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(54) Title: ENDOLYSIN FROM BACTERIOPHAGE AGAINST GEOBACILLUS AND METHODS OF USING

(57) Abstract: Bacteriophage against Geobacillus are provided, and methods of making and using the bacteriophage also are provided.



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ENDOLYSIN FROM BACTERIOPHAGE AGAINST GEOBACILLUS AND METHODS OF USING

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Serial No. 62/109,156 filed January 29, 2015. This disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

TECHNICAL FIELD

This disclosure generally relates to bacteriophage and, more specifically, relates to an endolysin from bacteriophage.

BACKGROUND

Bacteriophage destroy bacteria but are harmless to humans. They are strain and, usually, species-specific, and they are abundant in nature, in foods, and in the intestinal tract of animals. Bacteriophage are about 100 times smaller than bacteria, and they leave no ecological footprint. Bacteriophage are generally recognized as safe (GRAS).

The lytic lifecycle of bacteriophage typically includes adsorption to a bacterial cell, infection, which includes injecting their nucleic acid into the bacterial cell, replication, maturation, and assembly of bacteriophage inside the bacterial cell. The lytic lifecycle culminates in lysis of the bacterial cell to release all the progeny bacteriophage.

SUMMARY

This disclosure describes bacteriophage against *Geobacillus* and methods of making and using the bacteriophage.

In one aspect, an isolated bacteriophage having lytic activity against *Geobacillus* is provided. Such a bacteriophage typically includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to a nucleic acid

sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the nucleic acid sequence has a sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the endolysin encoded by the nucleic acid sequence has an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

In another aspect, an isolated bacteriophage having lytic activity against *Geobacillus* is provided. Such a bacteriophage typically includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12. In some embodiments, the endolysin has at least 99% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12. In some embodiments, the endolysin has an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

In still another aspect, an isolated nucleic acid molecule is provided. Typically, the nucleic acid molecule includes a nucleic acid sequence having at least 95% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the nucleic acid molecule includes a nucleic acid sequence having at least 99% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the nucleic acid molecules includes a nucleic acid sequence having a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11. In some embodiments, the nucleic acid molecule encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

In yet another aspect, a vector is provided that includes any of the isolated nucleic acids described herein. In another aspect, a host cell is provided that includes such a vector.

In one aspect, a purified polypeptide is provided. Typically, such a polypeptide includes an amino acid sequence having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12. In some embodiments, the amino acid sequence has at least 99% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12. In some

embodiments, the amino acid sequence has an sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

In another aspect, a method of making a polypeptide is provided. Such a method typically includes culturing a host cell as described herein under appropriate conditions.

In still another aspect, a method for reducing the number of viable *Geobacillus* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising any of the isolated bacteriophage described herein, any of the isolated nucleic acids described herein, any of the vectors described herein, any of the host cells described herein, or any of the purified polypeptides described herein. In some embodiments, the tobacco is reconstituted leaf.

In yet another aspect, reconstituted tobacco leaf is provided. Such reconstituted tobacco leaf typically includes any of the isolated bacteriophage described herein, any of the isolated nucleic acids described herein, any of the vectors described herein, any of the host cells described herein, or any of the purified polypeptides described herein.

In one aspect, a tobacco product is provided that includes such reconstituted leaf. In some embodiments, the tobacco product is a cigarette.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Figure 1 is a graph showing the effect of the G05 endolysin on *Geobacillus* in culture.

DETAILED DESCRIPTION

A number of bacteria are present on tobacco growing in a field and at various stages of processing. Some of those bacteria are beneficial and, for example, contribute to the

flavor profiles of tobacco, while some of those bacteria are undesirable and, for example, damage the tobacco and contribute to unwanted tobacco-specific nitrosamines (TSNAs). For example, there is at least one unwanted bacteria present on reconstituted leaf (RL) which oftentimes results in a biofilm. The presence of a biofilm on RL can cause holes and result in significant loss of yield. The primary genus of bacteria in the biofilm (about 95%) has been identified as *Geobacillus*.

