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(54) Title: BACTERIOPHAGE AND METHODS OF USING

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

BACTERIOPHAGE AND METHODS OF USING

TECHNICAL FIELD

This disclosure generally relates to 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 the progeny bacteriophage.

SUMMARY

This disclosure describes bacteriophage, as well as methods of making and using such bacteriophage.

In one aspect, an isolated bacteriophage having lytic activity against *Carnobacteriaceae* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:1. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:1. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:1. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:2.

In another aspect, an isolated bacteriophage having lytic activity against *Carnobacteriaceae* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:2. In some embodiments, the endolysin has at least 99%

sequence identity to the amino acid sequence shown in SEQ ID NO:2. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:2.

In one aspect, an isolated nucleic acid molecule is provided. Such a nucleic acid molecule typically includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:1. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:1. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:1. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:2.

In still another aspect, a vector comprising any of the isolated nucleic acids described herein is provided. In yet another aspect, a host cell comprising a vector as described herein is provided.

In another aspect, a purified polypeptide is provided. Such a polypeptide generally includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:2. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:2. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:2.

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

In another aspect, a method for reducing the number of viable *Carnobacteriaceae* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising an isolated bacteriophage as described herein, an isolated nucleic acid as described herein, a vector as described herein, a host cell as described herein, or a purified polypeptide as described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Virgibacillus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:3. In some embodiments, the nucleic acid

sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:3. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:3. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:4.

In another aspect, an isolated bacteriophage having lytic activity against *Virgibacillus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:4. In some embodiments, the endolysin has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:4. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:4.

In still another aspect, an isolated nucleic acid molecule is provided. Generally, the nucleic acid molecule includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:3. In some embodiments, the nucleic acid molecule includes a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:3. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:3. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:4.

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

In another aspect, a purified polypeptide is provided. Typically, such a polypeptide includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:4. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:4. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:4.

In one 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 one aspect, a method for reducing the number of viable *Virgibacillus* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition that includes any of the isolated bacteriophage described herein, the isolated

nucleic acids described herein, the vectors described herein, the host cells described herein, or the purified polypeptides described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Corynebacterium* is provided.

In yet another aspect, a method for reducing the number of viable *Corynebacterium* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition that 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 some embodiments, the tobacco is contacted with the composition including the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Staphylococcus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:5. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:5. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:5. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:6.

In another aspect, an isolated bacteriophage having lytic activity against *Staphylococcus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:6. In some embodiments, the endolysin has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:6. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:6.

In one aspect, an isolated nucleic acid molecule is provided. Such a nucleic acid molecule typically includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:5. In some embodiments, the nucleic acid

molecule comprises a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:5. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:5. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:6.

In still another aspect, a vector comprising any of the isolated nucleic acids described herein is provided. In yet another aspect, a host cell comprising a vector as described herein is provided.

In another aspect, a purified polypeptide is provided. Such a polypeptide generally includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:6. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:6. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:6.

In one aspect, a method of making a polypeptide is provided. Such a method generally includes culturing a host cell as described herein under appropriate conditions. In another aspect, a method for reducing the number of viable *Staphylococcus* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising an isolated bacteriophage as described herein, an isolated nucleic acid as described herein, a vector as described herein, a host cell as described herein, or a purified polypeptide as described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Staphylococcus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:7. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:7. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:7. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:8.

In another aspect, an isolated bacteriophage having lytic activity against *Staphylococcus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:8. In some embodiments, the endolysin has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:8. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:8.

In one aspect, an isolated nucleic acid molecule is provided. Such a nucleic acid molecule typically includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:7. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:7. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:7. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:8.

In still another aspect, a vector comprising any of the isolated nucleic acids described herein is provided. In yet another aspect, a host cell comprising a vector as described herein is provided.

In another aspect, a purified polypeptide is provided. Such a polypeptide generally includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:8. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:8. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:8.

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

In another aspect, a method for reducing the number of viable *Staphylococcus* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising an isolated bacteriophage as described herein, an isolated nucleic acid as described herein, a vector as described herein, a host cell as described herein, or a purified polypeptide as described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Carnobacteriaceae* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:9. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:9. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:9. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:10.

In another aspect, an isolated bacteriophage having lytic activity against *Carnobacteriaceae* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:10. In some embodiments, the endolysin has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:10. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:10.

In one aspect, an isolated nucleic acid molecule is provided. Such a nucleic acid molecule typically includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:9. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:9. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:9. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:10.

In still another aspect, a vector comprising any of the isolated nucleic acids described herein is provided. In yet another aspect, a host cell comprising a vector as described herein is provided.

In another aspect, a purified polypeptide is provided. Such a polypeptide generally includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:10. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:10. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:10.

In one aspect, a method of making a polypeptide is provided. Such a method generally includes culturing a host cell as described herein under appropriate conditions. In another aspect, a method for reducing the number of viable *Carnobacteriaceae* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising an isolated bacteriophage as described herein, an isolated nucleic acid as described herein, a vector as described herein, a host cell as described herein, or a purified polypeptide as described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In one aspect, an isolated bacteriophage having lytic activity against *Virgibacillus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin, wherein the nucleic acid sequence has at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:11. In some embodiments, the nucleic acid sequence has at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:11. In some embodiments, the nucleic acid sequence has the sequence shown in SEQ ID NO:11. In some embodiments, the endolysin encoded by the nucleic acid sequence has the amino acid sequence shown in SEQ ID NO:12.

In another aspect, an isolated bacteriophage having lytic activity against *Virgibacillus* is provided. Such a bacteriophage generally includes a nucleic acid sequence encoding an endolysin having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the endolysin has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the endolysin has the amino acid sequence shown in SEQ ID NO:12.

In one aspect, an isolated nucleic acid molecule is provided. Such a nucleic acid molecule typically includes a nucleic acid sequence having at least 95% sequence identity to the nucleic acid sequence shown in SEQ ID NO:11. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having at least 99% sequence identity to the nucleic acid sequence shown in SEQ ID NO:11. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence having the sequence shown in SEQ ID NO:11. In some embodiments, the nucleic acid molecule encodes a polypeptide having the amino acid sequence shown in SEQ ID NO:12.

In still another aspect, a vector comprising any of the isolated nucleic acids described herein is provided. In yet another aspect, a host cell comprising a vector as described herein is provided.

In another aspect, a purified polypeptide is provided. Such a polypeptide generally includes an amino acid sequence having at least 95% sequence identity to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the amino acid sequence has at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO:12. In some embodiments, the amino acid sequence has the sequence shown in SEQ ID NO:12.

In one aspect, a method of making a polypeptide is provided. Such a method generally includes culturing a host cell as described herein under appropriate conditions. In another aspect, a method for reducing the number of viable *Virgibacillus* in tobacco is provided. Such a method typically includes contacting tobacco with an effective amount of a composition comprising an isolated bacteriophage as described herein, an isolated nucleic acid as described herein, a vector as described herein, a host cell as described herein, or a purified polypeptide as described herein. In some embodiments, the tobacco is contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco. In some embodiments, the method reduces the level of TSNAs in the tobacco.

In another aspect, tobacco comprising one or more bacteriophages against a bacteria selected from the group consisting of *Carnobacteriaceae*, *Virgibacillus*, *Staphylococcus* and *Corynebacterium* is provided. In some embodiments, such a tobacco is aged and cured. In some embodiments, the bacteriophage is selected from any of the bacteriophage described herein.

In one aspect, a tobacco product is provided that includes such tobacco. Representative tobacco product 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.

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 photograph showing the plaques resulting from infection of *Carnobacteriaceae* with the bacteriophage described herein.

Figure 2 is a photograph showing the plaques resulting from infection of *Virgibacillus* with the bacteriophage described herein.

Figure 3 is a photograph showing the plaques resulting from infection of *Corynebacterium* with the bacteriophage described herein.

Figure 4 is a SDS-PAGE gel of cytosolic-expressed proteins in cell lysates and after IMAC purification.

Figure 5 is a SDS-PAGE gel of cytosolic-expressed proteins in cell lysates and after IMAC purification.

Figure 6 is a graph showing endolysin activity in a turbidity reduction assay of PlyStaph and derivatives against S. cohnii.

Figure 7 are photographs showing GFP-CBDStaph binding to S. cohnii.

Figure 8 is a graph showing PlyCarno activity in a turbidity reduction assay. Note that lysis could not be detected. The increase in turbidity in the control is likely to be the result of aggregate formation.

Figure 9 are photographs showing GFP-CBDCarno binding.

Figure 10 is a graph showing PlyVirgi activity in a turbidity reduction assay. Please note, that GFP-CBDVirgi protein was not found active. The reason is poor protein quality, rather than a lack of function.

Figure 11 is a graph showing PlyTet activity in a turbidity reduction assay.

Figure 12 are photographs showing GFP-CBDTet binding.

Figure 13A is a graph showing the effectiveness of a combination of endolysins from two different bacteriophage against *Staphylococcus* in culture.

Figure 13B is a graph showing the effectiveness of a combination of endolysins from two different bacteriophage against *Staphylococcus* in moist smokeless tobacco (MST) over 7 days in the fiberboard can.

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 the production of unwanted tobacco-specific nitrosamines (TSNAs).

Bacteriophage Compositions

A number of isolated bacteriophage 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.

