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Published:

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- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: METHOD FOR AWAKENING SILENT GENE CLUSTERS IN BACTERIA AND DISCOVERY OF CRYPTIC METABOLITES

(57) Abstract: The majority of clinically used antibiotics and anticancer agents are derived from bacterial small molecules. These molecules are produced by dedicated biosynthetic gene clusters, sets of genes that are responsible for the step-wise generation of the target small molecule. Recent investigations have indicated, to the surprise of many experts, that the majority of these biosynthetic genes are inactive or 'silent' for unknown reasons. Thus under typical bacterial culturing conditions, these genes are not expressed and consequently the bioactive small molecule products are not synthesized. Disclosed is a method for high throughput screening of elicitors of cryptic metabolites, a method for producing cryptic metabolites, and a new family of cryptic metabolites, the acybolins, as well as their complete structural elucidation.



WO 2015/138442 A1

METHOD FOR AWAKENING SILENT GENE CLUSTERS IN BACTERIA AND DISCOVERY OF CRYPTIC METABOLITES

[0001] CROSS-REFERENCE TO PRIOR FILED APPLICATIONS

[0002] This application claims priority to U.S. provisional application 61/950,291 which was filed on March 10, 2014, which is incorporated herein in its entirety.

[0003] BACKGROUND OF THE INVENTION

[0004] Recent genome sequencing efforts, which have revealed that our current discovery methods access, at best, 10% of the small molecule repertoire of bacteria. A detailed analysis of the sequenced genomes of actinomycetes, the group of bacteria responsible for over 50% of all antibiotics, has demonstrated that the great majority of biosynthetic gene clusters, the sets of genes responsible for production of bioactive compounds, remain inactive or 'silent' for unknown reasons. Given the track record of natural products as therapeutics, these clusters, dubbed silent or cryptic gene clusters, harbor an extensive supply of potential drug candidates, and successful approaches that systematically awaken them would have a profound impact on drug discovery.

[0005] The problem of silent gene clusters is challenging because an unknown signal activates an uncharacterized gene cluster leading to the production of a new metabolite. There are three variables in this process, two of which can be determined experimentally or computationally: bioinformatic methods allow for facile identification of genes that generate nonribosomal peptides, polyketides, and terpenes, and pinpointing gene assemblies of novel metabolites within these families can be performed with good fidelity. Once activated, the product of the gene cluster can be experimentally identified by differential metabolomics facilitating its isolation and structural elucidation via multi-dimensional NMR. Thus, the problem of crypticity may be reduced to the large variety of signals that may act as elicitors or activators of silent clusters.

[0006] Thus far, no method has been described that allows for identification of elicitors of a given silent gene cluster. An efficient platform that enables discovery of small molecule activators would allow scrutiny of the regulatory pathways that lead to induction of silent biosynthetic clusters as well as structural and functional elucidation of their products.

[0007] BRIEF SUMMARY OF THE INVENTION

[0008] A method for high-throughput screening to aid in discovering an agent able to activate silent bacterial gene clusters is disclosed. The method includes providing bacterial cells, which may be of a species existing naturally in soil or other environments, containing at least one gene cluster that is silent or lowly-expressed. Then, genetically modifying the gene cluster to include at least one reporter gene within the gene cluster. The reporter gene inserted into the gene cluster may include, but is not limited to, green fluorescent protein (GFP) or other fluorescent proteins (such as CFP, YFP, or RFP), the lux operon, and β -galactosidase (*lacZ*). One bacterial cell, group of cells, or cell culture is used as a control; at least one other cell, group, or culture is used as a test group. The test group is exposed to different stress conditions, including exposure to a test compound or a library of small molecules, of synthetic, semi-synthetic or natural origins. The expression of at least one of reporter gene is then measured for each group of bacterial cells, groups of cells, or cell cultures. An elicitor of a gene cluster has been identified when the expression of the reporter gene in the test group is a statistically significant amount greater than is expressed by the control group.

