and *V. natriegens*, but the V2lacI expression system was reported to leak, consequently, the lycopene produced after addition of aTc is directly transformed into B-carotene by the CrtY present in the cells due its minimal expression and high processivity.

[00139] B-carotene production in *E. coli* was observed no matter the absence or presence of the inducers aTc and IPTG (FIG. 13B), thus demonstrating that the V2TcR/V3LacI expression system failed to keep downregulated the B-carotene BGC in in this host. In *P. putida* and *V. natriegens* B-carotene production was only achieved when the cultures were induced with aTc or with aTc + IPTG (FIG. 13A). IPTG induction alone did not conduce to B-carotene production because the crtEBI genes were not transcribed by the V2TcR expression system. Addition of aTc resulted in B-carotene levels similar to induction with aTc + IPTG (FIG. 13A), thus indicating that the minimal leakage of the V2lacI-crtY is sufficient to metabolize the lycopene produced by the V2TcR-crtEBI into B-carotene.

[00140] Importantly, the yields of B-carotene reached by *E. coli* were always lower than the production achieved by *P. putida* and *V. natriegens*. Thus, *P. putida* can be used to produce complex biosynthetic gene clusters. Therefore, a dual expression system developed in this study expands the genetic tool kit of *P. putida* to produce complex proteins. Also, *V. natriegens* outperformed *E. coli*, and the dual expression system allowed higher production of B-carotene compared with the V2TcR by 1.2-fold. Together these results demonstrate the potential of the dual expression system presented here to fully capitalize the potential of heterologous host in industrial applications.

SEQUENCES

FIG. 2A Sequences:

Lac (SEQ ID NO: 3)

AGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC
TATG

lacUV5 (SEQ ID NO: 4)

AGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC
TATG

tacI (SEQ ID NO: 5)

GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGA
ATCATATG

V1lac (SEQ ID NO: 6)

AGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAACGCAGTAAGAGAGGAATG
TACATATG

V2lac (SEQ ID NO: 7)

GAGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGAGCGGATAACAACGCAGTAAGAGAGG
AATGTACATATG

V3lac (SEQ ID NO: 8)

AGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGAGCGGATAACAAGTGGAATTGTGAGCG
GATAACAATTTCACACAGGAAACAGAATCATATG

V4lac (SEQ ID NO: 9)

CTTTATGCTTCCGGCTCGTTGACAGTGTGGAATTGTGAGCGGATAACAATATAATGTGTGGAATTGTGAGCGGATAA
CAATTTCACACAGGAAACAGAATCATATG

FIG. 2B Sequences:

Tet (SEQ ID NO: 10)

GGATCCTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAATG

V1Tc (SEQ ID NO: 11)

GGATCCTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGACGCAGTAAGAGAGGAAT
GTACATATG

V2Tc (SEQ ID NO: 12)

GAGCTGTTGACAACTCTATCATTGATAGAGTTATAATGTTCCCTATCAGTGATAGAGACGCAGTAAGAGAGGAATGT
ACATATG

V3Tc (SEQ ID NO: 13)

GAGCTGTTGACAACTCTATCATTGATAGAGTTATAATGTTCCCTATCAGTGATAGAGAGTGGAATTGTGAGCGGATA
ACAATTTCACACAGGAAACAGAATCATATG

V4Tc (SEQ ID NO: 14)

TTGACACTCTATCATTGATAGAGTTTGACATCCCTATCAGTGATAGAGATATAATGTGTGGAATTGTGAGCGGATAA
CAATTTCACACAGGAAACAGAATCATATG

SEQ ID NOS: 15-38 are provided in Table 2.

SEQ ID NOS: 39-43 are provided in Example 2.

SEQ ID NOS: 44-53 are provided in the Detailed Description.

FIG. 14 Full-length Sequence:

V2Tc/tetR-V2lac/lacI (SEQ ID NO: 54)

GAGCTGTTGACAACTCTATCATTGATAGAGTTATAATGTTCCCTATCAGTGATAGAGACGCAGTAAGAGAGGAATGT
ACATATGAAGCTTCTCGGTACCAAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCCGAA
TTCTTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCGCAGTAAGAGAGGAATGTACACATGTCCCGCCTGGATA
AATCGAAAGTGATTAACTCGGCCCTCGAATTGCTGAATGAAGTCGGTATCGAGGGGCTGACGACCCGTAAATTGGCA
CAAAAGTTGGGGGTGGAGCAACCCACGTTGTATTGGCACGTCAAAAATAAGCGGGCATTGCTGGATGCCCTCGCTAT
TGAAATGTTGGATCGCCACCATACCCATTTCTGTCCACTGGAGGGCGAGTCCTGGCAGGACTTTCTCCGCAACAACG
CGAAATCCTTTCGCTGTGCACTCTTGTCCCATCGGGACGGTGCTAAGGTGCACTTGGGCACCCGTCCCACCGAAAAA
CAATACGAAACCTTGGAAAATCAATTGGCGTTTTTGTGCCAGCAAGGGTTTAGCTTGGAGAATGCTCTCTATGCGCT
CTCGGCTGTCGGGCACTTTACGTTGGGGTGCGTGTTGGAGGACCAGGAGCATCAAGTCGCAAAAGAGGAGCGTGAAA
CCCCAACCACGGACTCGATGCCACCTCTGCTCCGCCAAGCTATCGAACTCTTCGATCATCAGGGCGCGGAGCCAGCC
TTCCTCTTTGGGCTGGAGCTGATTATCTGCGGTTTGGAAAAACAACTCAAGTGTGAAAGCGGGTCCTAACTGCAGTC
ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCC
CTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCG
GGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCG
GTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAG
CATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGC
GAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATT
TGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGAT

GGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGT
CATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCG
ACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAAT
TTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTG
CCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTG
GCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTT
CACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGG
TGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAG
CACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGagtcaaaagcctc
cggtcggaggcttttgactTCTAGAGAGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGA
GCGGATAACAACGCAGTAAGAGAGGAATGTACCCATGGCCATGGCTCGAGgacgaacaataaggcctccctaacggg
gggccttttttattgataacaaaa

Additional sequences:

V2Tc/tetR-V3lac/lacI (SEQ ID NO: 55)

GAGCTGTTGACAACTCTATCATTGATAGAGTTATAATGTTCCCTATCAGTGATAGAGACGCAGTAAGAGAGGAATGT
ACATATGAAGCTTCTCGGTACCAAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCCGAA
TTCTTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCGCAGTAAGAGAGGAATGTACACATGTCCCGCCTGGATA
AATCGAAAGTGATTAACTCGGCCCTCGAATTGCTGAATGAAGTCGGTATCGAGGGGCTGACGACCCGTAAATTGGCA
CAAAAGTTGGGGGTGGAGCAACCCACGTTGTATTGGCACGTCAAAAATAAGCGGGCATTGCTGGATGCCCTCGCTAT
TGAAATGTTGGATCGCCACCATACCCATTTCTGTCCACTGGAGGGCGAGTCCTGGCAGGACTTTCTCCGCAACAACG
CGAAATCCTTTCGCTGTGCACTCTTGTCCCATCGGGACGGTGCTAAGGTGCACTTGGGCACCCGTCCCACCGAAAAA
CAATACGAAACCTTGGAAAATCAATTGGCGTTTTTGTGCCAGCAAGGGTTTAGCTTGGAGAATGCTCTCTATGCGCT
CTCGGCTGTCGGGCACTTTACGTTGGGGTGCGTGTTGGAGGACCAGGAGCATCAAGTCGCAAAAGAGGAGCGTGAAA
CCCCAACCACGGACTCGATGCCACCTCTGCTCCGCCAAGCTATCGAACTCTTCGATCATCAGGGCGCGGAGCCAGCC
TTCCTCTTTGGGCTGGAGCTGATTATCTGCGGTTTGGAAAAACAACTCAAGTGTGAAAGCGGGTCCTAACTGCAGTC
ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCC
CTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCG
GGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCG
GTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAG
CATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGC
GAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATT
TGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGAT
GGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGT
CATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCG
ACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAAT
TTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTG
CCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTG
GCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTT
CACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGG

TGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAG
CACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGagtcaaaagcctc
cggtcggaggcttttgactTCTAGAGAGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGA
GCGGATAACAAGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGAATCCCATGGCTCGAGgacgaacaat
aaggcctccctaacgggggggccttttttattgataacaaaa

Table 3. List of Plasmids

pσ^{70} V2TcR-mCardinal (SEQ ID NO: 56)

ggatccGAGCTGTTGACAACTCTATCATTGATAGAGTTATAATGTTCCCTATCAGTGATAGAGACGCAGTAAGAGAG
GAATGTACATATGGTGAGTAAGGGTGAGGAGCTCATTAAGGAGAACATGCACATGAAGCTGTATATGGAGGGCACCG
TAAACAACCACCACTTCAAGTGTACCACCGAGGGTGAAGGTAAACCCTACGAGGGGACGCAGACCCAACGCATCAAG
GTCGTGGAGGGCGGCCCGCTGCCTTTCGCATTCGACATTCTGGCGACCTGTTTTATGTACGGCTCGAAGACCTTCAT
CAACCACACCCAAGGCATCCCGGACTTCTTCAAGCAGAGCTTCCCTGAGGGCTTCACCTGGGAGCGCGTCACCACGT
ATGAAGACGGTGGGGTGCTCACCGTGACCCAGGACACGAGCTTGCAGGATGGCTGCTTGATTTACAACGTCAAGCTG
CGCGGGGTGAACTTCCCTAGCAACGGGCCAGTGATGCAGAAAAAGACGCTGGGTTGGGAGGCCACCACCGAGACCCT
GTACCCGGCCGACGGGGGGCTGGAAGGGCGGTGCGATATGGCCCTGAAATTGGTCGGCGGCGGTCATTTGCACTGCA
ATCTCAAGACCACGTACCGCTCCAAGAAACCCGCCAAAAACCTGAAGATGCCTGGTGTTTATTTTGTCGACCGGCGC
CTGGAGCGCATCAAGGAAGCGGACAATGAGACGTACGTGGAACAGCACGAAGTGGCCGTGGCTCGTTATTGCGATCT
GCCGTCGAAGCTGGGTCACAAACTGAACGGCATGGATGAGCTGTACAAAGATTATAAGGATGATGACGACAAGTAAA
AGCTTCTCGGTACCAAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCCGAATTCTTGAC
AGCTAGCTCAGTCCTAGGTATAATGCTAGCCGCAGTAAGAGAGGAATGTACACATGTCCCGCCTGGATAAATCGAAA
GTGATTAACTCGGCCCTCGAATTGCTGAATGAAGTCGGTATCGAGGGGCTGACGACCCGTAAATTGGCACAAAAGTT
GGGGGTGGAGCAACCCACGTTGTATTGGCACGTCAAAAATAAGCGGGCATTGCTGGATGCCCTCGCTATTGAAATGT
TGGATCGCCACCATACCCATTTCTGTCCACTGGAGGGCGAGTCCTGGCAGGACTTTCTCCGCAACAACGCGAAATCC
TTTCGCTGTGCACTCTTGTCCCATCGGGACGGTGCTAAGGTGCACTTGGGCACCCGTCCCACCGAAAAACAATACGA
AACCTTGGAAAATCAATTGGCGTTTTTGTGCCAGCAAGGGTTTAGCTTGGAGAATGCTCTCTATGCGCTCTCGGCTG
TCGGGCACTTTACGTTGGGGTGCGTGTTGGAGGACCAGGAGCATCAAGTCGCAAAAGAGGAGCGTGAAACCCCAACC
ACGGACTCGATGCCACCTCTGCTCCGCCAAGCTATCGAACTCTTCGATCATCAGGGCGCGGAGCCAGCCTTCCTCTT
TGGGCTGGAGCTGATTATCTGCGGTTTGGAAAAACAACTCAAGTGTGAAAGCGGGTCCTAACTGCAGTCTAGACCAT
GGctcgag

V2lacI-mCardinal (SEQ ID NO: 57)

GGATCCGAGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGAGCGGATAACAACGCAGTAA
GAGAGGAATGTACATATGGTGAGTAAGGGTGAGGAGCTCATTAAGGAGAACATGCACATGAAGCTGTATATGGAGGG
CACCGTAAACAACCACCACTTCAAGTGTACCACCGAGGGTGAAGGTAAACCCTACGAGGGGACGCAGACCCAACGCA
TCAAGGTCGTGGAGGGCGGCCCGCTGCCTTTCGCATTCGACATTCTGGCGACCTGTTTTATGTACGGCTCGAAGACC
TTCATCAACCACACCCAAGGCATCCCGGACTTCTTCAAGCAGAGCTTCCCTGAGGGCTTCACCTGGGAGCGCGTCAC
CACGTATGAAGACGGTGGGGTGCTCACCGTGACCCAGGACACGAGCTTGCAGGATGGCTGCTTGATTTACAACGTCA
AGCTGCGCGGGGTGAACTTCCCTAGCAACGGGCCAGTGATGCAGAAAAAGACGCTGGGTTGGGAGGCCACCACCGAG
ACCCTGTACCCGGCCGACGGGGGGCTGGAAGGGCGGTGCGATATGGCCCTGAAATTGGTCGGCGGCGGTCATTTGCA
CTGCAATCTCAAGACCACGTACCGCTCCAAGAAACCCGCCAAAAACCTGAAGATGCCTGGTGTTTATTTTGTCGACC

GGCGCCTGGAGCGCATCAAGGAAGCGGACAATGAGACGTACGTGGAACAGCACGAAGTGGCCGTGGCTCGTTATTGC
GATCTGCCGTCGAAGCTGGGTCACAAACTGAACGGCATGGATGAGCTGTACAAAGATTATAAGGATGATGACGACAA
GTAAAAGCTTCTCGGTACCAAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTTGGTCCGAATTC
CCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCA
ACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGAGATCCCGGACACCATCGAATGGCG
CAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAACGT
TATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCT
GCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGC
GGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGA
TTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAA
GCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCAT
TGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTA
TTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTA
GCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCA
GCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCA
TCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTG
CGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCAC
CATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGA
AGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCC
CGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGACTGCAGCTCGA
G

pσ^{70} V3LacI-mCardinal (SEQ ID NO: 60)

GGATCCGAGCTGTTGACACTTTATGCTTCCGGCTCGTATAATGTGTGTGGAATTGTGAGCGGATAACAAGTGGAATT
GTGAGCGGATAACAATTTCACACAGGAAACAGAATCATATGGTGAGTAAGGGTGAGGAGCTCATTAAGGAGAACATG
CACATGAAGCTGTATATGGAGGGCACCGTAAACAACCACCACTTCAAGTGTACCACCGAGGGTGAAGGTAAACCCTA
CGAGGGGACGCAGACCCAACGCATCAAGGTCGTGGAGGGCGGCCCGCTGCCTTTCGCATTCGACATTCTGGCGACCT
GTTTTATGTACGGCTCGAAGACCTTCATCAACCACACCCAAGGCATCCCGGACTTCTTCAAGCAGAGCTTCCCTGAG
GGCTTCACCTGGGAGCGCGTCACCACGTATGAAGACGGTGGGGTGCTCACCGTGACCCAGGACACGAGCTTGCAGGA
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pUC19-crtEBI (SEQ ID NO: 69)

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pσ^{70}V2TcR-crtEBI (SEQ ID NO: 70)

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TTTCTCCGCAACAACGCGAAATCCTTTCGCTGTGCACTCTTGTCCCATCGGGACGGTGCTAAGGTGCACTTGGGCAC
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AAAGAGGAGCGTGAAACCCCAACCACGGACTCGATGCCACCTCTGCTCCGCCAAGCTATCGAACTCTTCGATCATCA
GGGCGCGGAGCCAGCCTTCCTCTTTGGGCTGGAGCTGATTATCTGCGGTTTGGAAAAACAACTCAAGTGTGAAAGCG
GGTCCTAACTGCAGTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGC
CCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTG
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GCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAA
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GCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGC
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CCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTT
CGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGT
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CACCACGATGATTTGACTGAGAGCCAACATCGTTGGATAGCTCCGCTGGTGGTTCATCACTGGCCCGACTATCAGGT
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ATCAGCAAAATGGTTATCGCTTCGTGTACAGCCTGCCGCTCTCGCCGACCAGATTGTTAATTGAAGATACGCACTAT

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CGGCTACGTATTCTGAGCGGCAAGCCGCCTGTTCCGGTATTAGCAGCATTGCAAGCCATTATGACGACTCATCGTTG
ACTCGAG

cocE DNA sequence (SEQ ID NO: 74)

ATGGTGGACGGTAATTATTCGGTAGCGTCCAACGTTATGGTGCCGATGCGCGACGGGGTGCGCTTGGCTGTAGATCT
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GGAGTACGCAGAGCACGAACTGGCTGGAATTTGTGCGCGATGGGTACGCCGTCGTCATCCAAGACACCCGGGGCCTC
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CCATATCGTGCTGCCGATCATTAAGCGCGACTATAAGGACGACGACGATAAGTGA

cocE Amino acid sequence (SEQ ID NO: 75)

MVDGNYSVASNVMVPMRDGVRLAVDLYRPDADGPVPVLLVRNPYDKFDVFAWSTQSTNWLEFVRDGYAVVIQDTRGL
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EATTMHKAFFDRHLRGETDALAGVPKVRLFVMGIDEWRDETDWPLPDTAYTPFYLGGSGAANTSTGGGTLSTSISGT
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GVIAREQLEEMCTAVNRIHRGPEHPSHIVLPIIKRDYKDDDDK-

crtE (SEQ ID NO: 76)

ATGTATCCGTTTATAAGGACAGCCCGAATGACGGTCTGCGCAAAAAAACACGTTCATCTCACTCGCGATGCTGCGGA
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TAAATCGACGCTGGTCAATCTGTTAGGCCCGAGGGCGGTTGAAGAACGTCTGAGACAACATCTTCAGCTTGCCAGTG
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crtB (SEQ ID NO: 77)

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CTCTAG

crtI (SEQ ID NO: 78)

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CCTCAACTGGCGAAACTGCAGGCATGGAGAAGCGTTTACAGTAAGGTTGCCAGTTACATCGAAGATGAACATCTGCG
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GGAGGATCTGATTTGA

crtY (SEQ ID NO: 79)

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CATTTCACCACGATGATTTGACTGAGAGCCAACATCGTTGGATAGCTCCGCTGGTGGTTCATCACTGGCCCGACTAT
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TTTACAGCGACAGTTTGGCCCGCACTTGTGGATGGATACCGCGGTCGCAGAGGTTAATGCGGAATCTGTTCGGTTGA
AAAAGGGTCAGGTTATCGGTGCCCGCGCGGTGATTGACGGGCGGGGTTATGCGGCAAATTCAGCACTGAGCGTGGGC
TTCCAGGCGTTTATTGGCCAGGAATGGCGATTGAGCCACCCGCATGGTTTATCGTCTCCCATTATCATGGATGCCAC
GGTCGATCAGCAAAATGGTTATCGCTTCGTGTACAGCCTGCCGCTCTCGCCGACCAGATTGTTAATTGAAGATACGC
ACTATATTGATAATGCGACATTAGATCCTGAATGCGCGCGGCAAAATATTTGCGACTATGCCGCGCAACAGGGTTGG
CAGCTTCAGACACTGCTGCGAGAAGAACAGGGCGCCTTACCCATTACTCTGTCGGGCAATGCCGACGCATTCTGGCA
GCAGCGCCCCCTGGCCTGTAGTGGATTACGTGCCGGTCTGTTCCATCCTACCACCGGCTATTCACTGCCGCTGGCGG
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CGCGAGCGCTGGCAGCAGCAGGGCTTTTTCCGCATGCTGAATCGCATGCTGTTTTTAGCCGGACCCGCCGATTCACG
CTGGCGGGTTATGCAGCGTTTTTATGGTTTACCTGAAGATTTAATTGCCCGTTTTTATGCGGGAAAACTCACGCTGA
CCGATCGGCTACGTATTCTGAGCGGCAAGCCGCCTGTTCCGGTATTAGCAGCATTGCAAGCCATTATGACGACTCAT
CGTTGA

[00141] While exemplary embodiments have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will occur to those skilled in the art. It should be understood that various alternatives to the embodiments described herein may be employed. It is intended that

the following claims define the scope of the disclosure and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

What is claimed is:

1. A nucleic acid construct comprising a modified inducible promoter, wherein the modified inducible promoter comprises:

(a) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the promoter;

(b) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the promoter; and

(c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence.

2. The nucleic acid construct of claim 1, wherein the nucleic acid construct further comprises a transgene.

3. The nucleic acid construct of claim 1, wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a cell.

4. The nucleic acid construct of claim 3, wherein the expression of the transgene in the absence of an inducer is lower than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct.

5. The nucleic acid construct of claim 3, wherein the expression of the transgene in the presence of an inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct.

6. The nucleic acid construct of claim 2, wherein the transgene is selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, an ABA1 gene, an ABA2 gene, and a CocE gene.

