A composition comprising combinations of antimicrobial peptide biofilm enzyme combinations, preferably in the form of a fusion protein. The biofilm may be on an animate or inanimate surface and both medical and non-medical uses and methods are provided. In one aspect the invention provides a composition for use in the treatment or prevention of a biofilm infection in a subject, particularly in the oral cavity.
Compositions and Methods for Inhibiting Biofilm Deposition and Production

By

Henry Daniell

Hyun Koo

This application claims priority to US Provisional Application No. 62/335,650 filed May 12, 2016, the entire disclosure being incorporated herein by reference as though set forth in full.

This invention was made with government support under Grant Nos: ROI HL107904 and ROI HL109442 awarded by the National Institutes of Health. The government has certain rights in the invention.

Field of the Invention

The present invention relates to the fields of biofilm deposition and the treatment of disease. More specifically, the invention provides compositions and methods useful for the treatment of dental caries and other oral diseases. The invention also provides methods for coating biomedical devices for inhibiting undesirable biofilm deposition thereon.

Background of the Invention

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated by reference herein as though set forth in full.

Biopharmaceuticals produced in current systems are prohibitively expensive and are not affordable for large majority of the global population. The cost of protein drugs ($140 billion in 2013) exceeds GDP of >75% of countries around the globe [Walsh 2014], making them unaffordable. One third of the global population earns <$2 per day and can't afford any protein drug (including the underprivileged, elderly and lower socio-economic groups in the US). Such high costs are associated with protein production in prohibitively expensive fermenters, purification, cold transportation/storage, short shelf life and sterile delivery methods [Daniell et al 2015, 2016].
Biofilms are formed by a complex group of microbial cells that adhere to the exopolysaccharide matrix present on the surface of medical devices. Biofilm-associated infections associated with medical device implantation pose a serious problem and adversely affects the function of the device. Medical implants used in oral and orthopedic surgery are fabricated using alloys such as stainless steel and titanium. Surface treatment of medical implants by various physical and chemical techniques has been attempted in order to improve surface properties, facilitate biointegration and inhibit bacterial adhesion as bacterial adhesion is associated with surrounding tissue damage and often results in malfunction of the implant.

Many infectious diseases in humans are caused by biofilms, including those occurring in the mouth [Hall-Stoodley et al., 2004; Marsh, et al 2011]. For example, dental caries (or tooth decay) continues to be the single most prevalent biofilm-associated oral disease, afflicting mostly underprivileged children and adults in the US and worldwide, resulting in expenditures of >$81 billion annually [Beiker and Flemmig, 2011; Dye et al., 2015; Kassebaum et al, 2015]. Caries-causing (cariogenic) biofilms develop when bacteria accumulate on tooth-surfaces, forming organized clusters of bacterial cells that are firmly adherent and enmeshed in a extracellular matrix composed of polymeric substances such as exopolysaccharides (EPS) [Bowen and Koo, 2011]. Current topical antimicrobial modalities for controlling cariogenic biofilms are limited. Chlorhexidine (CHX) is considered the 'gold standard' for oral antimicrobial therapy, but has adverse side effects including tooth staining and calculus formation, and is not recommended for daily therapeutic use [Jones, 1997; Autio-Gold, 2008]. As an alternative, several antimicrobial peptides (AMPs) have emerged with potential antibiofilm effects against caries-causing oral pathogens such as *Streptococcus mutans* [da Silva et al., 2012; Guo et al., 2015]. Antimicrobial peptides (AMP) are an evolutionarily conserved component of the innate immune response and are naturally found in different organisms, including humans. When compared with conventional antibiotics, development of resistance is less likely with AMPs. They are potently active against bacteria, fungi and viruses and can be tailored to target specific pathogens by fusion with their surface antigens (Lee et al 2011; DeGray et al 2001; Gupta et al 2015). Linear AMPs have poor stability or antimicrobial activity when compared to AMPs with complex secondary structures. For example, retrocyclin or protegrin has high antimicrobial activity or stability when cyclized (Wang et al 2003) or when it forms a hairpin structure (Chen et al 2000) via disulfide bond formation. RC 101 is highly stable at pH 3, 4, 7 and temperature 25°C to 37°C.
as well as in human vaginal fluid for 48 hours (Sassi et al 201 la), while its antimicrobial activity was maintained for up to six months (Sassi et al 201 lb). Likewise, protegrin is highly stable in salt or human fluids (Lai et al 2002; Ma et al 2015) but lost potency when linearized. These intriguing characteristics of antimicrobial peptides with complex secondary structures may facilitate development of novel therapeutics. However, the high cost of producing sufficient amounts of antimicrobial peptides is a major barrier for their clinical development and commercialization.

**Summary of the Invention**

In accordance with the present invention, a multi-component composition comprising at least one antimicrobial peptide (AMP) and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures and inhibit biofilm deposition is provided. In certain embodiments, the AMP is selected from protegrin 1, RC-101-Dex, the AMPs listed in Table 1. The biofilm degrading enzyme, includes, for example, mutanase, dextranase, glucoamylase, deoxyribonuclease I, DNAase, dispersin B, glycoside hydrolases and the enzymes provided in Table 2. In certain embodiments, the coding sequences for these enzymes are codon optimized for expression in a plant chloroplast. In a particularly preferred embodiment, the at least one AMP and at least one biofilm degrading enzyme are produced recombinantly. In a particularly preferred embodiment the AMP and biofilm degrading enzyme(s) are expressed as a fusion protein. When the composition is for the treatment of oral diseases, the composition may optionally further comprise an antibiotic, fluoride, CHX or all of the above. The composition may be contained within chewing gum, hard candy, or within an an oral rinse. Preferred fusion proteins of the invention include, without limitation, PG-l-Mut, PG-l-Dex, PG-l-Mut-Dex, RC-101-Mut, RC-101-Dex, RC-101-Mut-Dex for use alone or in combination for the degradation of biofilms. Notably any of the AMPs listed in Table 1 can replace either PG-1 or RC-101 in the aforementioned fusion proteins to alter or improve the bacteriocidal action of the fusion protein. To alter the degradation activity of the fusion proteins, the enzymes listed above and hereinbelow may replace Mut, Dex or both in the fusion proteins of the invention. In another embodiment, when two different EPS enzymes are employed in the compositions, such enzymes may be delivered at different ratios, e.g., 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 etc. When Mut and Dex are delivered together in a gum or oral rinse for example, a preferred ratio is 5:1 Dex:Mut.
In another aspect, the invention provides a method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the compositions described above, the composition having a bactericidal effect, and reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination. In certain embodiments, the biofilm is present in the mouth. In other embodiments, the biofilm is present on an implanted medical device. The method may also be used to remove biofilms present in an internal or external body surface iselected from the group consisting of a surface in an urinary tract, a middle ear, a prostate, vascular intima, heart valves, skin, scalp, nails, teeth and an interior of a wound.

In yet another embodiment, the composition of the invention comprising said at least one AMP and said at least one biofilm degrading enzyme are produced in a plant plastid. The plant may be a tobacco plant and the sequences encoding said AMP and enzyme is codon optimized for expression in a plant plastid. In a preferred embodiment, the AMP and biofilm degrading enzyme are expressed in a lettuce plant as a fusion protein under the control of endogenous regulatory elements present in lettuce plastids.

**Brief Description of the Drawings**

**Figures 1A- 1D - Purification of GFP fused Retrocyclin (RC101) and Protegrin (PG1) expressed in tobacco chloroplasts** - Fig. 1A. Western blot analysis of purified GFP-RC101 fusion using Anti-GFP antibody. Fig. 1B. Native fluorescence gel of purified GFP-RC101 fusion. Fig.1C. Western blot of purified GFP-PG1 fusion using Anti-GFP antibody. Fig. 1D. Native fluorescence gel of purified GFP-PG1. Note - All the samples for Fig 1A -1D were loaded based on total protein values obtained from the Bradford method. Densitometry using Image J software was done to determine GFP concentration Expression level, purity and yield. Expression level and yield were calculated from GFP concentrations relative to total protein values. Yield was determined by multiplying GFP concentration with recovered volume after purification. Individual peptide yield was determined by dividing GFP yield with molar factor 14 (ratio of GFP MW to peptide MW). The fold enrichment was calculated by dividing % purity with % expression in plant crude extracts.
Figures 2A - 2E. Antimicrobial activity of AMPs (GFP-PGl and GFP-RC101) against Streptococcus mutans and other oral microbes. Cell viability was determined by absorbance \( \Lambda_{600\text{nm}} \) and counting colony forming units (CFU) over-time. (Fig. 2A) Time-killing curve of S. mutans treated with different concentrations of GFP-PGl and synthetic PG1 (A600 nm). (Fig. 2B) Viable cells (CFU/ml) of S. mutans treated with GFP-PGl and synthetic PG1 at each time point. (Fig. 2C) Time-killing curve of S. mutans treated with GFP-RC101 at different concentrations \( \Lambda_{600\text{nm}} \). (Fig. 2D) Viable cells (CFU/ml) of S. mutans treated with GFP-RC101 at each time point. (Fig. 2E) Viable cells (CFU/ml) of S. gordonii, A. naeslundii and C. albicans treated with GFP-PGl at 10 \( \mu \text{g/ml} \) for 1 h and 2 h.

Figures 3A - 3C. Bacterial killing by GFP-PGl as determined via confocal fluorescence and SEM imaging (Fig. 3A) Time-lapse killing of S. mutans treated with GFP-PGl at 10 \( \mu \text{g/ml} \). The control group (Fig. 3B) consisted of S. mutans cells treated with buffer only. Propidium iodide (PI) (in red) was used with confocal microscopy to determine the bacterial viability over time at single-cell level. PI is cell-impermeant and only enters cells with damaged membranes; in dying and dead cells a bright red fluorescence is generated upon binding of PI to DNA. GFP-PGl is shown in green. (Fig. 3C) Morphological observations of S. mutans subjected to GFP-PGl at a concentration of 10 \( \mu \text{g/ml} \) for 1 h using scanning electron microscopy. Red arrows show dimpled membrane and extrusion of intracellular content.

Figures 4A - 4C Inhibition of biofilm formation by a single topical treatment of GFP-PGl.

This figure displays representative images of three-dimensional (3D) rendering of S. mutans biofilm. Bacterial cells were stained with SYTO 9 (in green) and EPS were labeled with Alexa Fluor 647 (in red). Saliva-coated hydroxyapatite (sHA) disc surface was treated with a single topical treatment of GFP-PGl with a short-term 30 min exposure (Fig. 4B). The control group (Fig. 4A) was treated with buffer only. Then, the treated sHA disc was transferred to culture medium containing 1% (w/v) sucrose and actively growing S. mutans cells (10^5 cfu/ml) and incubated at 37°C, 5% CO_2 for 19 h. After biofilm growth, the biofilms were analyzed by two photon confocal microscopy. (Fig. 4C) Quantitative analysis of proportion of live and dead S. mutans cells via quantitative PCR (qPCR) with or without propidium monoazide (PMA) treatment (Klein et al., 2012). The combination of PMA and qPCR (PMA-qPCR) quantify viable cells with intact membrane. Before genomic DNA isolation and qPCR quantification, PMA is
added to selectively cross-link DNA of dead cells, and thereby prevent PCR amplification (Klein et al., 2012). Asterisks indicate that the values from GFP-PG1 treatment are significantly different from control (P<0.05).

**Figure 5. EPS-degrading enzymes digesting biofilm matrix.** Representative time-lapsed images of EPS degradation in *S. mutans* biofilm treated with combination of dextranase and mutanase. Bacterial cells were stained with SYTO 9 (in green) and EPS were labeled with Alexa Fluor 647 (in red). The white arrows show 'opening' of spaces between the bacterial cell clusters and 'uncovering' cells following enzymatic degradation of EPS.

**Figures 6A -6C. Biofilm disruption by synthetic PGI alone or in combination with EPS-degrading enzymes.** (Fig. 6A) Time-lapse quantification of EPS degradation within intact biofilms using COMSTAT. (Fig. 6B) The viability of *S. mutans* biofilm treated with synthetic PGI and EPS-degrading enzymes (Dex/Mut) either alone or in combination by ImageJ. (Fig. 6C) Antibiofilm activity of synthetic PGI was enhanced by EPS-degrading enzymes (Dex/Mut). Asterisks indicate that the values for different experimental groups are significantly different from each other (P<0.05).

**Figure 7. In vitro uptake of purified fused protein CTB-GFP, PTD-GFP, Protegrin-l-GFP (PG1-GFP) and RetrocyclinOl-GFP (RC101- GFP) in different human periodontal cell lines: human periodontal ligament stem cells (HPDLS), maxilla mesenchymal stem cells (MMS), human head and neck squamous cell carcinoma cells (SCC), gingiva-derived mesenchymal stromal cells (GMSC), adult gingival keratinocytes (AGK) and osteoblast cell (OBC) with confocal microscopy.** 2×10⁴ cells of human cell lines HPDLS, MMS, SCC, GMSC, AGK and OBC were cultured in 8-well chamber slides (Nunc) at 37°C for overnight, followed by incubation with purified GFP fusion proteins: CTB-GFP (8.8 µg), PTD-GFP (13 µg), PG1-GFP (1.2 µg), RC101-GFP (17.3 µg) in 100 µl PBS supplemented with 1% FBS, respectively, at 37°C for 1 hour. After fixing with 2% paraformaldehyde at RT for 10 min and washing with PBS for three times, the cells were stained with antifade mounting medium with DAPI. For negative control, cells were incubated with commercial GFP (2 µg) in PBS with 1% FBS and processed in the same condition. All fixed cells were imaged using confocal microscope. The green fluorescence shows GFP expression; the blue fluorescence shows DAPI labeled cell nuclei. The images were observed under 100X objective, and at least 10-15 GFP-positive cells or
images were observed in each cell line. Scale bar represent 10 \( \mu \text{m} \). All images studies have been analyzed in triplicate.