Bacteriophage Compositions

Isolated bacteriophages are provided herein, as well as progeny thereof. As used herein with respect to bacteriophage, “isolated” refers to a bacteriophage that has been separated from the environment in which it is naturally found (e.g., that does not contain a significant amount of other bacteriophage or of the bacterial host). As used herein, “progeny” refers to replicates of a bacteriophage, including descendants of a bacteriophage created by serial passage or other methods known in the art.

In addition to bacteriophage, a bacteriophage composition also can include media, buffers, one or more nutrients, one or more minerals, one or more co-factors, or any other component that is necessary to maintain viability of the bacteriophage. Additionally, components that are not related to the viability of the bacteriophage may be desirable in a bacteriophage composition such as, without limitation, a dye or color marker.

Bacteriophage Nucleic Acids and Polypeptides

Bacteriophage contain endolysins, a generic term for one or more enzymes that are involved in the degradation of the peptidoglycan in the bacterial cell wall, ultimately resulting in lysis of the bacteria. The specificity exhibited by the bacteriophage for a particular bacteria strain is typically attributed to the endolysin(s). Therefore, as described herein, isolated bacteriophage nucleic acids are provided that encode for the endolysins, and the purified endolysin polypeptides also are provided.

An endolysin gene from a bacteriophage described herein has a nucleic acid sequence shown in SEQ ID NO:1, 3, 5, 7, 9, or 11, and encodes an endolysin polypeptide having a sequence shown in SEQ ID NO:2, 4, 6, 8, 10, or 12, respectively. In addition to a nucleic acid sequence shown in SEQ ID NO:1, 3, 5, 7, 9 or 11, and the polypeptide sequence shown

in SEQ ID NO:2, 4, 6, 8, 10 or 12, nucleic acid and polypeptide sequences are provided that differ in sequence from SEQ ID NO:1, 3, 5, 7, 9, or 11, and SEQ ID NO:2, 4, 6, 8, 10, or 12, respectively. For example, nucleic acid sequences having at least 70% sequence identity (e.g., at least 75%, 80%, 85%, 90%, 95%, 99% or 100% sequence identity) to the nucleic acid sequence shown in SEQ ID NO:1, 3, 5, 7, 9, or 11 are provided. Similarly, amino acid sequences having at least 70% sequence identity (e.g., at least 75%, 80%, 85%, 90%, 95%, 99% or 100% sequence identity) to the amino acid sequence shown in SEQ ID NO:2, 4, 6, 8, 10, or 12 are provided.

To calculate the percent sequence identity of two sequences, the first and second sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It also will be appreciated that a single sequence can align differently with other sequences and hence, can have different percent sequence identity values over each aligned region. Two sequences can be aligned to determine percent sequence identity using the algorithm described by Altschul et al. (1997, *Nucleic Acids Res.*, 25:3389-3402), which is incorporated into BLAST (basic local alignment search tool) programs available at ncbi.nlm.nih.gov on the World Wide Web.

With respect to nucleic acids, an “isolated” nucleic acid refers to a nucleic acid that is separated from other nucleic acids that are usually associated with the isolated nucleic acid. Thus, an “isolated” nucleic acid includes, without limitation, a nucleic acid that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the organism from which the isolated nucleic acid is derived (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule. With respect to polypeptides, a “purified” polypeptide refers to a polypeptide that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered “purified” when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, or 99%) by dry weight, free from the proteins

and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is “purified.”

The nucleic acids described herein (e.g., encoding the bacteriophage endolysin polypeptide) can be introduced into vectors. Vectors, including expression vectors, are commercially available or can be produced by routine molecular biology methods. A vector containing a bacteriophage nucleic acid also can have elements necessary for expression operably linked to the bacteriophage nucleic acid, and a vector further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene) and/or sequences that can be used in purification of a polypeptide (e.g., 6xHis tag).

Elements necessary for expression include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences such as, for example, promoter sequences. Elements necessary for expression also can include introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid. As used herein, operably linked means that an element necessary for expression (e.g., a promoter and/or other regulatory element) is positioned in a vector relative to a nucleic acid coding sequence in such a way as to direct or regulate expression of the nucleic acid coding sequence.