7. The nucleic acid construct of claim 1, wherein the nucleic acid construct further comprises a sequence encoding a reporter gene.

8. The nucleic acid construct of claim 1, wherein the nucleic acid construct further comprises one or more regulatory element.

9. The nucleic acid construct of claim 1, wherein the nucleic acid construct further comprises a terminator sequence.

10. A nucleic acid construct comprising a sequence that is at least 85% identical to a sequence comprising any one of: SEQ ID NOS: 1-14, 39-73.

11. A nucleic acid construct comprising:

(a) a first modified inducible promoter sequence, wherein the first modified inducible promoter sequence comprises the modified inducible promoter of any one of claims 1 to 10; and

(b) a second modified inducible promoter sequence, wherein the second modified inducible promoter sequence comprises:

(i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the second modified inducible promoter sequence;

(ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the second modified inducible promoter sequence; and

(iii) a nucleic acid sequence that encodes a second bacterial ribosome binding sequence.

12. The nucleic acid construct of claim 11, wherein the nucleic acid construct further comprises a transgene.

13. The nucleic acid construct of claim 11, wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is in the presence of an inducer.

14. The nucleic acid construct of claim 11, further comprising a terminator sequence.

15. The nucleic acid construct of claim 11, further comprising one or more regulatory element.

16. The nucleic acid construct of claim 11, wherein the transgene comprises a carotenoid biosynthesis pathway gene.

17. The nucleic acid construct of claim 16, wherein the transgene is selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, an ABA1 gene, an ABA2 gene, and a CocE gene.

18. A composition comprising two or more of a nucleic acid construct of any one of claims 1 to 17.

19. An cell comprising the nucleic acid construct of any one of claims 1 to 17 or the composition of claim 18.

20. The cell of claim 19, wherein the cell is a prokaryotic cell.

21. The cell of claim 20, wherein the prokaryotic cell is a bacterial cell.

22. The cell of claim 21, wherein the bacterial cell is a Gram-negative bacterial cell.

23. The cell of claim 22, wherein the Gram-negative bacterial cell is a *P. putida* bacterium or a *V. natriegens* bacterium.

24. The cell of claim 21, wherein the bacterial cell is a Gram-positive bacterial cell.

25. The cell of claim 19, wherein the cell is a eukaryotic cell.

26. The cell of claim 19, wherein the cell is a mammalian cell.

27. A cell-free system comprising the nucleic acid construct of any one of claims 1 to 17 or the composition of claim 18; and an RNA polymerase.

28. The cell-free system of claim 27, further comprising a ribosome, an aminoacyl transfer RNA, a translation factor, and a buffer.

29. A method of expressing a protein encoded by a transgene in a cell, the method comprising:

(a) transforming a cell with a nucleic acid construct, wherein the nucleic acid construct comprises a modified inducible promoter that comprises:

(i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the modified inducible promoter;

(ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the modified inducible promoter;

(iii) a nucleic acid that encodes a bacterial ribosome binding sequence; and

(iv) a transgene; and

(b) contacting the cell with an inducer, thereby expressing the protein encoded by the transgene.

30. The method of claim 29, wherein the cell is a prokaryotic cell.

31. The method of claim 30, wherein the prokaryotic cell is a bacterium.

32. The method of claim 29, wherein the cell is a eukaryotic cell.

33. The method of claim 31, wherein the bacterium is a *P. putida* bacterium or a *V. natriegens* bacterium.

34. The method of any one of claims 29 to 33, wherein the cell does not comprise a T7 promoter or a T7 polymerase.

35. The method of claim 34, wherein when in the absence of the inducer, the expression of the protein is lower than the expression of the protein in a comparable cell comprising the transgene operably linked to a T7 promoter.

36. The method of any one of claims 31 to 35, wherein when in the presence of the inducer, the expression of the protein is greater than the expression of the protein in a comparable cell comprising the transgene operably linked to a T7 promoter.

37. A non-naturally occurring organism comprising:

a nucleic acid construct comprising:

(a) a first inducible promotor sequence comprising:

(i) a TATAATGT (SEQ ID NO: 44) sequence at position -10, relative to a transcriptional start site of the first inducible promoter sequence;

(ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the first inducible promoter sequence; and

(iii) a nucleic acid sequence that encodes a first bacterial ribosome binding sequence;

(b) one or more of a biosynthesis pathway transgene; and

(c) a second modified inducible promotor sequence comprising:

(i) a TATAATGT (SEQ ID NO: 44) consensus sequence at position -10, relative to a transcriptional start site of the second modified inducible promoter sequence;

(ii) a TTGACA (SEQ ID NO: 2) sequence at position -35, relative to a transcriptional start site of the second modified inducible promoter sequence; and

(iii) a nucleic acid sequence that encodes a second bacterial ribosome binding sequence.

38. The non-naturally occurring organism of claim 37, wherein the one or more biosynthesis pathway transgene is selected from the group consisting of: a crtW gene, a crtE gene, a crtY gene, a crtI gene, a crtZ gene, a crtEB gene, a crtEBI gene, a crtEBIY gene, a crtEBIYZ gene, a crtEBI-YZW gene, and a CocE gene.

39. The non-naturally occurring organism of claim 37 or claim 38, wherein the non-naturally occurring organism comprises a bacterium.

40. The non-naturally occurring organism of claim 39, wherein the bacterium is a Gram-negative bacterium.

41. The non-naturally occurring organism of claim 40, wherein the Gram-negative bacterium is a *P. putida* bacterium or a *V. natriegens* bacterium.

42. The non-naturally occurring organism of claim 39, wherein the bacterium is a Gram-positive bacterium.

43. The non-naturally occurring organism of claim 37 or claim 38, wherein the non-naturally occurring organism comprises a eukaryotic cell.

44. The non-naturally occurring organism of any one of claims 37 to 43, wherein the nucleic acid construct comprises a terminator sequence.

45. The non-naturally occurring organism of any one of claims 37 to 44, wherein the nucleic acid construct comprises one or more regulatory element.

46. The non-naturally occurring organism of any one of claims 37 to 45, wherein the nucleic acid construct comprises a reporter gene.

47. A composition comprising the non-naturally occurring organism of any one of claims 37 to 46; and an inducer.

48. The composition of claim 47, wherein the inducer comprises: anhydrotetracycline (aTc), isopropyl β- d-1-thiogalactopyranoside, cocaine, metallothionine, ecdysone, an antibiotic agent, galactose, a steroid, or a divalent cation.

49. A method for producing a protein, the method comprising: culturing the non-naturally occurring organism of any one of claims 37 to 46 for a period of time; and contacting the non-naturally occurring organism with an inducer, thereby producing the protein.

50. A method for producing a carotenoid, the method comprising: culturing the non-naturally occurring organism of any one of claims 37 to 46 for a period of time; and contacting the non-naturally occurring organism with an inducer, thereby producing the carotenoid.

51. A carotenoid produced by the method of claim 50, wherein the carotenoid is lycopene, beta-carotene, zeaxanthin, canthaxanthin, or astaxanthine.

52. A method for producing benzoic acid, the method comprising: culturing the cell of any one of claims 21 to 27 or the non-naturally occurring organism of any one of claims 37 to 46 for a period of time; and contacting the cell or the non-naturally occurring organism with an inducer, thereby producing benzoic acid.

53. A composition comprising benzoic acid, wherein the benzoic acid is produced by the method of claim 52.

54. A kit comprising the nucleic acid construct of any one of claims 1 to 19, packaging, buffers, and materials therefor.

55. A kit comprising the cell of any one of claims 21 to 27, packaging, culture medium, buffers, and materials therefor.

56. A kit comprising the non-naturally occurring organism of any one of claims 37 to 46, packaging, culture medium, buffers, and materials therefor.

57. A nucleic acid construct comprising a modified inducible promoter, wherein the modified inducible promoter comprises:

(a) a TATAAT consensus sequence at position -10, relative to a transcriptional start site of the promoter;

(b) a TTGACA sequence at position -35, relative to a transcriptional start site of the promoter;

(c) a bacterial ribosome binding sequence;

wherein the modified inducible promoter, when operatively linked to a transgene, facilitates expression of the transgene when the nucleic acid construct is inserted into a prokaryotic cell in the presence of an inducer; and wherein:

(i) the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a weak promoter in a comparable nucleic acid construct; and

(ii) the expression of the transgene in the presence of the inducer is at least equal to an amount of expression in the presence of the inducer of the transgene operatively coupled to the strong promoter in the comparable nucleic acid construct.

58. An isolated prokaryotic cell comprising the nucleic acid construct of claim 57.

59. A method of expressing a transgene in a prokaryotic cell that does not comprise a T7 RNA polymerase, the method comprising:

(a) transforming a nucleic acid construct into the prokaryotic cell, wherein the nucleic acid construct comprises a modified inducible promoter that comprises:

(i) a TATAAT consensus sequence at position -10, relative to a transcriptional start site of the promoter;

(ii) a TTGACA sequence at position -35, relative to a transcriptional start site of the promoter; and

(iii) a bacterial ribosome binding sequence; and

(b) contacting the prokaryotic cell with an inducer, thereby expressing the transgene;

wherein

(i) the expression of the transgene in the absence of the inducer is less than an amount of expression in the absence of the inducer of the transgene operatively coupled to a T7 promoter in a comparable nucleic acid construct; and

(ii) the expression of the transgene in the presence of the inducer is greater than an amount of expression in the presence of the inducer of the transgene operatively coupled to the T7 promoter in the comparable nucleic acid construct.

60. A nucleic acid construct comprising a modified inducible promoter, wherein the modified inducible promoter comprises:

(a) a TXTXXTGT (SEQ ID NO: 45) sequence at position -10, relative to a transcriptional start site of the promoter;

(b) a TXGXCX (SEQ ID NO: 46) sequence at position -35, relative to a transcriptional start site of the promoter; and

(c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence.

61. A nucleic acid construct comprising a modified inducible promoter, wherein the modified inducible promoter comprises:

(a) a TXTXXT (SEQ ID NO: 47) sequence at position -10, relative to a transcriptional start site of the promoter;

(b) a TXGXCX (SEQ ID NO: 46) sequence at position -35, relative to a transcriptional start site of the promoter; and

(c) a nucleic acid sequence that encodes a bacterial ribosome binding sequence.

62. Any nucleic acid, nucleic acid construct, composition, cell, non-naturally occurring organism, cell-free system, kit, or method provided herein.

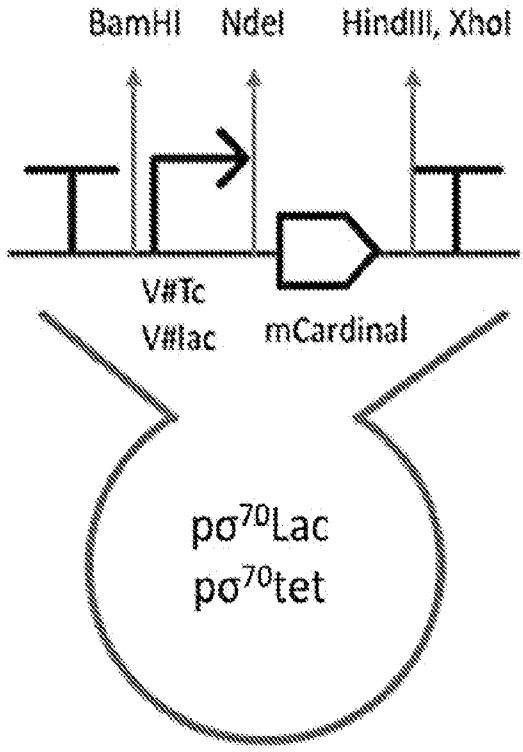

BamHI
NdeI
HindIII, XhoI
V#Tc
V#lac
mCardinal
pσ70Lac
pσ70tet

FIG. 1A

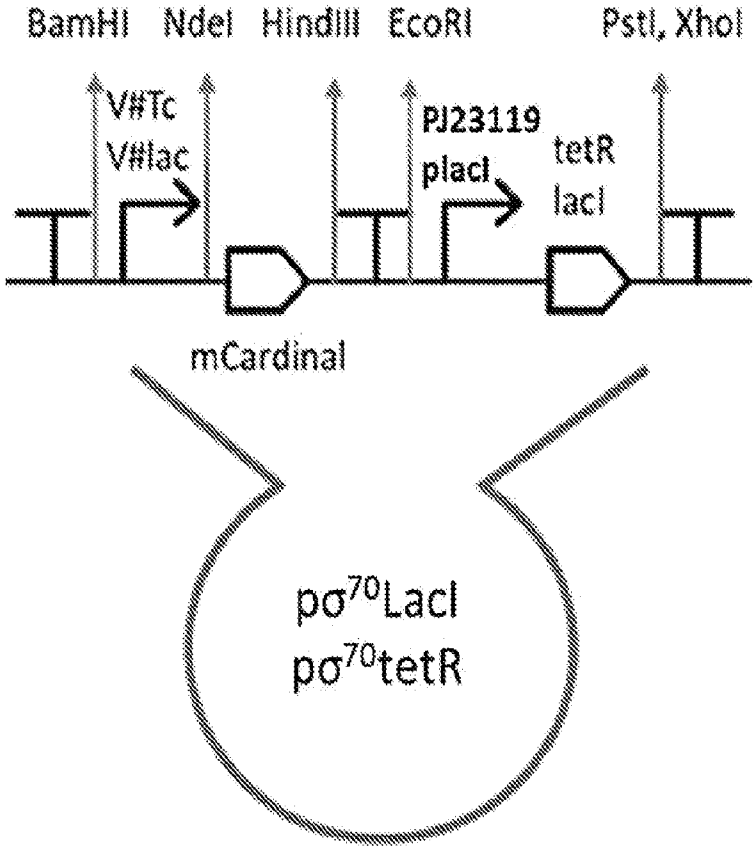

BamHI
NdeI
HindIII
EcoRI
PstI, XhoI
V#Tc
V#lac
PJ23119
placI
tetR
lacI
mCardinal
pσ70LacI
pσ70tetR

FIG. 1B

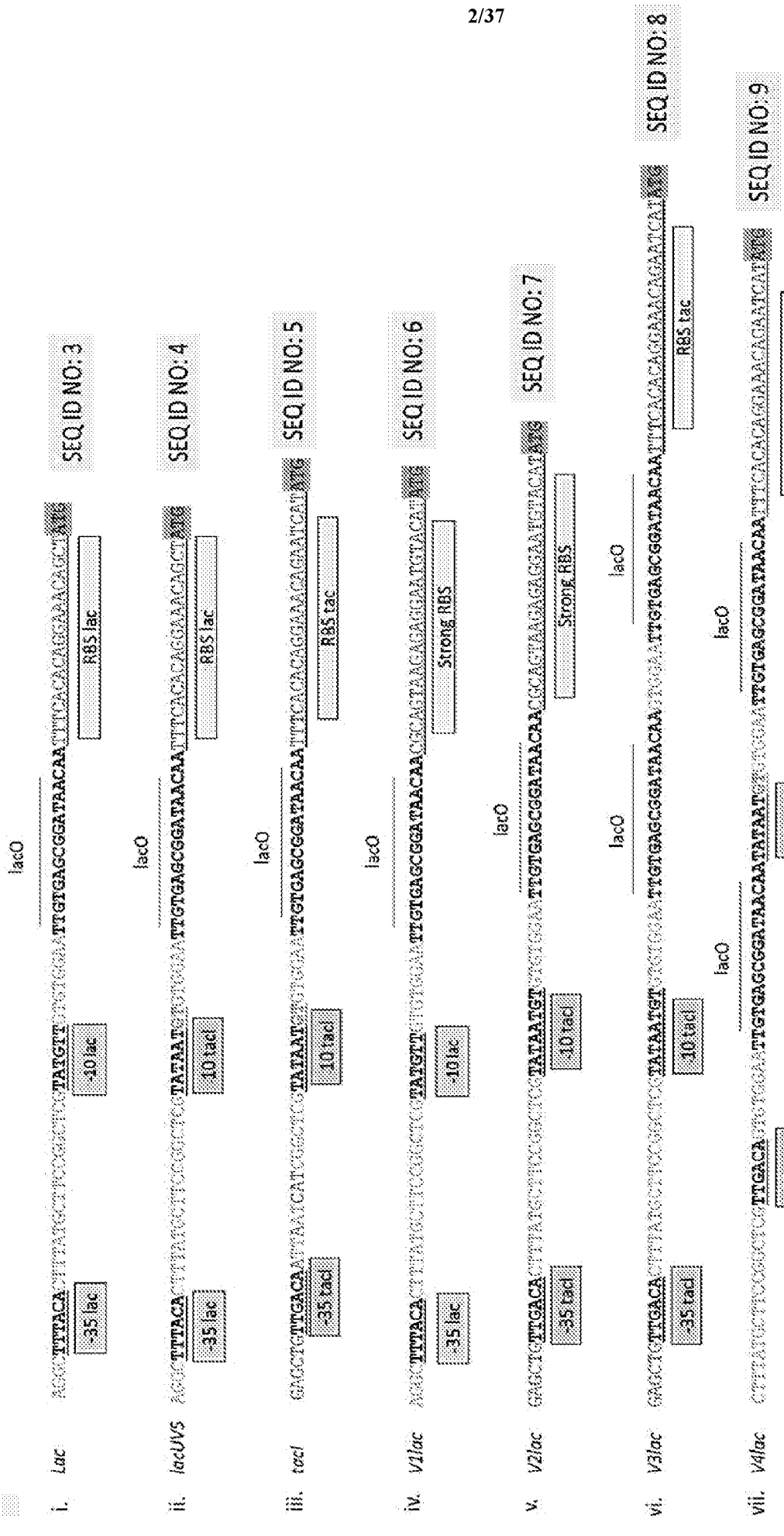

i. Lac
ii. lacUV5
iii. tacI
iv. V1lac
v. V2lac
vi. V3lac
vii. V4lac
lacO
-35 lac
-10 lac
RBS lac
-35 tacI
-10 tacI
RBS tac
Strong RBS
Strong RBS
RBS tac
SEQ ID NO: 3
SEQ ID NO: 4
SEQ ID NO: 5
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 8
SEQ ID NO: 9

FIG. 2A

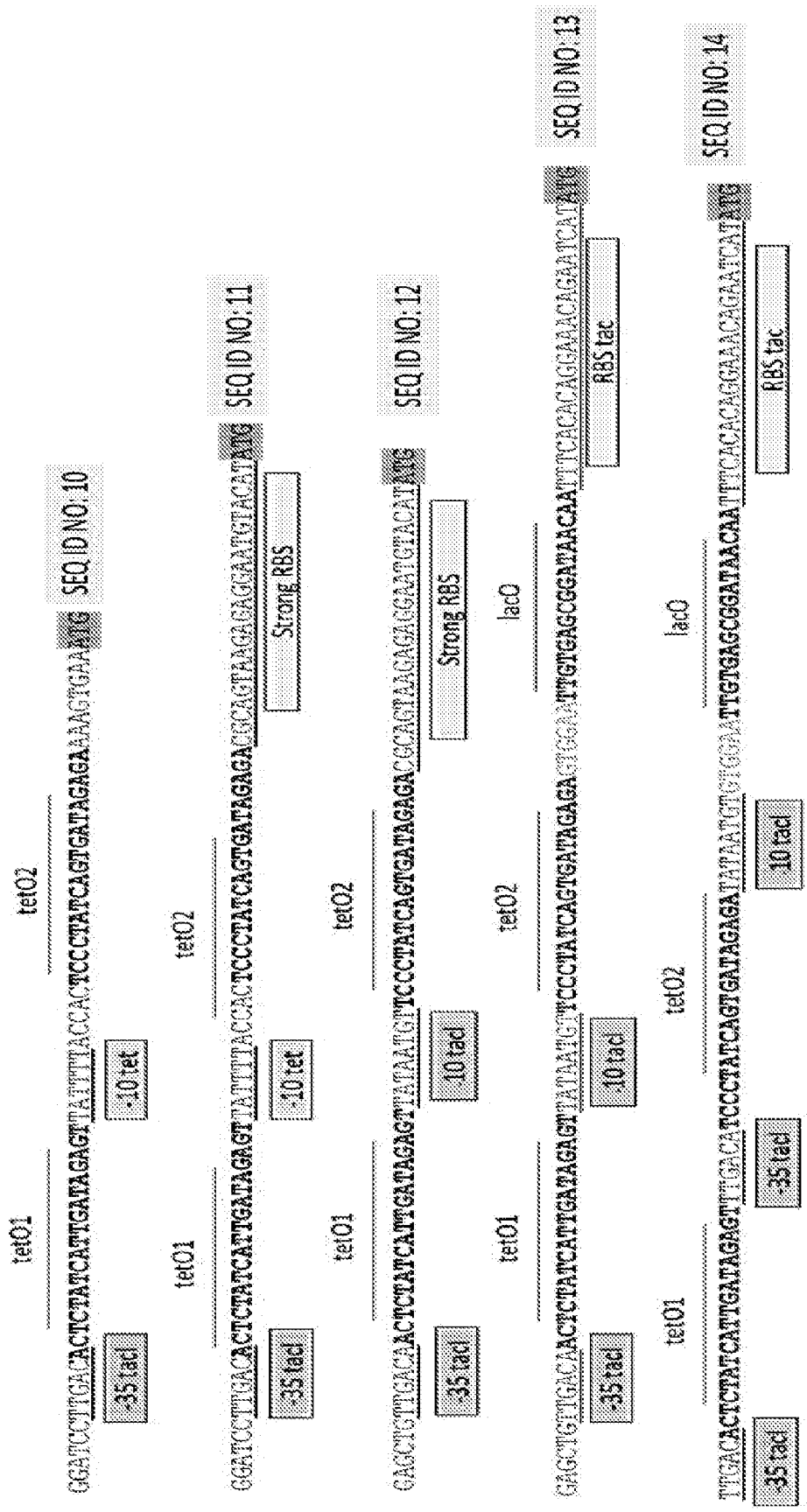

i. tet
ii. V1Tc
iii. V2Tc
iv. V3Tc
v. V4Tc
tetO1
tetO2
-35 tacI
-10 tet
-10 tacI
lacO
Strong RBS
RBS tac
SEQ ID NO: 10
SEQ ID NO: 11
SEQ ID NO: 12
SEQ ID NO: 13
SEQ ID NO: 14