**Figure 8. Downstream processing of GFP fusions from transplastomic tobacco:** Flow diagram illustrating the different steps involved in generation of purified GFP fusions from transplastomic tobacco plants grown in greenhouse.

**Figures 9A - 9B.** Vectors and codon optimized sequences for mutanase (Fig. 9A) and dextranase (Fig. 9B). Codon optimized mutanase: SEQ ID NO: 1. Codon Optimized dextranase: SEQ ID NO: 2.

**Figure 10.** A schematic diagram of a chloroplast vector expressing tandem repeats of AMPs fused with GVGVP for use alone or for expressing fusion proteins comprising the EPS proteins in Figure 9.

**Figure 11.** Novel purification strategy: inverse temperature cycling purification process demonstrates high yield.

**Figures 12A - 12B: Expression of functional codon optimized mutanase in E. coli.** Fig. 12 shows western blots showing mutanase expression in E. coli. Fig. 12B shows E. coli spread on 0.5% blue dextran plates. Transformed clones are able to produce recombinant dextranase normally made in S. mutans and able to clear a blue halo around the colony. Fig. 12C represents a gel diffusion assay comparing the degradation activity of recombinant dextranase present in the crude lysate (Total Protein loading) from the transformed E. coli against blue dextran as compared to commercially purified enzyme from Penicillin.

**Figure 13.** A flow diagram of the steps for engineering lettuce plants for AMP/biofilm degrading enzyme production.

**Figure 14.** Chewing gum tablet preparation is shown. While GFP is exemplified herein, chewing gum comprising the AMP-enzyme fusion proteins (e.g., those provided in Figs. 9 and 10) is also within the scope of the invention.
Figure 15. Gum tables were evaluated via fluorescence, and by western blot to ascertain the concentration of GFP. Quantification of the GFP release from chewing gum based on (i) Western blotting (ii) Fluorometer (Fluoroskan Ascent™ Microplate Fluorometer - Thermo; $\lambda_{ex}$ 485nm; $\lambda_{em}$ 538nm). Commercial GFP (Vector Laboratories, Cat# MB-0752) was used as standard. The chewing gum was ground in the protein extraction buffer.

Figure 16. A chewing simulator is shown which uses artificial saliva for assessing release kinetics of biofilm degrading agents from the gum tablets of the invention.

Figure 17. A graph showing quantification of GFP released from chewing gum. Gum tablets comprising increasing concentrations of GFP expressed in lettuce leaves were assessed in a chewing simulator in the presence of artificial saliva to determine GFP release kinetics.

Figure 18. A graph demonstrating that crude extracts comprising enzymes expressed from chloroplast vectors are as efficacious for inhibiting CFU formation as commercial enzymes, when mixed with Listerine. Enzymatic degradation of *in vitro* *S. mutans* biofilms using *E. coli* derived Mutanase and Dextranase (ratio 1:5) supplemented with listerine. Commercial Mutanase (from *Bacillus* sp., Amano) and Dextranase (from *Penicillium* sp., Sigma) was used as positive control while the crude *E. coli* extract served as negative control. CFU/ml is expressed as mean ± standard deviation (n = 2). ***, P < 0.001 versus *E. coli* extract.

Detailed Description of the Invention

Many infectious diseases in humans are caused by virulent biofilms, including those occurring within the mouth (e.g. dental caries and periodontal diseases). Dental caries (or tooth decay) continues to be the single most costly and prevalent biofilm-associated oral disease in the US and worldwide. It afflicts children and adults alike, and is a major reason for emergency room visits leading to absenteeism from work and school. Unfortunately, the prevalence of dental caries is still high (>90% of US adult population) and it remains the most common chronic disease afflicting children and adolescents, particularly from a poor socio-economic background. Furthermore, poor oral health often leads to systemic consequences and impacts overall health. Importantly, the cost to treat the ravages of this disease (e.g. carious lesions and pulpal infection) exceeds $40 billion/yr in the US alone. Fluoride is the mainstay of dental caries prevention. However, its widespread use offers incomplete protection against the disease.
Fluoride is effective in reducing demineralization and enhancing demineralization of early carious lesions, but has limited effects against biofilms. Conversely, current antimicrobial modalities for controlling caries-causing biofilms are largely ineffective.

There is an urgent need to develop efficacious therapies to control virulent oral biofilms. In accordance with the present invention, methods for low-cost production and delivery of therapeutically effective plant-expressed biopharmaceuticals superior to current antibiofilm/anticaries modalities are provided.

**Definitions:**

As used herein, antimicrobial peptides are small peptides having any bacterial activity. "RC-101" is an analogue of retrocyclin, a cyclic octadecapeptide, which can protect human CD4+ cells from infection by T- and M-tropic strains of HIV-1 in vitro and prevent HIV-1 infection in human cervicovaginal tissue. The ability of RC-101 to prevent HIV-1 infection and retain full activity in the presence of vaginal fluid makes it a good candidate for other topical microbicide applications, especially in oral biofilms. The sequence of RC-101 is provided in Plant Biotechnol J. 2011 Jan; 9(1): 100-115 which is incorporated herein by reference.

"C16G2" is a novel synthetic antimicrobial peptide with specificity for *S. mutans*, "Protegrin-1 (PG)" is a cysteine-rich, 18-residue β-sheet peptide. It has potent antimicrobial activity against a broad range of microorganisms, including bacteria, fungus, virus, and especially some clinically relevant, antibiotic-resistant bacteria. For example, bacterial pathogens *E. coli* and fungal opportunist *C. albicans* are effectively killed by PG in laboratory testing. The sequence of PG-1 is provided in Plant Biotechnol J. 2011 Jan; 9(1): 100-115 which is incorporated herein by reference.

Additional antimicrobial peptides include those set forth below in Table 1 below.

**Table 1.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Molecular wt (observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSP</td>
<td>SGSLSTFFRLFNRSFTQALGK</td>
<td>2,364.9</td>
</tr>
<tr>
<td>CSP&lt;sub&gt;C16&lt;/sub&gt;</td>
<td>TFFRLFNRSFTQALGK (SEQ ID NO: 3)</td>
<td>1,933.3</td>
</tr>
<tr>
<td>G2</td>
<td>KNLRIIRKGIHIKKY&lt;sub&gt;b&lt;/sub&gt; (SEQ ID NO: 4)</td>
<td>1,993.5</td>
</tr>
</tbody>
</table>
**Amino acid sequences**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequences</th>
<th>Molecular w t (observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16G2</td>
<td>TFFRLFNRSFTQALGKGGGKtNLRIRKGIHIIKYYi</td>
<td>4,079.0</td>
</tr>
<tr>
<td>CSP_M8</td>
<td>TFFRLFNR</td>
<td>1,100.6</td>
</tr>
<tr>
<td>M8G2</td>
<td>TFFRLFNRIKKGKtNLRIRKGIHIIKYYi</td>
<td>3,246.9</td>
</tr>
<tr>
<td>S6L3-33</td>
<td>FKKFWKWFRRF</td>
<td>1,677.5</td>
</tr>
<tr>
<td>iC16-33</td>
<td>TRRRLFNRSFTQALGKSSGGFFKKFWKWFRRF</td>
<td>3,849.0</td>
</tr>
<tr>
<td>M8-33</td>
<td>TFFRLFNRSGGGFKKFWKWFRRF</td>
<td>3,016.9</td>
</tr>
</tbody>
</table>

* Linker regions between targeting and killing peptides are underlined.
* Peptide C-terminal amidation.

A "biofilm" is a complex structure adhering to surfaces that are regularly in contact with water, consisting of colonies of bacteria and usually other microorganisms such as yeasts, fungi, and protozoa that secrete a mucilaginous protective coating in which they are encased. Biofilms can form on solid or liquid surfaces as well as on soft tissue in living organisms, and are typically resistant to conventional methods of disinfection. Dental plaque, the slimy coating that fouls pipes and tanks, and algal mats on bodies of water are examples of biofilms. Biofilms are generally pathogenic in the body, causing such diseases as dental caries, cystic fibrosis and otitis media.

"Biofilm degrading enzymes" include, without limitation, exo-polysaccharide degrading enzymes such as dextranase, mutanase, DNase, endonuclease, deoxyribonuclease I, dispersin B, and glycoside hydrolases, such as 1→3)-α-D-glucan hydrolase, although use of chloroplast codon optimized sequences encoding dextranase and mutanase are preferred, the skilled person is well aware of many different biofilm degrading enzymes in the art. Additional enzyme sequences for use in the fusion proteins of the invention are provided below.

As used herein, the terms "administering" or "administration" of an agent, drug, or peptide to a subject includes any route of introducing or delivering to a subject a compound to
perform its intended function. The administering or administration can be carried out by any suitable route, including orally, topically, intranasally, parenterally (intravenously, intramuscularly, intraperitoneally, or subcutaneously), or rectally. Administering or administration includes self-administration and the administration by another.

As used herein, the terms "disease," "disorder," or "complication" refers to any deviation from a normal state in a subject.

As used herein, by the term "effective amount" "amount effective," or the like, it is meant an amount effective at dosages and for periods of time necessary to achieve the desired result.

As used herein, the term "inhibiting" or "preventing" means causing the clinical symptoms of the disease state not to worsen or develop, e.g., inhibiting the onset of disease, in a subject that may be exposed to or predisposed to the disease state, but does not yet experience or display symptoms of the disease state.

As used herein, the term "expression" in the context of a gene or polynucleotide involves the transcription of the gene or polynucleotide into RNA. The term can also, but not necessarily, involves the subsequent translation of the RNA into polypeptide chains and their assembly into proteins.

A plant remnant may include one or more molecules (such as, but not limited to, proteins and fragments thereof, minerals, nucleotides and fragments thereof, plant structural components, etc.) derived from the plant in which the protein of interest was expressed. Accordingly, a composition pertaining to whole plant material (e.g., whole or portions of plant leaves, stems, fruit, etc.) or crude plant extract would certainly contain a high concentration of plant remnants, as well as a composition comprising purified protein of interest that has one or more detectable plant remnants. In a specific embodiment, the plant remnant is rubisco.

In another embodiment, the invention pertains to an administrable composition for treating or preventing biofilm formation in situ (e.g., in the mouth) and on biomedical devices useful for surgical implantation such as stents, artificial joints, and the like. In this embodiment, the devices are coated with the composition to inhibit unwanted biofilm deposition on the device. The composition comprises a therapeutically-effective amount of one or more antimicrobial peptides (AMP) and one or more enzymes having biofilm degrading activity in combination, each of said AMP and enzyme thereof having been expressed by a plant and a plant remnant and acting synergisticall to degrade said biofilm. In certain embodiments the AMP(s) and
enzymes(s) are expressed from separate plastid transformation vectors. In other embodiments, the plastid transformation vectors comprising polycistronic coding sequences where both the AMP and the enzymes are expressed from a single vector.

Proteins expressed in accord with certain embodiments taught herein may be used in vivo by administration to a subject, human or animal in a variety of ways. The pharmaceutical compositions may be administered orally, topically, subcutaneously, intramuscularly or intravenously, though oral topical administration is preferred.

Oral compositions produced by embodiments of the present invention can be administered by the consumption of the foodstuff that has been manufactured with the transgenic plant producing the plastid derived therapeutic protein. The edible part of the plant, or portion thereof, is used as a dietary component. The therapeutic compositions can be formulated in a classical manner using solid or liquid vehicles, diluents and additives appropriate to the desired mode of administration. Orally, the composition can be administered in the form of tablets, capsules, granules, powders, gums, and the like with at least one vehicle, e.g., starch, calcium carbonate, sucrose, lactose, gelatin, etc. The therapeutic protein(s) of interest may optionally be purified from a plant homogenate. The preparation may also be emulsified. The active ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, e.g., water, saline, dextrose, glycerol, ethanol or the like and combination thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants. In a preferred embodiment the edible plant, juice, grain, leaves, tubers, stems, seeds, roots or other plant parts of the pharmaceutical producing transgenic plant is ingested by a human or an animal thus providing a very inexpensive means of treatment of disease.

In a specific embodiment, plant material (e.g. lettuce material) comprising chloroplasts expressing AMPs and biofilm degrading enzymes and combinations thereof, is homogenized and encapsulated. In one specific embodiment, an extract of the lettuce material is encapsulated. In an alternative embodiment, the lettuce material is powderized before encapsulation. As mentioned previously, the biofilm degrading proteins may also be purified from the plant following expression.
In alternative embodiments, the compositions may be provided with the juice of the transgenic plants for the convenience of administration. For said purpose, the plants to be transformed are preferably selected from the edible plants consisting of tomato, carrot and apple, among others, which are consumed usually in the form of juice.

According to another embodiment, the subject invention pertains to a transformed chloroplast genome that has been transformed with a vector comprising a heterologous gene that expresses a combination of peptides as disclosed herein.

Of particular present interest is a transformed chloroplast genome transformed with a vector comprising a heterologous gene that expresses one or more AMP and biofilm degrading enzyme or a combination thereof, polypeptide. In a related embodiment, the subject invention pertains to a plant comprising at least one cell transformed to express a peptide as disclosed herein.