Vectors containing a bacteriophage nucleic acid can be introduced into host cells. Methods of introducing nucleic acids into host cells are known in the art and include, without limitation, calcium phosphate precipitation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer. The term “host cell” refers not only to the particular cell but also to the progeny or potential progeny of such a cell. A host cell can be any prokaryotic or eukaryotic cell. For example, nucleic acids can be expressed in bacterial cells such as, without limitation, *E. coli*, or in insect cells, yeast cells, or mammalian cells such as Chinese hamster ovary (CHO) cells or COS cells. It would be appreciated by those skilled in the art that the natural infection process of bacteriophage can be used to introduce a nucleic acid or nucleic acid vector into a bacterial cell.

Methods of Using A Bacteriophage Composition and Bacteriophage Nucleic Acid and Polypeptide

The bacteriophage described herein, or the bacteriophage endolysin nucleic acid or polypeptide described herein, can be used in methods of reducing the number and/or growth of the *Geobacillus* bacteria on reconstituted leaf (or on any of the tobacco materials used to make reconstituted leaf), which reduces the resulting *Geobacillus*-produced biofilm on the reconstituted leaf. For example, reconstituted leaf can be contacted with the bacteriophage at any point during the process of making the reconstituted leaf or after the reconstituted leaf has been produced. In certain instances, the tobacco material (e.g., tobacco stems, tobacco leaves, tobacco solubles) can be contacted with the bacteriophage prior to being used in or made into reconstituted leaf. Contacting reconstituted leaf (or tobacco material prior being made into reconstituted leaf) with the bacteriophage described herein reduces the amount of biofilm present on the reconstituted leaf. Since the presence of biofilm results in holes in the reconstituted leaf, the treated reconstituted leaf has fewer holes, which increases yield and decreases waste.

Since biofilm is present in a number of different environments (e.g., hospitals, kitchens, bathrooms, in fluid-carrying pipes (e.g., carrying water, milk, oil, fuel, or sewage), on boat hulls, on plants or trees, in the oral cavities of animals, and/or in paper- or pulp-making facilities), and since at least a portion of this biofilm is *Geobacillus*-produced biofilm, the bacteriophage described herein can be used to reduce or eliminate the biofilm that is present in these different environments.

As used herein, a reduction (e.g., a statistically significant reduction) in the number of viable bacteria means a reduction in the number of bacteria that are alive and capable of, for example, replication. For example, lysed bacteria or bacteria in the process of lysing are not considered viable. The viability of bacteria can be determined using methods routinely used in microbiology. In addition, preventing or reducing the amount of biofilm means that the surface area containing biofilm is reduced or the volume of the biofilm on a surface is reduced relative to a “control” surface that has not been contacted with a bacteriophage. These reductions (i.e., in the number of viable bacteria or the amount of biofilm) in the presence of any of the bacteriophage (or endolysin nucleic acid or polypeptide) described herein are a result of the lytic activity exerted by the bacteriophage (or endolysin nucleic acid

or polypeptide) on the bacteria. As used herein, an “effective amount” of a bacteriophage or of an endolysin nucleic acid or polypeptide is an amount that results in lysis of bacteria in an amount or at a rate that is sufficient to reduce the number of viable bacteria or the amount of biofilm present to a desired level. As used herein, “statistically significantly” refers to a p-value of less than 0.05 (e.g., less than 0.025 or 0.01) using an appropriate measure of statistical significance (e.g., a one-tailed two-sample t-test).

Methods of Obtaining Bacteriophage Compositions

Methods of obtaining bacteriophage are known in the art. See, for example, *Bacteriophages: Methods and Protocols*, Volume 1: Isolation, Characterization, and Interactions (Methods in Molecular Biology), Eds, Clokie & Kropinski, 2010, Humana Press; Seeley et al., 1982, J. Applied Bacteriol., 53:1-17; Pope et al., 2011, PLoS ONE, 6:e16329; and Hendrix et al., 1999, PNAS USA, 96:2192-7. Briefly, bacteria of interest (e.g., the target bacteria) are obtained, generally using standard culture methods. Typically, bacteria are cultured in such a way so as to activate the lytic phase of bacteriophage native to the bacteria and cause lysis. Following lysis of the bacteria, the bacteriophage is collected and can be characterized using any number of known methods such as, without limitation, nucleic acid sequencing, electron microscopy, burst size, and/or attachment rate. Bacteriophage also can be described based on their host (i.e., host profiling).