FIG. 2B

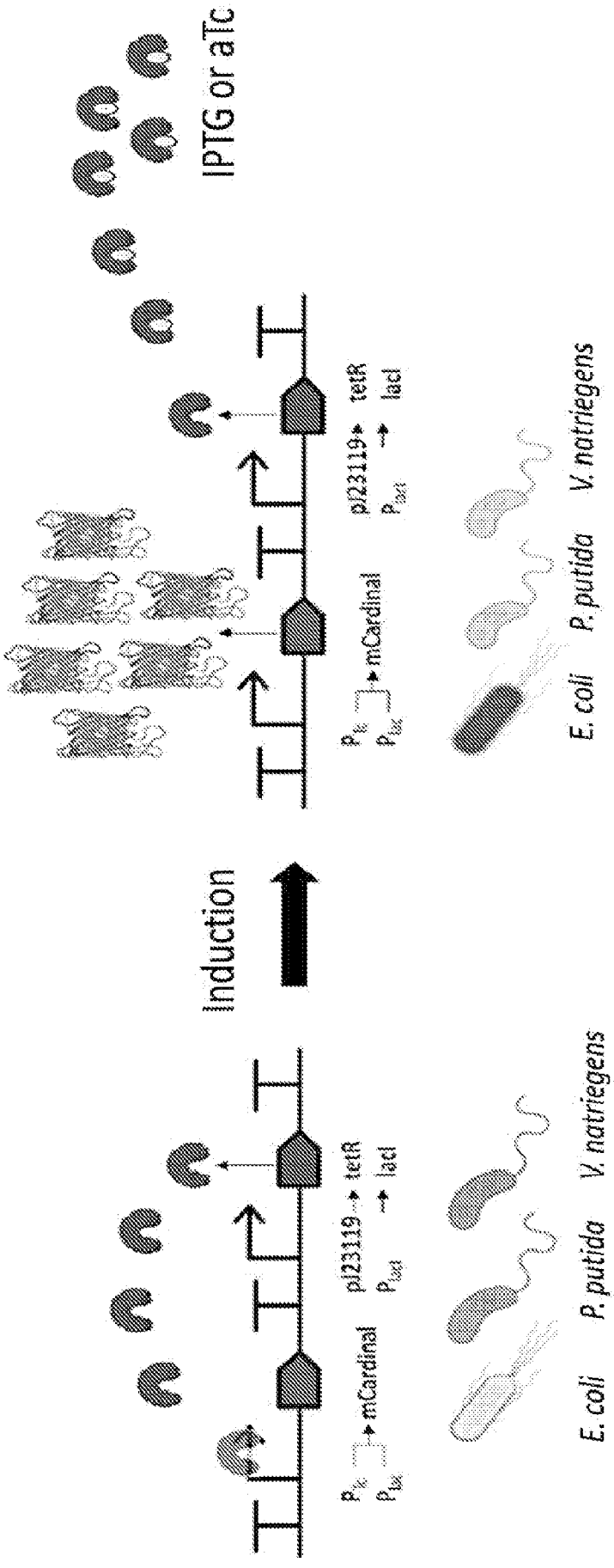

IPTG or aTc
Induction
mCardinal
pJ23119→ tetR
→ lacI
E. coli
P. putida
V. natriegens

FIG. 2C

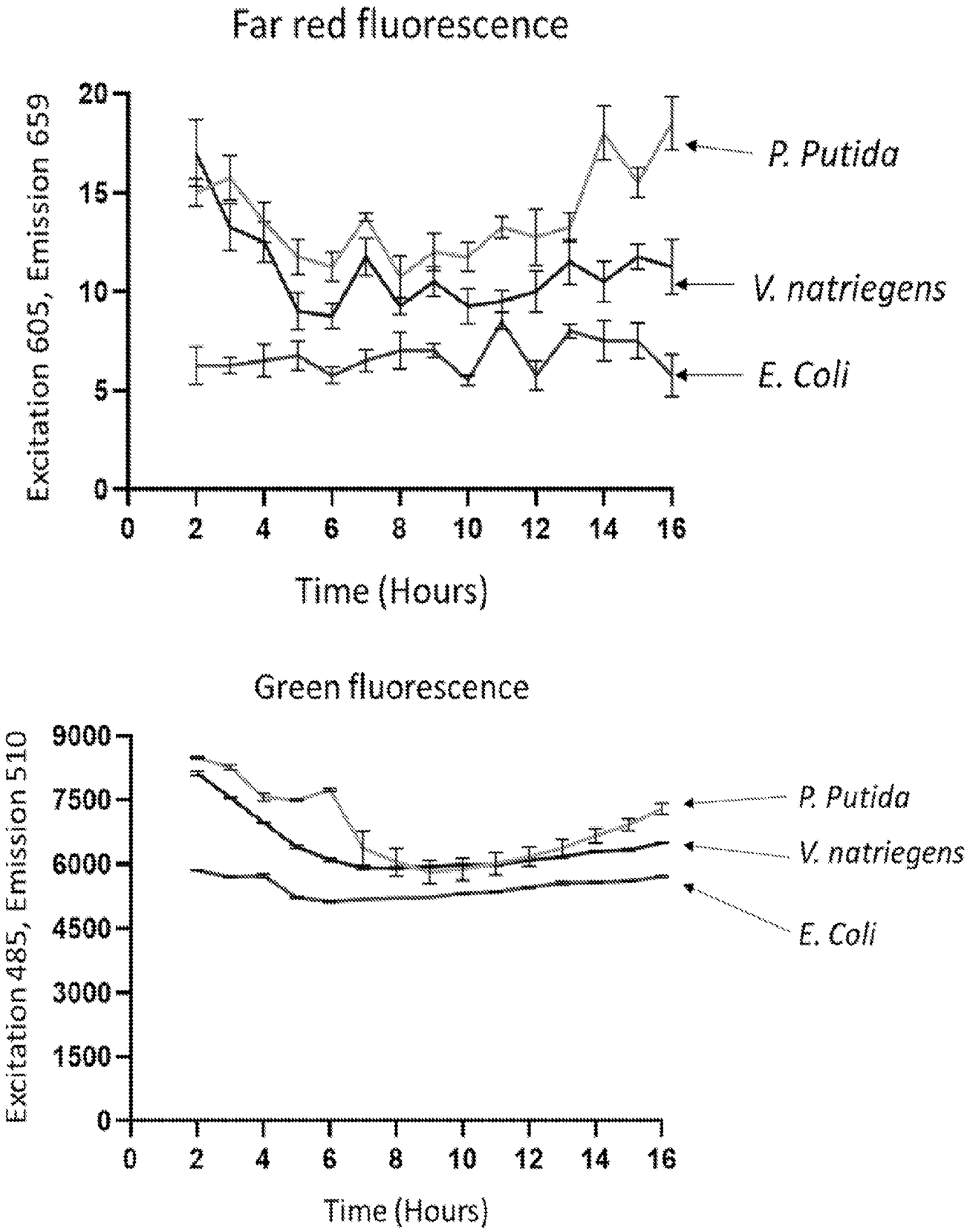

Far red fluorescence
Excitation 605, Emission 659
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Time (Hours)
P. Putida
V. natriegens
E. Coli
Green fluorescence
Excitation 485, Emission 510
9000
7500
6000
4500
3000
1500
0
0
2
4
6
8
10
12
14
16
Time (Hours)
P. Putida
V. natriegens
E. Coli

FIG. 3A

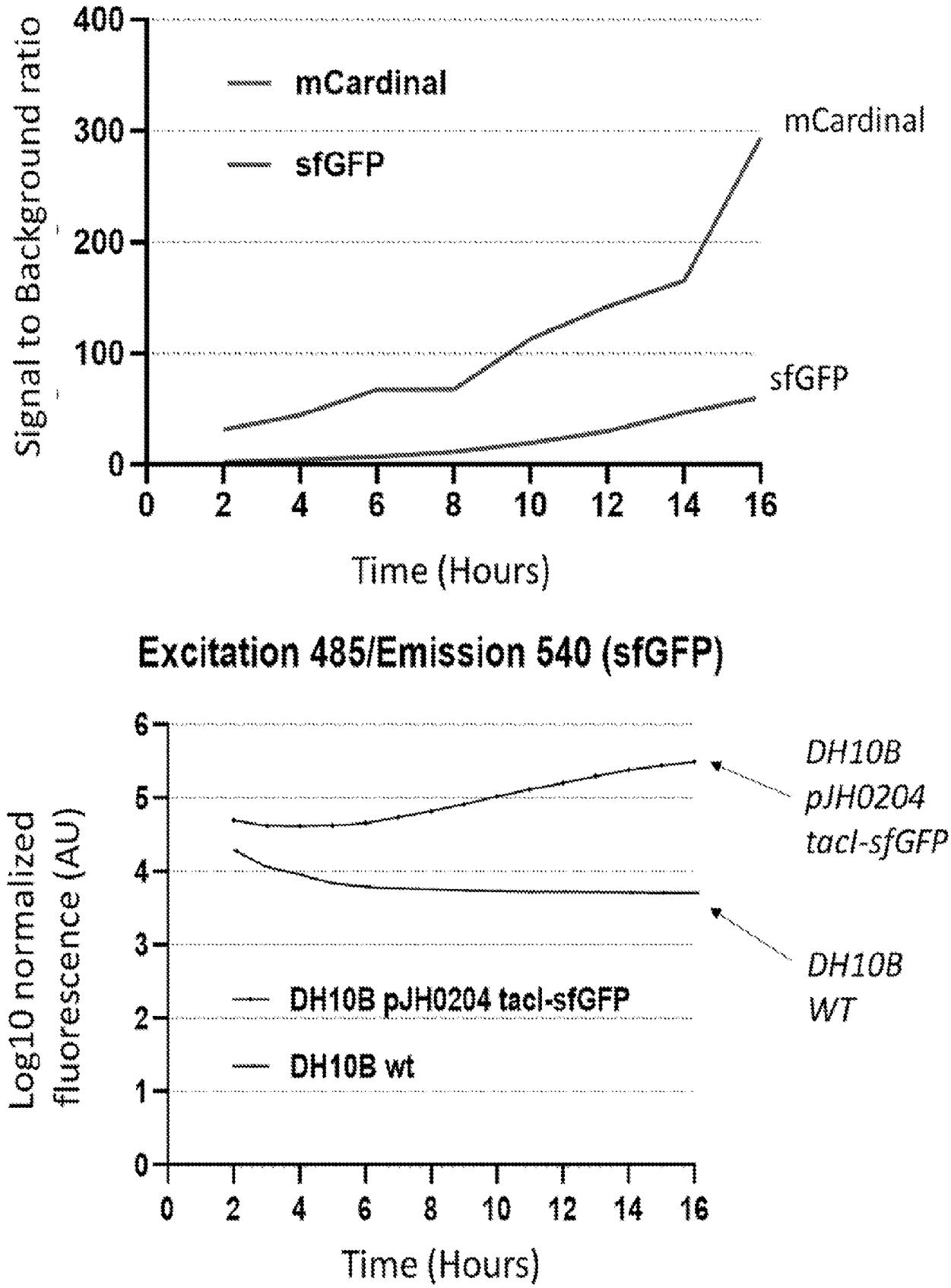

Signal to Background ratio
400
300
200
100
0
mCardinal
sfGFP
0 2 4 6 8 10 12 14 16
Time (Hours)
mCardinal
sfGFP
Excitation 485/Emission 540 (sfGFP)
Log10 normalized fluorescence (AU)
6
5
4
3
2
1
0
DH10B pJH0204 tacI-sfGFP
DH10B wt
0 2 4 6 8 10 12 14 16
Time (Hours)
DH10B pJH0204 tacI-sfGFP
DH10B WT

FIG. 3B

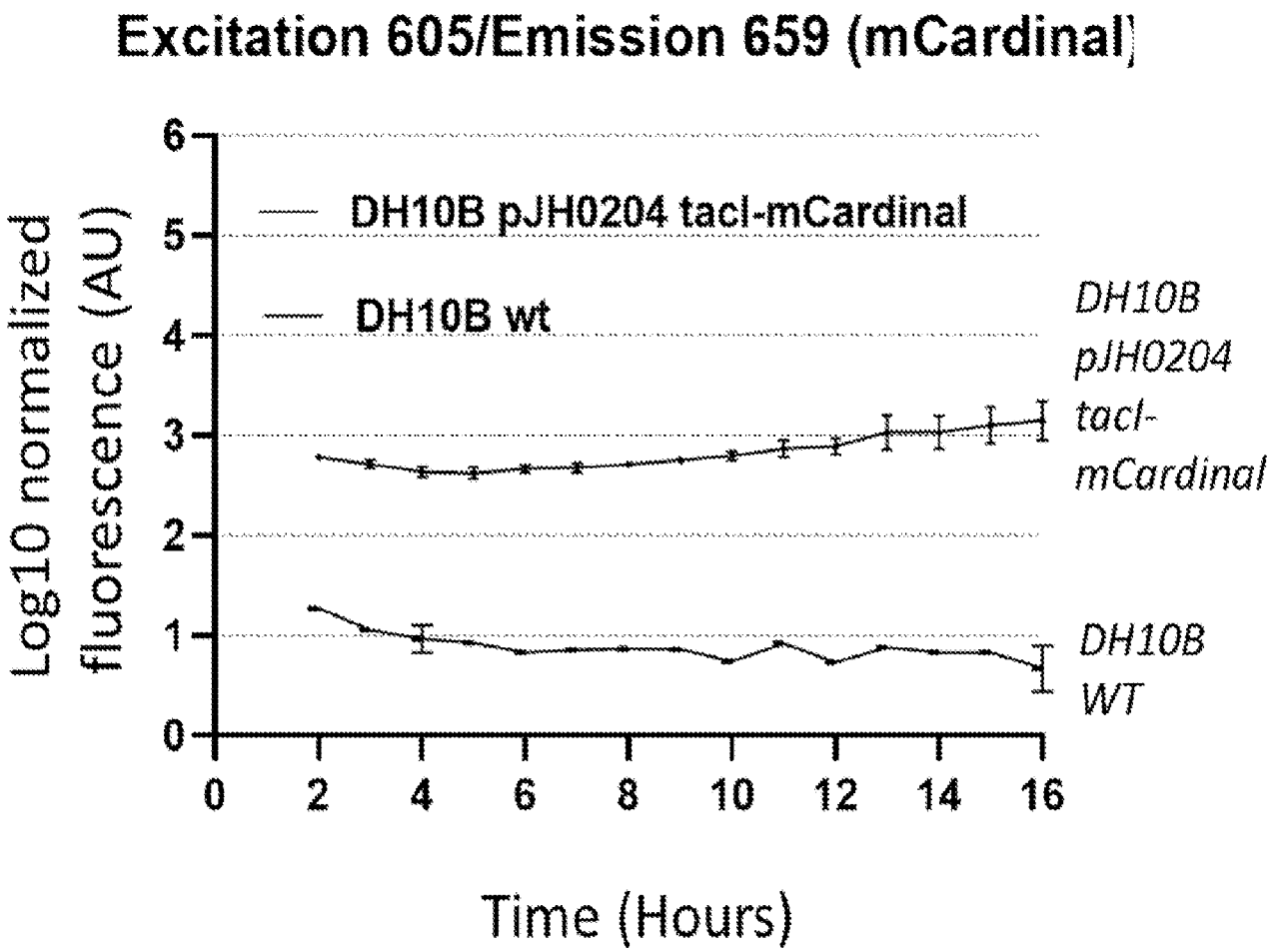


FIG. 3B (Continued)

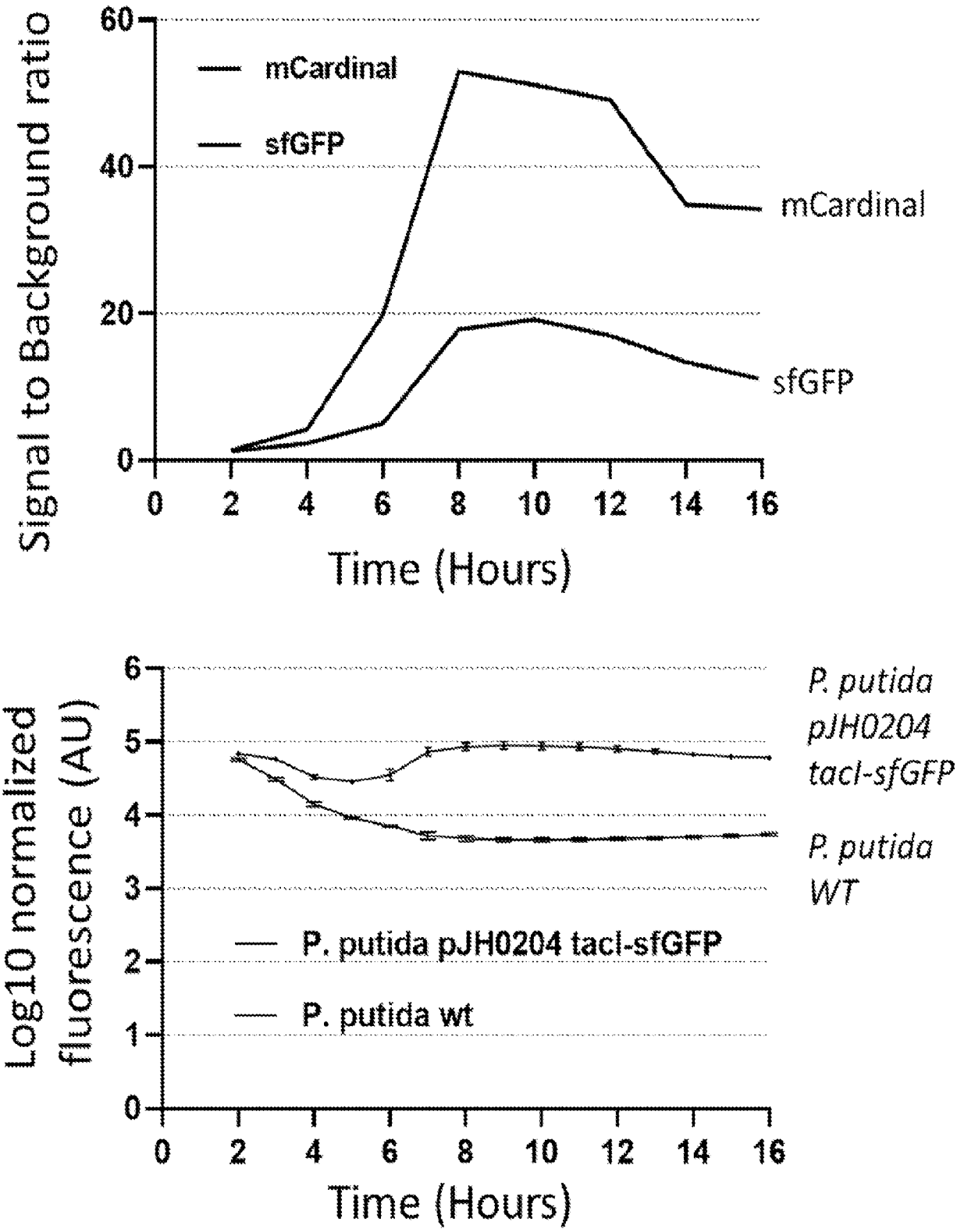

Signal to Background ratio
60
40
20
0
mCardinal
sfGFP
0 2 4 6 8 10 12 14 16
Time (Hours)
mCardinal
sfGFP
Log10 normalized fluorescence (AU)
6
5
4
3
2
1
0
P. putida pJH0204 tacI-sfGFP
P. putida wt
0 2 4 6 8 10 12 14 16
Time (Hours)
P. putida pJH0204 tacI-sfGFP
P. putida WT