Reference to genetic sequences herein refers to single- or double-stranded nucleic acid sequences and comprises a coding sequence or the complement of a coding sequence for polypeptide of interest. Degenerate nucleic acid sequences encoding polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 60, preferably about 75, 90, 96, or 98% identical to the cDNA may be used in accordance with the teachings herein polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of nucleic acid sequences which encode biologically active polypeptides also are useful polynucleotides.

Variants and homologs of the nucleic acid sequences described above also are useful nucleic acid sequences. Typically, homologous polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions: 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50° C. once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.
Species homologs of polynucleotides referred to herein also can be identified by making suitable probes or primers and screening cDNA expression libraries. It is well known that the Tm of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., J. Mol. Biol. 81, 123 (1973). Nucleotide sequences which hybridize to polynucleotides of interest, or their complements following stringent hybridization and/or wash conditions also are also useful polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., 1989, at pages 9.50-9.51.

The following materials and methods are provided to facilitate the practice of the present invention.

**Microorganisms and growth conditions**

*Streptococcus mutans* UA159 serotype c (ATCC 700610), *Actinomyces naeslundii* ATCC 12104, *Streptococcus gordonii* DL-1 and *Candida albicans* SC5314 were used in present study. These strains were selected because *S. mutans* is a well-established virulent cariogenic bacteria [Ajdic D et al, 2002]. *S. gordonii* is a pioneer colonizer of dental biofilm, and *A. naeslundii* is also detected during the early stages of dental biofilm formation and may be associated with development of root caries [Dige I et al, 2009]. *C.albicans* is a fungal organism that colonizes human mucosal surfaces, and it is also detected in dental plaque from toddlers with early childhood caries [Hajeshengallis E et al, 2015]. All strains were stored at -80°C in tryptic soy broth containing 20% glycerol. Blood agar plates were used for cultivating *S.mutans*, *S.gordonii* and *A.naeslundii*. Sabouraud agar plates were used for *C.albicans*. All these strains were grown in ultra-filtered (10 kDa molecular-weight cut-off membrane; Prep/Scale, Millipore, MA) buffered tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose to mid-exponential phase (37°C, 5% CO2) prior to use.

**Creation of transplastomic lines expressing different tagged GFP fusion proteins**

The transplastomic plants expressing GFP fused with CTB, PTD, retrocyclin and protegrin were created as described in previous studies [Limaye et al 2006; Kwon et al 2013; Xiao et al 2016; Lee et al 2011]. Transplastomic lines expressing GFP fusion proteins were
confirmed using Southern blot assay as described previously [Verma et al 2008]. Also, expression of GFP tagged proteins were confirmed by visualizing green fluorescence from the leaves of each construct under UV illumination.

**Purification of tag-fused GFP proteins**

Purification of GFP fusions Protegrin-1 (PG1) and Retrocyclin (RC101) from transplastomic tobacco was accomplished by organic extraction followed by hydrophobic chromatography done previously (Lee et al, 2011). About 0.2-1 gm of lyophilized leaf material was taken and reconstituted in 10-20 ml of plant extraction buffer (0.2M Tris HCl pH 8.0, 0.1M NaCl, 10mM EDTA, 0.4M sucrose, 0.2 % Triton X supplemented with 2% Phenylmethylsulfonylfluoride and 1 protease inhibitor cocktail). The resuspension was incubated in ice for 1 hour with vortex homogenization every 15 min. The homogenate was then spun down at 75000g at 4°C for 1 hour (Beckman LE-80K optima ultracentrifuge) to obtain the clarified lysate. The lysate was subjected to pretreatment with 70% saturated ammonium sulfate and 1/4th volume of 100% ethanol, followed by vigorous shaking for 2 min (Yakhnin et al, 1998). The treated solution was spun down at 2100 g for 3 min. The upper ethanol phase was collected and the process was repeated with 1/16th volume of 100% ethanol. The pooled ethanol phases were further treated with 1/3rd volume of 5M NaCl and 1/4th volume of 1-butanol, homogenized vigorously for 2 min and spun down at 2100 g for 3 min. The lowermost phase was collected and loaded onto a 7kDa MWCO zeba spin desalting column (Thermo scientific) and desalted as per manufacturer’s recommendations.

The desalted extract was then subjected to hydrophobic interaction chromatography during the capture phase for further purification. The desalted extract was injected into a Toyopearl butyl - 650S hydrophobic interaction column (Tosoh bioscience) which was run on a FPLC unit (Pharmacia LKB-FPLC system). The column was equilibrated with 2.3 column volumes of salted buffer (10mM Tris-HCl, 10mM EDTA and 50% saturated ammonium sulfate) to a final 20% salt saturation to facilitate binding of GFP onto the resin. This was followed by a column wash with 5.8 column volumes of salted and unsalted buffer mix and then eluted with unsalted buffer (10mM Tris-HCl, 10mM EDTA). The GFP fraction was identified based on the peaks observed in the chromatogram and collected. The collected fractions were subjected to a
final polishing step by overnight dialysis. After dialysis the purified proteins were lyophilized (labconco lyophilizer) in order to concentrate the finished product and then stored in -20°C.

Quantification of purified GFP fusions

Quantification of GFP-RC101 and GFP-PGl was done by both western blot and fluorescence based methods. The lyophilized purified proteins were resuspended in sterile 1X PBS and the total protein was determined by Bradford method. The purified protein was then quantified by SDS-PAGE method by loading denatured protein samples along with commercial GFP standards (Vector labs) onto a 12 % SDS gel and then western blotting was done using 1:3000 dilution of mouse Anti-GFP antibody (Millipore) followed by probing with 1:4000 dilution of secondary HRP conjugated Goat-Anti Mouse antibody (Southern biotech).

The purified proteins were also quantified using GFP fluorescence. The protein samples were run on a 12 % SDS gel under native conditions. After the run, the gel was placed under a UV lamp and then photographed. The GFP concentration in both western and native fluorescence methods was determined by densitometric analysis using Image J software with commercial GFP standards in order to obtain the standard curve. Purity was determined based on GFP quantitation with respect to total protein values determined in Bradford method.

Uptake of purified tag-fused GFP proteins by human periodontal cell lines

As previously described (Xiao, et al 2016), to determine the uptake of four tags, CTB, PTD, PG1 and RC101, in different human periodontal cell lines, including human periodontal ligament stem cells (HPDLS), maxilla mesenchymal stem cells (MMS), human head and neck squamous cell carcinoma cells (SCC-1), gingiva-derived mesenchymal stromal cells (GMSC), adult gingival keratinocytes (AGK) and osteoblast cells (OBC), briefly, each human cell line cells (2x10^4) were cultured in 8 well chamber slides (Nunc) at 37°C overnight, followed by incubation with purified GFP-fused tags: CTB-GFP (8.8 µg), PTD-GFP (13 µg), GFP-PGl (1.2 µg) and GFP-RC101 (17.3 µg) in 100 µl PBS supplemented 1% FBS at 37°C for 1 hour. After fixing with 2% paraformaldehyde at RT for 10 min and washing with PBS for three times, all cells were stained with antifade mounting medium with DAPI (Vector laboratories, Inc). For negative control, cells were incubated with commercial GFP (2 µg) in PBS with 1% FBS at 37°C.
for 1 hour. All fixed cells were imaged using confocal microscopy. The images were observed under 100x objective, and at least 10-15 GFP-positive cells were recorded for each cell line in three independent analysis.

**Evaluation of antibacterial activity**

The killing kinetics of AMPs (Gfp-PG1 and Gfp-RC101) against S. mutans were analyzed by time-lapse killing assay. S. mutans were grown to log phase and diluted to $10^5$ CFU/ml in growth medium. GFP-PG1 and GFP-RC101 were added to S. mutans suspensions at concentrations of 0 to 10 µg/ml and 0 to 80 µg/ml, respectively. At 0, 1, 2, 4, 8 and 24 h, samples were taken and serially diluted in 0.89% NaCl, then spread on agar plates and colonies were counted after 48 h. Absorbance at 600 nm was also checked at each time point. S. gordonii, A. naeslundii and C. albicans suspensions were mixed with Gfp-PG1 at concentration of 10 µg/ml and at 0, 1 and 2 h, aliquots were taken out for enumeration of CFU.

The effects of AMP on the viability of S. mutans cells were also assessed by time-lapsed measurements. S. mutans were grown to log phase and harvested by centrifugation (5500 g, 10 min) and the pellet was washed once with sodium phosphate-buffered saline (PBS) (pH 7.2), re-suspended in PBS and adjusted to a final concentration of $1 \times 10^5$ CFU/ml. GFP-PG1 was added to S. mutans suspensions at concentrations of 10 µg/ml and 2.5 µM propidium iodide-PI (Molecular Probe Inc., Eugene, OR, USA) was added for labeling dead cells. 5 µl of mixtures were loaded on an agarose pad for confocal imaging. Confocal images were acquired using Leica SP5-FLIM inverted single photon laser scanning microscope with a 100X (numerical aperture, 1.4) Oil immersion objective. The excitation wavelengths were 488 nm and 543 nm for GFP and PI, respectively. The emission filter for GFP was a 495/540 OlyMPFCl filter, while PI was a 598/628 OlyMPFC2 filter. For the time-lapse series, images in the same field of view were taken at 0, 10, 30, and 60 min and created by ImageJ 1.44 (Http://rsbweb.nih.gov/ij/download.html).

Morphological observations of S. mutans treated with AMP were also examined by scanning electron microscopy (SEM). S. mutans were grown to log phase and diluted to $10^5$ CFU/ml in PBS. Bacteria suspension was mixed with GFP-PG1 (final concentration of 10 µg/ml) for 1 h at 37°C. After treatment, the bacteria were collected by filtration using 0.4 µm Millipore filters. The deposits were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate
buffer (pH 7.4) for 1 hour at room temperature and processed for SEM (Quanta FEG 250, FEI, Hillsboro, OR) observation. Untreated or bacteria treated with buffer only served as controls.

**Evaluation of anti-biofilm activity**

A well-characterized EPS-matrix producing oral pathogen, *S. mutans* UA159, was used to form biofilms on saliva-coated hydroxyapatite disc surfaces. Briefly, hydroxyapatite discs (1.25 cm in diameter, surface area of 2.7 ± 0.2 cm², Clarkson, Chromatography Products, Inc., South Williamsport, PA) were coated with filter-sterilized, clarified human whole saliva (sHA) [Xiao et al., 2012]. *S. mutans* was grown in UFTYE medium with 1% (w/v) glucose to mid-exponential phase (37°C, 5% C0₂). Each sHA disc was inoculated with 10⁵ CFU of actively growing *S. mutans* cells per ml in UFTYE medium containing 1% (w/v) sucrose, and inoculated at 37°C and 5% C0₂ for 19 h. Before inoculation, the sHA discs were topically treated with GFP-PG1 solution (10 μg) for 30min. The inhibition effect of GFP-PG1 treatment on 3D biofilm architectures were observed via confocal imaging. Briefly, EPS was labeled using 2.5 μM Alexa Fluor 647-labeled dextran conjugate (10 kDa; 647/668 nm; Molecular Probes Inc.), while the bacteria cells were stained with 2.5 μM SYTO9 (485/498 nm; Molecular Probes Inc.). The imaging was performed using Leica SP5 microscope with 20X (numerical aperture, 1.00) water immersion objective. The excitation wavelength was 780 nm, and the emission wavelength filter for SYTO 9 was a 495/540 OlyMPFEC1 filter, while the filter for Alexa Fluor 647 was a HQ655/40M-2P filter. The confocal image series were generated by optical sectioning at each selected positions and the step size of z-series scanning was 2 μm. Amira 5.4.1 software (Visage Imaging, San Diego, CA, USA) was used to create 3D renderings of biofilm architecture [Xiao et al., 2012, Koo et al., 2010].

To examine the effects of the PG1 on biofilms formed with *S. mutans* for 19 h on sHA discs, we examined the 3D architecture of the EPS-matrix and in situ cell viability using time-lapse confocal microscopy following biofilms incubation with 1) Control, 2) EPS-degrading enzymes only, 3) PG1 only, or 4) PG1 and EPS-degrading enzymes for up to 60 minutes. The EPS-degrading enzymes used here were dextranase and mutanase, which were capable of digesting the EPS derived from *S. mutans* by hydrolyzing α-1,6 glucosidic linkages and α-1,3 glucosidic linkages [Hayacibara et al., 2004]. Dextranase produced from *Penicillium sp.* was commercially purchased from Sigma (St. Louis, MO) and mutanase produced from *Trichoderma*
*harzianum* was kindly provided by Dr. William H. Bowen (Center for Oral Biology, University of Rochester Medical Center). Dextranase and mutanase were mixed at ratio of 5:1 before applying to biofilms [Mitsue F. Hayacibara et al. 2004]. Alexa Fluor 647-labeled dextran conjugate was used to label the EPS-matrix, while SYTO 9 and PI were used to label live cells and dead cells. Biofilms were examined using confocal fluorescence imaging at 0, 10, 30 and 60 min, and subjected to AMIRA/COMSTAT/Imagel analysis. The total biomass of EPS matrix, live and dead cells in each series of confocal images was quantified using COMSTAT and ImageJ. The ratio of live to the total bacteria at each time point was calculated, and the survival rate of live cells (relative to live cells at 0 min) was plotted. The initial number of viable cells at time point 0 min was considered to be 100%. The percent-survival rate was determined by comparing to time point 0 min.