Tobacco Products

Tobacco products for adult tobacco consumers are provided that contain tobacco (e.g., whole leaf, stems, cut, chopped or comminuted leaf or stem, or reconstituted leaf) that has been contacted with one or more bacteriophage (or endolysin nucleic acids or polypeptides). In some instances, the one or more bacteriophage include the bacteriophage described herein.

Under certain circumstances, the tobacco or reconstituted leaf can undergo one or more treatments in order to remove or inactivate the bacteriophage once the amount and/or growth of the respective bacteria has reached an acceptable level. However, since bacteriophage are in the generally recognized as safe (GRAS) category, the bacteriophage may be present in the final tobacco product.

Tobacco products are known in the art and include any product made or derived from tobacco that is intended for human consumption, including any component, part, or accessory of a tobacco product. Representative tobacco products include, without limitation, smokeless tobacco products, tobacco-derived nicotine products, cigarillos, non-ventilated recess filter cigarettes, vented recess filter cigarettes, cigars, snuff, pipe tobacco, cigar tobacco, cigarette tobacco, chewing tobacco, leaf tobacco, shredded tobacco, and cut tobacco. Representative smokeless tobacco products include, for example, chewing tobacco, snus, pouches, films, tablets, coated dowels, rods, and the like. Representative cigarettes and other smoking articles include, for example, smoking articles that include filter elements or rod elements, where the rod element of a smokeable material can include cured tobacco within a tobacco blend. In addition to the tobacco described herein (i.e., that includes one or more bacteriophages), tobacco products also can include other ingredients such as, without limitation, binders, plasticizers, stabilizers, and/or flavorings. See, for example, US 2005/0244521, US 2006/0191548, US 2012/0024301, US 2012/0031414, and US 2012/0031416 for examples of tobacco products. Suitable packaging is known for the various types of tobacco products.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

EXAMPLES

Example 1—Bacteriophage Endolysins

Geobacillus bacteriophages isolated by Mircleos BV (The Netherlands), designated G01 – G09, were sequenced to determine the sequence identity among the bacteriophages and to identify the endolysin sequence from each. Six of the nine bacteriophages had readily identifiable endolysin sequences. Out of the 6 endolysins identified, 3 were 99% similar (G05, G08, G09). The sequences of the endolysins are shown in the attached Appendix A.

Example 2—Cloning and Expression of the Endolysin Gene

The endolysin gene from G05 was then cloned into an expression vector using conventional PCR techniques, and the expression vector was transformed into BL21-AI™ ONESHOT® Chemically Competent *E. coli* cells. Following expression, the protein was isolated and run on an SDS-PAGE gel.

Given the homology between the endolysin gene of G05 and the endolysin gene of G08 and G09, the same PCR conditions, including primer sequences, were used to clone the endolysin gene from each of the G05, G08 and G09 bacteriophages.

Example 3—Evaluating Endolysin Activity

The transformed organisms containing the endolysin gene are applied to *Geobacillus* in pure culture, in strong brown water (SBW), and on tobacco matrices to test the efficacy of the endolysin on bacterial cell lysis. The purified protein is used in various tobacco matrices such as reconstituted leaf (RL) to prevent biofilm formation.

Example 4—Use of Bacteriophage in Reconstituted Leaf (RL)

Geobacillus is a biofilm-producing bacterial organism that is obligately thermophilic and facultatively anaerobic. When it produces a biofilm on tobacco (e.g., reconstituted leaf), the congealed material interferes with the further processing of the tobacco.

To determine the effectiveness of the bacteriophage against *Geobacillus* as described herein, the bacteriophage was inoculated into fresh 1/10 Tryptic Soy Broth (TSB) with Yeast Extract (YE), collectively “TSBYE,” and incubated at 55°C for up to four hours (OD between 0.5 and 0.9). The culture was then inoculated into fresh TSBYE containing either 1:1 or 1:10 ratio of Phage:TSBYE, SM Buffer:TSBYE (negative control), and TSBYE. The mixtures were incubated at 55°C overnight and then serially diluted 1/10 to 10⁻⁵, and plated in duplicate on Tryptic Soy Agar with Yeast Extract (TSAYE). The plates were incubated at 60°C overnight. The addition of the phage inhibited the growth of *Geobacillus*.