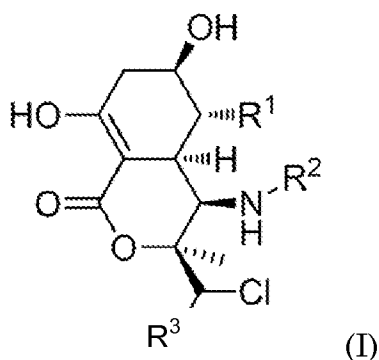
[0009] The method may also include identifying a molecule that results from the activation of said gene clusters. One type of molecule that could result is a cryptic metabolite.

[0010] A kit for discovering an agent which is able to activate silent bacterial gene clusters is also disclosed. The kit includes a bacteria configured with a reporter gene located within a silent or lowly-expressed gene cluster in the bacteria.

The reporter gene inserted into the gene cluster may include, but is not limited to, green fluorescent protein (GFP), the lux operon, and β -galactosidase (*lacZ*).

[0011] A method for producing cryptic metabolites is also disclosed. The method includes providing a bacterial cell containing at least one gene cluster, that is silent or lowly-expressed, then exposing the bacterial cell to a small molecule modulator or small molecule modulator library. The small molecule modulator library may consist of functionally and structurally diverse molecules, any of which may include, but are not limited to, an antibiotic.

[0012] A cryptic metabolite resulting from the above method is also disclosed. The metabolite is a molecule having a formula:



wherein R^1 consists of H or OH, R^2 comprises a plurality of amino acids and at least one functional group, and R^3 consists of H or Cl. The plurality of amino acids utilized may include, but are not limited to, alanine, glycine, or both. Additionally, the functional group may include an acyl group, which may comprise a carbon chain having between about 2 to about 20 carbons in length, or preferably between about 6 to about 12 carbons in length.

[0013] DETAILED DESCRIPTION OF THE INVENTION

[0014] The present disclosure generally relates to cryptic metabolites and awakening silent gene clusters in bacteria. Specifically, it discloses a method for screening of elicitors or activators of silent gene clusters in bacteria, a kit to enable such screenings, a method for activating silent gene clusters, and a cryptic metabolite resulting from such activation.

[0015] The disclosed screening method begins with acquiring bacteria having at least one silent or lowly-expressed gene cluster. The bacteria are then genetically modified to include a reporter gene within the silent or lowly-expressed gene cluster. One quantity of bacteria is used as a control group, and at least one other quantity of bacteria is used as one or more test groups. The test group or groups are exposed to at least one test compound. While only one test compound may be tested, and thus, only one test group is required, this method allows for any number of test compounds to be tested. Additionally, any class of compound may be tested, which include but are not limited to, vitamins, muscle relaxants, general agonists, estrogens, antifungals, lipid biosynthesis inhibitors, and antibacterials. The expression of the reporter genes are measured in the control group and each test group. A test compound will be considered an elicitor or an activator if the measured expression of the test group is a statistically significant amount greater than that of the control group.

[0016] In one example of the high throughput screening method, the silent malleilactone (*mal*) cluster in *Burkholderia thailandensis* E264 (hereafter E264), was targeted. To monitor expression of this cluster, a translational *lacZ* fusion to *malL*, a gene essential for the biosynthesis of malleilactone, was utilized (hereafter *malL-lacZ*). *MalL-lacZ* is not expressed under standard growth conditions and served as a negative control. A *lacZ* reporter in *btaK*, which is quorum sensing-regulated and expressed at high cell densities, provided a positive control.

[0017] Bacterial growth was carried out in lysogeny broth (LB) supplemented with 50 mM Mops, pH 7.0 (hereafter, LB-Mops). The pH was adjusted with a 5–6 N NaOH solution. To commence the screen, *malL-lacZ* from an LB agar plate was used to inoculate 5 mL of LB-Mops in a sterile 14 mL bacterial culture tube. The culture was grown overnight at 30°C and 250 rpm. After 12–16 h, its OD_{600 nm} was determined on an Ultraspec 5300 Pro Spectrophotometer (Amersham Biosciences). The culture was diluted into 100 mL of LB-Mops to give a final OD_{600 nm} of 0.05. Subsequently a volume of 45 µL was dispensed into each of four sterile, white 384-