**Microbiological assays**

At selected time point (19 h), biofilms were removed, homogenized via sonication and subject to microbiological analyses as detailed previously [Xiao I et al. 2012, Koo H et al. 2010]; our sonication procedure does not kill bacteria cells while providing optimum dispersal and maximum recoverable counts. Aliquots of biofilm suspensions were serially diluted and plated on blood agar plates using an automated Eddy Jet Spiral Plater (IUL, SA, Barcelona, Spain). Meanwhile, propidium monoazide (PMA) combined with quantitative PCR (PMA-qPCR) was used for analysis of *S. mutans* cell viability as describe Klein MI et al. [Klein MI et al. 2012]. The combination of PMA and qPCR will quantify only the cells with intact membrane (i.e. viable cells) because the PMA cross-linked to DNA of dead cells and extracellular DNA modifies the DNA and inhibits the PCR amplification of the extracted DNA. Briefly, biofilm pellets were resuspended with 500 µl TE (50 mM Tris, 10 mM EDTA, pH 8.0). Using a pipette, the biofilm suspensions were transferred to 1.5 ml microcentrifuge tubes; then mixed with PMA. 1.5 µl PMA (20 mM in 20% dimethyl sulfoxide; Biotium, Hayward, CA) was added to the biofilm suspensions. The tubes were incubated in the dark for 5 min, at room temperature, with occasional mixing. Next, the samples were exposed to light for 3 min (600-W halogen light source). After photo-induced cross-linking, the biofilm suspensions were centrifuged (13,000 g/10 min/4°C) and the supernatant was discarded. The pellet was resuspended with 100 µl TE,
following by incubation with 10.9 µl lysozyme (100 mg/ml stock) and 5 µl mutanylysin (5µl/µl stock) (37°C/30 min). Genomic DNA was then isolated using the MasterPure DNA purification kit (Epicenter Technologies, Madison, WI). Ten pictograms of genomic DNA per sample and negative controls (without DNA) were amplified by MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad Laboratories Inc., CA) and S.mutans specific primer (16S rRNA) [Klein MI et al 2010].

**Statistical Analysis**

Data are presented as the mean ± standard deviation (SD). All the assays were performed in duplicate in at least two distinct experiments. Pair-wise comparisons were made between test and control using Student's t-test. The chosen level of significance for all statistical tests in present study was P<0.05.

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

**Example I**

**Creation and characterization of transplastomic lines**

All fusion tags (CTB, PTD, protegrin, retrocyclin) were fused to the green fluorescent protein (smGFP) at N-terminus to evaluate their efficiency and specificity. Fusion constructs encoding these fusion proteins were cloned into chloroplast transformation vectors which were then used to transform plants of interest as described in US Patent application no. 13/101,389 which is incorporated herein by reference. To create plants expressing GFP fusion proteins, tobacco chloroplasts were transformed using biolistic particle delivery system. As seen in the Fig. IB, each tag-fused GFP is driven by identical regulatory sequences - the psbA promoter and 5' UTR regulated by light and the transcribed mRNA is stabilized by 3' psbA UTR. The psbA gene is the most highly expressed chloroplast gene and therefore psbA regulatory sequences are used for transgene expression in our lab [7, 34]. To facilitate the integration of the expression cassette into chloroplast genome, two flanking sequences, isoleucyl-tRNA synthetase (trnl) and alanyl-tRNA synthetase (trnA) genes, flank the expression cassette, which are identical to the native chloroplast genome sequence. The emerging shoots from selection medium were
investigated for specific integration of the transgene cassette at the trnl and trnA spacer region and then transformation of all chloroplast genomes in each plant cell (absence of untransformed wild type chloroplast genomes) was confirmed by Southern blot analysis. Thus, stable integration of all GFP expression cassettes and homoplasm of chloroplast genome with transgenes were confirmed before extracting fusion proteins. In addition, by visualizing the green fluorescence under UV light, GFP expression of was phenotypically confirmed. Confirmed homoplasmic lines were then transferred and cultivated in an automated greenhouse to increase biomass.

To scale up the biomass of each GFP tagged plant leaf material, each homoplasmic line was grown in a temperature- and humidity-controlled greenhouse. Fully grown mature leaves were harvested in late evenings to maximize the accumulation of GFP fusion proteins driven by light-regulated regulatory sequences. To further increase the content of the fusion proteins on a weight basis, frozen leaves were freeze-dried at -40°C under vacuum. In addition to the concentration effect of proteins, lyophilization increased shelf life of therapeutic proteins expressed in plants more than one year at room temperature [Daniell et al 2015; 2016]. Therefore, in this study, lyophilized and powdered plant cells expressing GFP-fused tag proteins were used for oral delivery to mice.

Expression and purification of GFP fused antimicrobial peptides from transplastomic tobacco.

Tobacco leaves expressing GFP fused antimicrobial peptides RC101 and PG1 were harvested from greenhouse and subsequently lyophilized for protein extraction and purification. The average expression level of GFP-RC101 was found to be 8.8% of total protein in crude extracts while expression of GFP-PG1 was that of 3.8% of total protein based on densitometry. The difference in expression levels was similar to what was reported previously (Lee et al 2011, Gupta et al, 2015).

Purification of GFP fused to different antimicrobial peptides (RC101 and PG1) was done in order to test the microbicidal activity against both planktonic and biofilm forming S. mutans. Lyophilized tobacco material expressing different GFP fusions was used for extractions and subsequent downstream processing (See Figure 8) to obtain the finished purified product which was subsequently quantified to determine concentration of GFP fused peptides. Quantitation of purified GFP-RC101 and GFP-PG1 was done by both western blot and Native GFP fluorescence
method where purified GFP-RC101 show 94% average purity with an average yield of 1624 µg of GFP (116 µg of RC101 peptide) per gram of lyophilized leaf material (Fig 1A and IB). In GFP-PGl both methods (Fig 1C and ID) show 17% average purity with an average yield of 58.8 µg of GFP (4^g PG1 peptide) per gram of lyophilized leaf material. The difference in purity can be attributed to difference in the type of tags fused to GFP as seen in previous studies (Xiao et al 2015, Skosyrev et al 2003). The fold enrichment of purified GFP-RC101 and GFP-PGl from plant extracts was 10.6 and 4.5 respectively. The western blots also show GFP standards at 27 kDa which corresponds to the monomer fragment along with a 54kDa GFP dimer with loadings ranging from 6-8 ng of GFP. In GFP-RC101 western blots, 29 kDa and 58 kDa fragments are clearly visible which correspond to the monomer and dimer forms of the fusion (Fig. 1A). This could be attributed to the ability of GFP to form dimers (Ohashi et al, 2007). Western blots of GFP-PGl (Fig ID) clearly show the 29 kDa monomer along with a 40 kDa fragment could be due to mobility shift caused by GFP-PGl bound to other non-specific plant proteins which could have been co-purified as described previously (Morassuttia et al 2002). Native fluorescence of GFP-RC101 and GFP-PGl (Fig IB and ID) show multimeric bands with some of them visible below the 27 kDa GFP standard size which could be because of GFP being fused to cationic peptides causing an electrophoretic mobility shift with each GFP fragment as described in previous studies (Lee et al, 2011).

20 Antibacterial Activity of AMPs

We first examined the antimicrobial activity of GFP-PGl using dose-response time-kill studies as shown in Fig. 2 (A-E). GFP-PGl displays potent antibacterial activity against Streptococcus mutans, a proven biofilm-forming and caries-causing pathogen, rapidly killing the bacterial cells within 1h at low concentrations (Fig. 2A). GFP-PGl also killed the early oral colonizers Streptococcus gordonii and Actinomyces naeslundii, but showed limited antifungal activity against Candida albicans at the concentrations tested (Fig 2E). Time-lapse confocal imaging shows that S. mutans viability is affected as early as 10 minutes as shown in Fig 3A relative to the untreated controls (Fig. 3B). SEM imaging revealed disruption of S. mutans membrane surface, causing extrusion of the intracellular content as well as irregular cell morphology, while untreated bacteria showed intact and smooth surfaces without any visible cell lysis or debris (Fig 3C). Having shown the antimicrobial efficacy of GFP-PGl against S. mutans,
we have examined the potential of this antimicrobial peptide to prevent biofilm formation or disrupt pre-formed biofilms.

**Inhibition of Biofilm Initiation by AMPs**

Preventing the formation of pathogenic oral biofilms is challenging because drugs need to exert therapeutic effects following topical applications. To determine whether GFP-PGl can disrupt the initiation of the biofilm, we treated saliva coated apatitic (sHA) surface (tooth surrogate) with a single topical treatment of GFP-PGl for 30 min, and then incubated with actively growing *S. mutans* cells in cariogenic (sucrose-rich) conditions. We observed substantial impairment of biofilm formation by *S. mutans* with minimal accumulation of EPS-matrix on the GFP-PGl treated sHA surface (Figs. 4B and AC). The few adherent cell clusters were mostly non-viable compared to control (Fig. 4A), demonstrating potent effects of GFP-PGl on biofilm initiation despite topical, short-term exposure.

**Disruption of pre-formed biofilm by AMP with or without EPS-degrading enzymes**

Disruption of formed biofilms on surfaces is challenging. Disruption of cariogenic biofilms is particularly difficult because drugs often fail to reach clusters of pathogenic bacteria (such as *S. mutans*) because of the surrounding exopolysaccharides (EPS)-rich matrix that enmeshes and protects them [Bowen and Koo, 2011]. EPS-degrading enzymes such as dextranase and mutanase could help digest the matrix of cariogenic biofilms, although they are devoid of antibacterial effects. We first optimized the dextranase and/or mutanase required for EPS-matrix disruption without affecting the cell viability (data not shown). As shown in Figure 5, the combination of dextranase and mutanase can digest the EPS (in red) and ‘open spaces’ (see arrows) between the bacterial cell clusters (in green) and ‘uncover’ cells (see arrows). Thus, the combination of GFP-PGl and EPS-degrading enzymes synergistically potentiate the overall antibiofilm effects.

To explore this concept, *Streptococcus mutans* biofilms were pre-formed on sHA surface, and treated topically with GFP-PGl and EPS-degrading enzymes (Dex/Mut) either alone or in combination. Time-lapsed confocal imaging and quantitative computational analyses were conducted to analyze EPS-matrix degradation and live/dead bacterial cells within biofilms (Fig 6A). The enzymes-peptide combination resulted in more than 60% degradation of the EPS-
matrix, while increasing the bacterial killing when compared to either GFP-PG or Dex/Mut alone. These findings were further validated via standard culturing assays by determining colony forming units. The antibacterial activity of PG against S. mutans biofilms combined with Dex/Mut was significantly enhanced than either one alone. Topical exposure of Dex/Mut alone showed no effects on biofilm cell viability, whereas GFP-PG-1 alone showed limited killing activity (Fig 6B). Together, the data demonstrate potential of this combined approach to synergistically enhance antimicrobial efficacy of GFP-PG-1 against established biofilms (Fig. 6C).

Uptake of GFP fused with different tags by human periodontal cells.

Purified GFP fusion proteins when incubated with human cultured cells, including HPDLSC, MMS, SCC-1, GMSC, AGK and osteoblast cells (OBC) revealed interesting results. Although only one representative image of each cell line is presented, uptake studies were performed in triplicate and at least 10-15 images were recorded under confocal microscopy. Without a fusion tag, GFP did not enter any tested human cell line. Both CTB-GFP and PTD-GFP effectively penetrated all tested cell types, although their localization patterns differed. Upon incubation with CTB-GFP, GFP signals localized primarily to the periphery of HPDLSC and MMS, uniformly small cytoplasmic puncta in SCC-1, AGK, OBC and large cytoplasmic foci in GMSC. PTD-GFP was observed as small cytoplasmic foci in MMS, variably sized cytoplasmic puncta in HPDLSC, GMSC, AGK, OBC and both the cytoplasm and the periphery of SCC-1 cells. PGl-GFP is the most efficient tag in entering all tested human cells because GFP could be localized at tenfold lower concentrations than any other fusion proteins. PGl-GFP showed exclusively cytoplasmic localization in HPDLSC, SCC-1, GMSC and AGK cells and localized to both the periphery and cytosol in MMS, but it is only localized to the periphery of OBC. RC101-GFP was localized in SCC-1, GMSC, AGK and OBC, but its localization in HPDLSC was negligible and was undetectable in MMS cells.
Discussion and Conclusions

The assembly of cariogenic oral biofilms is a prime example of how pathogenic bacteria accumulate on a surface (teeth), as an extracellular EPS matrix develops. Prevention of cariogenic biofilm formation requires disruption of bacterial accumulation on the tooth surface with a topical treatment. Chlorhexidine (CHX) is considered 'gold standard' for topical antimicrobial therapy (Flemming and Beikler 2011; Marsh et al 2011; Caufield et al 2001). CHX effectively suppresses mutans streptococci levels in saliva, but it has adverse side effects including tooth staining and calculus formation, and is not recommended for daily preventive or therapeutic use (Auto-Gold 2008). As an alternative, several antimicrobial peptides (AMP) have been developed and tested against oral bacteria, and have shown potential effects against biofilms (albeit with reduced effects vs planktonic cells) (as reviewed by Silva et al., 2012) Unfortunately, most of these studies tested antibiofilm efficacy using continuous, prolonged biofilm exposure to AMPs (several hours) rather than topical treatment regimen as used clinically. Furthermore, synthetic AMPs are expensive to produce making them unaffordable for dental applications. Here, we show a plant-produced AMP, which demonstrates potent effects in controlling biofilm formation with a single, short-term topical treatment of a tooth-surrogate surface.