The endolysin gene from the bacteriophage against *Carnobacteriaceae* has the nucleic acid sequence shown in SEQ ID NO:1 and encodes an endolysin polypeptide having the sequence shown in SEQ ID NO:2; the endolysin gene from the bacteriophage against *Virgibacillus* has the nucleic acid sequence shown in SEQ ID NO:3 and encodes a polypeptide having the sequence shown in SEQ ID NO:4; the endolysin gene from the bacteriophage against *Staphylococcus* has the nucleic acid sequence shown in SEQ ID NO:5 and encodes an endolysin polypeptide having the sequence shown in SEQ ID NO:6; the endolysin gene from the bacteriophage against *Staphylococcus* has the nucleic acid sequence shown in SEQ ID NO:7 and encodes a polypeptide having the sequence shown in SEQ ID NO:8; the endolysin gene from the bacteriophage against *Carnobacteriaceae* has the nucleic acid sequence shown in SEQ ID NO:9 and encodes an endolysin polypeptide having the sequence shown in SEQ ID NO:10; and the endolysin gene from the bacteriophage against *Virgibacillus* has the nucleic acid sequence shown in SEQ ID NO:11 and encodes a polypeptide having the sequence shown in SEQ ID NO:12.

In addition to the nucleic acid sequences shown in SEQ ID NOs:1, 3, 5, 7, 9 and 11, and the polypeptide sequences shown in SEQ ID NOs: 2, 4, 6, 8, 10 and 12, nucleic acid and polypeptide sequences are provided that differ in sequence from SEQ ID NOs: 1, 3, 5, 7, 9 and 11, and SEQ ID NOs: 2, 4, 6, 8, 10 and 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 any of the nucleic acid sequences shown in SEQ ID NOs: 1, 3, 5, 7, 9 and 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 any of the amino acid sequences shown in SEQ ID NOs: 2, 4, 6, 8, 10 and 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 polypeptides) 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 Bacteriophage Compositions and Bacteriophage Nucleic Acids and Polypeptides

Any of the bacteriophages described herein (i.e., bacteriophage against *Carnobacteriaceae* sp., *Virgibacillus* sp., *Staphylococcus* sp. and *Corynebacterium* sp.), or the endolysin nucleic acids or polypeptides from any of the bacteriophage described herein, can be used in methods of reducing the number and/or growth of *Carnobacteriaceae* sp., *Virgibacillus* sp., *Staphylococcus* sp. or *Corynebacterium* sp. bacteria. For example, tobacco (e.g., blends of tobacco used to manufacture smokeless tobacco products) can be contacted with an effective amount of any of the bacteriophages described herein, or any of the bacteriophage endolysin nucleic acids or polypeptides described herein. The tobacco can be contacted with an effective amount of one or more of the indicated bacteriophage, or an endolysin nucleic acid or polypeptide, prior to, during and/or after fermentation of the tobacco, and/or at the finishing stage.

Briefly, after harvesting, tobacco can be cured using conventional means, e.g., air curing, fire curing, barn curing, sun curing. See, for example, Tso (1999, Chapter I in Tobacco, Production, Chemistry and Technology, Davis & Nielsen, Eds., Blackwell Publishing, Oxford). Optionally, cured tobacco then can be conditioned and/or fermented. Conditioning includes, for example, a heating, sweating or pasteurization step as described in

U.S. Publication Nos. 2004/0118422 or 2005/0178398. Fermenting typically is characterized by high initial moisture content, heat generation, and a 10 to 20% loss of dry weight. See, for example, US Patent Nos. 4,528,993; 4,660,577; 4,848,373; and 5,372,149. Cured or cured and fermented tobacco then can be further processed (e.g., cut, expanded, blended, milled or comminuted).

Contacting tobacco during the processing and finishing of the products with any of the bacteriophage or bacteriophage endolysins described herein (e.g., bacteriophage against *Carnobacteriaceae*, *Virgibacillus*, or *Corynebacterium*) results in a number of benefits or improvements to the tobacco including, without limitation, a reduction in the level of TSNAs in the tobacco, and an increased shelf-life of the tobacco product. A reduction in the level of TSNAs is defined as a reduction in at least 10% (e.g., at least 15%, 20%, 25%, 30%, 40%, 50% or more) TSNAs in bacteriophage-contacted tobacco relative to tobacco not contacted with bacteriophage. The shelf life of a tobacco product is increased if the tobacco in the tobacco product maintains its sensory characteristics (e.g., mouth feel, flavor profile, etc.) for a longer period of time than a comparable tobacco product containing tobacco cured and processed under comparable conditions but without bacteriophage (a "control" tobacco product). Under certain circumstances, the shelf life of the tobacco product containing the bacteriophage-contacted tobacco is statistically significantly longer than the shelf life of a control tobacco product. 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).

As used herein, a 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. These reductions (i.e., in the number of viable bacteria) 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 to a desired level.

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 as 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, and cut, chopped or comminuted leaf or stem) that has been contacted with one or more bacteriophage (i.e., bacteriophage against *Carnobacteriaceae* sp., *Virgibacillus* sp., *Staphylococcus* sp. or *Corynebacterium* sp., or endolysin nucleic acids or polypeptides from any of such bacteriophages).

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. For example, in certain embodiments, one or more bacteriophage (or one or more endolysin proteins) can be present in a final tobacco product, such as, without limitation, a container of moist smokeless tobacco, in loose form or in a pouch.

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—Isolation of Bacteriophages from Tobacco

Smokeless tobacco products and tobacco materials were used to isolate bacteriophages. 30 grams of the solid tobacco samples was added to 270 g of the low salt (5%) diluent in a filtered stomacher bag. The sample was mixed using a stomacher for 3 minutes at 200 RPM. The sample was then poured from the filtered side of the stomacher bag into a centrifuge tube and centrifuged for 30 minutes at 11,000 xg. The supernatant was poured off and passed sequentially through a 0.45 micron and 0.22 micron filter. The sterile filtrate was subjected to ultracentrifugation. 15 ml of the filtrate was added to the Amicon Ultra-15 Centrifugal Filter Device. The devices were centrifuged for 30 minutes at 1,500 xg

to concentrate and separate the phages from the filtrate. 15 ml of the filtrate was then concentrated to 250 to 500 µl.

The filtrates were then combined 1:1 with 2X Tryptic Soy Broth (TSB), 2X low salt broth, 2X high salt broth, 2X 15% salt broth (pH 8), 2X 10% salt broth (pH 9 and pH 7.4). Each of the filtrate broth combinations, now referred to as enrichments, were then inoculated with 1 ml of a turbid culture of interest. *Carnobacteriaceae, Virgibacillus, Staphylococcus* and *Corynebacterium* were separately inoculated into each of the enrichments and incubated for 21, 24 and 18 days, respectively. 2 ml of the enrichment was removed after incubation and centrifuged for 1 minute at 13,000 RPM. The supernatant was passed through a sterile 0.22 micron filter and placed into a sterile microcentrifuge tube. 10 µl of the sterile filtrates were then dropped on to the appropriate agars with the corresponding soft agars on top. The soft agars contained 100 µl of the appropriate culture for which it was enriched. The spot plates were left to absorb into the agar and then incubated at 32°C until clear lysis zones developed. The enrichments were placed back into the incubator and processed 4 to 6 more times as stated above before the enrichment series was stopped.

Upon observation of a clear lysis zone (plaque), the plate was removed from the incubator and the plaque was harvested for isolation. The wide end of a 1000 µl tip was placed over the plaque and gently dug into the soft agar overlay of the plate. The soft agar plug was then placed into 1 ml of SM buffer and refrigerated at 4°C overnight to allow for diffusion of the bacteriophage. 10 µl of the SM buffer containing the phage was then dropped onto the appropriate soft agar with the appropriate strain in the soft agar. The plate was then incubated at 32°C to confirm lysis of the bacterial strain.

Using these methods, several bacteriophage were identified that are specific against the *Carnobacteriaceae, Virgibacillus, Staphylococcus* and *Corynebacterium* bacteria.

Example 2—Bacteriophage Plaque Formation

Figures 1, 2, and 3 show the inhibition of the target bacterial strains in the presence of the phage on soft agar plates. Figure 1 shows a lawn of *Carnobacteriaceae* from tobacco ultrafiltrate growing on 10% salt agar. The circled area shows a plaque. Figure 2 shows a lawn of *Virgibacillus* from tobacco ultrafiltrate and Great Salt Lake sterile filtrate (0.22 micron) growing on 5% salt agar. The circled areas show plaques. Figure 3 shows a lawn of

Corynebacterium and Great Salt Lake ultrafiltrate growing on 10% salt agar. The circled areas show plaques. Great Salt Lake water samples originally were used as a source of halophilic bacteria and their corresponding bacteriophage; once isolated, use of water samples continued in the culture.

Example 3—*In-silico* Analysis of Bacteriophage Genomes

Phage sequence contigs were screened for potential endolysin sequences. All six reading frames of all sequences were translated into amino acid sequences. The obtained amino acid sequences were searched using PFAM domain homology for the identification of potential lytic domain and cell wall binding domains.

Example 4—Cloning of Candidate Endolysin Sequences

Artificial *E. coli* codon optimized gene sequences for candidate proteins were designed and synthesized. The sequences were cloned into BamHI/SalI sites of pQE30 protein expression plasmids and transformed into *E. coli* XL1BlueMRF` hosts. Sequence integrities were confirmed by sequencing.

Several derivative endolysin sequences were constructed for comparison purposes. Inter-domain linker sequences were estimated and protein expression vectors harboring the putative cell wall binding domain (CBD) fused to green fluorescence protein (GFP) were constructed. The GFP-CBD fusions were generated to evaluate binding properties of the endolysins to their target cells. CBD sequences were in-frame ligated into SacI/SalI sites of pHGFP vector (Loessner et al., 2002, Mol. Microbiol., 44:335-49).

Example 5—Protein Over-Expression and Partial Purification

Recombinant protein production was performed in Luria Bertani-broth under IPTG induction at 20°C for 16 h. Cells were lysed in a cell pressure homogenizer and 6x-His tagged proteins were partially purified from cell lysates by immobilized metal affinity chromatography (IMAC) using Zinc loaded IMAC sepharose (GE Healthcare).