well plates (Corning) using a WellMate automated dispenser (Matrix Technologies) at the ICCB-L. Using a Compound Transfer Robot (Seiko) equipped with a calibrated stainless steel pin transfer tool (V&P Scientific), each well was supplemented with 0.1 μ L of the at least one compound from a 640-member-containing library. The compounds were dispensed into columns 3 through 22 on each plate, while columns 1 and 2 contained the negative control (*malL-lacZ* in the absence of any library compounds) and columns 23 and 24 contained the positive control (*btaK-lacZ*). Between each dispense cycle, the transfer tool pins were washed in MeOH, sonicated in a MeOH/water mixture, and dried with pressurized air. Each plate was covered with a Breathe-Easy sealing membrane (Sigma), vortexed briefly on a plate vortexer, and cultured at 30°C and 230 rpm for 12 h in a Multitron Shaker (ATR) equipped with a green sealing tray. To maintain constant humidity, several 1 L Erlenmeyer flasks containing 200 mL of water were also placed inside the shaker. After 12 h, the plates were removed from the shaker and rested at room temperature for 5 minutes. The β -Glo reagent (Promega) was used to monitor *lacZ* activity. The reagent was diluted 2:1 with water. Then, each well was supplemented with 30 μ L of the diluted β -Glo reagent using the WellMate automated dispenser, and subsequently vortexed, and incubated in the dark at room temperature for 45 minutes. Total end-point luminescence was then determined on an EnVision Multilabel Reader (PerkinElmer). The optimization wizard in the EnVision software was used to optimize plate dimensions and minimize cross-talk yielding a software-derived correction factor of 0.27. The Z'-score was calculated according to equation 1, where σ_p and σ_n correspond to the standard deviation of the positive (*btaK-lacZ*) and negative (*malL-lacZ* in the absence of elicitors) controls, respectively, while μ_p and μ_n correspond to the mean *lacZ* activity for the positive and negative controls. Standard Z-scores for each compound in the library in the actual screen were calculated using equation 2, where μ_c corresponds to the mean *lacZ* activity for each compound in the library, μ_n corresponds to the mean of the negative control on the same assay plate (columns 1 and 2), and σ_n corresponds to the standard deviation of the negative control.

[0018]
$$Z' = 1 - \left(\frac{3(\sigma_p + \sigma_n)}{\mu_p - \mu_n} \right) \quad (1)$$

[0019]
$$Z = \left(\frac{\mu_p - \mu_n}{\sigma_n} \right) \quad (2)$$

[0020] This method provided an optimized, robust screening assay with a Z' value of 0.51 in a 384-well format. A total of nine potential elicitors having Z -scores between 6 and 21 were observed.

[0021] It should be understood by a practitioner that while *lacZ* was utilized in this example, any appropriate reporter gene, or combination of reporter genes would produce similar results. This includes, without limitation, green fluorescent proteins (GFP), red fluorescent proteins (RFP), β -Galactosidase (GUS), and luciferase. It also includes combinations of reporter genes within the same strain.

[0022] Once the above screening has occurred, identification of molecules may occur, utilizing standard analytical techniques, including but not limited to RT-qPCR, GC, HPLC, Mass Spectrometry, and NMR.

[0023] In another embodiment of the invention, a kit containing a bacteria, a group of bacteria, or a bacterial cell culture configured with a reporter gene located within a silent or lowly-expressed gene cluster in the bacteria is disclosed to facilitate the high throughput screenings. This includes application of this approach to any gene cluster in any bacterium, regardless of the source of the bacterium.

[0024] One example of such a kit includes a kit targeting the burkholdac (*bhc*) cluster in E264. *Bhc* is lowly-expressed under typical growth conditions and produces the histone deacetylase inhibitor. In this case, a kit comprising E264 bacteria having a translational *lacZ* fusion to *bhcF* was produced, enabling screenings for *bhc* production.