Developed cariogenic biofilms are characterized by bacteria embedded in EPS matrix, making biofilm treatment and removal extremely difficult (Paes Leme et al 2006; Koo et al 2013). EPS-rich matrix promotes microbial adhesion, cohesion and protection as well as hindering diffusion (Koo et al 2013; Flemming and Wingender 2010. EPS matrix creates spatial and microenvironmental heterogeneity in biofilms, modulating the growth and protection of pathogens against antimicrobials locally as well as a highly adhesive scaffold that ensures firm attachment of biofilms on tooth surfaces (Flemming and Wingender 2010; Peterson et al. 2015). CHX is far less effective against formed cariogenic biofilms (Hope and Wilson, 2004; Van Strydonck et al 2012; Xiao et al., 2012). The EPS are comprised primarily of a mixture of insoluble (with high content of α,3 linked glucose) and soluble (mostly α,6 linked glucose) glucans (Bowen and Koo 2011). Thus, the possibility of using EPS-matrix degrading dextranase or mutanase (from fungi) to disrupt biofilm and prevent dental caries has been explored and included in commercially available over-the-counter mouthwashes (e.g. Biotene PBF). However, topical applications of enzyme alone have generated moderate anti-biofilm/anti-caries effects
clinically (Hull 1980), possibly due to lack of antibacterial action and reduced enzymatic activity in the mouth (Balakrishnan et al 2000). Interestingly, a recent *in vitro* study has shown that a chimeric glucanase comprised of fused dextranase and mutanase is more effective in disrupting plaque-biofilms than either enzymes alone (Jiao et al 2014). However, an approach of combining antimicrobial agents with both EPS-matrix degrading enzymes into a single therapeutic system has not yet been developed, likely due to difficulties associated with cost and formulations. In this study we demonstrate that PG1 together with matrix-degrading enzymes act synergistically and effectively to disrupt cariogenic biofilms. This feasible and efficacious topical antibiofilm approach is capable of simultaneously degrading the biofilm matrix scaffold and killing embedded bacteria using antimicrobial peptides combined with EPS-digesting enzymes.

Retention of high level antimicrobial activity by protegrin along with GFP fusion opens the door for a number of clinical applications to enhance oral health, beyond disruption of biofilms. In addition to biofilm disruption, enhancing wound healing in the gum tissues is an important clinical need. We recently reported that both protegrin and retrocyclin can enter human mast cells and induce degranulation, an important step in the wound healing process (Gupta et al 2015). Therefore, antimicrobial peptides protegrin and retrocyclin play an important role in killing bacteria in biofilms and initiate wound healing through degranulation of mast cells. In addition, it is important to effectively deliver growth hormones or other proteins to enhance cell adhesion, stimulate osteogenesis, angiogenesis, bone regeneration, differentiation of osteoblasts or endothelial cells. Previously identified cell penetrating peptides have several limitations. CTB enters all cell types via the ubiquitous GM1 receptor and this requires pentameric form of CTB. PTD on the other hand does not enter immune cells (Xiao et al 2016).

In this study we tested ability of PG1-GFP or RC101-GFP to enter periodontal and gingival cells. PG1-GFP is the most efficient tag in entering periodontal or gingival human cells because GFP signal could be detected even at ten-fold lower concentrations than any other fusion proteins. Although there were some variations in intracellular localization, PG1-GFP effectively entered HPDLSC, SCC-1, GMSC, AGK, MMSC and OBC. In contrast RC101-GFP entered SCC-1, GMSC, AGK and OBC but its localization in HPDLSC and MMSC cells were poor or undetectable. Therefore, this study has identified a novel role for protegrin in delivering drugs to osteoblasts, periodontal ligament cells, gingival epithelial cells or fibroblasts to enhance oral health. It is feasible to release protein drugs synthesized in plant cells by mechanical grinding
and protein drugs bioencapsulated in lyophilized plant cells embedded in chewing gums provides an ideal mode of drug delivery for their slow and sustained release for longer duration. This overcomes a major limitation of current oral rinse formulations - short duration of contact of antimicrobials on the gum/dental surface.

Beyond topical applications, protein drugs fused with protegrin expressed in plant cells can be orally delivered to deeper layers of gum tissues in a non-invasive manner and increase patient compliance. Protein drugs bioencapsulated in plants can be stored for many years at room temperature without losing their efficacy (Su et al 2015; Daniell et al 2016). The high cost of current protein drugs is due to their production in prohibitively expensive fermenters, purification, cold transportation/storage, short shelf life and sterile delivery methods. All these challenges could be eliminated using this novel drug delivery concept to enhance oral health. Recent FDA approval of plant cells for production of protein drugs (Walsh 2014) augurs well for clinical advancement of this novel concept.

References


prevention of oral mucositis in patients receiving stomatotoxic chemotherapy (PROMPT-CT trial). Leuk Lymphoma. 44:1165-72.


Ohashi, Tomoo, Stephane D. Galiacy, Gina Briscoe, and Harold P. Erickson. An Experimental Study of GFP-Based FRET, with Application to Intrinsically Unstructured Proteins. Protein


Example II

**Creation of chloroplast vectors expressing AMP, biofilm degrading enzymes and fusion proteins thereof**

Effective treatment of biofilm-associated infections is problemantic as antimicrobials often fail to reach clusters of microbes present within the surrounding extracellular matrix that enmeshes and protects them. Furthermore, development of novel therapies against biofilm-related oral diseases and maintenance of oral health needs to be cost-effective and readily accessible.

To ensure a continued supply of reagents, dextranase/mutanase and protegrin/retrocyclin are expressed independently and as fusion proteins in tobacco and other plant chloroplasts, such as lettuce. Proteins will be produced and used in low cost purification strategies. Tobacco plants produce a million seeds, and thus, it is feasible to scale up production easily. Each acre of tobacco will produce up to 40 metric tons of biomass, facilitating low cost large scale production.
of AMP, enzymes and fusion constructs encoding the same. In another approach, the proteins are produced in an edible plant such as lettuce.

Several dextranases (Dex) and mutanases (Mut) have been isolated from fungi and bacteria and characterized for their enzymatic activity. Optimal dextranase and mutanase enzymes should have enzymatic properties suitable for human oral environment. Based on short duration of oral treatments, strong binding/retention property to plaque-biofilms and catalytic activity to both types of EPS (dextrans and mutans) are highly desirable. The enzymes added in commercial dextranase-containing mouthwashes (e.g. Biotene) are largely derived from fungi (*Penicillium* sp. and *Chaetomium erraticum*). However, fungal dextranases show higher temperature optima (50-60°C) than bacterial dextranases (35-40°C). Furthermore, bacterial dextranases are more stable and effective at oral temperature (-37°C) and are suitable for dental caries-prevention. Recently, a dextranase from *Arthrobacter* sp. strain Arfb410 showed superior dextran degradation properties at optimal temperatures (35-45°C) and pH values (pH 5-7) found in mouth and in cariogenic biofilms when compared to fungal dextranases. In addition, topical applications of bacterial dextranase are more effective in reducing dental caries *in vivo* than fungal dextranase. Likewise, a bacterial mutanase from *Paenibacillus* sp. strain RM1 shows that biofilm was effectively degraded by 6 hr incubation even after removal of the mutanase, preceded by first incubation with the biofilms for 3 min. Also, when compared to other microbial species, RM1 mutanase shows enhanced biofilm-degrading property. Notably, fungal enzymes require glycosylation, which precludes their expression in chloroplasts. In addition, immunogenicity of glycoproteins in human system may raise additional regulatory concerns. Therefore, the present invention involves use of bacterial dextranase and mutanase for expression in chloroplasts.

In order to increase the production of Arfb410 dextranase and RM1 mutanase protein in chloroplasts, both sequences have been codon optimized for chloroplast expression. See Figures 9A and 9B.

**Retrocyclin and Protegrin.**

In order to maximize synthesis and reduce toxicity of AMPs, ten tandem repeats of PG1 or RC101, separated by protease cleavage sites as shown in Figure 10 are employed. For each copy of expressed gene, ten functional copies of PG1 or RC 101 will be made. For this purpose we have chosen the Tobacco Etch Virus (TEV) protease, which has high specificity and a short
cleavage site of seven amino acids. Alternatively, furin cleavage sites can also be employed. This vector can also be engineered to include a nucleic acid encoding a biofilm degrading enzyme. The coding region can be expressed under the promoter utilized to express the AMP or can be ligated into the vector operably linked to a second promoter region. The biofilm degrading enzyme coding sequence may also contain TEV protease cleavage sites to facilitate release of the enzyme. This approach provides a safer and cleaner option than chemical cleavage methods. Most importantly, individual PG1 peptides in the fusion protein will not form secondary structures before cleavage, thereby avoiding accumulation of functional peptides which can be lethal to the host production systems. Antimicrobial activity of the cleaved PG1/RC101, biofilm degrading enzymes or fusion proteins thereof can be used to degrade biofilms using the methods disclosed in Example 1.

As mentioned above, the sequences encoding the AMP/biofilm degrading enzymes are optionally codon-optimized prior to insertion into chloroplast transformation vectors, such as pLD. Chloroplast transformation relies upon a double homologous recombination event. Therefore, chloroplast vectors comprise homologous regions to the chloroplast genome which flank the expression cassette encoding the heterologous proteins of interest, which facilitate insertion of the transgene cassettes into the intergenic spacer region of the chloroplast genome, without disrupting any functional genes. Although any intergenic spacer region could be used to insert transgenes, the most commonly used site of transgene integration is the transcriptionally active intergenic region between the \textit{trnl-trnA} genes (in the \textit{rrn} operon), located within the IR regions of the chloroplast genome (Figure 10). Because of similar protein synthetic machinery between \textit{E. coli} and chloroplasts, efficiency of codon-optimization can also be assessed in \textit{E.coli} and then plants can be created. Both systems could be used for expression of AMPs, biofilm degrading enzymes or fusion proteins thereof, as well as for purification and evaluation of AMPs or enzymatic activities.

**Purification strategies**

A hydrophobic interaction column (HIC; TOSOH Butyl Toyopearl 650m) can be used to purify PG1 fused with Green Florescent Protein (GFP). The GFP selectively binds to the HIC and facilitates RclOl/PG1 to >90% purity. Despite using the expensive HIC chromatography method, recovery is very poor (<20%). To address this problem and enhance yield, 10 tandem
repeats of PG1 with an elastin like biopolymer (GVGVP (SEQ ID NO: 11); Fig. 10) are engineered into the vector. This biopolymer, has a unique thermal property of precipitating out of solution upon increasing temperature above its inverse transition temperature (Tt). GVGVP remains in soluble monomeric state below Tt and form insoluble aggregates above it. This phase transition from soluble to insoluble state is reversible by changing the temperature of the solution and this facilitates protein purification. Subsequently fused protein is re-solubilized by cooling below Tt and to remove any insoluble contaminants that have co-precipitated as shown in Figure 11. The process of heating (37°C ) and cooling (4°C) is known as Inverse Transition Cycling (ITC) and performing 3-5 rounds of ITC results in highly purified proteins (>98% purity, Figure 11).

In an alternative approach, a signal peptide is fused with dextranase or mutanase for expression in E. coli, where the signal peptide will result in secretion of the enzymes into the extracellular media. In addition, secretory proteins should pass through two membrane systems of E.coli, during which they pass through the periplasmic environment where disulfide isomerases, foldases and chaperones are present. Therefore, correct folding and disulfide bond formation of secretory proteins are facilitated by the enzymes, resulting in enhancement of biological activity of proteins (ideal for AMPs). Another merit of this production strategy is the low level of proteolytic activity in the culture medium which serves to enhance the stability of the recombinant protein. The signal sequence of the secreted protein is cleaved during the export process, creating an authentic N-terminus to the native protein. There are several molecules useful for translocating proteins to extracellular media, such as TAT, SRP, or SecB-dependent pathways. However, rather than working independently, the different pathways closely interact with each other. Both SRP and SecB-dependent pathways can work together in targeting of a single protein. Also, under Sec-deficient conditions, translocation of Sec pathway substrates can be rescued by TAT systems.

Among numerous signal sequences, outer membrane protein A (OmpA) and Seq X (derived from lac Z) signal peptide demonstrate superior export functions and are capable of exporting fused protein into extracellular medium at up to 4g/L and 1g/L, respectively. Therefore, these signal sequences are used for efficient exporting of Arth 410 Dex and RMI Mut to extracellular milieu. Accumulation of the dextranase and mutanase exported into media will be determined by protein quantitation and enzyme assays.
Successful expression of these proteins in E. coli has been achieved. See Western blot results shown in Figure 12. Chloroplast vectors harboring these sequences will be bombarded into tobacco or lettuce leaves to create plants capable of large scale production of extranase/mutanase/AMP proteins. After harvesting large scale biomass, leaves will be lyophilized and stored at room temperature. In another approach, clinically-proven anti-caries compounds such as (fluoride 250ppm) and a broad-spectrum bactericidal, chlorhexidine 0.12% can be included to assess whether these agents increase efficacy.

The AMP-enzyme combination effectively disrupts cariogenic biofilm formation and the onset of cavitation in vivo. Furthermore, AMP-enzyme fusion protein appears to be superior to current chemical modalities for antimicrobial therapy and caries prevention.