Samples from the reconstituted leaf process were collected and stored at 4°C (native) or sterilized by passing sequentially through 0.45 micron and 0.22 micron filters and stored at 4°C (sterile). The samples were then inoculated with mid-log phase *Geobacillus*, SM buffer (negative control) or the bacteriophage. The samples were also supplemented with TSBYE

to allow for growth. The mixtures were incubated at 55°C overnight and then serially diluted 1/10 to 10^{-5} , and plated in duplicate on TSAYE. The plates were incubated at 60°C overnight. Results showed that the phage inhibited the growth of *Geobacillus*.

To determine the effectiveness of the cloned endolysin against *Geobacillus* described herein was inoculated into fresh TSBYE and incubated at 55C for up to four hours (i.e., to an OD of between 0.5 and 0.9). A 1:10 inoculation of the culture was transformed into fresh TSBYE containing endolysin with a range of 0 to 200 µg/ml. The prepared samples were then placed in a microplate reader and monitored for growth at an OD₆₀₀ for 24 hours at 50C. Readings were taken every 20 minutes with shaking between each reading. See Figure 1.

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

WHAT IS CLAIMED IS:

1. An isolated bacteriophage having lytic activity against *Geobacillus*, wherein the bacteriophage comprises a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

2. The isolated bacteriophage of claim 1, wherein the nucleic acid sequence has at least 99% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

3. The isolated bacteriophage of claim 1, wherein the nucleic acid sequence has a sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

4. The isolated bacteriophage of claim 1, wherein the endolysin encoded by the nucleic acid sequence has an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

5. An isolated bacteriophage having lytic activity against *Geobacillus*, wherein the bacteriophage comprises a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

6. The isolated bacteriophage of claim 5, wherein the endolysin has at least 99% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

7. The isolated bacteriophage of claim 5, wherein the endolysin has an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

8. An isolated nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleic acid sequence having at least 95% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

9. The isolated nucleic acid molecule of claim 8, wherein the nucleic acid molecule comprises a nucleic acid sequence having at least 99% sequence identity to a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

10. The isolated nucleic acid molecule of claim 8, wherein the nucleic acid molecules comprise a nucleic acid sequence having a nucleic acid sequence selected to the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11.

11. The isolated nucleic acid molecule of claim 8, wherein the nucleic acid molecule encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

12. A vector comprising the isolated nucleic acid of any of claims 8-11.

13. A host cell comprising the vector of claim 12.

14. A purified polypeptide comprising an amino acid sequence having at least 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

15. The purified polypeptide of claim 14, the amino acid sequence having at least 99% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

16. The purified polypeptide of claim 14, the amino acid sequence having an sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, and 12.

17. A method of making a polypeptide, comprising:
culturing the host cell of claim 13 under appropriate conditions.
18. A method for reducing the number of viable *Geobacillus* in tobacco,
comprising:
contacting tobacco with an effective amount of a composition comprising the
isolated bacteriophage of any of claims 1-7, the isolated nucleic acid of any of claims 8-11,
the vector of claim 12, the host cell of claim 13, or the purified polypeptide of any of claims
14-16.
19. The method of claim 18, wherein the tobacco is reconstituted leaf.
20. Reconstituted tobacco leaf comprising the isolated bacteriophage of any of
claims 1-7, the isolated nucleic acid of any of claims 8-11, the vector of claim 12, the host
cell of claim 13, or the purified polypeptide of any of claims 14-16.
21. A tobacco product comprising the reconstituted leaf of claim 20.
22. The tobacco product of claim 21, wherein the tobacco product is a cigarette.