[0025] Another embodiment of this invention discloses a method of generating a cryptic metabolite. The disclosed method begins by providing a bacterial cell containing at least one gene cluster that is silent or lowly-expressed. Preferably,

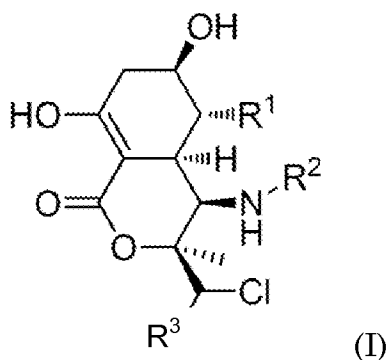
this gene cluster will consist of a quorum sensing-regulated biosynthetic gene cluster. More preferably, this quorum sensing regulated biosynthetic gene cluster will generate a non-cryptic metabolite under normal growth conditions. Once the bacterial cell has been provided, the bacterial cell is exposed to a small molecular modulator, typically previously determined to be an elicitor of a cryptic metabolite. The small molecular modulator may comprise an antibiotic, typically introduced at a quantity below the inhibitor level of that antibiotic. Following standard practices for growing cultures of the particular bacteria provided, a cryptic metabolite may be produced.

[0026] As an example, wild type *B. thailandensis* bacterial cells were first provided. It is well-known that the *bta* quorum sensing-regulated gene cluster on *B. thailandensis* normally generates bactobolins. Using the disclosed method, *B. thailandensis* E264 grown on an LB agar plate was used to inoculate 5 mL of LB medium in a 14 mL sterile culture tube. After overnight growth at 30°C and 250 rpm, the culture was diluted to an OD_{600 nm} of 0.05 into 50 mL of LB in a 250 mL Erlenmeyer flask. This culture was grown overnight at 30°C and 250 rpm and used to inoculate 650 mL LB-Mops (LB + 50 mM Mops, pH 7) in each of 12 x 4 L Erlenmeyer flasks. The initial OD_{600 nm} of the large cultures was 0.05 and the cultures contained 30 µM trimethoprim (a bacteriostatic antibiotic), prepared as a 10 mM stock in DMSO. After 26 h growth at 30°C and 200 rpm, the cultures were extracted twice with one volume of ethyl acetate. To verify that the cryptic metabolites had been produced, the organic layers were then combined, dried over Na₂SO₄, and evaporated completely *in vacuo*. The remaining residue was resolved by solid-phase extraction using a 10 g Seppak-C18 column, which had been washed with MeCN and equilibrated with 15% MeCN in H₂O. Step-wise elution was performed with 100 mL of 15%, 35%, 55%, 75%, and 100% MeCN (in H₂O), all containing 0.1% formic acid. The 55% MeCN fraction contained the previously unknown cryptic metabolites.

[0027] These were further purified on a manual Hypercarb column (Fisher Scientific), which had been equilibrated with 20% MeCN in H₂O. Step-wise elution

was performed with 15 mL of 20%, 35%, 50%, 75%, and 100% MeCN (in H₂O+0.1% (v/v) formic acid). The 35% and 50% MeCN fractions were combined, dried *in vacuo*, resuspended in MeOH, and purified by reverse-phase HPLC on a preparative Eclipse XDB-C8 column (Agilent, 7 μ m, 21.2 x 250 mm) operating at 12 mL/min. The elution program started with an isocratic step (5 min, 20% MeCN in H₂O), followed by a gradient from 20-100% MeCN (+0.1% formic acid) over 30 min. The desired cryptic metabolites eluted at ~62-68% MeCN, and fractions containing the cryptic metabolites were combined, dried *in vacuo*, and further purified by reverse-phase HPLC on a preparative Luna C18 column (Phenomenex, 5 μ m, 21.2 x 250 mm) operating at 12 mL/min. The elution program included an isocratic step (30 min, 30% MeCN in H₂O), followed by a gradient from 30-100% MeCN (+0.1% formic acid) over 20 min. The desired cryptic metabolites eluted at approximately 74-80% MeCN. Fractions containing this desired cryptic metabolite were combined and further purified by reverse-phase HPLC on an analytical Synergi Fusion-RP column (Phenomenex, 4 μ m, 4.6 x 250 mm) operating at 1 mL/min. These cryptic metabolites were eluted isocratically at 32% MeCN in H₂O (+0.1% formic acid) over 50 min, and they eluted in separate fractions between 33 and 46 minutes, yielding 0.6–3 mg of pure material.