As mentioned previously, effective AMP-enzyme (independently or in combination) can be expressed in lettuce chloroplasts under the control of endogenous lettuce regulatory elements, for large scale GLP production and stability assessment. A key advantage is the lower production cost by elimination of prohibitively expensive purification processes. Freeze-dried leaf material expressing AMP/enzymes can be stored at ambient temperatures for several months or years while maintaining their integrity and functionality. See Figure 13. In addition to long-term storage, increase of protein drug concentration and decrease of microbial contamination are other advantages. Lettuce leaves, after lyophilization showed 20-25 fold increase in protein drug concentration when compared to fresh leaves, thereby reducing the amount of materials used for oral or topical delivery. Following lyophilization, the plant material can be incorporated into a chewing gum to deliver the biofilm degrading compositions contained therein.

The steps for producing the AMP/enzymes or fusions thereof are shown in Figure 12. The lettuce chloroplast vectors useful for expressing the proteins of the invention have been previously described in US Patent Application No. 12/059,376, which is incorporated herein by reference. Expression levels of up to 70% of total protein in case of therapeutic proteins like proinsulin in lettuce chloroplasts can be achieved using this system.

AMP-enzyme(s) expressed in the edible plants are preferably orally delivered (topically) when used for treatment of oral diseases and the prevention and inhibition of dental carie formation. For enhanced lysis of plant cells within the oral cavity, AMP/enzyme expressing plant cells are optionally mixed with plant cells expressing cell wall degrading enzymes, described in US Patent Application, 12/396,382, also incorporated herein by reference.
Chewing gum tablet preparation is shown in Figure 14. Using GFP as an example of the protein of interest, this data shows the amounts of GFP that can be incorporated in to a chewing gum tablet. GFP levels were assessed both via fluorescence and by western blot. The results are shown in Figure 15. The present inventors employed the chewing simulator shown in Figure 16 and artificial saliva to assess GFP release kinetics from the gum tablets comprising GFP. Figure 17 shows a graph illustrating the release kinetics over time from gum tablets comprising different amounts of GFP present in recombinant lettuce.

It is clear from these data that gum tablets comprising the AMP-enzyme fusion proteins of the invention will deliver the active material for a suitable time period to achieve bacterial kill and plaque or biofilm degradation. However, oral rinses such as Listerine can also be employed to deliver the AMP-enzyme fusion proteins or combinations of the invention. Figure 18 demonstrates that crude extracts comprising the enzymes of the invention mixed with Listerine are as effective as commercially produced and purified enzymes that are quite costly to prepare. The data reveal that the dual-enzyme at various combinations (both different ratio and amounts) markedly reduced the biomass of *S. mutans* biofilm, in a dose-dependent manner. Among different combinations, 25U Dex and 5U Mut (5:1, Dex:Mut ratio) was the most effective, resulting on more than 80% of the total biomass degradation within 120 minutes. Further experiments confirmed that 5:1 Dex/Mut activity ratio displayed the highest effectiveness for both EPS degradation and bacterial killing by Listerine. Excitingly, the dual-enzyme pre-treatment dramatically enhanced the efficacy of Listerine-mediated bacterial killing (> 3 log reduction vs vehicle pre-treatment and Listerine). The inclusion of a third enzyme further enhanced the overall anti-biofilm activity. Furthermore, results from the mixed-species model indicated that the dual-enzyme combination was capable of not only enhancing the overall antibacterial activity, but also inducing targeted reduction of *S. mutans* dominance (while increasing the proportion of commensal/probiotic *S. oralis*) when Listerine was used after enzymes pre-treatment. Accordingly, the enzyme+Listerine strategy should selectively target the pathogen *S. mutans*, while increasing the proportion of commensal *S. oralis*, thereby preventing microecological imbalance within mixed-species biofilm.

AMPS have the ability to stimulate innate immunity and wound healing, in addition to antimicrobial activity. Harnessing this novel mast cell host defense feature of AMPs in addition to their antimicrobial properties expands their clinical applications.
the most challenging model for development of topical therapeutics. When developed, such topical drug delivery can be easily adapted to other biofilms, as matrix formation hinders drug efficacy in many other biofilm-associated diseases. Matrix is inherent in all biofilms thus the application goes beyond the biofilm in the mouth. The biofilm inhibiting compositions described herein can also be employed in coating stents, artificial joints, implants, valves and other medical devices inserted into the human body for the treatment of disease.

As discussed above, the AMP/enzymes, or leaves expressing the same can be incorporated into a chewing gum for effective topical application of the same for the treatment of oral disease. The compositions may also be incorporated into an oral rinse, such as Listerine. As mentioned previously, other anti dental carrie agents such as fluoride or CHX may included in such gums or oral rinses.

The references below in Table 2 describe a number of different mutanases from a variety of biological sources. Each of these references incorporated herein by reference.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Mutanase resource</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2014</td>
<td>Paenibacillus humicus NA1123</td>
</tr>
<tr>
<td>2</td>
<td>2013</td>
<td>Trichoderma harzianum CCM F-340</td>
</tr>
<tr>
<td>3</td>
<td>2013</td>
<td>Trichoderma harzianum strain CCM F-340</td>
</tr>
<tr>
<td>4</td>
<td>2012</td>
<td>Paenibacillus humicus</td>
</tr>
<tr>
<td>5</td>
<td>2012</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>6</td>
<td>2011</td>
<td>Paenibacillus humicus</td>
</tr>
<tr>
<td>7</td>
<td>2008</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>8</td>
<td>2008</td>
<td>Paenibacillus sp. strain RM1</td>
</tr>
<tr>
<td>9</td>
<td>2008</td>
<td>Paenibacillus sp. strain RM1</td>
</tr>
<tr>
<td>10</td>
<td>2005</td>
<td>Trichoderma harzianum CCM F-340</td>
</tr>
<tr>
<td>11</td>
<td>2004</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>12</td>
<td>2001</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>13</td>
<td>1997</td>
<td>Trichoderma harzianum CCM F-341</td>
</tr>
<tr>
<td>14</td>
<td>1996</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>15</td>
<td>1993</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>16</td>
<td>1990</td>
<td>Pseudomonas sp. strain</td>
</tr>
<tr>
<td>17</td>
<td>1988</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>18</td>
<td>1978</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>19</td>
<td>1978</td>
<td>Trichoderma harzianum</td>
</tr>
<tr>
<td>20</td>
<td>1972</td>
<td>Trichoderma harzianum OMZ 779</td>
</tr>
</tbody>
</table>
Additional biofilm degrading enzyme encoding sequences useful in the practice of the invention, include without limitation,

1) *Paenibacillus humicus* **NA1123**

See also http://www.ncbi.nlm.nih.gov/nuccore/AB489092

<table>
<thead>
<tr>
<th>Genbank</th>
<th>AB489092</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length</strong></td>
<td>:1,146</td>
</tr>
<tr>
<td><strong>Mass (Da):</strong></td>
<td>119,007</td>
</tr>
</tbody>
</table>


2. The protein sequence of mutanase from *Paenibacillus humicus* NA1123


3. Sequence of mRNA from *Paenibacillus humicus* NA1123

| >gil257153265ldbj IAB123187. Ilputative mutanase [Paenibacillus humicus] |
|-----------------------------|------------------|
| 1 | aaagaggtcatgcaaaacctaacctctcaccagaagaaggtagtggcagcccagaaagggaa |
| 6 | 1aagaggtcatgcaaaacctaacctctcaccagaagaaggtagtggcagcccagaaagggaa |
| 12 | 61aagaggtcatgcaaaacctaacctctcaccagaagaaggtagtggcagcccagaaagggaa |
| 18 | 121aagaggtcatgcaaaacctaacctctcaccagaagaaggtagtggcagcccagaaagggaa |
| 24 | 181aagaggtcatgcaaaacctaacctctcaccagaagaaggtagtggcagcccagaaagggaa |

41
241 atcgctcccg ccaccgctgc aggaggcgcg aatctgacgc tcggcaaaac cgtcaccgcc
301 agcggccagt cgcagacgta cagccccgac aatgtcaagg acagcaatca gggaacttac
361 tgggaaagca cgaacaacgc cttcccgcag tggatccaag tcgaccttgg cgcctagcagc
421 acatccactc aatcccgcaa ccggcaatac ggtgacgatt ccggtctccg cgacggtcaa gcgcctccag
481 gcagcgcgaa cggctcgacg ttcacgaaca ggtcgtcggat ggcctggctat
541 acatccactc ctcgctgcgcg gtcacagatg tgggagtcgg cagcaacttc ctctctcacgc
601 ggcacgccag cgcctaatcc ggactgacgc attaccggca tgtcctggac tcgctccggta
661 ccgactcctc cggcaggcag caacatcgcc gtagggaaat cgattacagc gctcttccagc
721 ccgactcctc cggcaggcag caacatcgcc gtagggaaat cgattacagc gctcttccagc
781 ccgactcctc cggcaggcag caacatcgcc gtagggaaat cgattacagc gctcttccagc
841 acagcaaccg cgcctaaat ccgctactca ccgctaaaac gcgctctctgc gcgctctctgc
901 gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc
961 gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc
1021 gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc gcgctctctgc
1081 ggcgcgcttg cggcaggagc ttctgcaaat gtatcgatca atgcaggcgc caaagcagcc
1141 ctctcccttgc cggcaggacgc ggagcgggct cggcaggacgc ggagcgggct cggcaggacgc
1201 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1261 cggtctgacg cggcaggacgc ggagcgggct cggcaggacgc ggagcgggct cggcaggacgc
1321 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1381 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1441 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1501 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1561 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1621 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1681 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
1741 gcgacgccag cgcctacctc ggacctgac gcgccggttc ttctctctgc gcgctctctgc
ACCTATACTGG TACGTGGCAG CAGGCTGACG CTACGGCCTG GACGGCAATG AAATTCCGGC CAAGCAAGGC

AACAAATTCACT GCCATATTGC CGCGAGCAAC CCTACGCTC GCACGGCGAG CACCGGCTCC GCACGGCAG

AACAGCAGAC ACATCATCAA CTCACGGCGG CAGACCGGAG CAGCGGCGAG CACGCGGAGC
II) *Paenibacillus curdlanolyticus* MP-1

1. General information of of mutanase from *Paenibacillus curdlanolyticus* MP-1


Genbank HQ640944; **Length:** 1,261; **Mass (Da):** 131,631


2. The protein sequence of mutanase from *Paenibacillus curdlanolyticus* MP-1

>gil315201261lgblADT91063.1l alpha-1,3-glucanase [Paenibacillus curdlanolyticus]

3. Sequence of mRNA from *Paenibacillus curdlanolyticus* MP-1

1 atgcgcaaca agatgtcatc atggcaggct gcgcctgct aatcgttttct cgctgacagc 

61 ctgctctgct gccggtgttc agactgagct gcgcctgct aatcgttttct cgctgacagc
168 1 gcgaacaaca gttatacgaa cgcatcctcg ctcgtcgtcg ctcctgtcgc aagctctgac
1741 ttggttggcg cgacgacgtg gacgcctagc acgccggttg ...
2041 gggacaacgc taagcacttc gaacatcgtt gttatgcac aagatgcgtcgaat
2101 tacagcgttt atgacaacgc agacgccttc cgtgacccccagatacgc
2161 ccaacacctta accaagcgcg aatgctctcg gacacaatcg gacaaatcct gacagactgc
2221 ccttcgaacg gtcattgcctt ccaagctgaa gacaagcgggg aagctgtcgtt
2281 acagatgctg tcacagtggc ggttacgtaa gacgatgggt gttgaagttc ttcgcgctgc
2941 ctggcggttt ggacagtaa cagcaatggc gcgccagcag gcgtgaacaa cacggttcag
III) Paenibacillus sp. strain RM1.