FIG. 3C

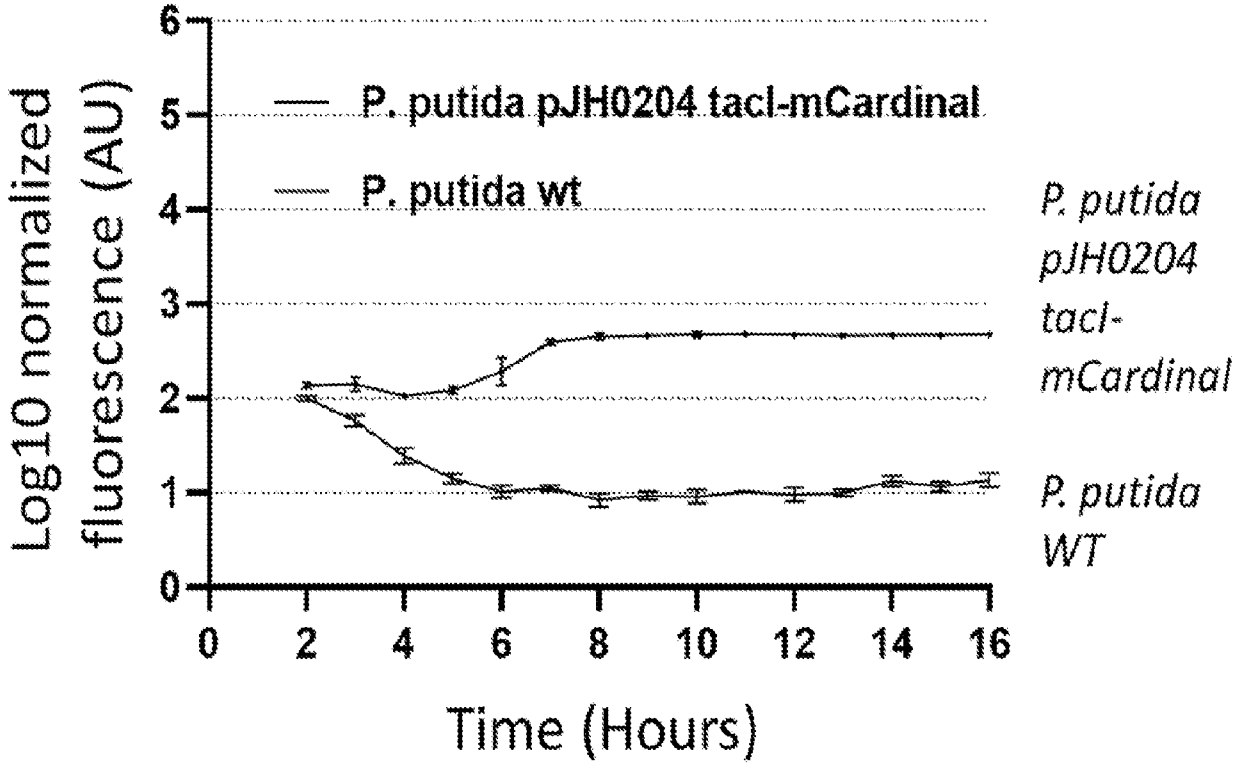


FIG. 3C (Continued)

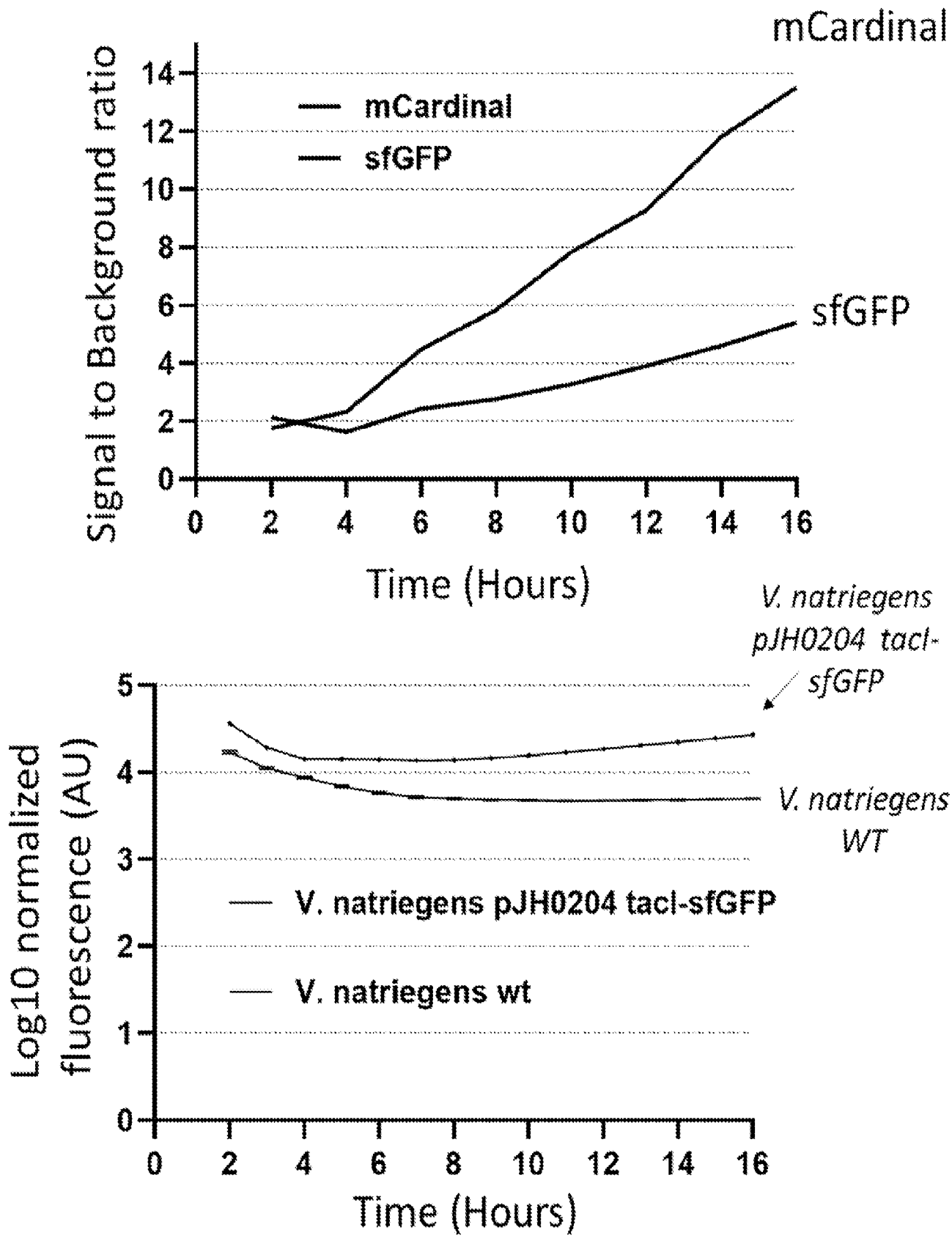

mCardinal
Signal to Background ratio
mCardinal
sfGFP
sfGFP
Time (Hours)
V. natriegens pJH0204 tacI-sfGFP
V. natriegens WT
Log10 normalized fluorescence (AU)
V. natriegens pJH0204 tacI-sfGFP
V. natriegens wt
Time (Hours)

FIG. 3D

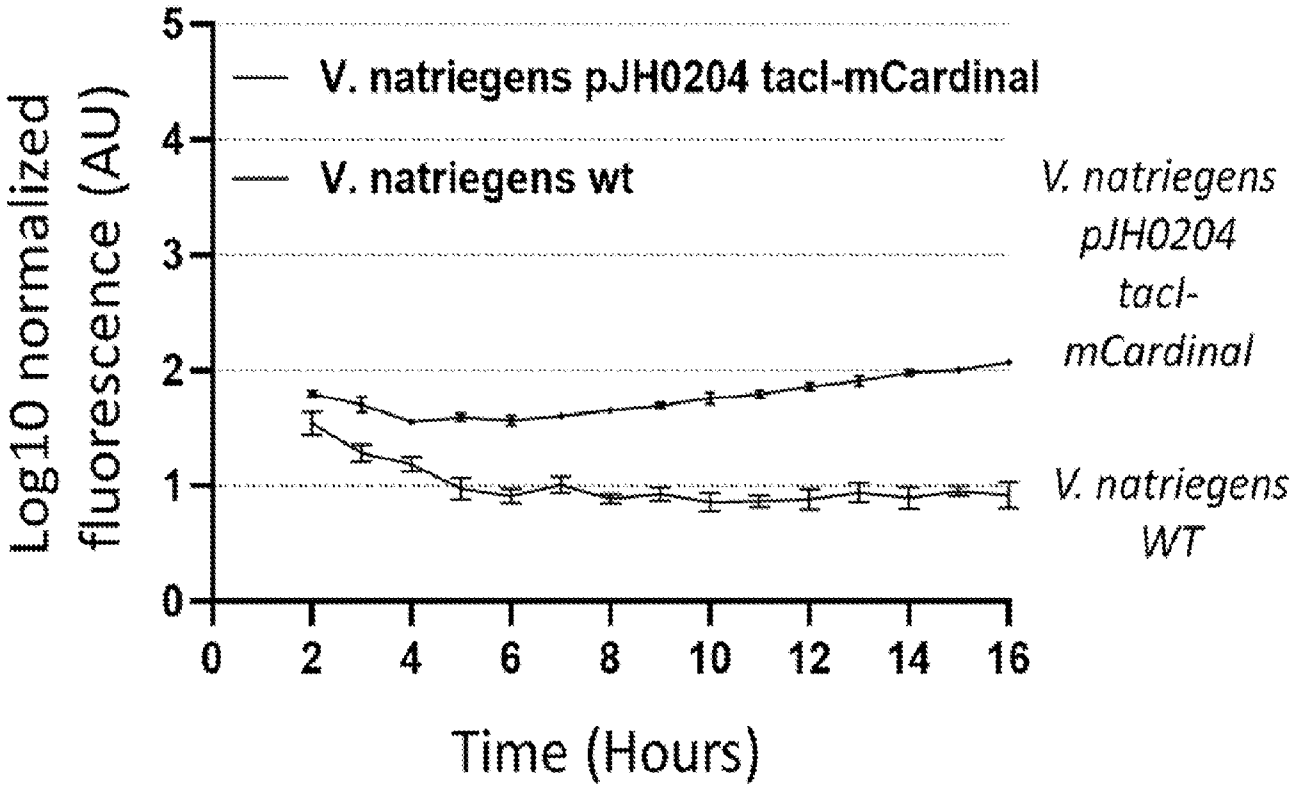


FIG. 3D (Continued)

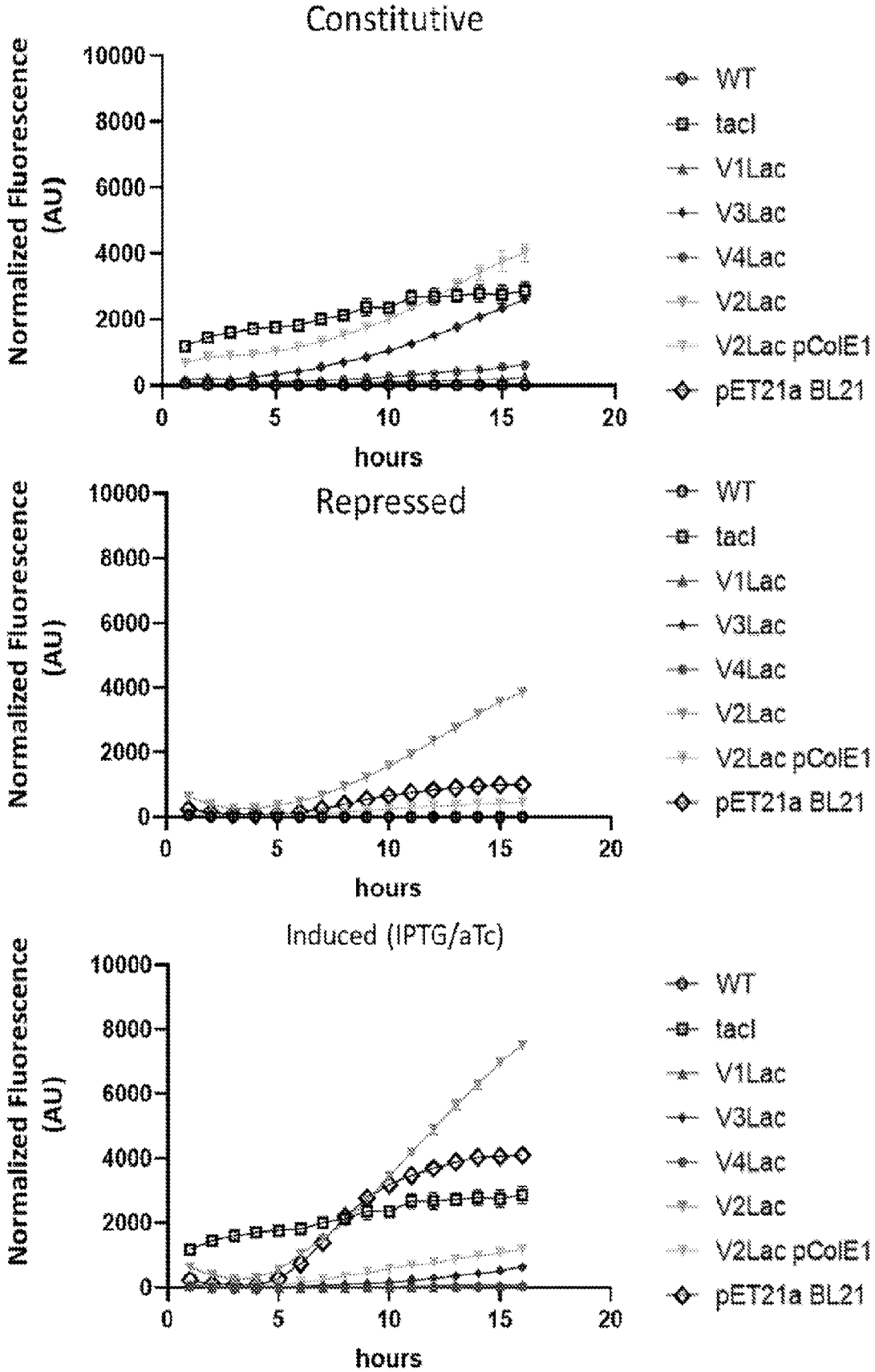

Constitutive
Normalized Fluorescence (AU)
10000
8000
6000
4000
2000
0
0
5
10
15
20
hours
WT
lacI
V1Lac
V3Lac
V4Lac
V2Lac
V2Lac pColE1
pET21a BL21
Repressed
Normalized Fluorescence (AU)
hours
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
hours

FIG. 4A

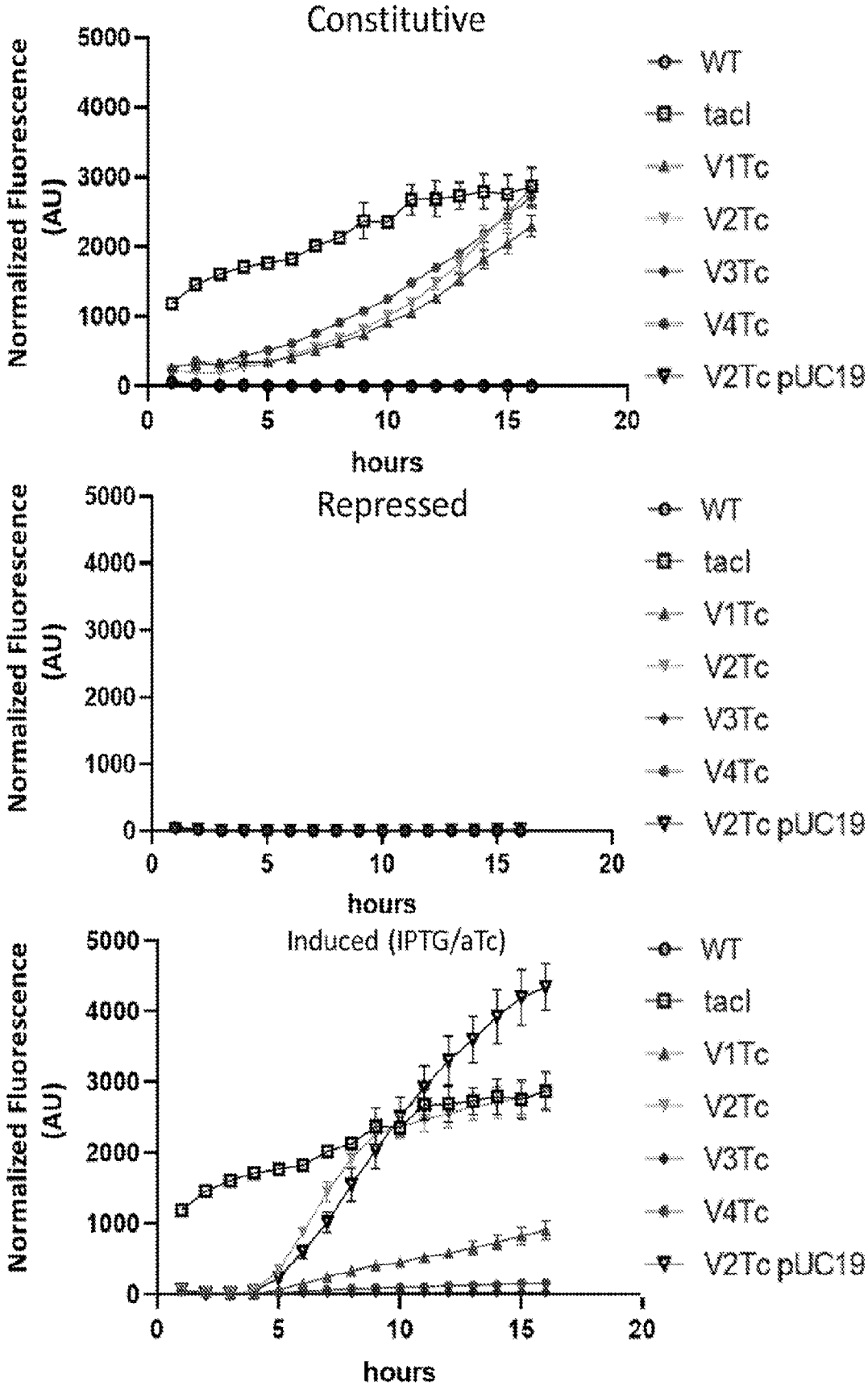
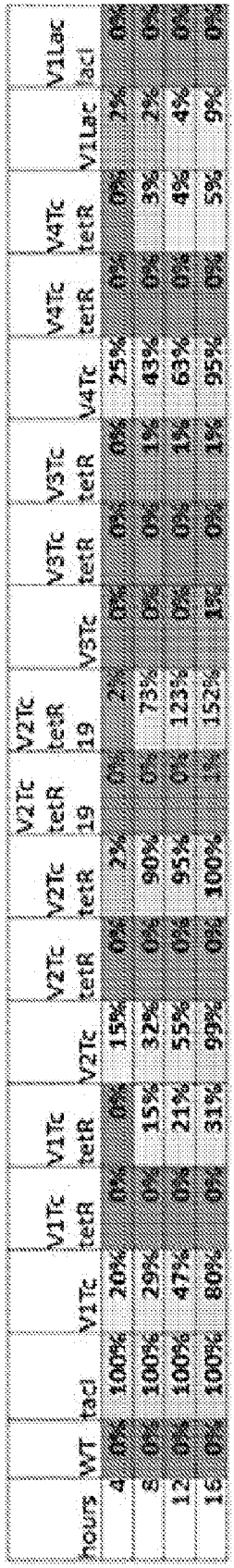

Constitutive
Normalized Fluorescence (AU)
5000
4000
3000
2000
1000
0
0
5
10
15
20
hours
WT
tacI
V1Tc
V2Tc
V3Tc
V4Tc
V2Tc pUC19
Repressed
Normalized Fluorescence (AU)
hours
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
hours

FIG. 4B



				aTc			aTc			aTc			aTc			
hours	WT	lacI	V1Tc	V1Tc tetR	V1tc tetR	V2Tc	V2Tc tetR	V2Tc tetR	V2Tc tetR 19	V2Tc tetR 19	V3Tc	V3Tc tetR	V3Tc tetR	V4Tc	V4Tc tetR	V4Tc tetR	V1Lac	V1Lac lacI
4	0%	100%	20%	0%	0%	15%	0%	3%	0%	2%	0%	0%	0%	25%	0%	0%	2%	0%
8	0%	100%	29%	0%	15%	32%	0%	90%	0%	73%	0%	0%	1%	43%	0%	3%	2%	0%
12	0%	100%	47%	0%	21%	55%	0%	95%	0%	123%	0%	0%	1%	63%	0%	4%	4%	0%
16	0%	100%	80%	0%	31%	99%	0%	100%	1%	152%	1%	0%	1%	95%	0%	5%	9%	0%