[0028] Disclosed also is the class of materials called acybolins resulting from use of the method described above. This class of materials typically has a molecular formula of:



wherein R^1 consists of H or OH, R^2 comprises a plurality of amino acids and at least one functional group, and R^3 consists of H or Cl. While this invention encompasses a range of amino acids, a preferred embodiment consists solely of alanine, glycine, or both. Further, the functional group is preferably an acyl group, and more preferably an acyl group having a carbon chain of between around 2 to around 20 carbons in length, and more preferably between around 6 to around 12 carbons in length.

[0029] As an example, six acybolins were generated using the example method above. In each, the acyl group was 3-hydroxydecanoyl. Those acybolins had structures as follows: Acybolin A was found to have $R^1 = \text{H}$, $R^2 = \text{Ala-Ala-Ala-Gly-Acyl}$, and $R^3 = \text{Cl}$; Acybolin B was found to have $R^1 = \text{OH}$, $R^2 = \text{Ala-Ala-Ala-Gly-Acyl}$, and $R^3 = \text{Cl}$; Acybolin C was found to have $R^1 = \text{H}$, $R^2 = \text{Ala-Ala-Gly-Ala-Gly-Acyl}$, and $R^3 = \text{Cl}$; Acybolin D was found to have $R^1 = \text{H}$, $R^2 = \text{Gly-Ala-Ala-Gly-Acyl}$, and $R^3 = \text{Cl}$; Acybolin E was found to have $R^1 = \text{H}$, $R^2 = \text{Ala-Ala-Gly-Gly-Acyl}$, and $R^3 = \text{Cl}$; and Acybolin F was found to have $R^1 = \text{OH}$, $R^2 = \text{Ala-Ala-Gly-Acyl}$, and $R^3 = \text{Cl}$.

What is claimed is:

1. A high-throughput screening method for discovering an agent which is able to activate silent bacterial gene clusters, comprising the steps of:

providing at least a first and second bacterial cell, wherein the bacterial cells contains at least one gene cluster that is silent or lowly-expressed and wherein the gene cluster is genetically modified to include at least one reporter gene within the gene cluster;

exposing the second bacterial cell to a test compound;

measuring the expression of at least one reporter gene in the first bacterial cell;

measuring the expression of at least one reporter gene in the second bacterial cell; and

identifying activation of gene clusters by determining whether at least one reporter gene has been expressed by the second bacterial cell by a statistically significant amount greater than is expressed by the first bacterial cell.

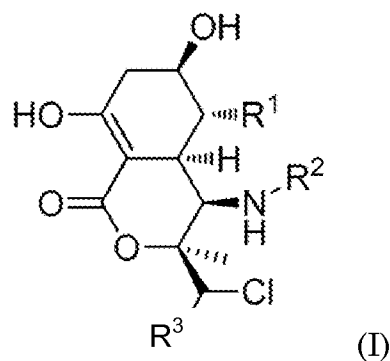
2. The high throughput screening method of claim 1, wherein the reporter gene is lacZ.
3. The high throughput screening method of claim 1, wherein the reporter gene is a fluorescent protein, including but not limited to green fluorescent protein (GFP).
4. The high throughput screening method of claim 1, wherein the at least a first and second bacterial cell are of a species that occurs naturally in soil.

5. The high throughput screening method of claim 1, wherein the test compound comprises an antibiotic.
6. The high throughput screening method of claim 1, further comprising identification of a molecule that results from the activation of said gene clusters.
7. The method of claim 6, wherein the molecule that is identified is a cryptic metabolite.
8. A kit for discovering an agent which is able to activate silent bacterial gene clusters comprising a bacteria configured with a reporter gene located within a silent or lowly-expressed gene cluster in the bacteria.
9. The kit of claim 8, wherein the reporter gene is lacZ.
10. The kit of claim 8, wherein the reporter gene is a fluorescent protein, including but not limited to green fluorescent protein (GFP).
11. A method of generating cryptic metabolites, comprising the steps of:

providing a bacterial cell containing at least one gene cluster that is silent or lowly-expressed; and

exposing the bacterial cell to a small molecule modulator or small molecule modulator library.
12. The method of claim 11, wherein the gene cluster is a quorum sensing-regulated biosynthetic gene cluster.
13. The method of claim 12, wherein the gene cluster generates a non-cryptic metabolite under normal growth conditions.
14. The method of claim 11, wherein the small molecule modulator comprises an antibiotic.
15. The method of claim 11, further comprising detecting a cryptic metabolite.
16. The method of claim 15, wherein the metabolite is acybolin.