1. General information of mutanase

Genbank E16590; Length: 1291; Mass (Da): 135kD
The protein sequence of mutanase from Paenibacillus sp. strain RMI

Deduced amino acid sequence of mutanase RMI. The signal peptide region is underlined, and the linker region is boxed. The arrow indicates the cleavage site for the N-terminal domain of the protein. The DNA sequence was registered as GenBank accession number E16590. (SEQ ID NO: 16)

3. Sequence of mRNA from Paenibacillus sp. strain RMI

1 cccgggtacc agaacatcg ggaaacaagc gacgcccct tgcgctata tcgcctacgg
   61 acgggtcgg cgagggtt ctggggcttc acatcctec tcgggatcg
   121 tatatccct tttgcgct gcggggtcgc ggacaggggt gagaagcgct gcttcg
   181 cagaatttc gcgtgctatg ctataagggc cccagcaagc cacctgctgc
cgaggtcg
   241 tcgtggtgg ggctgcctag ccaagagcgc atcgctggtgc gagaagtcgg
cgaggtcg

15 301 cccgggaanaa tgcggagcat gctggggtgc ggacatcgcg cgggtgtgtg gcgcggcag
361 attgcggtaa tcgcggccct tcgctcggcc tggaagaagc gggaccggag catgaccgtc
421 gtcttcgttg gaatcgcctc gtcttatctt ccgtgggttt ...
541 aaaaatggaat agegttaagcc gggatatcgg attgcgaacgc tccttaacgc aggegeggtt
546 ctgcggcttg tcgctcggcc tggaagaagc gggaccggag catgaccgtc
601 attgcggtaa tcgcggccct tcgctcggcc tggaagaagc gggaccggag catgaccgtc
661 ggcgaccgcg ttctgaagtg gttcccgcca gggatatcgg aggegeggtt
721 acaaggagc aggagggagc aggegeggtt gggatcgcctc ttcttaacgc
781 ggcgaccgcg ttctgaagtg gttcccgcca gggatatcgg aggegeggtt
841 ggtatcccaag caagaaagagcg gttcgaacgc tccttaacgc aggegeggtt
901 attgcggtaa tcgcggccct tcgctcggcc tggaagaagc gggaccggag catgaccgtc
961 ggcgaccgcg ttctgaagtg gttcccgcca gggatatcgg aggegeggtt
1021 ctgcggcttg tcgctcggcc tggaagaagc gggaccggag catgaccgtc
1081 cccaaacctg caegcttcag aacggaagcg gggattcgcctc tccttaacgc
1141 ggcgaccgc gggacgacgc tcctgaagtg gttcccgcca gggatatcgg aggegeggtt
1201 acaaggagc aggagggagc aggegeggtt gggatcgcctc tccttaacgc
1261 gtcgcggcttg tcgctcggcctggaagaagc gggaccggag catgaccgtc
1321 acaaggagc aggagggagc aggegeggtt gggatcgcctc tccttaacgc
1381 ggcgaccgcg ttctgaagtg gttcccgcca gggatatcgg aggegeggtt
1441 acgcgggtcg ccgaccagacgc ggctgctgcttggaagaagc gggaccggag catgaccgtc
1501 cccaaacctg caegcttcag aacggaagcg gggattcgcctc tccttaacgc
1561 gtcgcggcttg tcgctcggcctggaagaagc gggaccggag catgaccgtc
1621 acaaggagc aggagggagc aggegeggtt gggatcgcctc tccttaacgc
1681 cccaaacctg caegcttcag aacggaagcg gggattcgcctc tccttaacgc
1741 acaaggagc aggagggagc aggegeggtt gggatcgcctc tccttaacgc
1801 acgcgggtcg ccgaccagacgc ggctgctgcttggaagaagc gggaccggag catgaccgtc
1861 gtcgcggcttg tcgctcggcctggaagaagc gggaccggag catgaccgtc
1921
1921 cgcctacacc cacacctact ccgaccccga cgcctacgcc tacacctaca cctacaccta
1981 cgccgacgcc tcctccgggc ggcaacatcg ccatcggcaa ... aaagtcaaaa ccgtatcgct gacctcctac tacagctggc
3421 agtatttctc gggcgatatg cccggagacg ctcccagcgc gggccgtccg ctcttccgct
348 lttgacgaagt gcactggaag ctggatactc cgctcaaacc cggagacacg attcgcatcc
3541 agaagaacaa cggcgacagc ctggaatacg gtgtcgactt ... ggtgtggtt cagggagagc
4981 taattgcgtt gcgctcactg cccgctttcc agtcgggaaa ctgtcgtgcc agctgcatta

5 3721 acaagatctt gtacatcgg gaaggaacct tccacctgg caacatgtgg gagaatctt
10 4021 agacatctta atgcgtgttt tggggtggg gactacggcta taacgcgggcc atccgcgcga
15 4321 gacacaagcg caacaccaac tacatcttg gactgctttg cggtccgctg atecggtatg
20 4621 atctgcctgaac cggcgggtgcc tggcggaggc atggcgtgttt cggcgggttt cacaagatctt
25 4921 taacttcgct gcatctgatg cagccgacac gactacggcta taacgcgggcc atccgcgcga
IV) *Trichoderma harzianum* (CCM F-470)

1. General information of of mutanase from *Trichoderma harzianum*

Also see [http://www.uniprot.org/uniprot/Q8WZM7](http://www.uniprot.org/uniprot/Q8WZM7), Length: 635

Mass (Da): 67,726

Last modified: March 1, 2002 - v1

Checksum: iBB0D864E2F432C58

2. The protein sequence of mutanase from *Trichoderma harzianum*

[http://www.uniprot.org/uniprot/08WZM7.fasta](http://www.uniprot.org/uniprot/08WZM7.fasta)

3. Sequence of mRNA (Trichoderma harzianum)

[http://www.ebi.ac.uk/ena/dawview/AJ243799&display=fasta](http://www.ebi.ac.uk/ena/dawview/AJ243799&display=fasta)

4. [http://www.uniprot.org/uniprot/08WZM7](http://www.uniprot.org/uniprot/08WZM7)
V) Trichoderma harzianum

1. General information of mutanase from Trichoderma harzianum

Also see http://www.uniprot.org/uniprot/Q8WZM7;

2. The protein sequence of mutanase from Trichoderma harzianum

See: http://www.uniprot.org/uniprot/08WZM7.fasta

GACGCGAGTCTGGCAACTACATCGGCCTGTGCCAATTCAGCTGCAACTACGGTTACTGC
CCACCAGGACCCTGTAAGTGCACCGCCTTTGGTGCTCCCATCTCGCCACCCGCATCCAAC
GGCCGCAACGGCTGCCCTCTGCCGGAGAGAGCGATGTTATCTGGGCTGTGCCAGTTTC
AGTTGTAACCATATAACTGCCCGCCTTTGGTGCTCCCATCTCGCCACCCGCATCCAAC

A (SEQ ID NO: 19)

(There is a polyA tail since Trichoderma harzianum is fungi)
3. Sequence of mRNA (Trichoderma harzianum) Further information can be found at http://www.ebi.ac.uk/ena/data/view/AJ243799&display=fasta

>ENAIAJ243799IAJ243799.1 Trichoderma harzianum mRNA for alpha-1,3-ghicanase (p_3 gene)
ATGTTGGCCTTTCCCGCCCGCTCAAGCTCGCCCGCCCTTGCGCCCGAGCGCTGTGTCTCTCT
CTCGGCCATCCGCGCCCTATGTTGCTATCCTGCTGATGGGTAGGATCGGAGCGCGCTCTCT
GCTGACCTGTCGAATATCCTGATGGGTAGGATCGGAGCGCGCTCTCT
GCAGATTATGATGACGATATGCAACGTGCCAAAGCCGCTGGCATTGACGCCTTCGCCCTG
AACATCGGCCCGCTGCTAATACGACAGCGCTCCTCTTCTTCTGCCGCTGGCATTGGTTTGG
GGCAAGCCTTACCTGGCAGCCTTGCTCAACTTGGGTTTCTCAACCATTTCGGCGCCCGAAGTT
TCTATTCCAAAGAACTGGGTTTCCCAAGTGGGCCTCTGATCTATAACCGGTGGCAACAA
GTCTTGCAGCAAGGGTCTCCAAAGGGTTGAGATCGTTACCTGGGAAATCTACGCGGGGTCTT
CACATCGTCGCTCCCTGAAGTCTAATGGGAAATGGGTCGCAATATTCCAAGAACTGGGTTTCC
AGGATACCGACATCTCAGAATAGTGGTAATTTCTTCCAAGCCGCTGATAGGACCGGCCAG
AAGGATACCGACATCTCAGAATAGTGGTAATTTCTTCCAAGCCGCTGATAGGACCGGCCAG
AACTTTAACGCTGGGACGCTGGCAGCGCCAGCAACACCTTAACCGCCGCTGCTAAAT
GGAAGGCAATTACTTGGAGGGACGCCCCGCTAGGGTGCAACACTAGATGATACGGTTACGTG
GGCGCACTTCTCAAGACTGCCGCTAGCCTACGCCTGCTCGTCGTGCTGCTGCGGCAACCAC
CAAACGTTTCCAGGCCAAGCGCGGAGGAATCCACCTTCTTCCAATCCCGGCGAGCATCGCGG
CAGCAGGATTTGCTCTGCTGCTGTCAAGCGCCACCTTCTTCCAATCCCGGCGAGCATCGCGG
CAGCAGGATTTGCTCTGCTGCTGTCAAGCGCCACCTTCTTCCAATCCCGGCGAGCATCGCGG
CAGCAGGATTTGCTCTGCTGCTGTCAAGCGCCACCTTCTTCCAATCCCGGCGAGCATCGCGG
CAGCAGGATTTGCTCTGCTGCTGTCAAGCGCCACCTTCTTCCAATCCCGGCGAGCATCGCGG
(There is a polyA tail since *Trichoderma harzianum* is fungi)

**Dextranase (Dex)** gene from *Penicillium minioluteum*

GenBank: L41562.1

(http://www.ncbi.nlm.nih.gov/nuccore/L41562.1)

The mature protein has 574 amino acids with MW at 67KD. The optimum reaction condition is pH 5.5 and 40°C. The pH range is 3-6.

**Amino acid sequence**

MATMLKLLALTALASEAIGAVMHPGNSHPGTNHMGTTTNGC

ADFCTWVHSDEINTQTPVQPGVRSHSHYAVVQVSLAGTTNHFDSFVFYESIPRNGR

IYAPTDPNSNLDSSVDDGISEPSILMNMAWQFEYSHDVKILATDSSGLSPS

DVVIRPVISYASIQLSDGDGIVIRPADANGRKSVEFKTDLYTFLSDGNEYVTSKGS

VVGVGEPNTALVIFASPFLPSGMIPHMTDPDNTQTMTPPIMGDGGKSILFPPGVY

MNQDDQSGNSKGGLSNHRLNSNTYWWYLAPAGAVKGAIEYFTKQNYFATGHGILS

ENVYQQANAGDNYIAVKSDSTSLRMWWHHNLGGQQTWYCVGPTINAPPNTMDFNNGS

GISSQISDYKVQGAFQFTDGPQEIYPSVVDVFHVNDDAIKYSGAVSRSATIW

NDPIIQMGWTSRDISGVTIDTNLVIIHRYIKSETVVPASIAFSPFYASGMPDSRK

ISMTVSNVVCGLCPSLFRITPLQNYKNNVFVKNAFDPGLQTNISGTGIESIPAASGL

TMGLNISNWTGGQKVTMENFQANSLGQFIDGSYWGEWQIS  (SEQ ID NO: 22)
DNA sequence

1 ggcatagtaa tccecgacace cgagatagat ggagctttcg aactctaaccc
6 1 agaccttgct tgagctggag agctaaaaca ... caagtcaaat tagcgactat aagcaggtgg gagccttctt
2341 cttccagacg gatggaccag aaatatatcc caatagtgtc gtgcacgacg tcttctggca

5 1 caagatacgat cccttggaggt gtatgatact cccagatcct cccttggcgagt
241 agcttacccct eattgacgtg accttggat gtcggagctt cccttggcttt
301 acggactacc cttttggcct ttttgtggct tgtgtggacg
361 atgaggtttaagctcttc ccacttcctc cctcttccttcctttttgtgctt
gtaaaactcatag ttttctggcc cctgtgtgtt cccttggcttt
421 cttgagtttact gcttgacactt gaaacccgtt ggcggtttttcttctttttttttt
481 caaatgtgct gtcggcgacg gcgggtttttcttctttttttttt
541 ttgagtttact gcgtgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
2. **Dextranase** (Dex) gene from *Penicillium acuicatum* (Talaromyces *aculeatus* strain zOl)

GenBank: KF999646.1 ([link](http://www.ncbi.nlm.nih.gov/nuccore/KF999646.1))

The optimum pH is around 5. The pH range is 3-6.

**Amino acid sequence**

```
MATMLKLLTTLALAIASEAIGAVLPPPSSHPSSTRTDDTNTHCG
ADFTCWWDSEINTQTYPVQPGNRQSHKYSVQVSLAGANNFQDSFVYESIRPNNGR
IYAPTDPPNALTLDSSVDGIESHEISGLNMAWQSFEPQVDIKIADGSSLGPS
DVVIPVSISYAIQSGDDGIVRVPADANGRKFSVEFKNDPYTFLSDGNEYVTSGGS
VVGVEPTNALVIFASPFLPSGMPHMTDPNQTMTPGPNNGDWGSKLYFPPGVYW
MNQDQSGNSGLGSHNLNSNTYWYFAPAYVKGAEYFTKQNFYATGHGVLSGEN
YYQANAGEVNYAVKDSTSLRMWWHNNLGGGQTWYCVGTKTDNPFMDFNNSGIG
SSQIDYKQVQAGFFQTGDPEIPEVNSVHDVFVYNDDAIKYYSGASVSRATWCH
NDPIIQMGWTSRDQTVTIDLNVITHRYKSETVPSAIRGASPYASGMPSDDKSK
ISMTVSNVCEGLCSLFRTTPLQNYKNFVVKNVAFDGLQTNSIGTGESIIPAAAGL
TMGLDISHSWVGGKVMQNFQANSLGQFDIDGSYWGEWQIN (SEQ ID NO: 23)
```
DNA sequence

1 atggccacaa tgctaaagct acttacgttg gcccttgcaa ttagcgagtc tgccattgga
6 1 gcagtcctgc acccacctgg cagttctcat cccagtaccc gtacggacac tacgaataat
11 acccattgcg gtgccgactt ctgtacctgg tggcatgatt caggcgagat caacacacag
16 gctggtgcga aacacctgtcc aaccggggaa cgtgcgccaa tctcacaagt attccgtaca
21 agtgagccta tgcagtcctgc acccacctgg cagttctcat cccagtaccc gtacggacac tacgaataat

3. Penicillium funiculosum dexA gene for dextranase


The optimum pH is around 5.5. The optimum temperature is 60°C. The pH range is 5-7.5 (http://www.sciencedirect.com/science/article/pii/S0032959298001277)

Amino acid sequence

MATMLKLLATLALAEISAIGAMHVHPGVSHSHPGTHTGTGTNTNCHG
ADFCWTWHDSEGIEINTQTPVQPGNVRQSHKYSVQVSLAGTNNFHDSFYVESIPRNGRNG
IYAPTDPNSNTLDSVDDGISIEPSGLNMAWSQFEYSQVDIKIATDGDLSGPS
DVVRVPVISYAIQNSNGGIVIRVPADANGRKFSEVFKNLDYTLSDGNEYVTSGGS
VVGVEPTNALVIFASPFLPSGMIPHMKPHNTQTMTPGPINNGDWGAKSILYFPPGVYW
MNQDQSGNSGKLNSHRLNSXYWVYLAPGAYVKGAIEYFTKQNFYATGHGVLSGEN

(SEQ ID NO: 25)
While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such
embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.
What is claimed is:

1. A multi-component composition comprising at least one antimicrobial peptide (AMP) and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures and inhibit biofilm deposition, said composition biologically acceptable carrier for delivery of said composition.