FIG. 4C

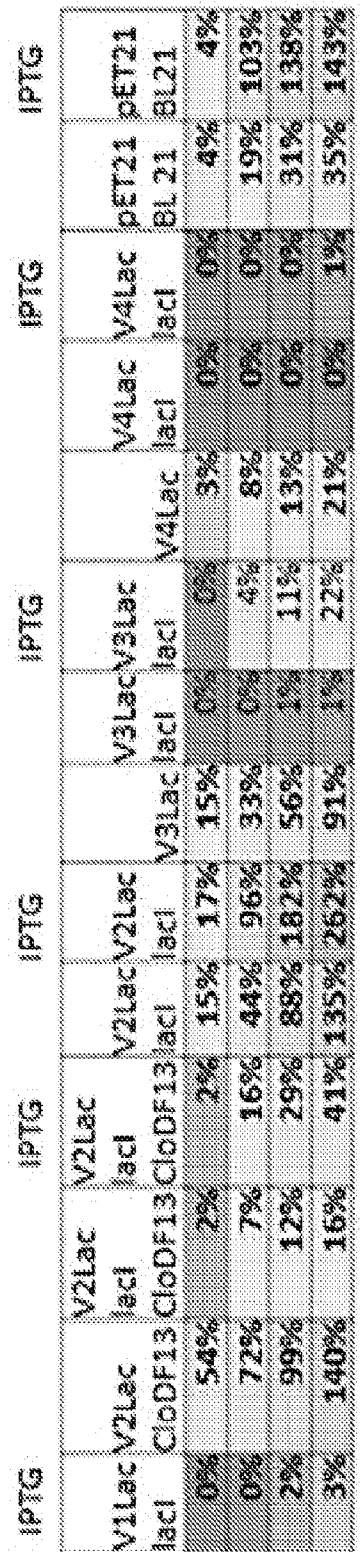

IPTG
V1Lac lacI
V2Lac ClоDF13
V2Lac lacI ClоDF13
IPTG
V2Lac lacI
V2Lac lacI
IPTG
V3Lac lacI
V3Lac lacI
IPTG
V4Lac lacI
V4Lac lacI
IPTG
pET21 BL 21
pET21 BL21
0% 54% 2% 2% 15% 17% 15% 0% 0% 3% 0% 0% 4% 4%
0% 72% 7% 16% 44% 96% 33% 0% 4% 8% 0% 0% 19% 103%
2% 99% 12% 29% 88% 182% 56% 1% 11% 13% 0% 0% 31% 138%
3% 140% 16% 41% 135% 262% 91% 1% 22% 21% 0% 1% 35% 143%

FIG. 4C (Con't)

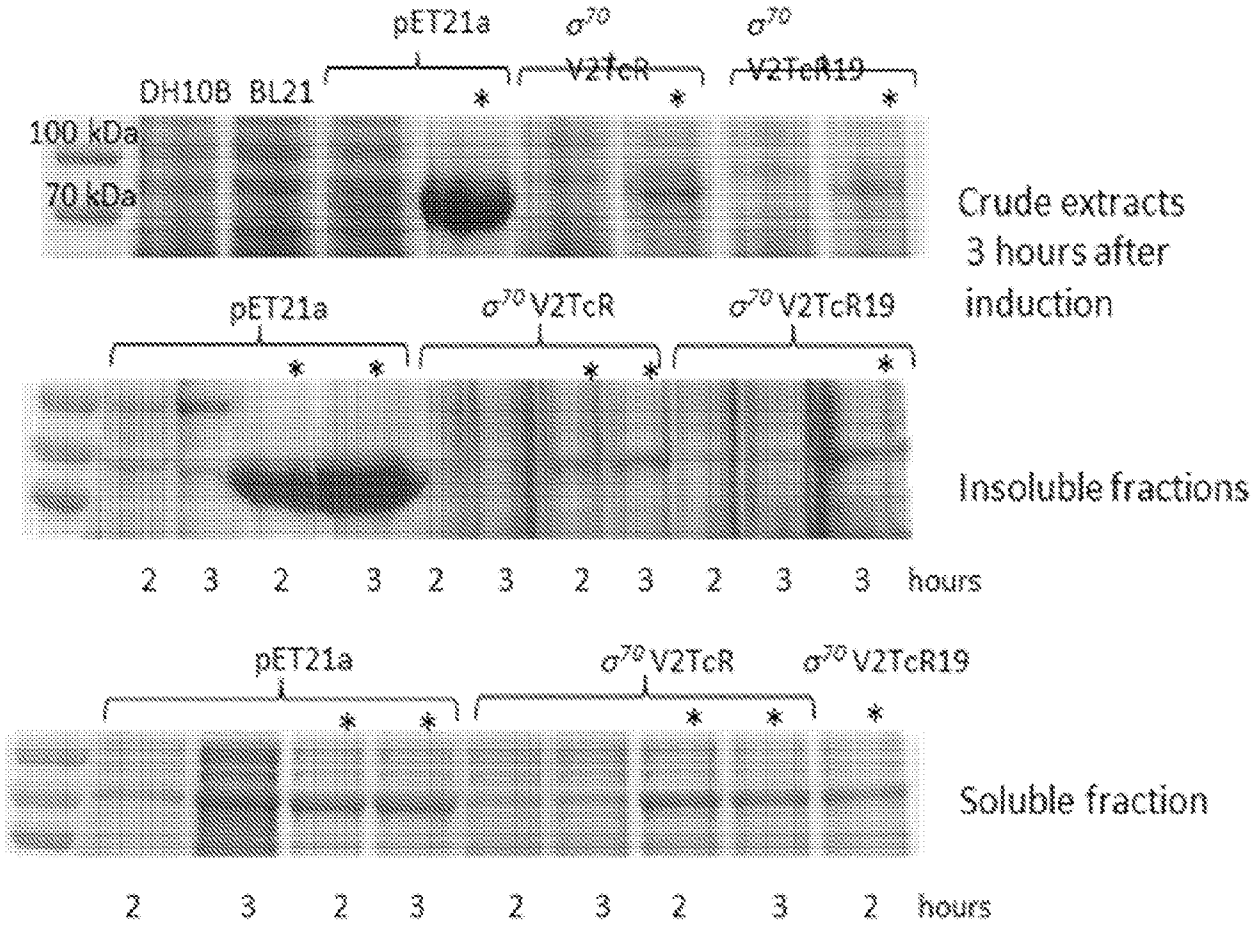

pET21a
σ70 V2TcR
σ70 V2TcR19
DH10B BL21
100 kDa
70 kDa
Crude extracts 3 hours after induction
pET21a
σ70V2TcR
σ70V2TcR19
Insoluble fractions
2 3 2 3 2 3 2 3 2 3 3 hours
pET21a
σ70V2TcR
σ70V2TcR19
Soluble fraction
2 3 2 3 2 3 2 3 2 hours

FIG. 5A

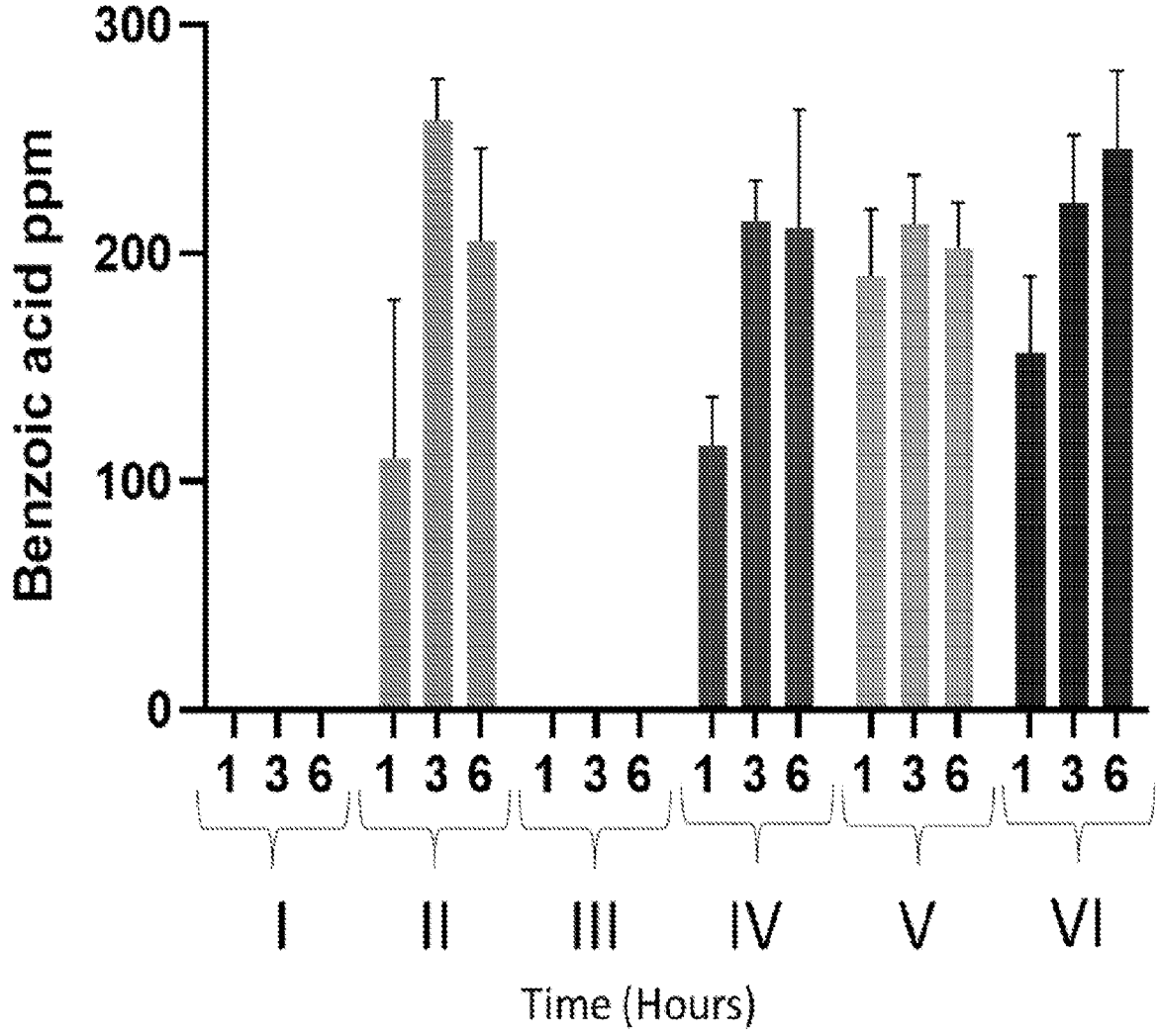

300
200
100
0
Benzoic acid ppm
1 3 6 1 3 6 1 3 6 1 3 6 1 3 6 1 3 6
I II III IV V VI
Time (Hours)

FIG. 5B

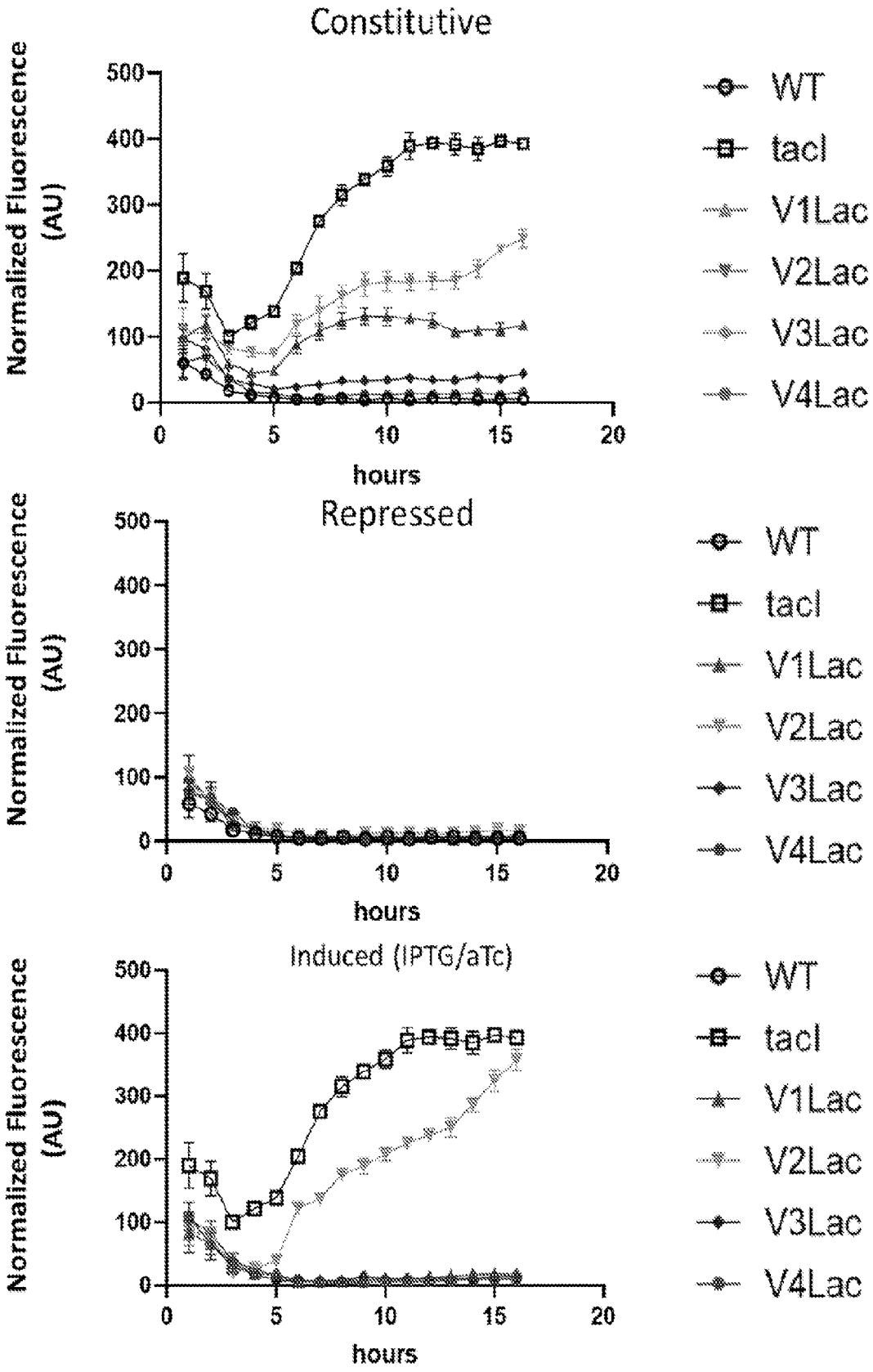

Constitutive
Normalized Fluorescence (AU)
500
400
300
200
100
0
0
5
10
15
20
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac
Repressed
Normalized Fluorescence (AU)
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac

FIG. 6A

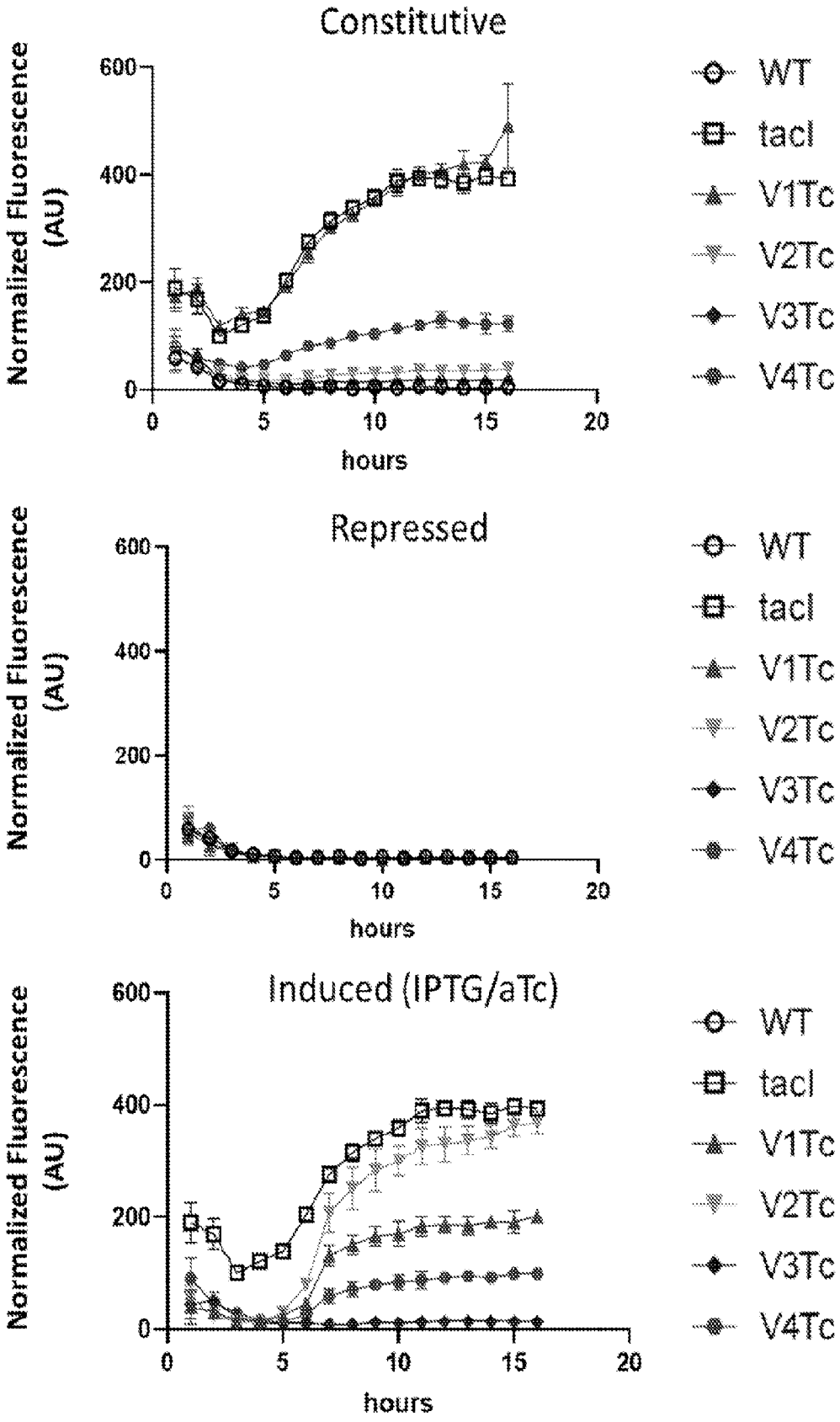
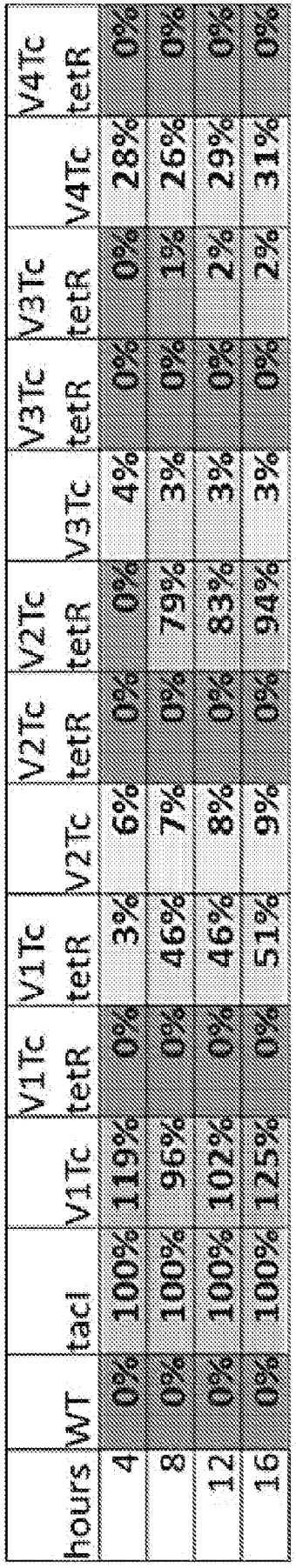
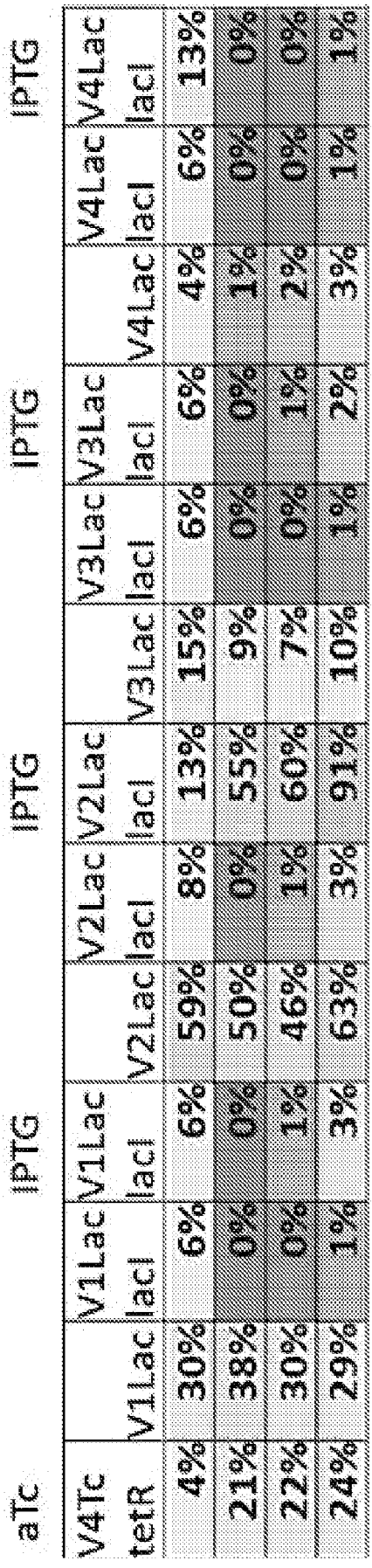