Example 6—Lysis and Binding Assays

Purified recombinant endolysins were diluted with PBS buffer (pH 7.4) to a concentration of 2 μM and mixed in a 1:1 ratio with substrate cells adjusted to an optical density at 600 nm (OD600nm) with the same buffer. Drop in OD600nm was subsequently monitored for up to 1 hour.

Binding of GFP_CBD proteins to target cells was done by mixing 20 μg proteins with cells from 1 ml culture with an OD600nm of about 1. Cells were washed twice with 1 ml PBS pH 7.4. Protein binding was evaluated with epi-fluorescence microscopy and images were taken using a confocal laser scanning microscope.

Example 7—Results

PlyStaph is composed of two domains with the N-terminal domain having homology to Amidase_2 (PFAM01510) domains and the C-terminal domain having homology to SH3_5 (PFAM08460) domains. Usually, endolysins with Staphylococcal background are built of 3 individually folded domains with a Cysteine-Histidine-dependent Amidase/Peptidase domain (CHAP) at the N-terminal end (e.g., CHAP-Amidase-SH3b). In many cases, the CHAP domain contributes most to catalytic activity when applied as exolysins, whereas the amidase domain seems to be virtually inactive. To account for this, a CHAP domain was fused to the N-terminal of PlyStaph and designated "Artificial Phage lysine Staph" or "ArtPlyStaph"). The CHAP domain was identified in a putative structural phage protein found in the same genome. This protein construct was also IMAC purified, but seemed to have some contaminating proteins or degradation products (Figure 4). Finally, this CHAP domain was also directly fused to the cell wall binding domain of PlyStaph ("TCHAP-CBDStaph").

All proteins were successfully expressed and partially purified (with the exception of GFP-CBDVirgi, which was not detectable in purified samples). The protein data are provided in Table 1, and Figures 4 and 5.

Activity data were collected in a turbidity reduction setup in PBS buffer with 0.1% Tween20 (pH 7.4). Buffer only served as control. Binding of GFP-tagged CBDs to target strains was evaluated under epi-fluorescent light and imaged using a confocal scanning light microscopy setup. The results are presented in Figures 6 to 12.

Table 1: Protein concentrations after recombinant expression in 700 ml LB-PE medium and IMAC purification

Protein	Concentration [mg/ml]	Volume [ml]	Total amount [mg]
PlyMarini	2.57	3	7.7
GFP-CBDMarini	4.51	3.2	14.43
PlyVirgi	3.33	2.7	8.99
GFP-CBDVirgi	3.16 (poor quality)	1.2	3.8
PlyStaph	1.23	4	4.92
ArtPlyStaph	1.5	4	6
TCHAP-CBDStaph	1.97	2	3.94
GFP-CBDStaph	4.55	2.5	11.38
PlyTet	7.75	2.8	21.69
GFP-CBDTet	1.96	3.2	6.26

Example 8—Endolysin Sequences

Endolysin sequences are provided in SEQ ID NOs: 1-12.

SEQ ID NOs: 1 and 2 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Carnobacteriaceae* bacteria.

SEQ ID NOs: 3 and 4 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Virgibacillus* bacteria.

SEQ ID NOs: 5 and 6 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Staphylococcus* bacteria.

SEQ ID NOs: 7 and 8 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Staphylococcus* bacteria.

SEQ ID NOs: 9 and 10 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Carnobacteriaceae* bacteria.

SEQ ID NOs: 11 and 12 are the nucleic acid and polypeptide sequences, respectively, of an endolysin from phage against *Virgibacillus* bacteria.

Example 9—Endolysin Application in Pure Cultures

To determine the effectiveness of the cloned endolysins against *Carnobacteriaceae, Virgibacillus, and Staphylococcus,* the bacteria was inoculated into fresh 2X low salt (*Virigibacillus* and *Staphylococcus*) or medium salt broth (*Carnobacteriaceae*) and incubated at 32ºC for 1-7 days to achieve mid-log growth. The cultures were normalized to an OD_{600} of 1 using Phosphate Buffered Saline (PBS) with 0.1% Tween 20 at a pH of 7.4. The respective endolysin was added at a concentration of 1 μM and incubated for 24 hours at 32ºC. Samples were taken at various time points for OD_{600} readings and for microbial enumeration. Representative data with *Staphylococcus* and a combination of endolysins from two different bacteriophage against *Staphylococcus* (referred to in Table 1 as "Ply" + "TCHAP-CBD", which correspond to SEQ ID NOs: 6 and 8, respectively) is shown in Table 2 and Figure 13A.

Table 2

	OD600		
Sample Point	Negative Control	*Staphylococcus*	*Staphylococcus* Ply+TCHAP-CBD
T0	0	0.79	0.1
30 min	0	1.12	0.03
2 hrs	0	1.85	0.09
24 hrs	0	1.76	0.08

Example 10- Bacteriophage or Endolysin Application in Moist Smokeless Tobacco

Moist smokeless tobacco (MST) was inoculated with *Staphylococcus* sp. at a final concentration of 1.81×10^6 cfu/g (log 6.26). Bacteriophage against *Staphylococcus* as described herein was added to tobacco at a final concentration of 1×10^{11} pfu/g (log 11). The tobacco was mixed for three minutes on medium speed using a kitchen aid mixer to ensure complete mixing and contact of the bacteria and the phage. The endolysins from each of the bacteriophage against *Staphylococcus* described herein (referred to in Table 2 as "Ply" + "TCHAP-CBD", which correspond to SEQ ID NOs: 6 and 8, respectively) were added to the tobacco at a final concentration of 120 μg/g (total). The tobacco was mixed for three minutes on medium speed using a kitchen aid mixer to ensure complete mixing and contact of the bacteria and the endolysin. The tobacco samples were monitored for growth of

Staphylococcus for 7 days after packing in fiberboard cans. Results demonstrated that *Staphylococcus* was inhibited by the bacteriophage and endolysin over 7 days in the fiberboard can. See Table 3 and Figure 13B.

Table 3

Sample	Sample Point	Dilution	Plate 1	Plate 2	CFU/g	Log
Control MST	T0	10	0	0	0.00E+00	0.00
MST+ *Staphylococcus*	T0	1000	94	87	1.81E+06	6.26
MST+*Staphylococcus*+P4	T0	1000	60	66	1.26E+06	6.10
MST+*Staphylococcus*+Ply+TCHAP-CBD	T0	10000	13	13	2.60E+06	6.41
MST Control	Day 1	10	0	0	0.00E+00	0.00
MST+ *Staphylococcus*	Day 1	1000	27	26	5.30E+05	5.72
MST+*Staphylococcus*+P4	Day 1	100	2	2	4.00E+03	3.60
MST+*Staphylococcus*+Ply+TCHAP-CBD	Day 1	1000	38	32	6.99E+05	5.84
MST Control	Day 2	10	0	0	0.00E+00	0.00
MST+ *Staphylococcus*	Day 2	1000	14	15	2.90E+05	5.46
MST+*Staphylococcus*+P4	Day 2	100	3	9	1.20E+04	4.08
MST+*Staphylococcus*+Ply+TCHAP-CBD	Day 2	1000	18	21	3.90E+05	5.59
MST Control	Day 5	10	0	0	0.00E+00	0.00
MST+ *Staphylococcus*	Day 5	1000	15	13	2.80E+05	5.45
MST+*Staphylococcus*+P4	Day 5	100	2	4	6.00E+03	3.78
MST+*Staphylococcus*+Ply+TCHAP-CBD	Day 5	100	64	60	1.24E+05	5.09
MST Control	Day 7	10	0	0	0.00E+00	0.00
MST+ *Staphylococcus*	Day 7	1000	10	6	1.60E+05	5.20
MST+*Staphylococcus*+P4	Day 7	10	31	36	6.69E+03	3.83
MST+*Staphylococcus*+Ply+TCHAP-CBD	Day 7	100	30	47	7.69E+04	4.89

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, 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 from the group consisting of SEQ ID NO: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 from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and 11.

3. The isolated bacteriophage of claim 1, wherein the nucleic acid sequence has a sequence selected from the group consisting of SEQ ID NO: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 NO:2, 4, 6, 8, 10 and 12.

5. An isolated bacteriophage having lytic activity against *Carnobacteriaceae*, 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 NO: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 NO: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 NO: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 from the group consisting of SEQ ID NO: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 from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and 11.

10. The isolated nucleic acid molecule of claim 8, wherein the nucleic acid molecule comprises a nucleic acid sequence having a sequence selected from the group consisting of SEQ ID NO: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 NO: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 NO: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 NO:2, 4, 6, 8, 10 and 12.

16. The purified polypeptide of claim 14, the amino acid sequence having an amino acid sequence selected from the group consisting of SEQ ID NO: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 bacteria 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 contacted with the composition comprising the bacteriophage prior to fermentation of the tobacco.