17. A molecule of the formula:



wherein R^1 consists of H or OH, R^2 comprises a plurality of amino acids and at least one functional group, and R^3 consists of H or Cl.

18. The molecule of claim 17, wherein the plurality of amino acids consist of alanine, glycine, or both.
19. The molecule of claim 17, wherein the functional group is an acyl group.
20. The molecule of claim 19, wherein the acyl group contains a carbon chain of between around 2 to around 20 carbons in length.
21. The molecule of claim 19, wherein the acyl group contains a carbon chain of between around 6 to around 12 carbons in length.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/19696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68, C12N 15/00, C12N 15/74, C07H 21/02 (2015.01)

CPC - C12Q 1/6883, C12N 15/102, C12N 15/70, A61K38/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8)- C12Q 1/68, C12N 15/00, C12N 15/74, C07H 21/02 (2015.01)

CPC- C12Q 1/6883, C12N 15/102, C12N 15/70, A61K38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/6.1, 435/440, 435/471, 536/23.1 (keyword search, terms limited)

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

PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar

Search Terms Used: Silent gent, cryptic metabolite, weakly expressed, GFP, LacZ, reporter, bacteria, secondary metabolite, transcription factor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/171158 A1 (Wink et al.) 21 November 2013 (21.11.2013) pg 1, ln 9, pg 2, ln 27-31, pg 4, ln 5, pg 3, ln 17-20, pg 24, ln 4-7, pg 40, ln 22	1-10
Y	US 7,026,460 B2 (Roberts et al.) 11 April 2006 (11.04.2006) col 17, ln 38 to col 18, ln 11	1-10
Y	US 8,222,434 B1 (Greenberg et al.) 17 July 2012 (17.07.2012) abstract	7

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 June 2015 (24.06.2015)

Date of mailing of the international search report

10 JUL 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/19696

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

Group I: claims 1-10, drawn to a high-throughput screening method for discovering an agent which is able to activate silent bacterial gene clusters and a kit comprising the same.

Group II: claims 11-16, drawn to a method of generating cryptic metabolites.

Group III: claims 17-21, drawn to a molecule of the formula (I).

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

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/19696

Continuation of:

Box NO III. Observations where unity of invention is lacking

Special Technical Features

Group I+ includes the special technical feature of a gene cluster that is silent or lowly-expressed and wherein the gene cluster is genetically modified to include at least one reporter gene within the gene cluster, not required by Groups II and III.

Group II includes the special technical feature of generating cryptic metabolites, comprising exposing the bacterial cell to a small molecule modulator, not required by Groups I and III.

Group III includes the special technical feature of a molecule of the formula (I).

Common Technical Features

The inventions of Groups I-III share the technical feature of a cryptic metabolite.

The inventions of Groups I and II share the technical feature of providing a bacterial cell containing at least one gene cluster that is silent or lowly-expressed; and exposing the bacterial cell to a compound.

However, these shared technical feature do not represent a contribution over prior art in view of US 2008/0010025 A1 to FARNET et al. (hereinafter 'Farnet'). Farnet teaches providing a bacterial cell containing at least one gene cluster that is silent or lowly-expressed; and exposing the bacterial cell to a compound to generate a cryptic metabolite (para [0005], prediction of conditions that may induce expression of a cryptic pathway; [0041], the microorganism is induced to express the target gene cluster and to synthesize the corresponding metabolite by growing the microorganism under multiple culture conditions. Minor modifications in medium composition and culture conditions can have a major influence of the range of secondary metabolites produced by a microorganism. In some embodiments, the culture conditions are selected ? antibiotics and/or exposure to radiation can be used in combination with screening to select for alternate or enhanced natural product production in this invention.). As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the groups.

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