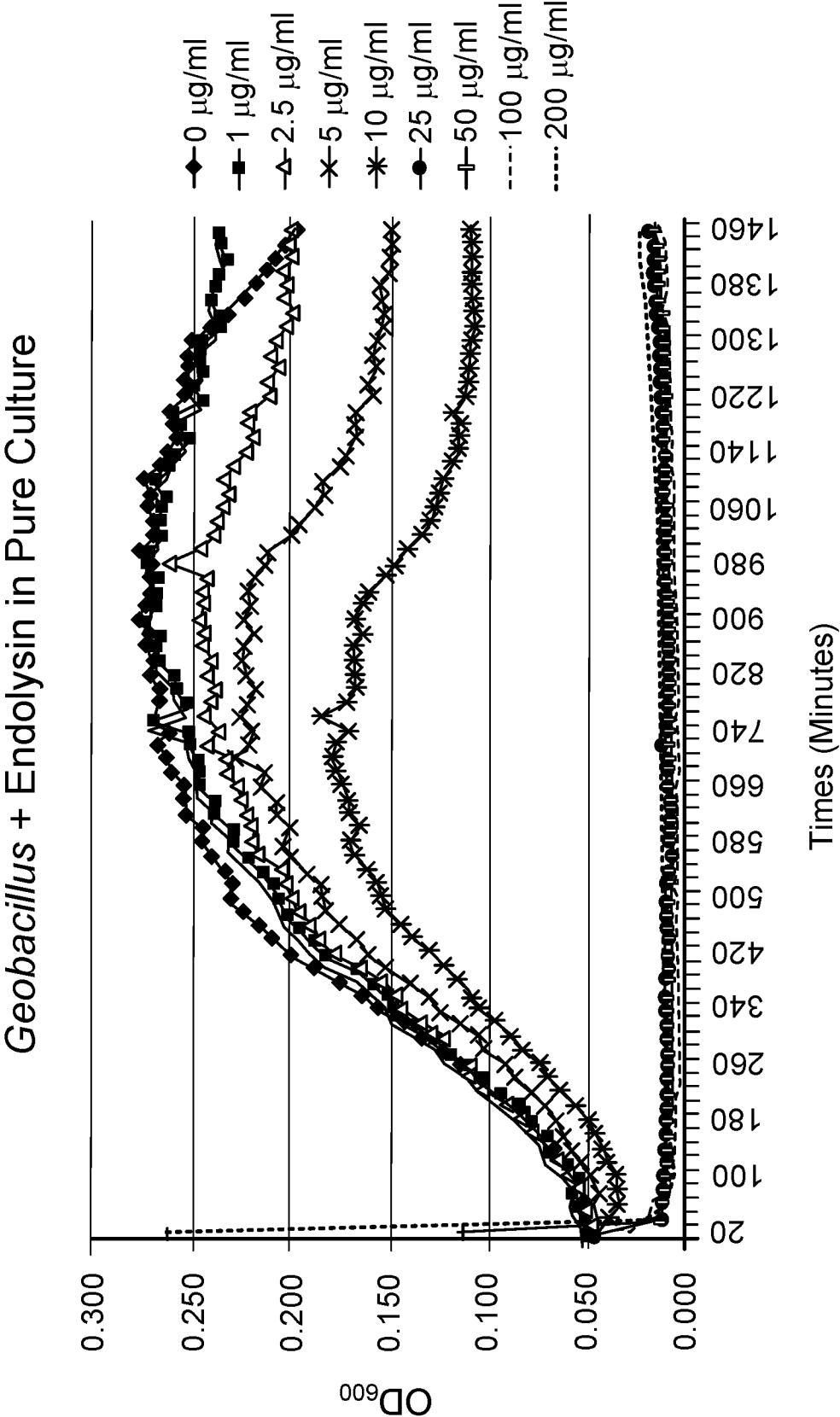


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/015516

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N7/00 C07K14/005 C12N15/33 C12N5/10 A24B15/20
C12N9/80

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/150870 A2 (ALTRIA CLIENT SERVICES INC [US]) 25 September 2014 (2014-09-25) the whole document	1-22
A	WO 2009/012481 A1 (INTEGRATED PLANT GENETICS INC [US]; UNIV FLORIDA [US]; REDDY JOSEPH D) 22 January 2009 (2009-01-22) the whole document	1-22
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/015516

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