20. The method of claim 18, wherein the method reduces the level of TSNAs in the tobacco.

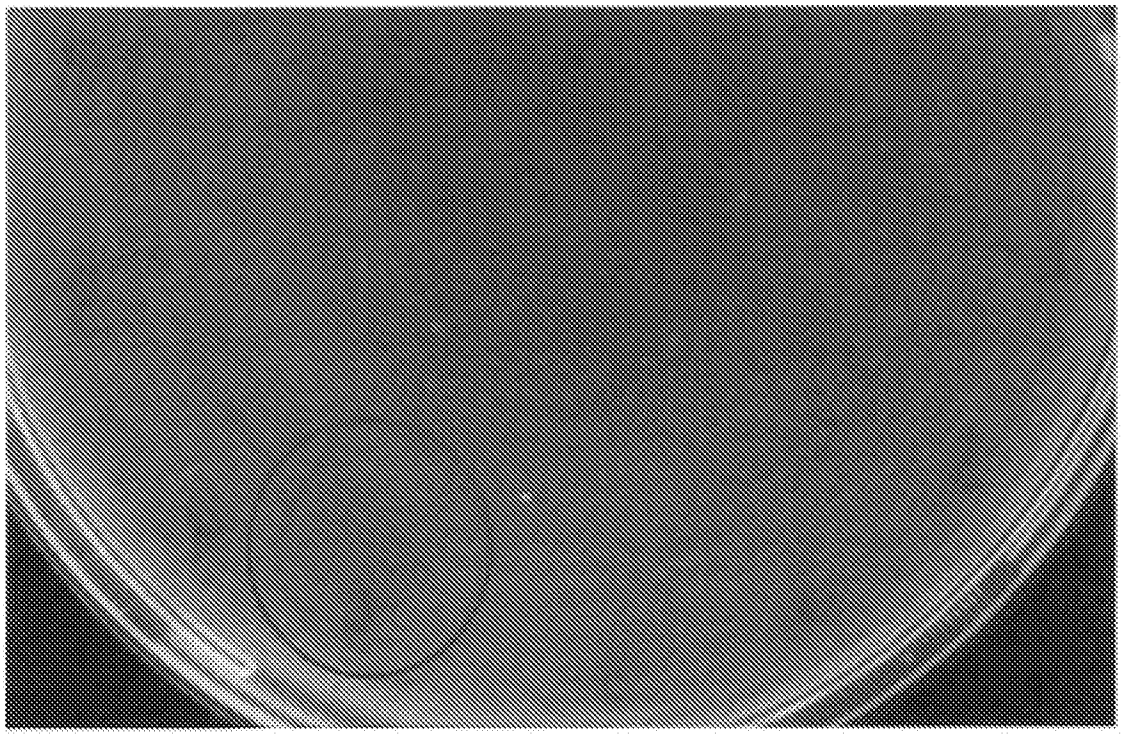


FIGURE 1

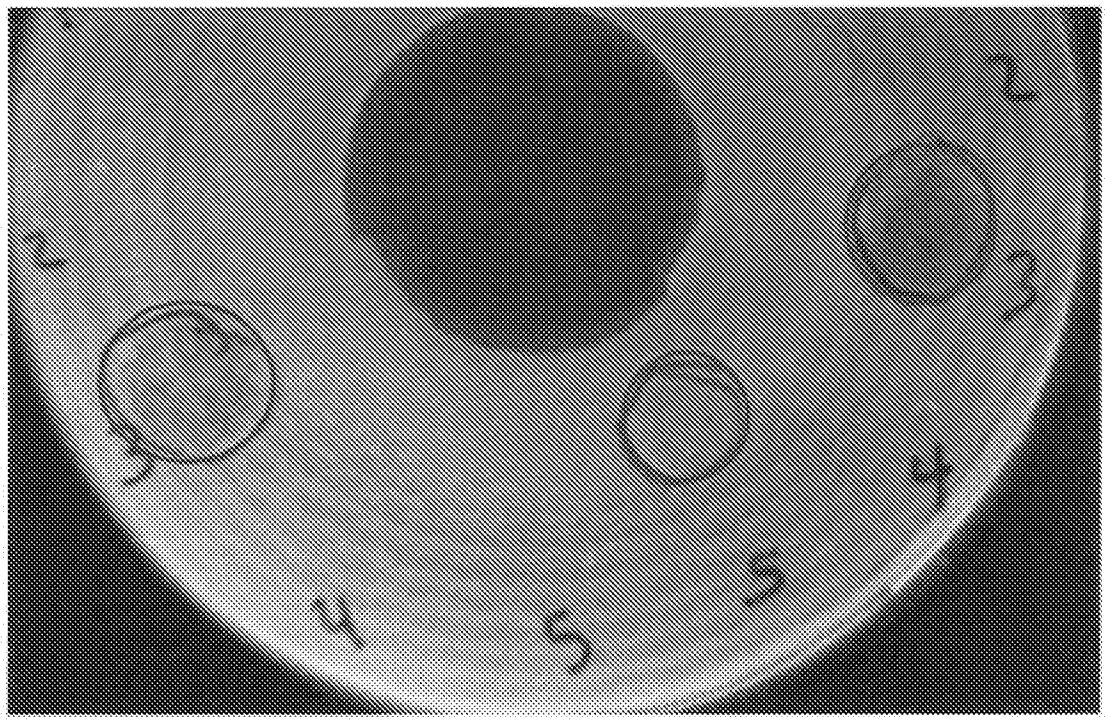


FIGURE 2

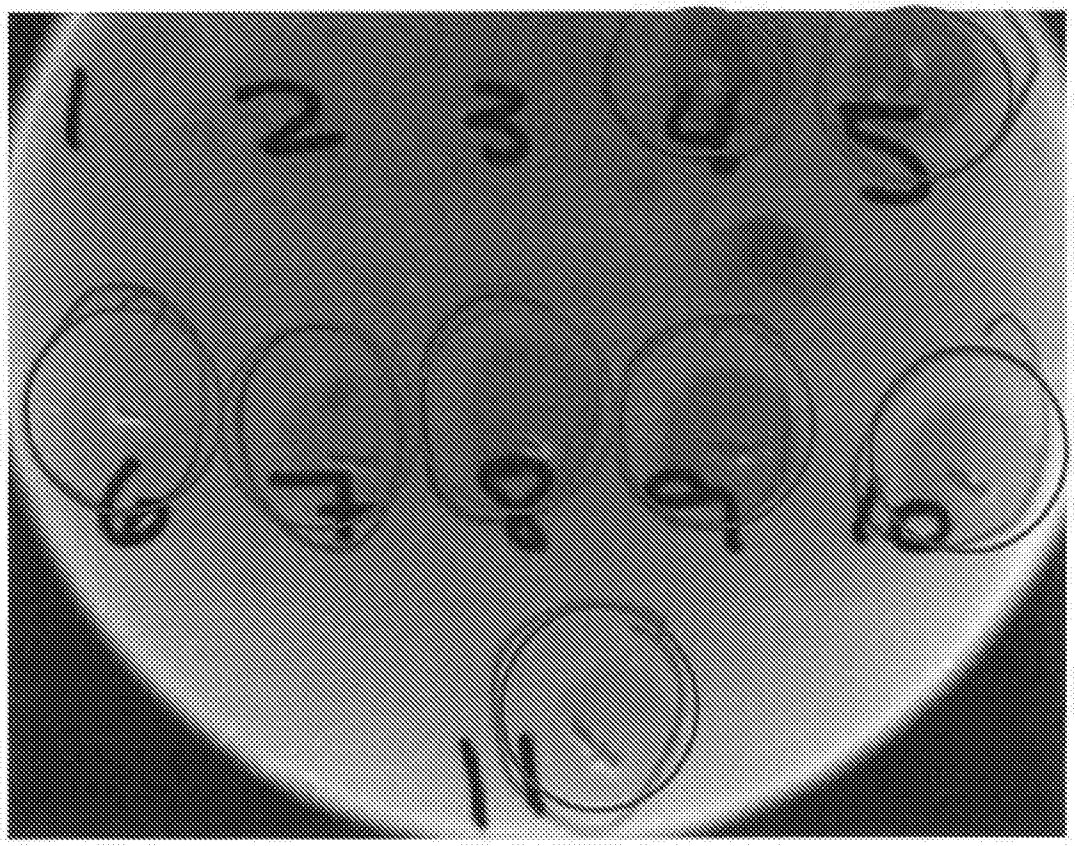
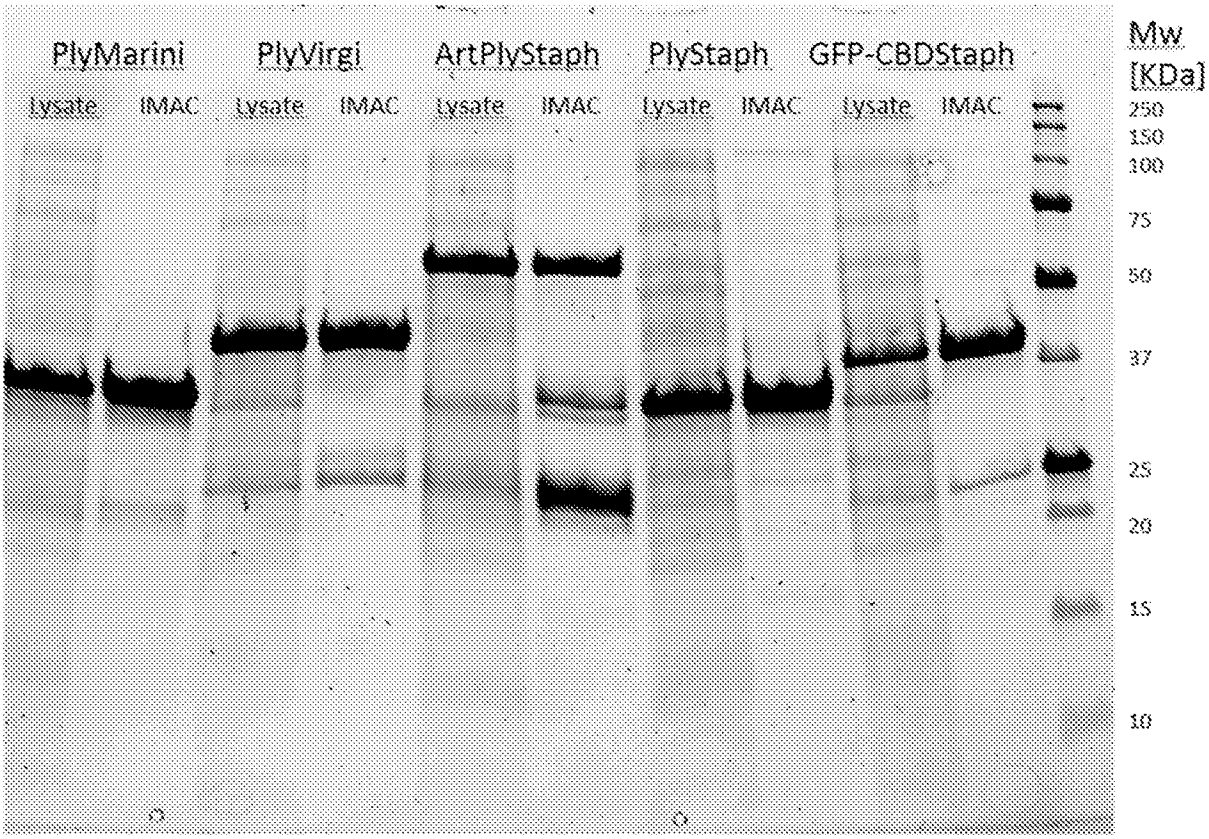