2. The composition as claimed in claim 1 wherein the AMP is selected from protegrin 1, RC-101 and the AMPs listed in Table 1.

3. The composition as claimed in claim 1 wherein the biofilm degrading enzyme is selected from mutanase, dextranase, glucoamylase, glucanase, deoxyribonuclease I, DNAase, dispersin B, glycoside hydrolases, and the enzymes listed in Table 2.

4. The composition as claimed in claim 1 wherein said AMP and said biofilm degrading enzyme are produced recombinantly and form a fusion protein.

5. The composition of claim 1, wherein said composition comprises 2 biofilm degrading enzymes comprises mutanase and dextranase and said AMP is PG-1.

6. The composition of claim 5 wherein said dextranase to mutanase ratio in said composition is 5:1.

7. The composition of claim 6 further comprising glucoamylase.

8. The composition as claimed in claim 1 further comprising an antimicrobial/antibiotic.

9. The composition as claimed in claim 1, further comprising fluoride and, or CHX.

10. The composition of claim 1, wherein said carrier is chewing gum.

11. The composition of claim 1, wherein said carrier is an oral rinse.

12. The composition of claim 1, wherein said carrier is a biologically compatible buffer.

13. A chewing gum comprising the composition of claim 1, claim 5 or claim 6.
14. An oral rinse comprising the composition of claim 1, claim 5 or claim 6.

15. The oral rinse of claim 11, comprising Listerine.

16. A method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the multicomponent composition of claim 1, said composition having a bactericidal effect, and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination.

17. A method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the multicomponent composition of claim 5, said composition having a bactericidal effect, and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination.

18. A method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the multicomponent composition of claim 6, said composition having a bactericidal effect, and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination.

19. The method of claim 16, claim 17 or claim 18, wherein said biofilm is present in the mouth.

20. The method of claim 16, claim 17 or claim 18, wherein said biofilm is present in an internal or external body surface selected from the group consisting of a surface in a urinary tract, a middle ear, a prostate, vascular intima, heart valves, skin, scalp, nails, teeth and an interior of a wound.

21. The composition of claim 1, wherein said AMP and said enzyme are produced in a plant plastid, said composition further comprising a plant remnant.

22. The composition of claim 21, wherein said plant is a tobacco or a lettuce plant.
23. The composition of claim 21, wherein said at least one AMP and said at least one enzyme are expressed in a lettuce plant as a fusion protein.
### FIG. 1A

**GFP STANDARDS (ng)**
- 2
- 4
- 6
- 8

**GFP – RC101 (ng TP)**
- 5
- 10
- 15
- 20
- 25

### FIG. 1B

**GFP STANDARDS (ng)**
- 125
- 250
- 500

**GFP – RC101 (µg TP)**
- 0.25
- 0.5
- 1.0
- 1.5

### FIG. 1C

**GFP STANDARDS (ng)**
- 150 kDa
- 100 kDa
- 75 kDa
- 50 kDa
- 37 kDa
- 25 kDa

**GFP-PG1 (ng TP)**
- 10
- 20
- 30
- 40
- 50

### FIG. 1D

**GFP STANDARDS (ng)**
- 125
- 250
- 500

**GFP-PG1 (µg TP)**
- 0.25
- 0.5
- 1.0
- 1.5

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Average Expression Level (%)</th>
<th>Average Purity (%)</th>
<th>GFP Yield [mg/mg lysate]</th>
<th>Purification Factor Yield [mg/mg lysate]</th>
<th>Fold Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-RC101</td>
<td>8.80%</td>
<td>94%</td>
<td>1624</td>
<td>116</td>
<td>10.6</td>
</tr>
<tr>
<td>GFP-PG1</td>
<td>3.80%</td>
<td>17%</td>
<td>58.8</td>
<td>4.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Uptake of GFP fused with different tags by human periodontal cell lines

- Standard GFP
- RC-101
- PG1-GFP
- PTD-GFP
- CTB-GFP

Periodontal ligament stem cell
Maxilla mesenchymal stem cell
Head and neck squamous carcinoma cell
Gingiva-derived mesenchymal stromal cell
Adult gingival keratinocytes
Osteoblast cells
FIG. 9A

Construction of codon-optimized mutanase sequence from Paenibacillus sp. Strain RM1 into chloroplastic transformation vector

Flanking sequence

16S trnl

Selection marker

aadA

PpsB A

Mutagen (CD): His tag

3' Terminator

Flanking sequence

tRNA

[3801 bp, 133kDa]

Substitute Sheet (Rule 29)
Construction of Dextranase sequence from Streptococcus mutans into chloroplast transformation vector

**Flanking sequence**

**Selection marker**

**5' promoter**

**Dextranase (Nat)**

**5' Terminator**

**Flanking sequence**

**16S trn**

**aadA**

**PpsA**

**trnA**

(2553 bp, 94.5kDa)

ATGAAAAGGCTCGTAAAGGCTCAAGACCTTTATTGAGTCAATTGAAATGCAAGGTGGATTCGCCATATTACCTTTTAAGGCAAGAGCAGCAAGCCTTAAGGTGGACTAACAGGAAAGAGG
ATGACGCTCGCTGGAAACGCTAACAGAGAAAGAATCCGGCTGCAAGAGATTTATAGCAATAGGAATAGGCAAAGCTGCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
- Two different E.coli strains (α-select and Top10) were tested for expression of Mutanase
- Each lane was loaded with 0.3 μg of total E.coli protein
- Immunoprobred with anti-His antibody
Activity test of Dextranase expressed in E.coli

FIG. 12C

FIG. 12B

E coli producing recombinant dextranase

Empty Vector Control

+ve Control Recombinant Dextranase from S. mutans made in E.coli

+ve Control Commercial Dextranase from Penicillin

-ve Control Untransformed E.coli

0.1 ug 1 ug 5 ug 10 ug 100 ug 1000 ug
**FIGURE 14**

Chewing Gum tablet preparation

<table>
<thead>
<tr>
<th>Lyophilized powder</th>
<th>25mg</th>
<th>50mg</th>
<th>75mg</th>
<th>100mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>0.45mg</td>
<td>0.9mg</td>
<td>1.35mg</td>
<td>1.8mg</td>
</tr>
<tr>
<td>Total Weight of the tablet</td>
<td>~2g</td>
<td>~2g</td>
<td>~2g</td>
<td>~2g</td>
</tr>
<tr>
<td>Weight of Sample taken for analysis and GFP concentration</td>
<td>250mg (56μg)</td>
<td>250mg (112μg)</td>
<td>250mg (168μg)</td>
<td>250mg (225μg)</td>
</tr>
</tbody>
</table>
### FIGURE 15

**Evaluation of gum tablet**

<table>
<thead>
<tr>
<th>Gum tablet</th>
<th>GFP Concentration (In 250mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected</td>
</tr>
<tr>
<td>25 mg</td>
<td>39 µg</td>
</tr>
<tr>
<td>50 mg</td>
<td>78.12 µg</td>
</tr>
<tr>
<td>75 mg</td>
<td>117.18 µg</td>
</tr>
<tr>
<td>100 mg</td>
<td>156.25 µg</td>
</tr>
</tbody>
</table>

**Fluorescence**

<table>
<thead>
<tr>
<th>Gum tablet</th>
<th>GFP Concentration (In 250mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected</td>
</tr>
<tr>
<td>25 mg</td>
<td>56 µg</td>
</tr>
<tr>
<td>50 mg</td>
<td>112 µg</td>
</tr>
<tr>
<td>75 mg</td>
<td>168 µg</td>
</tr>
<tr>
<td>100 mg</td>
<td>225 µg</td>
</tr>
</tbody>
</table>
FIGURE 16
Chewing Simulator - artificial saliva

SUBSTITUTE SHEET (RULE 26)
FIGURE 17

GFP release kinetics from gum tablets

Protein release kinetics from chewing gum
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC - A61 K 38/00, 38/04, 38/08 (2017.01)
CPC - A61 K 38/00, 38/04, 38/08, 38/1729; A61 L 29/16

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)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2012/0189682 A1 (O’NEIL et al.) July 26, 2012; abstract, paragraphs [0057], [0061] - [0063], [0107]-[0109], [0115], [0125], [0151], [0176]; claim 39</td>
<td>1, 8, 12, 16, 19/16, 18B/16, 20/16</td>
</tr>
<tr>
<td>Y</td>
<td>US 2011/0302675 A1 (DANIELL) December 8, 2011; abstract; paragraph [0063]; claims 3, 5</td>
<td>2, 21-23</td>
</tr>
<tr>
<td>Y</td>
<td>US 5,741,487 A (ASAHI et al.) April 21, 1998; abstract; column 15, lines 18-20; column 19, lines 28 to 48; claim 3</td>
<td>3, 9-11, 13/1, 14/1, 15</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* "A" 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

"Q" member of the same patent family

Date of the actual completion of the international search
29 September 2017 (29.09.2017)

Date of mailing of the international search report
19 OCT 2017

Name and mailing address of the ISA:
Mail Stop PCT, Attn: ISA-US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer
Shane Thomas
PCT Helpdesk: 571-272-4300
PCT GDS: 571-272-7774

Form PCT/ISA/2 10 (second sheet) (January 2015)
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).

This International Searching Authority found multiple inventions in this international application, as follows:

-**-Please See Supplemental Page-**-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-4, 8-12, 13/1, 14/1, 15/1, 16, 19/16, 20/16, 21-23; protegrin 1; mutanase

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

Groups I+ Claims 1-23, protegrin 1 and mutanase are directed toward a multi-component composition comprising at least one antimicrobial peptide (AMP) and at least one biofilm degrading enzyme; chewing gum and oral rinses comprising the composition; and a method of degrading or removing a biofilm therewith.

The composition, gum, oral rinse and method will be searched to the extent they encompass an antimicrobial peptide (AMP) encompassing protegrin 1 (first exemplary AMP), and a biofilm degrading enzyme encompassing mutanase (first exemplary biofilm degrading enzyme). Applicant is invited to elect additional AMP(s) and/or biofilm degrading enzyme(s) to be searched. Additional AMP(s) and/or biofilm degrading enzyme(s) will be searched upon the payment of additional fees. It is believed that claims 1, 2 (in-part), 3 (in-part), 4, 8-12, 13 (in-part), 14 (in-part), 15, 16, 19 (in-part), 20 (in-part) and 21-23 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass protegrin 1 (AMP) and mutanase (biofilm degrading enzyme). Applicants must specify the claims that encompass any additionally elected AMP(s) and/or biofilm degrading enzyme(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an AMP encompassing RC-101 (first exemplary elected AMP).

No technical features are shared between the antimicrobial peptides and/or biofilm degrading enzymes of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a multi-component composition comprising at least one antimicrobial peptide (AMP) and at least one biofilm degrading enzyme which act synergistically to degrade biofilm structures and inhibit biofilm deposition, said composition biologically acceptable carrier for delivery of said composition; a chewing gum comprising the composition; an oral rinse comprising the composition; a method of degrading and/or removing biofilm comprising contacting a surface harboring said biofilm with the multicomponent composition, said composition having a bactericidal effect, and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms, wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination.

However, these shared technical features are previously disclosed by US 2012/0189682 A1 to O'Neil et al. (hereinafter "O'Neil").

O'Neil discloses a multi-component composition (a product comprising at least two antibiotic agents (a multi-component composition); abstract) comprising at least one antimicrobial peptide (AMP) (comprising at least one antimicrobial peptide (AMP); abstract) and at least one biofilm degrading enzyme (and at least one dispersant enzyme (and at least one biofilm degrading enzyme); abstract, paragraphs [0107], [0108]) which act synergistically to degrade biofilm structures and inhibit biofilm deposition (which act synergistically to treat (degrade) biofilm structures and inhibit biofilm deposition; paragraph [0125]), said composition biologically acceptable carrier for delivery of said composition (said composition biologically acceptable carrier for delivery of said composition; paragraph [0115]); a chewing gum comprising the composition (a chewing gum comprising the composition; paragraph [0121]); an oral rinse comprising the composition (a mouthwash (an oral rinse) comprising the composition; paragraph [0121]); a method of degrading and/or removing biofilm (a method of degrading and/or removing biofilm; paragraphs [0057], [0061]) comprising contacting a surface harboring said biofilm with the multicomponent composition (comprising contacting a surface harboring said biofilm with the multicomponent composition; paragraphs [0061]-[0063], [0109]), said composition having a bactericidal effect (said composition having a bactericidal effect; paragraph [0176]), and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms (and synergistically reducing or eliminating said biofilm comprising one or more undesirable microorganisms; paragraph [0125]), wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination (wherein when said biofilm is present in or on an animal subject in need of said reduction or elimination; paragraphs [0061]-[0063], [0125]).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by O'Neil, the invention is lacking.