Constitutive
Normalized Fluorescence (AU)
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Tc
V2Tc
V3Tc
V4Tc
Repressed
Normalized Fluorescence (AU)
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Tc
V2Tc
V3Tc
V4Tc
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Tc
V2Tc
V3Tc
V4Tc

FIG. 6B



	aTc																	
hours	WT	tacI	V1Tc	V1Tc tetR	V1Tc	V2Tc tetR	V2Tc	V2Tc tetR	V2Tc	V3Tc tetR	V3Tc	V3Tc tetR	V3Tc	V4Tc tetR	V4Tc	V4Tc tetR	V4Tc	
4	0%	100%	119%	0%	3%	0%	6%	0%	0%	0%	4%	0%	0%	0%	28%	0%		
8	0%	100%	96%	0%	46%	0%	7%	79%	0%	0%	3%	0%	1%	0%	26%	0%		
12	0%	100%	102%	0%	46%	0%	8%	83%	0%	0%	3%	0%	2%	0%	29%	0%		
16	0%	100%	125%	0%	51%	0%	9%	94%	0%	0%	3%	0%	2%	0%	31%	0%		

FIG. 6C



aTc		IPTG				IPTG				IPTG				IPTG			
V4Tc tetR	V1Lac	V1Lac lacI	V1Lac	V2Lac	V2Lac lacI	V2Lac	V2Lac lacI	V3Lac	V3Lac lacI	V3Lac	V3Lac lacI	V4Lac	V4Lac lacI	V4Lac	V4Lac lacI
4%	30%	6%	6%	59%	8%	13%	13%	15%	6%	6%	6%	4%	6%	13%	1%
21%	38%	0%	0%	50%	0%	55%	0%	9%	0%	0%	0%	1%	0%	0%	0%
22%	30%	0%	1%	46%	1%	60%	1%	7%	0%	1%	0%	2%	0%	0%	0%
24%	29%	1%	3%	63%	3%	91%	3%	10%	1%	2%	1%	3%	1%	1%	0%

FIG. 6C (Con't)

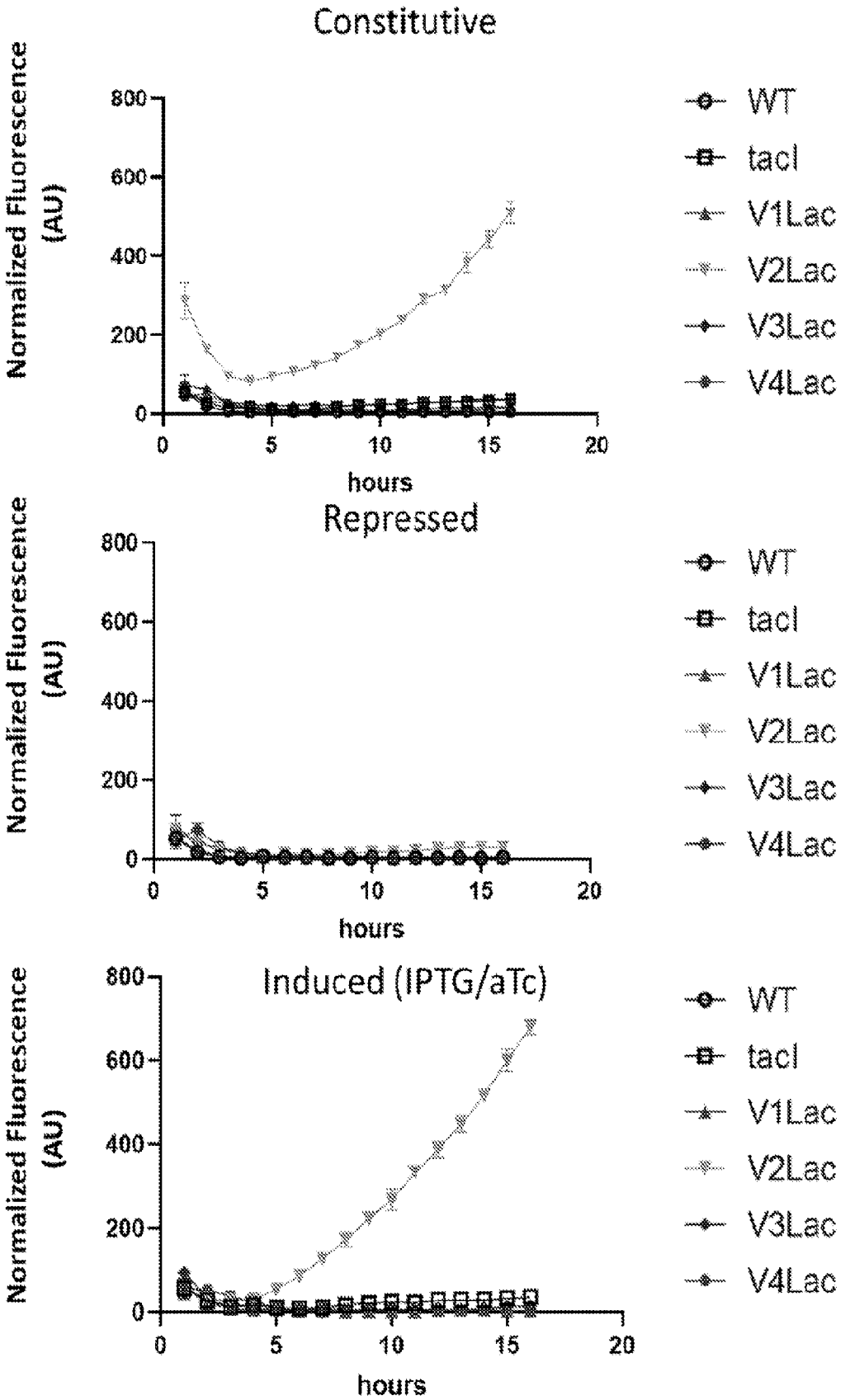

Constitutive
Normalized Fluorescence (AU)
800
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac
Repressed
Normalized Fluorescence (AU)
800
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
800
600
400
200
0
0
5
10
15
20
hours
WT
tacI
V1Lac
V2Lac
V3Lac
V4Lac

FIG. 7A

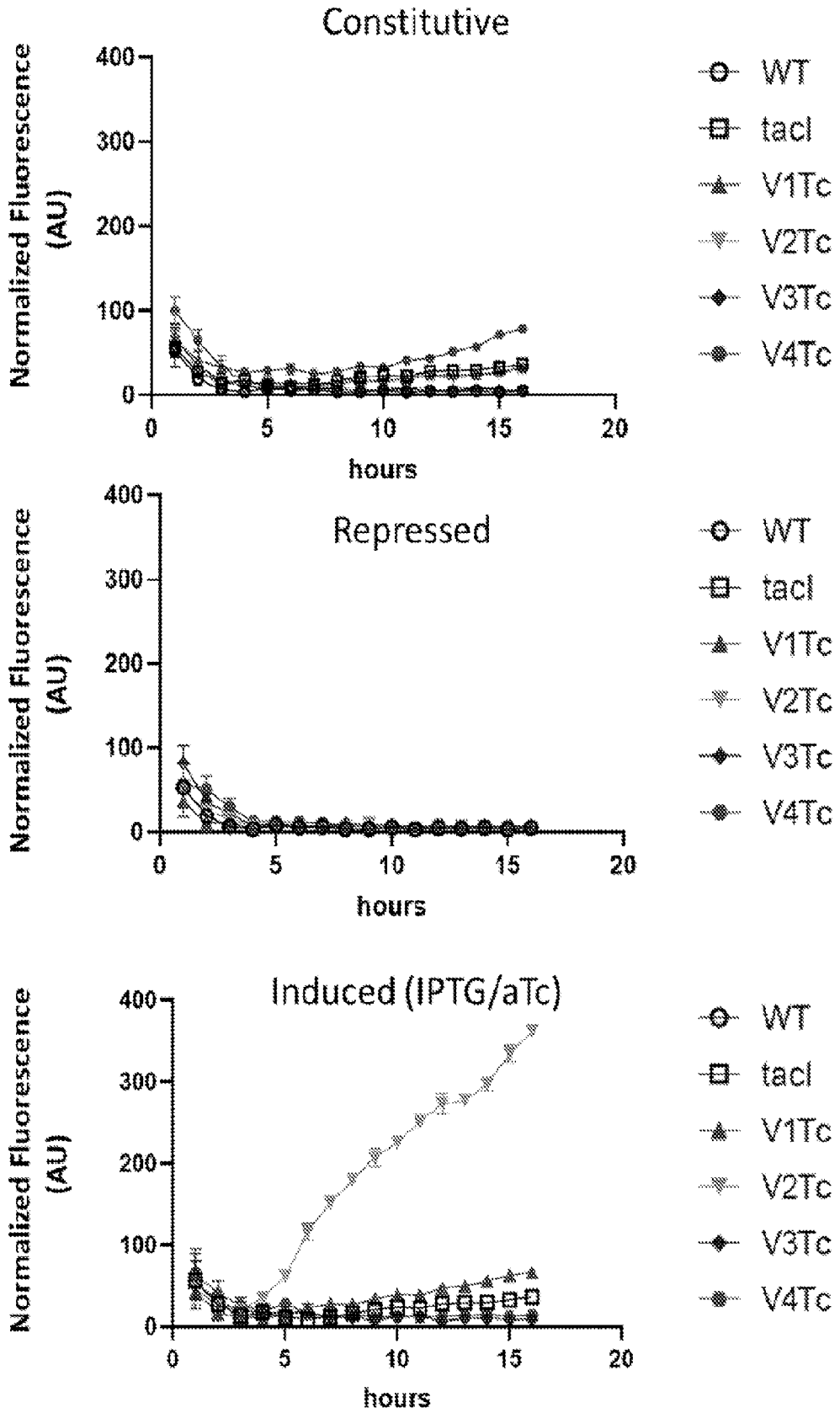
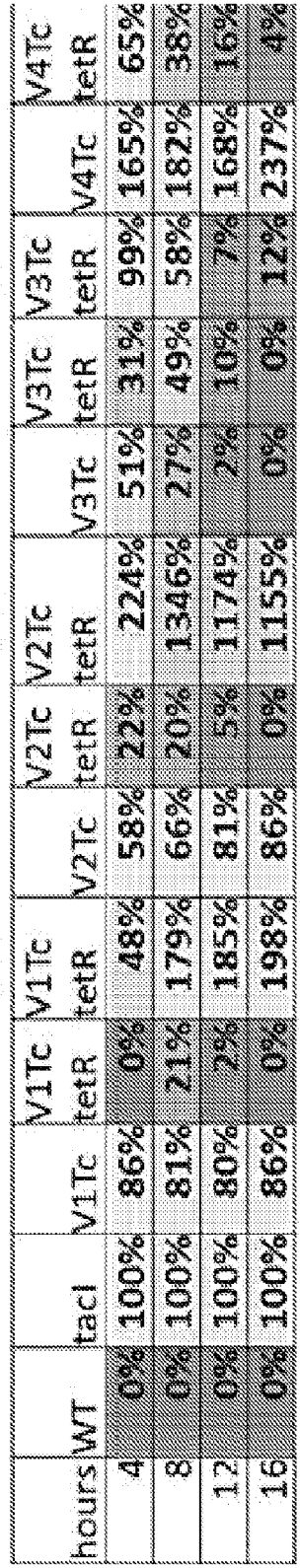
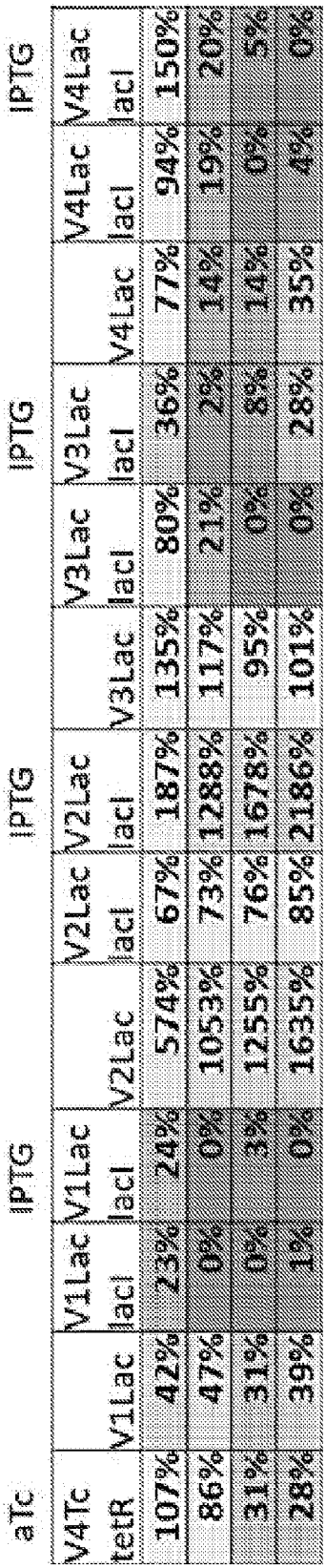

Constitutive
Normalized Fluorescence (AU)
400
300
200
100
0
0
5
10
15
20
hours
WT
tacI
V1Tc
V2Tc
V3Tc
V4Tc
Repressed
Normalized Fluorescence (AU)
hours
Induced (IPTG/aTc)
Normalized Fluorescence (AU)
hours

FIG. 7B



	aTc					aTc					aTc				
hours	WT	tacI	V1Tc	V1Tc tetR	V1Tc tetR	V2Tc	V2Tc tetR	V2Tc tetR	V3Tc	V3Tc tetR	V3Tc tetR	V4Tc	V4Tc tetR
4	0%	100%	86%	0%	48%	58%	22%	224%	51%	31%	99%	165%	65%
8	0%	100%	81%	21%	179%	66%	20%	1346%	27%	49%	58%	182%	38%
12	0%	100%	80%	2%	185%	81%	5%	1174%	2%	10%	7%	168%	16%
16	0%	100%	86%	0%	198%	86%	0%	1155%	0%	0%	12%	237%	4%

FIG. 7C



aTc	IPTG					IPTG					IPTG				
V4Tc tetR	V1Lac		V1Lac lacI	V1Lac lacI	V2Lac	V2Lac	V2Lac lacI	V2Lac lacI	V3Lac	V3Lac lacI	V3Lac lacI	V4Lac	V4Lac lacI	V4Lac lacI
107%	42%		23%	24%	574%	67%	187%	1288%	135%	80%	36%	77%	94%	150%
86%	47%		0%	0%	1053%	73%	1288%	2186%	117%	21%	2%	14%	19%	20%
31%	31%		0%	3%	1255%	76%	1678%	2186%	95%	0%	8%	14%	0%	5%
28%	39%		1%	0%	1635%	85%	2186%	2186%	101%	0%	28%	35%	4%	0%

FIG. 7C (Con't)

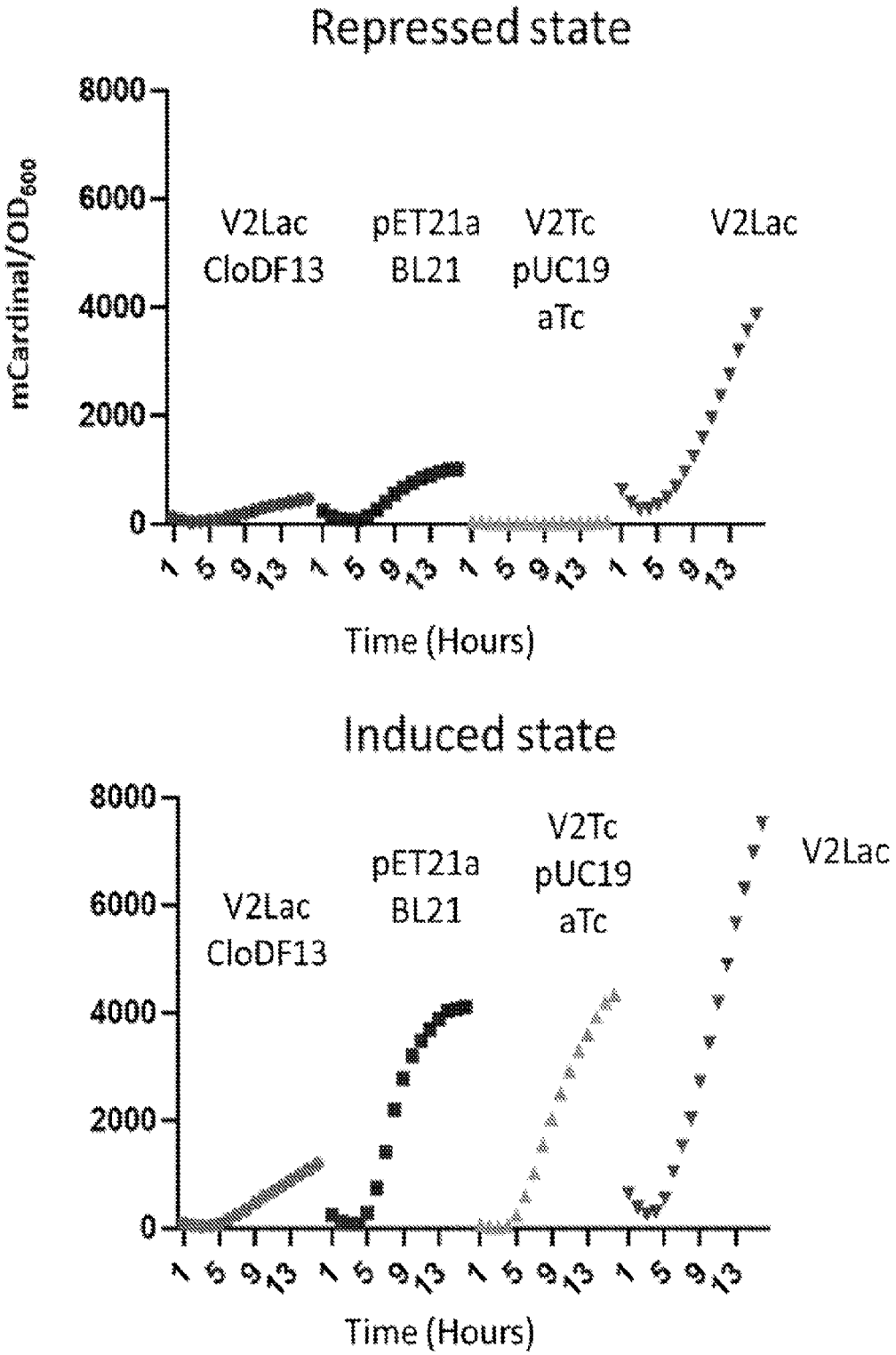

Repressed state
8000
6000
4000
2000
0
mCardinal/OD600
V2Lac
CloDF13
pET21a
BL21
V2Tc
pUC19
aTc
V2Lac
1
5
9
13
Time (Hours)
Induced state
8000
6000
4000
2000
0
V2Lac
CloDF13
pET21a
BL21
V2Tc
pUC19
aTc
V2Lac
1
5
9
13
Time (Hours)