FIGURE 3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PlyVirgi
ArtPlyStaph
PlyStaph
GFP-CBDStaph
Lysate
IMAC
Lysate
IMAC
Lysate
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Lysate
IMAC
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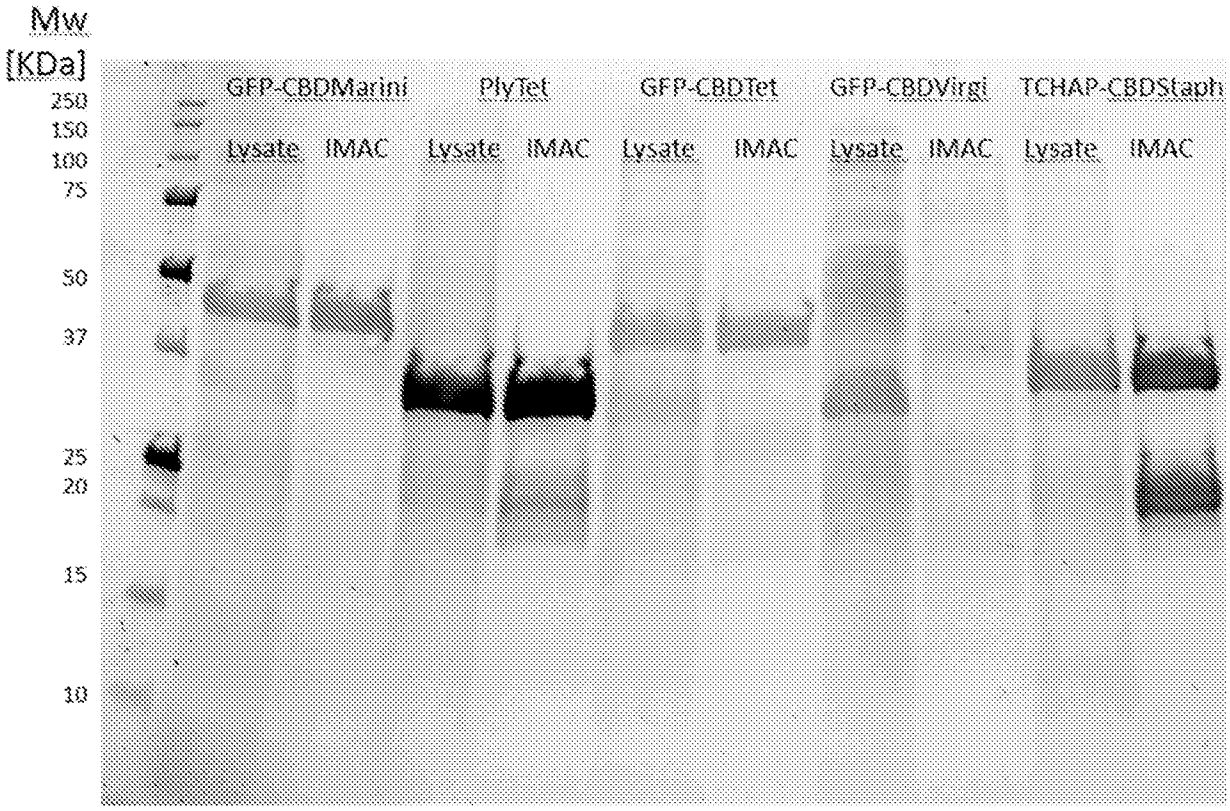
10

FIGURE 4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[KDa]
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150
100
75
50
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15
10
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PlyTet
GFP-CBDTet
GFP-CBDVirgi
TCHAP-CBDStaph
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IMAC
Lysate
IMAC
Lysate
IMAC
Lysate
IMAC
Lysate
IMAC

FIGURE 5

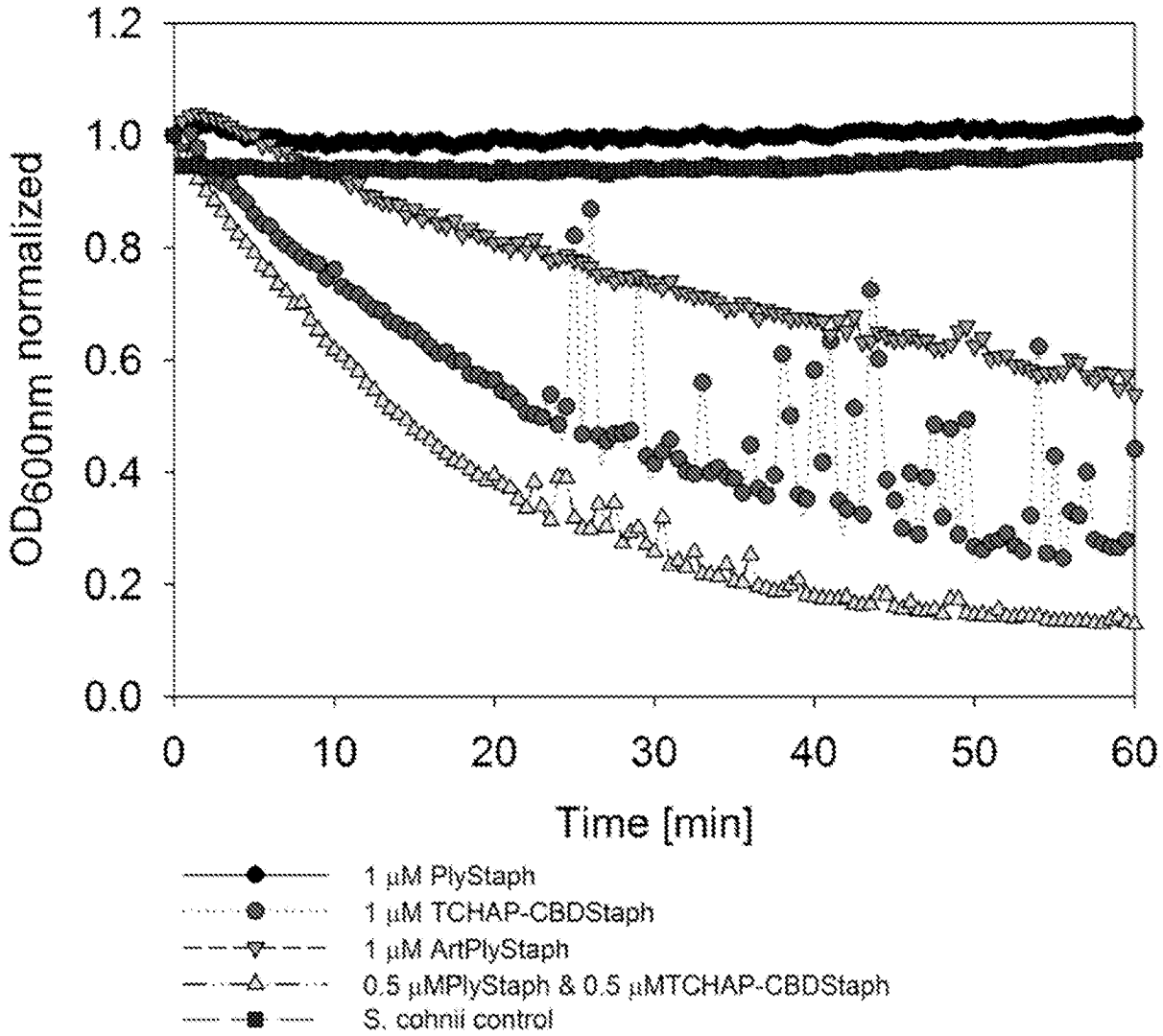

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm normalized
0
10
20
30
40
50
60
Time [min]
1 μM PlyStaph
1 μM TCHAP-CBDStaph
1 μM ArtPlyStaph
0.5 μMPlyStaph & 0.5 μMTCHAP-CBDStaph
S. cohnii control

FIGURE 6

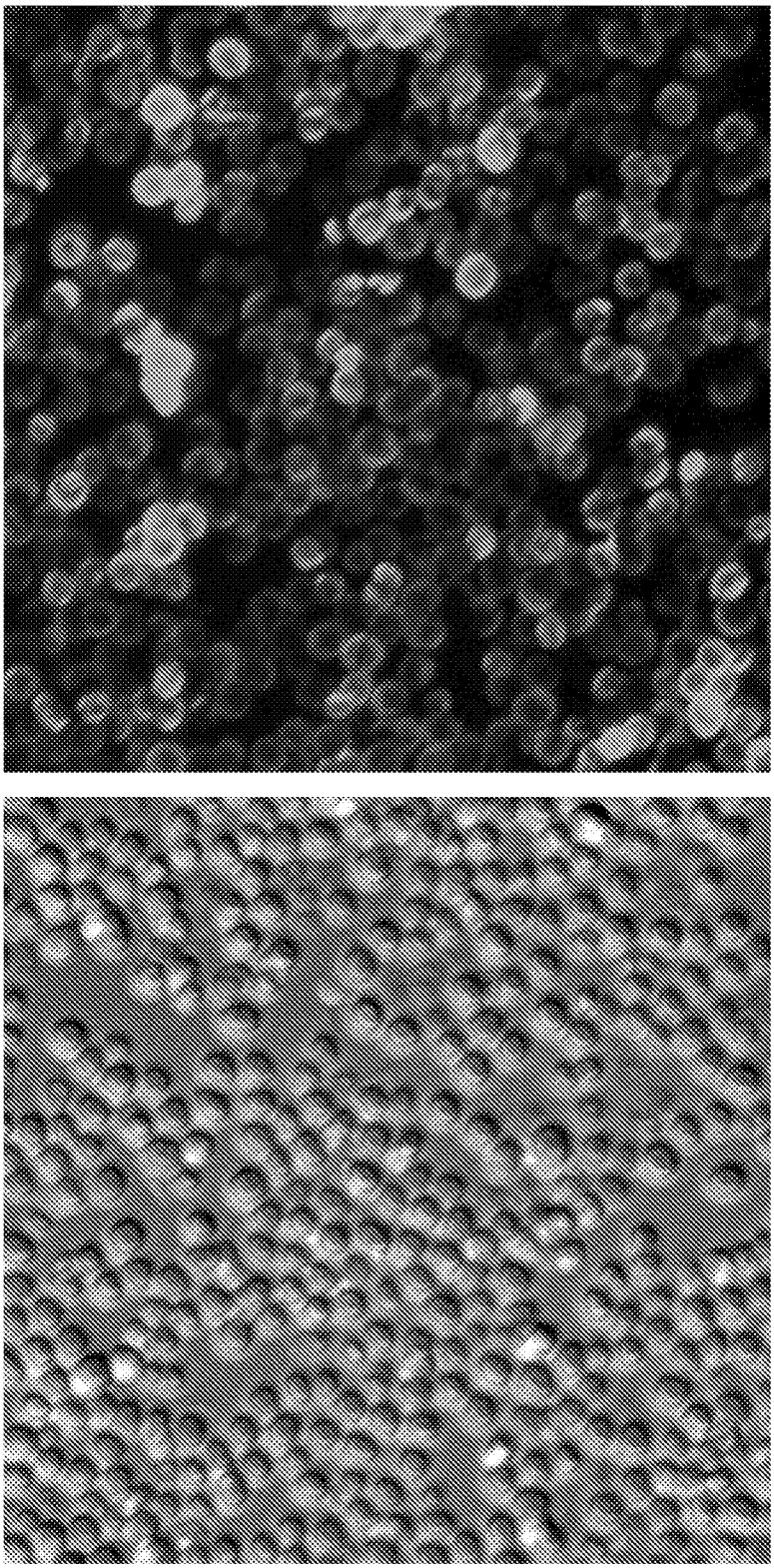
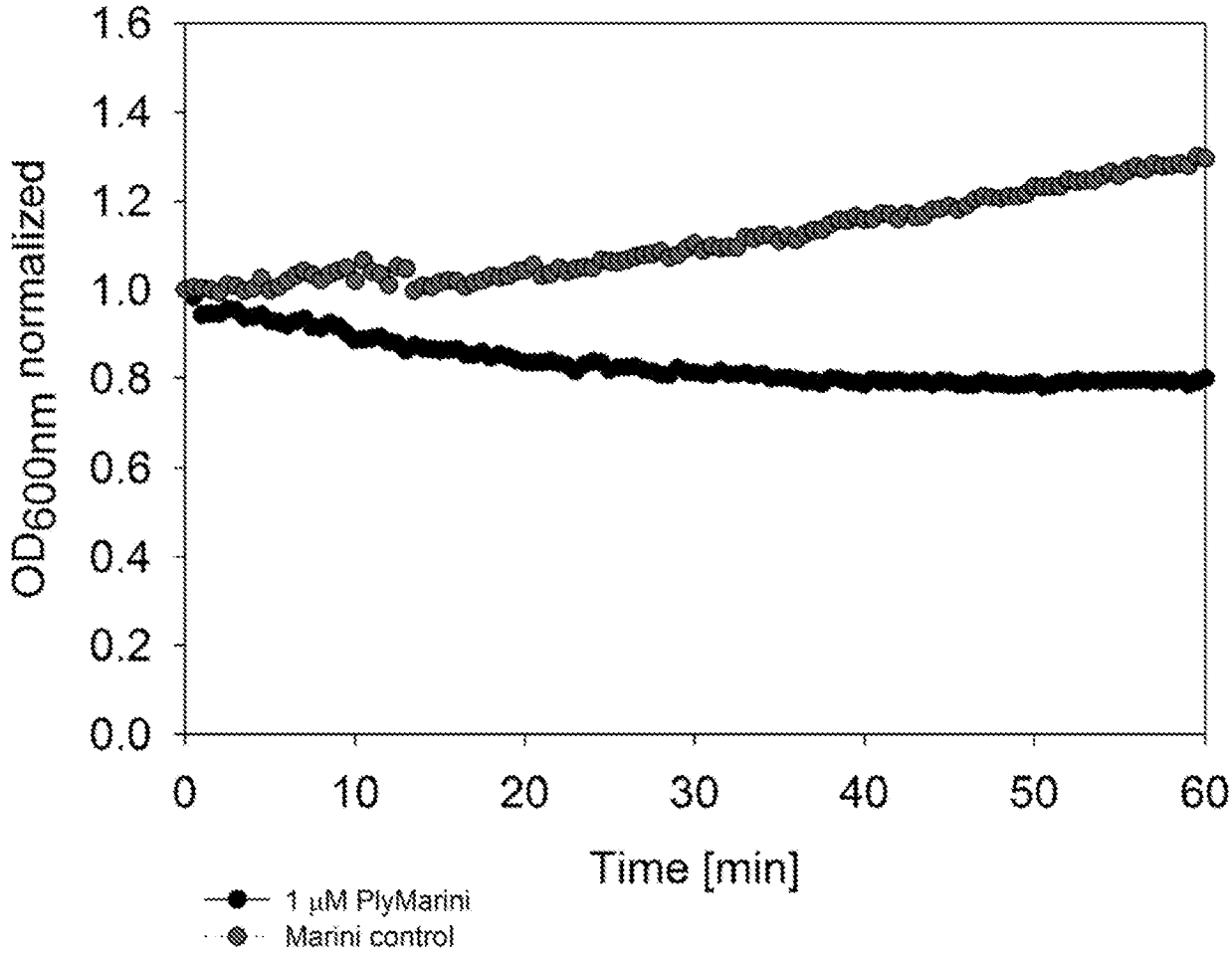


FIGURE 7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1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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0
10
20
30
40
50
60
Time [min]
1 μM PlyMarini
Marini control