FIG. 8

FIG. 9A

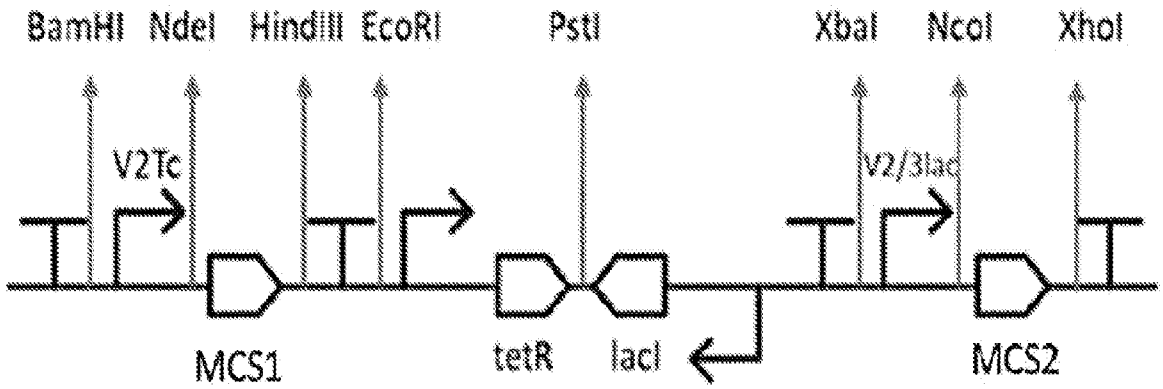


FIG. 9B

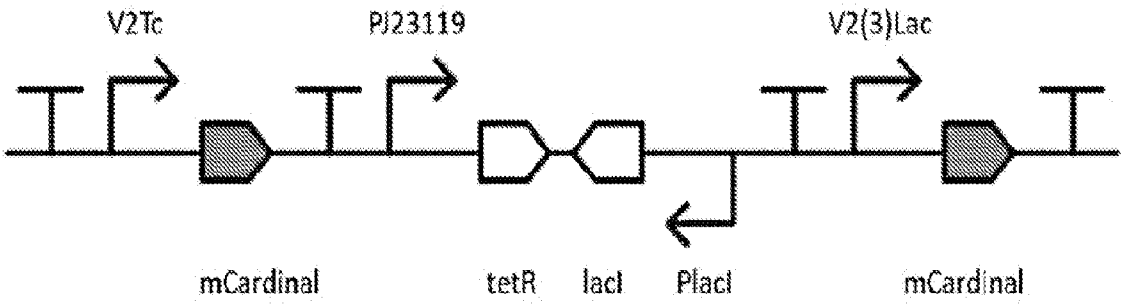


FIG. 9C

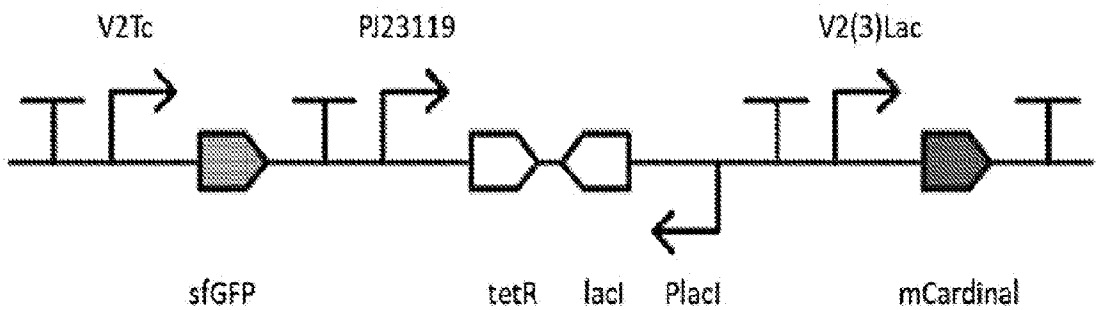


FIG. 9D

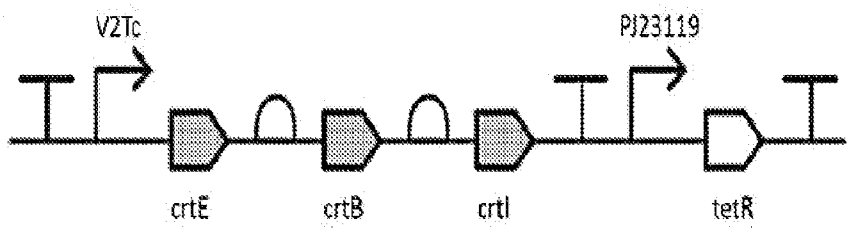


FIG. 9E

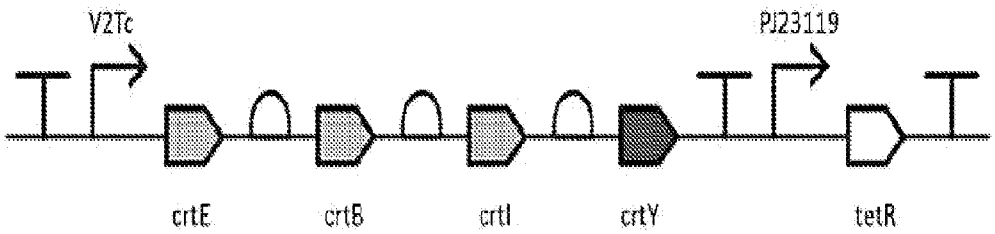
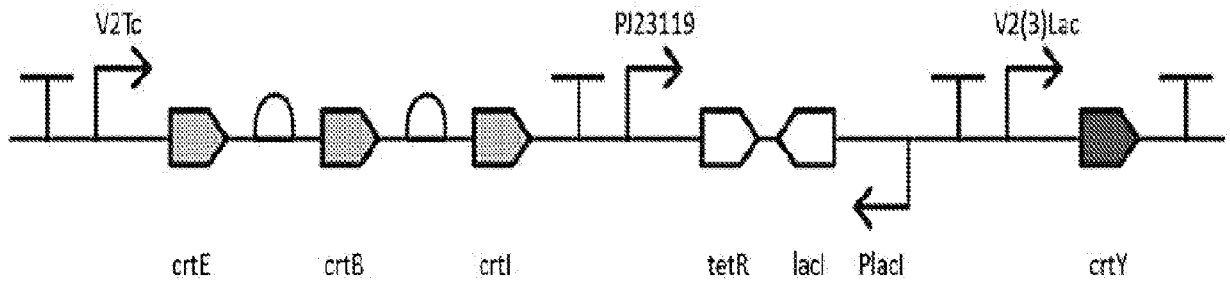


FIG. 9F



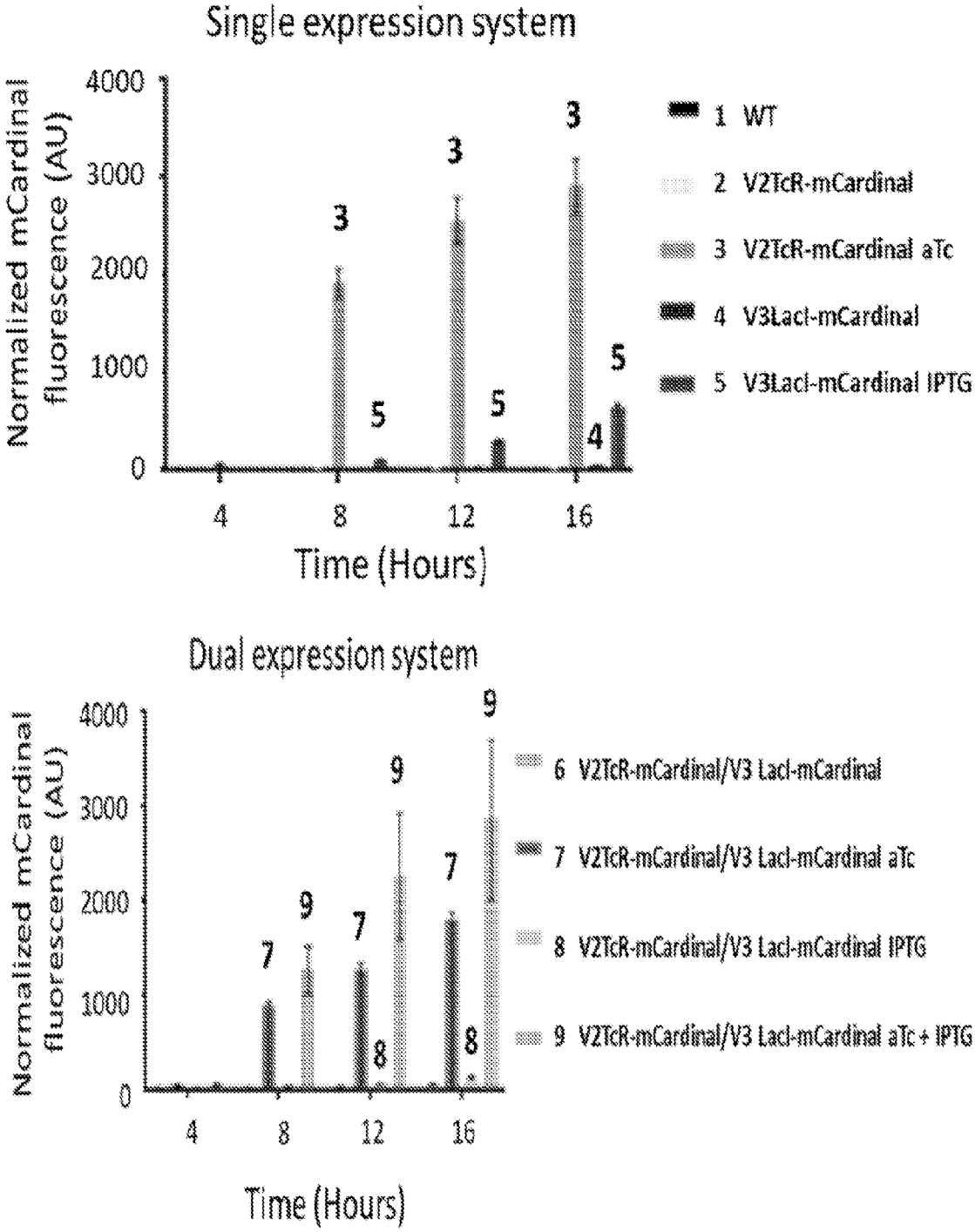

Single expression system
Normalized mCardinal fluorescence (AU)
4000
3000
2000
1000
0
4
8
12
16
Time (Hours)
1 WT
2 V2TcR-mCardinal
3 V2TcR-mCardinal aTc
4 V3LacI-mCardinal
5 V3LacI-mCardinal IPTG
Dual expression system
Normalized mCardinal fluorescence (AU)
Time (Hours)
6 V2TcR-mCardinal/V3 LacI-mCardinal
7 V2TcR-mCardinal/V3 LacI-mCardinal aTc
8 V2TcR-mCardinal/V3 LacI-mCardinal IPTG
9 V2TcR-mCardinal/V3 LacI-mCardinal aTc + IPTG

FIG. 10A

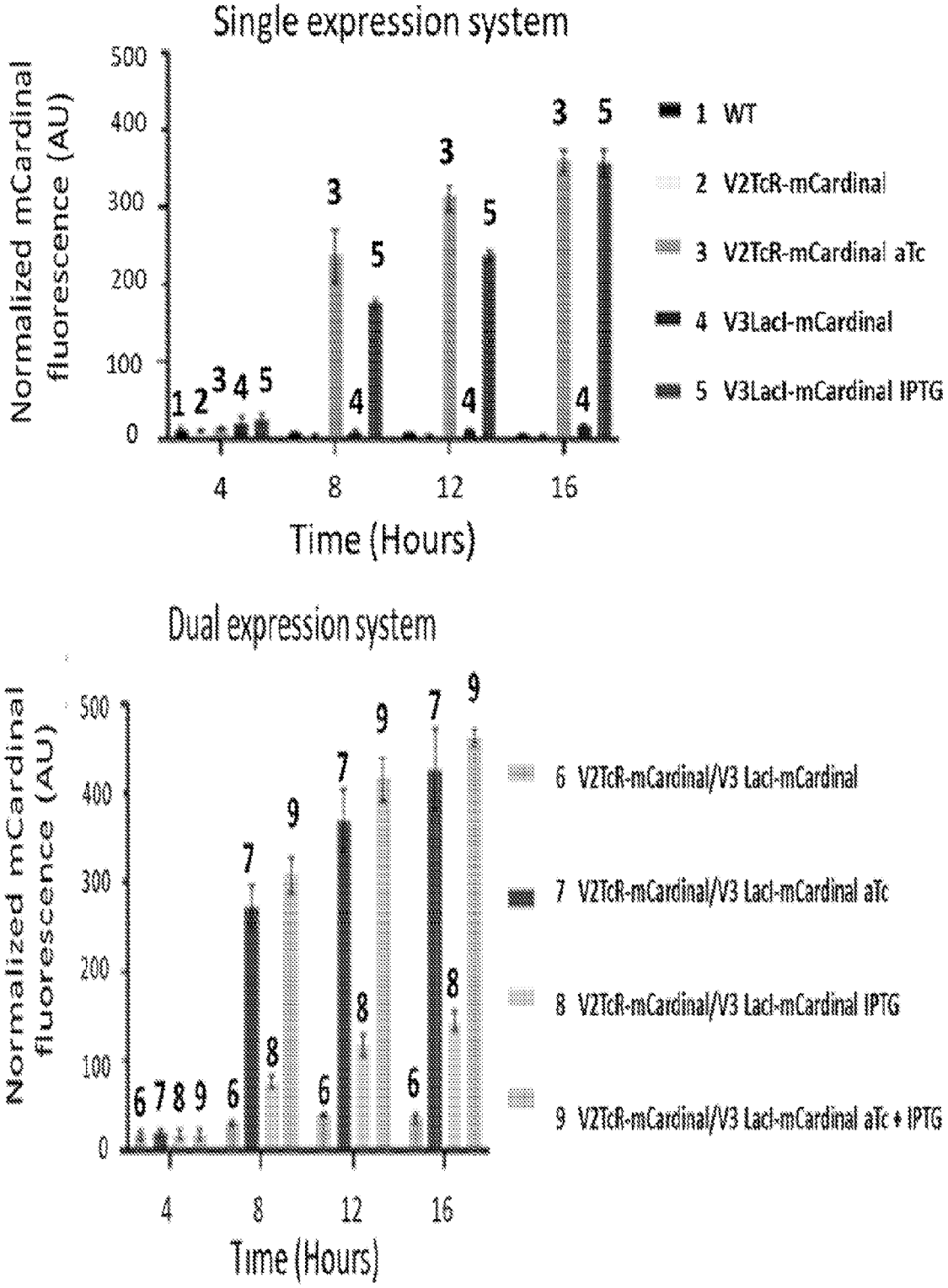

Single expression system
Normalized mCardinal fluorescence (AU)
500
400
300
200
100
0
4
8
12
16
Time (Hours)
1 WT
2 V2TcR-mCardinal
3 V2TcR-mCardinal aTc
4 V3LacI-mCardinal
5 V3LacI-mCardinal IPTG
Dual expression system
Normalized mCardinal fluorescence (AU)
Time (Hours)
6 V2TcR-mCardinal/V3 LacI-mCardinal
7 V2TcR-mCardinal/V3 LacI-mCardinal aTc
8 V2TcR-mCardinal/V3 LacI-mCardinal IPTG
9 V2TcR-mCardinal/V3 LacI-mCardinal aTc + IPTG

FIG. 10B

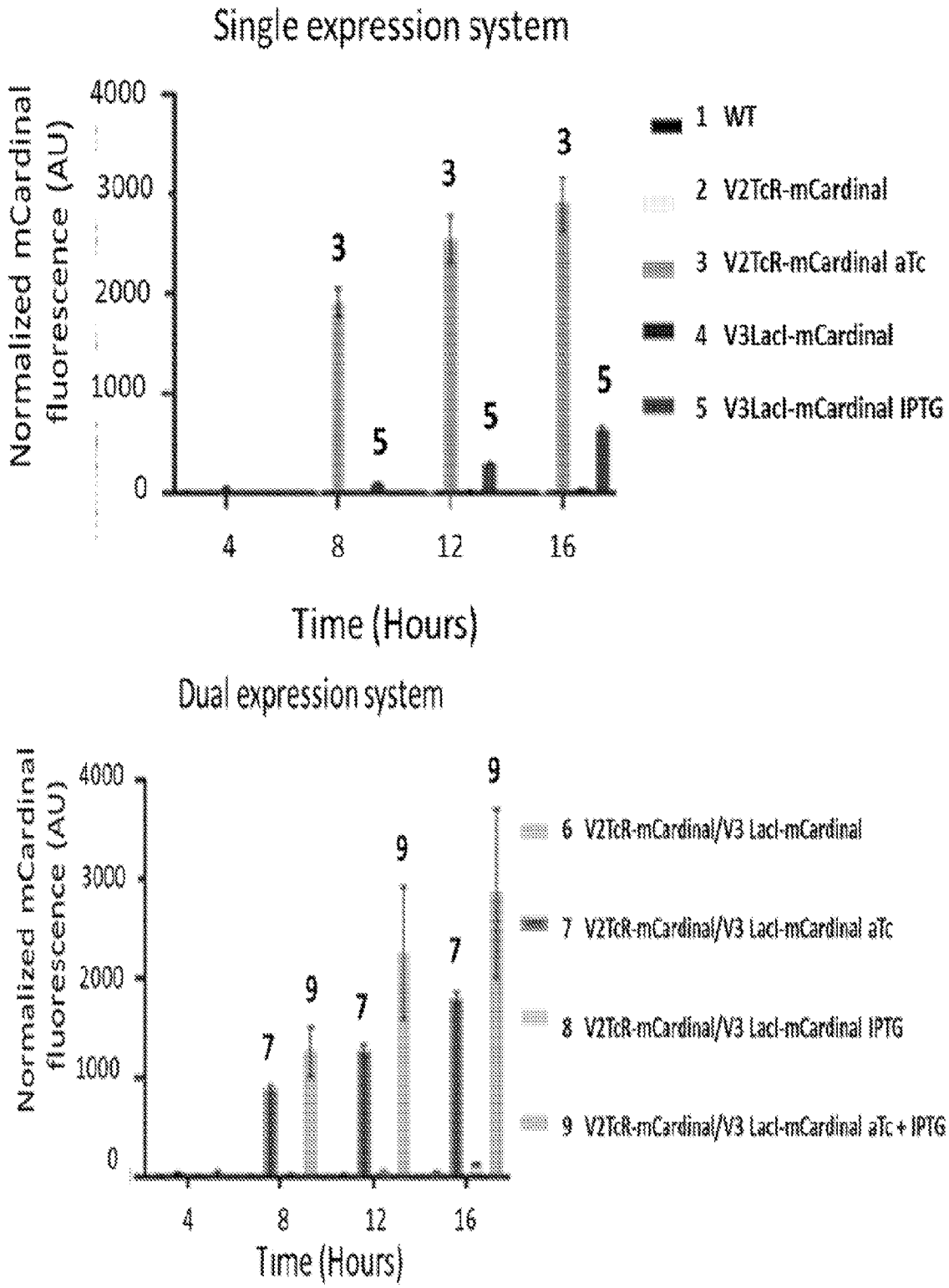

Single expression system
Normalized mCardinal fluorescence (AU)
4000
3000
2000
1000
0
4
8
12
16
Time (Hours)
1 WT
2 V2TcR-mCardinal
3 V2TcR-mCardinal aTc
4 V3LacI-mCardinal
5 V3LacI-mCardinal IPTG
Dual expression system
6 V2TcR-mCardinal/V3 LacI-mCardinal
7 V2TcR-mCardinal/V3 LacI-mCardinal aTc
8 V2TcR-mCardinal/V3 LacI-mCardinal IPTG
9 V2TcR-mCardinal/V3 LacI-mCardinal aTc + IPTG

FIG. 10C

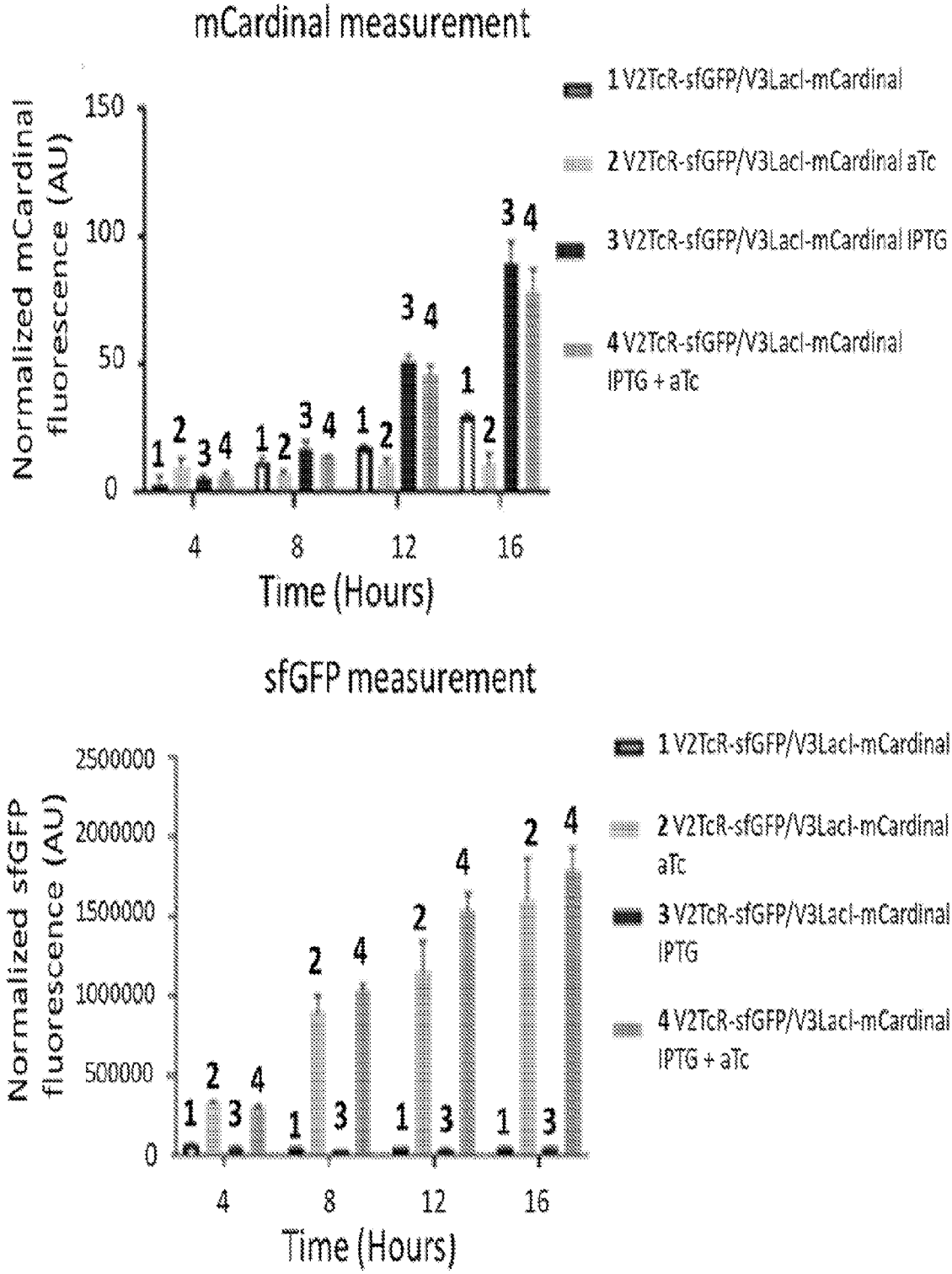

mCardinal measurement
Normalized mCardinal fluorescence (AU)
150
100
50
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc
sfGFP measurement
Normalized sfGFP fluorescence (AU)
2500000
2000000
1500000
1000000
500000
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc

FIG. 11A

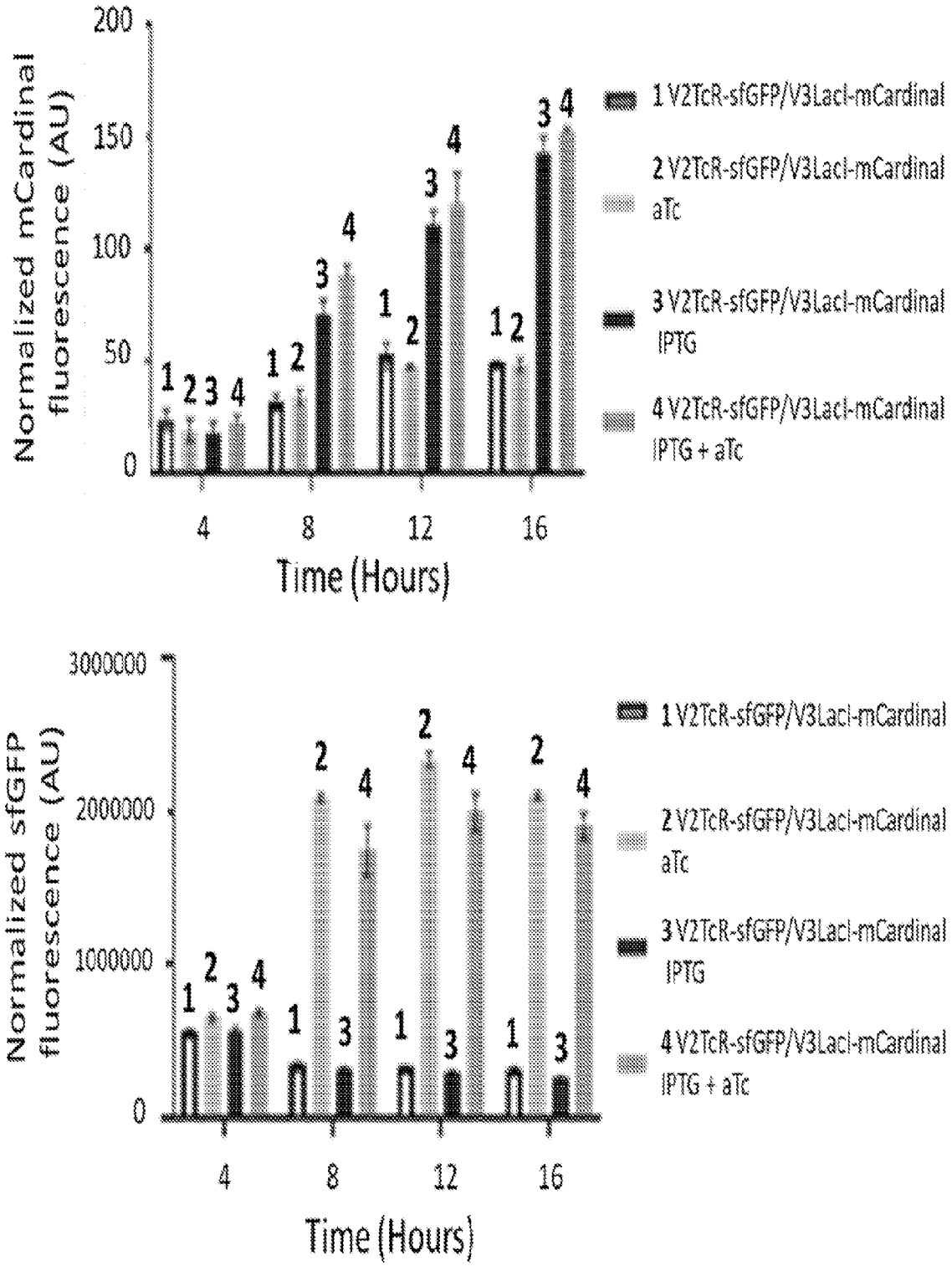

Normalized mCardinal fluorescence (AU)
200
150
100
50
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc
Normalized sfGFP fluorescence (AU)
3000000
2000000
1000000
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc

FIG. 11B

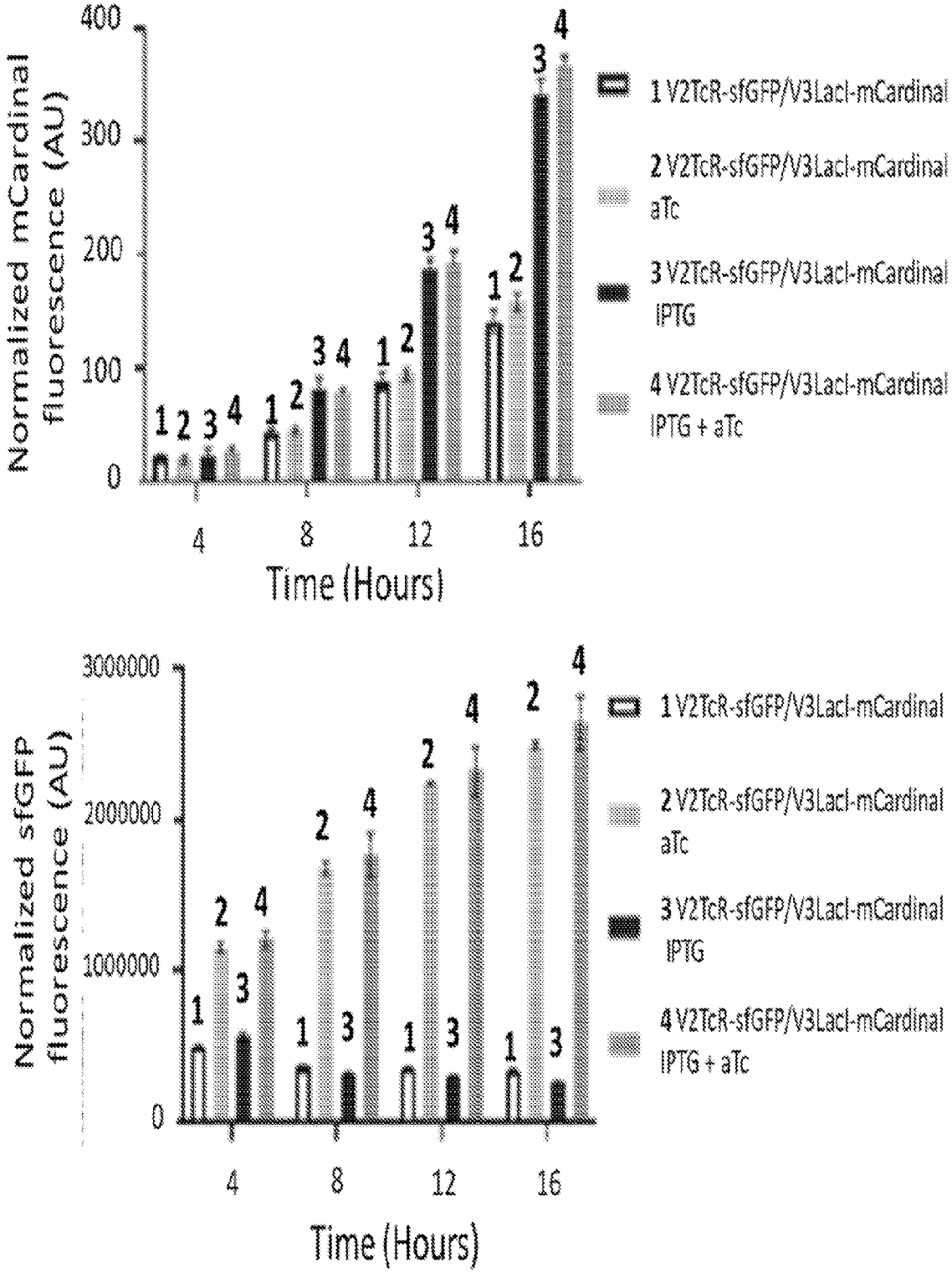

Normalized mCardinal fluorescence (AU)
400
300
200
100
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc
Normalized sfGFP fluorescence (AU)
3000000
2000000
1000000
0
4
8
12
16
Time (Hours)
1 V2TcR-sfGFP/V3LacI-mCardinal
2 V2TcR-sfGFP/V3LacI-mCardinal aTc
3 V2TcR-sfGFP/V3LacI-mCardinal IPTG
4 V2TcR-sfGFP/V3LacI-mCardinal IPTG + aTc

FIG. 11C

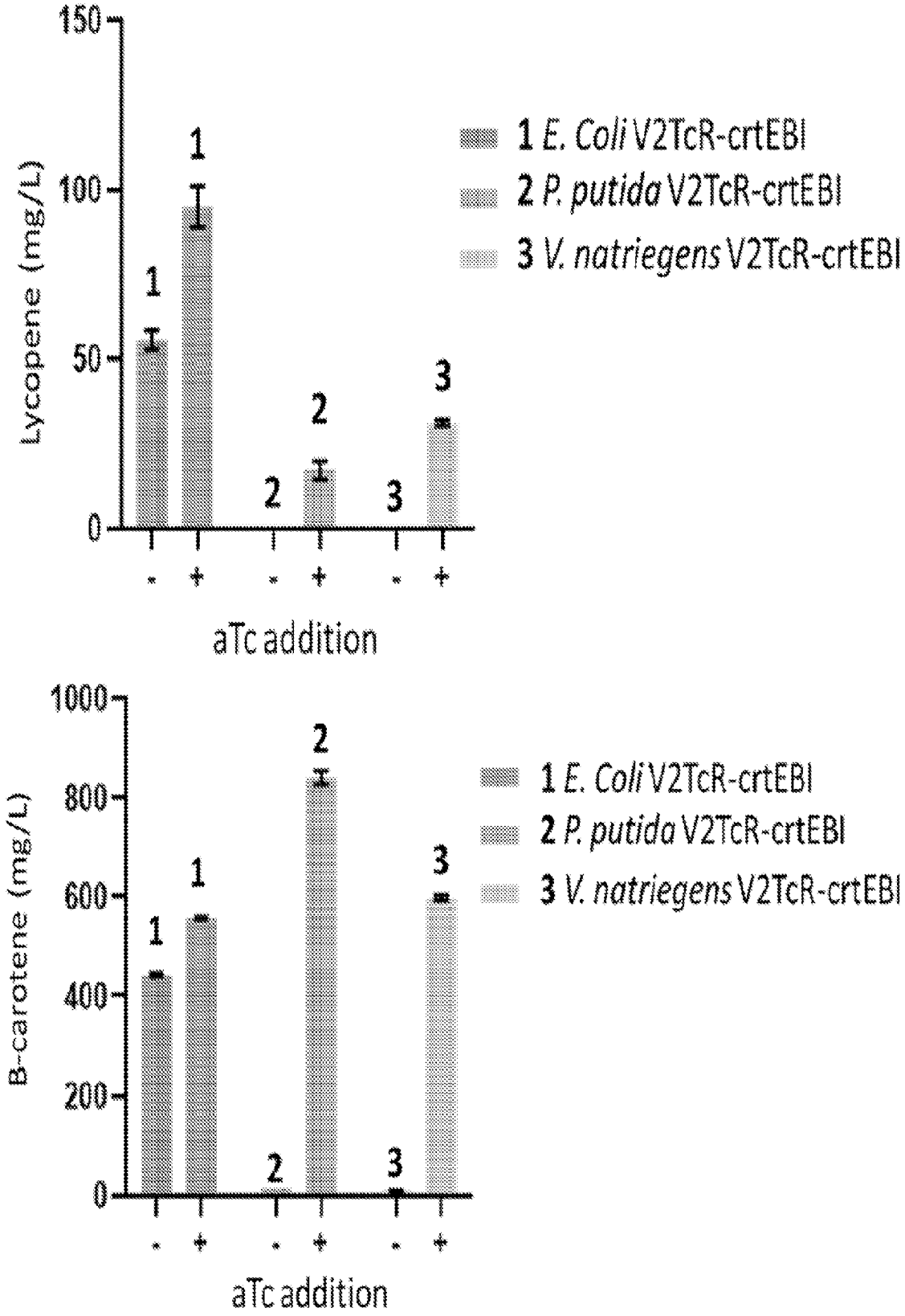

Lycopene (mg/L)
150
100
50
0
1
2
3
- +
aTc addition
1 E. Coli V2TcR-crtEBI
2 P. putida V2TcR-crtEBI
3 V. natriegens V2TcR-crtEBI
B-carotene (mg/L)
1000
800
600
400
200
0
aTc addition

FIG. 12B

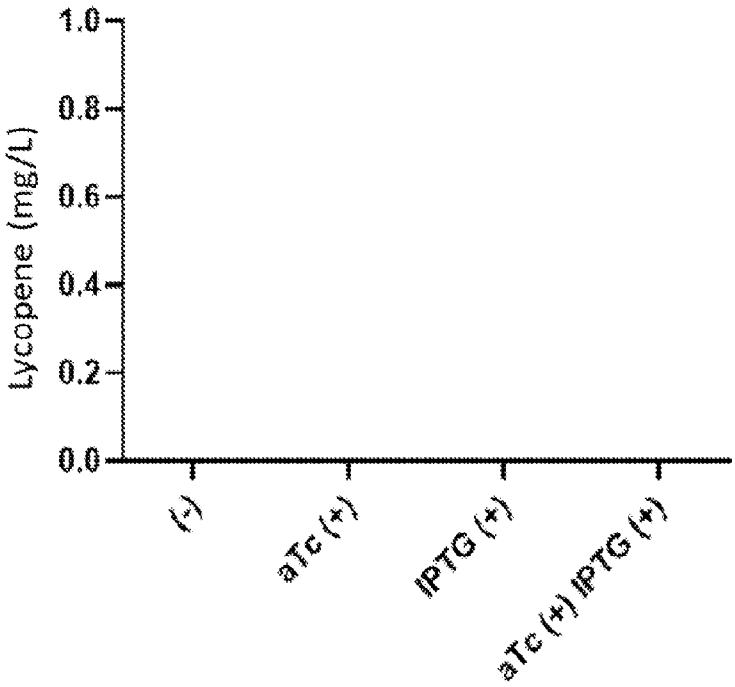


FIG. 13A

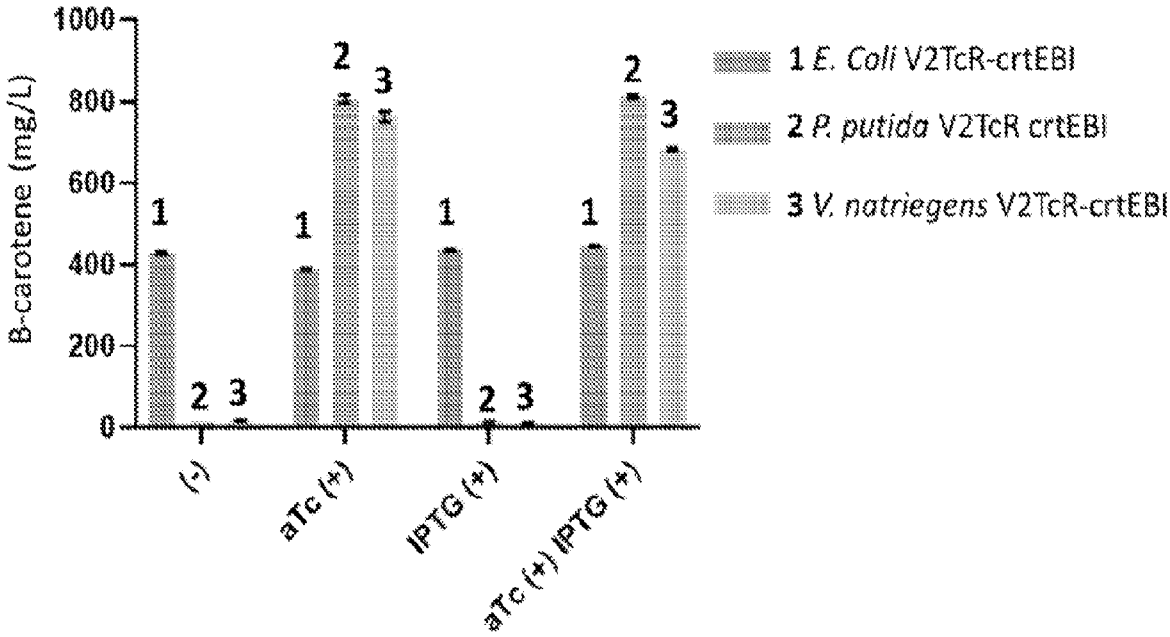


FIG. 13B

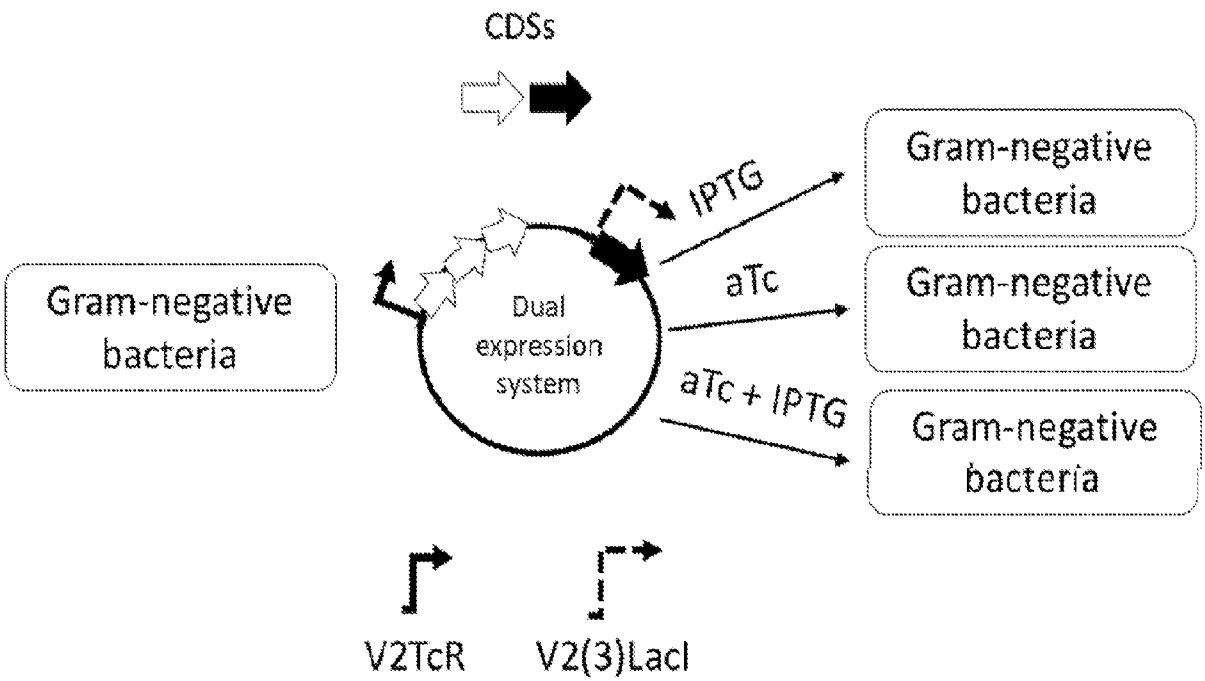

CDSs
Gram-negative bacteria
Dual expression system
IPTG
aTc
aTc + IPTG
Gram-negative bacteria
Gram-negative bacteria
Gram-negative bacteria
V2TcR
V2(3)LacI

FIG. 14