FIGURE 8

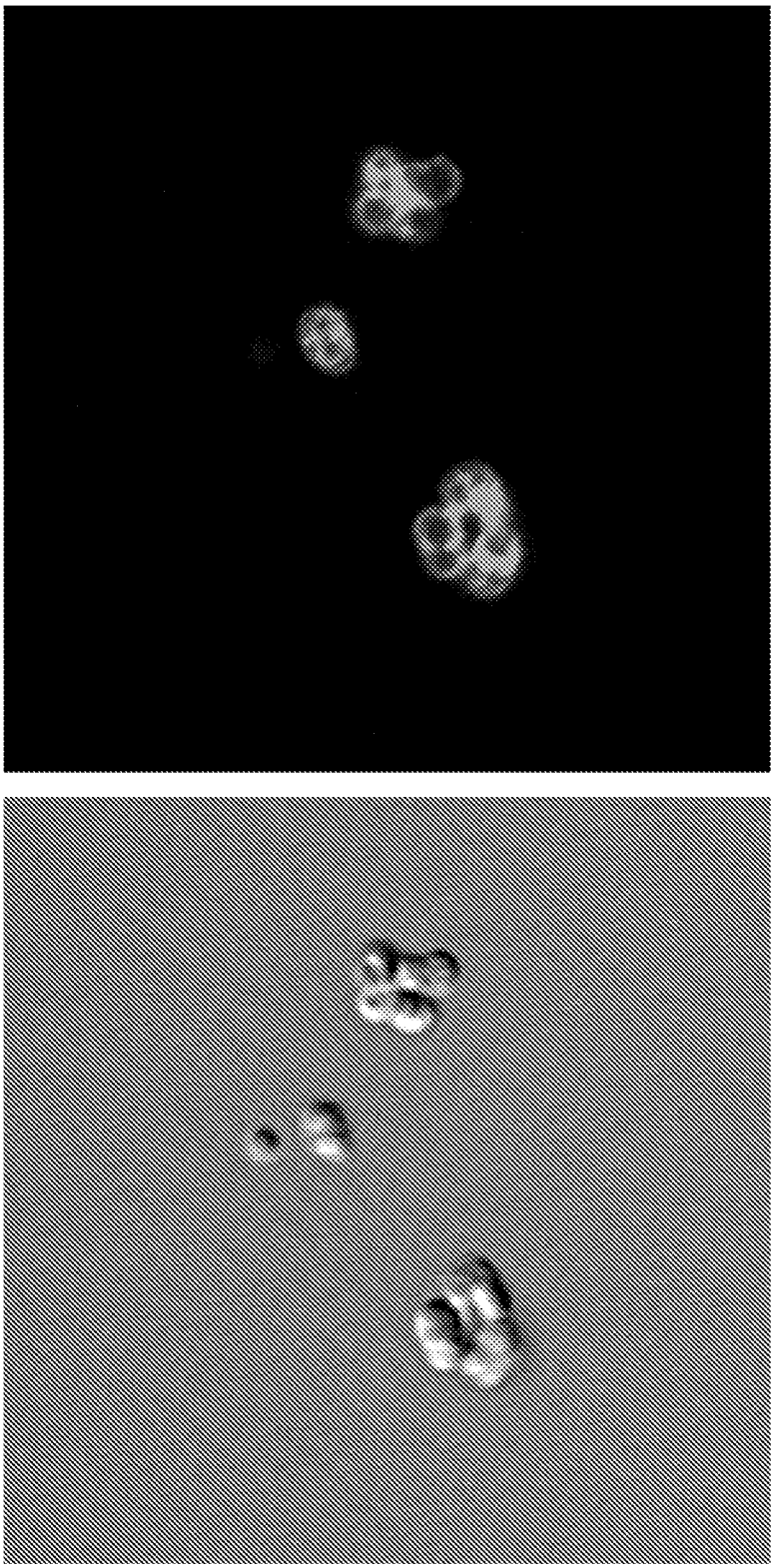

FIGURE 9

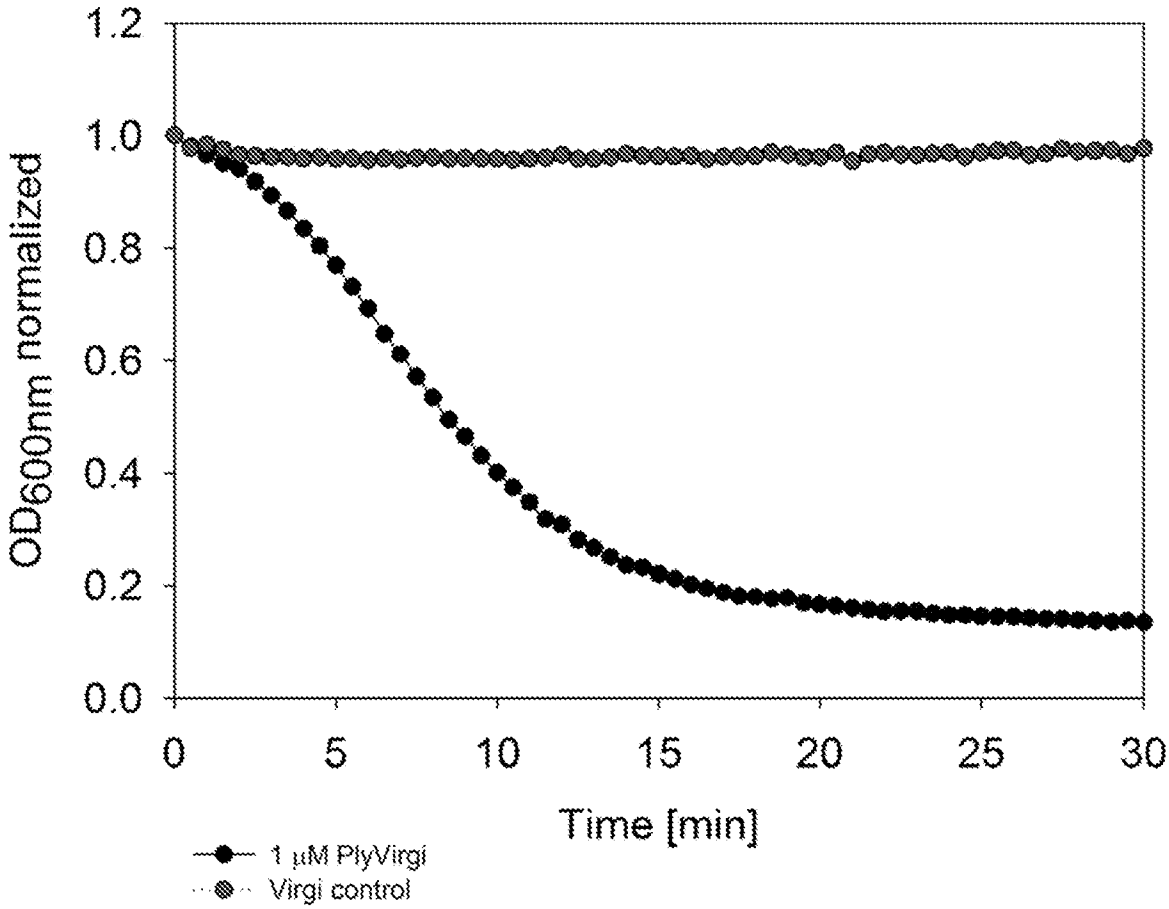
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm normalized
0
5
10
15
20
25
30
Time [min]
1 μM PlyVirgi
Virgi control

FIGURE 10

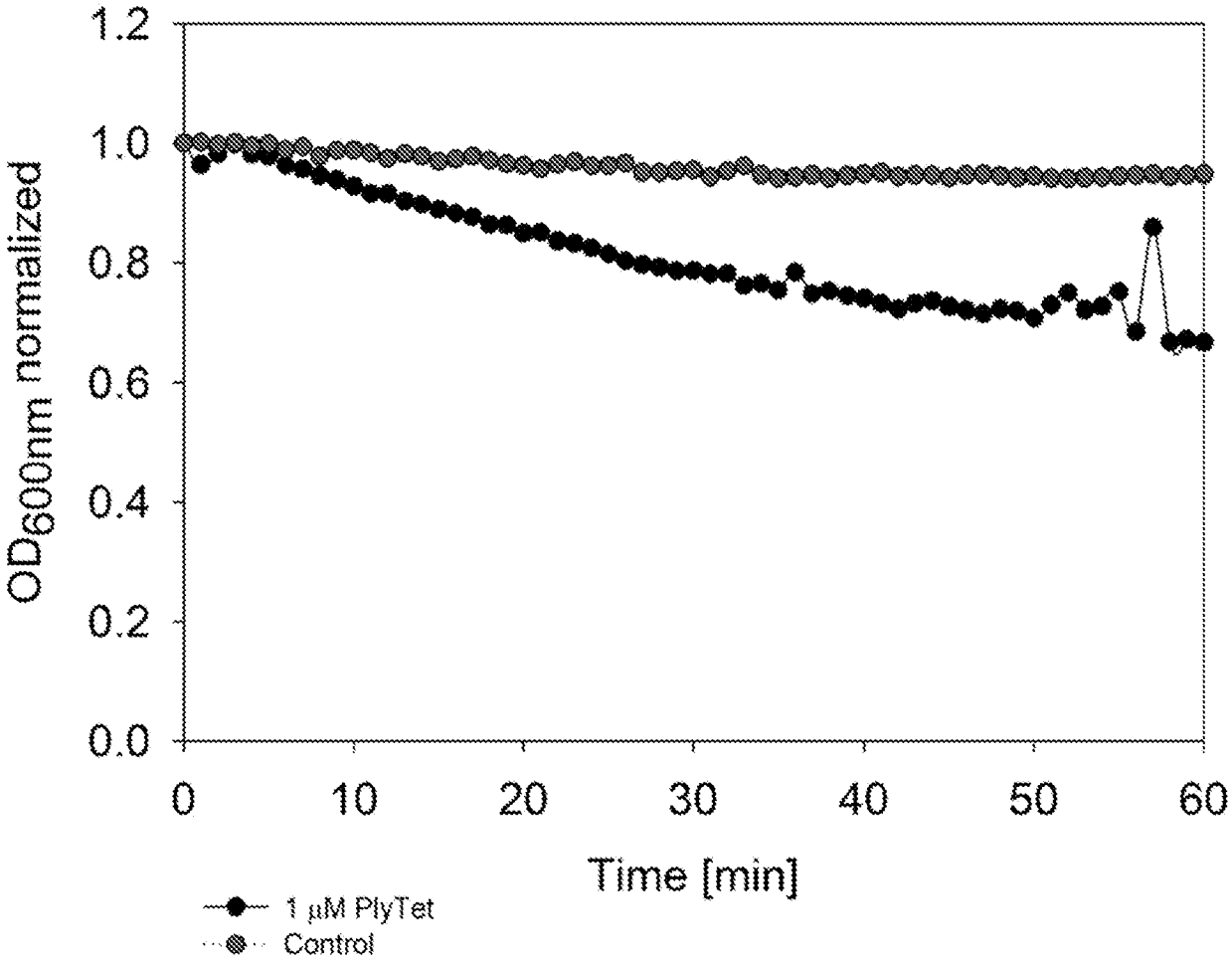
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm normalized
0
10
20
30
40
50
60
Time [min]
1 µM PlyTet
Control

FIGURE 11

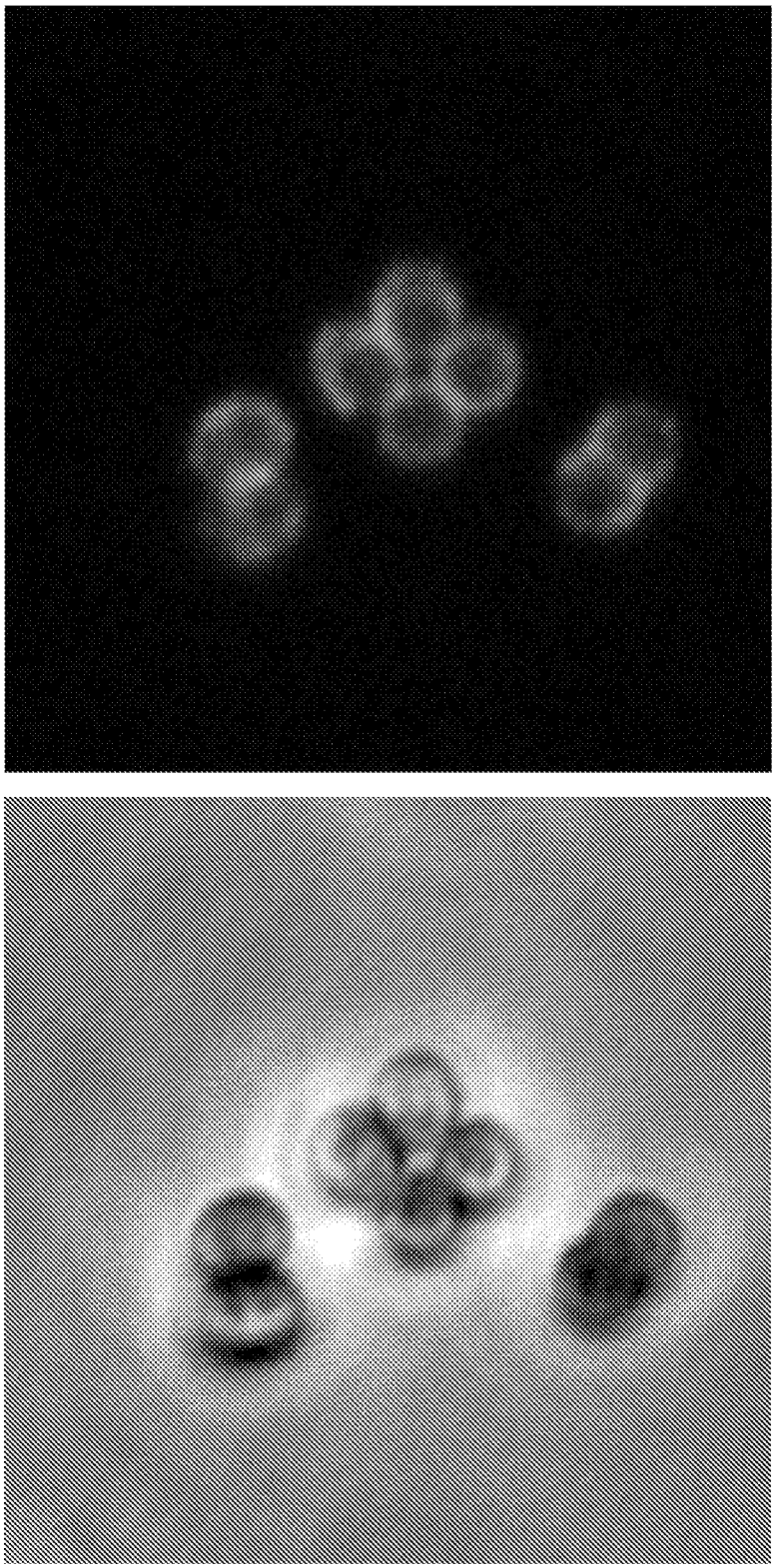

FIGURE 12

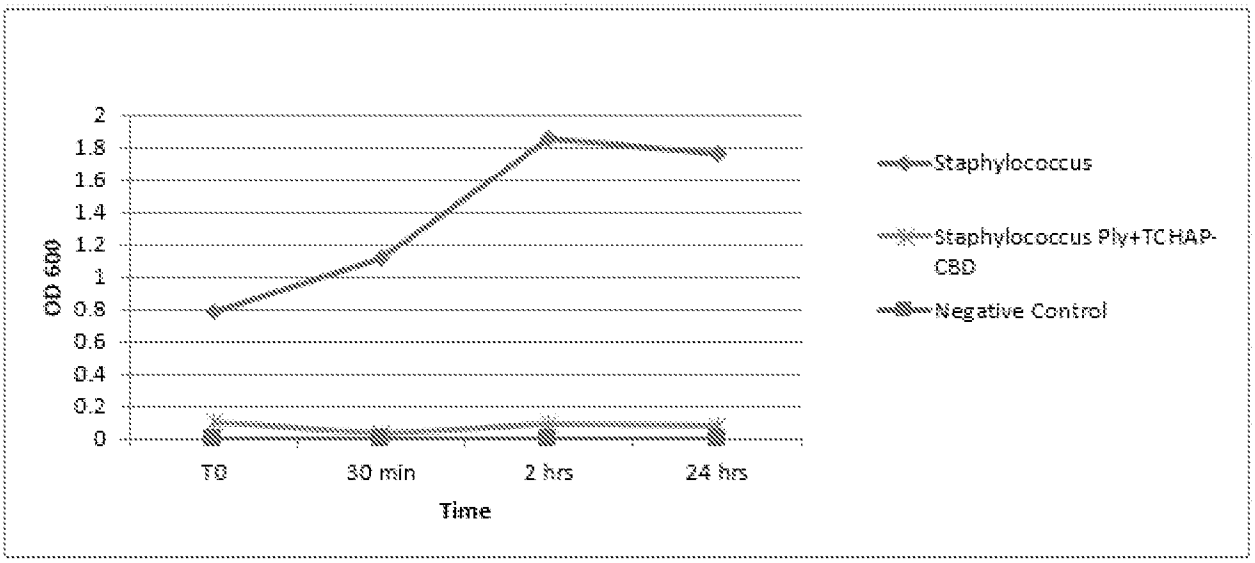

FIGURE 13A

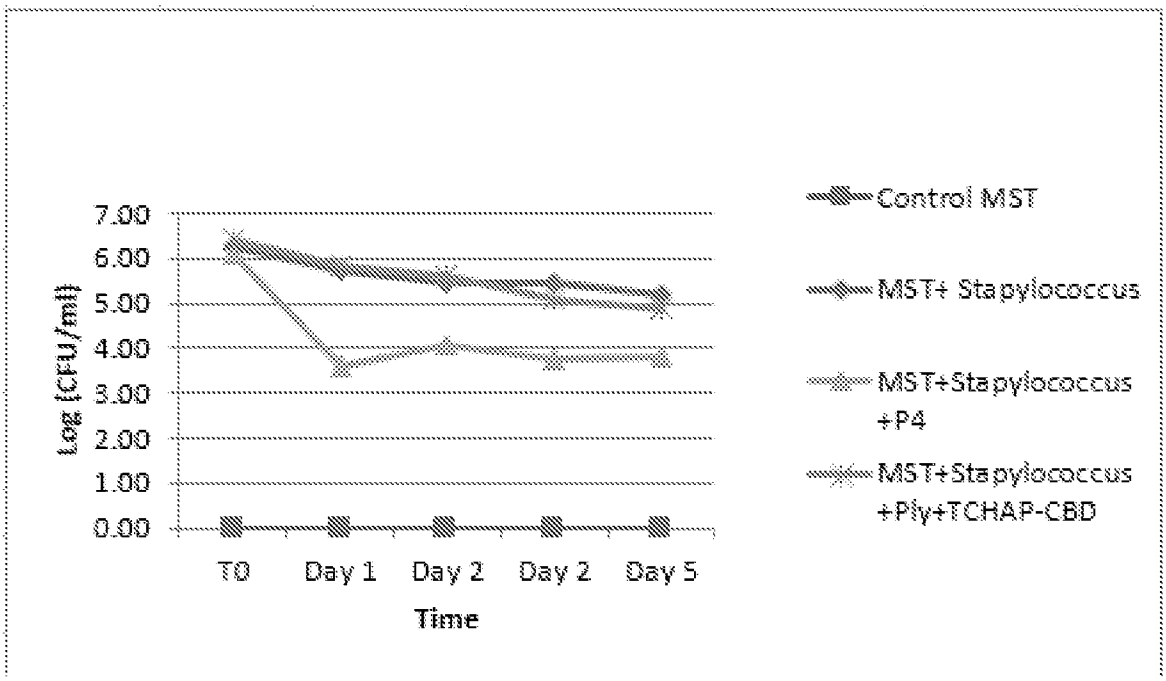

FIGURE 13B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/015520

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/005 A24B15/20 C12N7/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A24B C12N

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

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

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) 25 September 2014 (2014-09-25) examples 1-5 page 12, paragraph 1 - paragraph 3 claim ALL -----	1-20

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

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2016	22/06/2016

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

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/015520

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:

see additional sheet

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

2. ☐ As all searchable claims could be searched without effort justifying an 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.:

1-20(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-20(partially)

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 1, or a sequence with 99% or 95% identity with SEQ ID NO:1 or encoding an amino acid sequence as depicted in SEQ ID NO: 2 or a sequence with 99% or 95% identity with SEQ ID NO: 2.

---

2. claims: 1-20(partially)

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 3, or a sequence with 99% or 95% identity with SEQ ID NO:3 or encoding an amino acid sequence as depicted in SEQ ID NO: 4 or a sequence with 99% or 95% identity with SEQ ID NO: 4.

---

3. claims: 1-20(partially)

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 5, or a sequence with 99% or 95% identity with SEQ ID NO:5 or encoding an amino acid sequence as depicted in SEQ ID NO: 6 or a sequence with 99% or 95% identity with SEQ ID NO: 6.

---

4. claims: 1-20(partially)

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 7, or a sequence with 99% or 95% identity with SEQ ID NO:7 or encoding an amino acid sequence as depicted in SEQ ID NO: 8 or a sequence with 99% or 95% identity with SEQ ID NO: 8.

---

5. claims: 1-20(partially)

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 9, or a sequence with 99% or 95% identity with SEQ ID NO:9 or encoding an amino acid sequence as depicted in SEQ ID NO: 10 or a sequence with 99% or 95% identity with SEQ ID NO: 10.

---

6. claims: 1-20(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Subject-matter relating to an isolated bacteriophage comprising a nucleic acid encoding an endolysin which has the sequence as depicted in SEQ ID NO: 11, or a sequence with 99% or 95% identity with SEQ ID NO:11 or encoding an amino acid sequence as depicted in SEQ ID NO: 12 or a sequence with 99% or 95% identity with SEQ ID NO: 12.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/015520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014150870 A2	25-09-2014	CA 2907234 A1	25-09-2014
		EP 2970917 A2	20-01-2016
		US 2014261478 A1	18-09-2014
		WO 2014150870 A2	25-09-2014