Title

Compositions and methods for enzymatic detachment of bacterial and fungal biofilms

International Patent Classification(s)

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Applicant(s)

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Related Art

EMBL DATABASE Accession No. AC127237
EMBL DATABASE Accession No. AF546864
Somerville et al, (1993), PNAS, 90, pp 6751-6755
In response to the Office Action of July 11, 2007, Applicant timely submits the following Amendment. Entry of this Amendment and reconsideration of the claims is respectfully requested.

**Amendments to the Specification** begin on page 2 of this paper.

**Amendments to the Claims** are reflected in the listing of claims that begins on page 3 of this paper.

**Remarks** begin on page 5 of this paper.
Amendments to the Claims:

This listing of claims will replace all prior versions and listings of claims in the application.

Listing of Claims:

1-6. Cancelled.

7. (Currently Amended) An isolated Dispersin B (DspB) polypeptide An isolated amino acid sequence comprising a polypeptide encoded by the nucleic acid sequence of at least 95% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1, wherein the DspB polypeptide cleaves \( \beta \)-substituted \( N \)-acetylglucosaminidase claim 1, 2, 3 or 4.


10. (Currently Amended) A fusion protein comprising the DspB polypeptide of claim 7 amino acid sequence of claim 8 or 9 and a second polypeptide.

11. (Currently Amended) A pharmaceutical composition comprising an effective amount of the DspB polypeptide of claim 7 isolated soluble, \( \beta \)-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9 and a pharmaceutically acceptable carrier.


15. (Currently Amended) A liquid antiseptic solution comprising the DspB polypeptide of claim 7 isolated soluble, \( \beta \)-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9 and an antimicrobial.

16-20. Cancelled.

21. (Withdrawn and Currently Amended) A method for promoting of modulating detachment of bacterial or fungal cells from a biofilm comprising contacting bacterial cells with the DspB polypeptide of claim 7 a protein of claim 9, so that detachment of wherein bacterial or fungal cells from a the biofilm detach is promoted.
22-25. Cancelled

26. (Withdrawn and Currently Amended) A method for inhibiting, preventing or treating a bacterial or fungal infection infections comprising administering the DspB polypeptide of claim 7, wherein biofilm growth or activity is inhibited to an organism a protein of claim 9 so that detachment of bacterial or fungal cells from a biofilm is promoted.

27-34. Cancelled.

35. (New) A composition comprising the DspB polypeptide of claim 7 and an antibiotic.

36. (New) The pharmaceutical composition of claim 11, wherein the composition is an ointment, cream, or lotion.
REMARKS

Applicant respectfully requests entry of the Amendment and reconsideration of the claims.

Please cancel claims 1-6, 8-9, 12-14, 16-20, 22-25, and 27-34 without prejudice or disclaimer. Applicant reserves the right to pursue the cancelled subject matter in one or more continuation or divisional applications.

Claims 7, 10-11, 15, 21, and 26 have been amended. New claims 35 and 36 have been added. The amendments and support for these amendments and new claims are discussed in detail below.

Applicants respectfully request reconsideration and withdrawal of the objection to the claims and the pending rejections under 35 U.S.C. § 102(b) and § 112, first and second paragraphs.

Examiner Interview

Applicant thanks Examiner Slobodyansky for her time spent on a telephone interview with Applicant's representative, Brian R. Dorn, on August 10, 2007. Patentability of the claims was discussed. No agreement was reached. This amendment and response is consistent with the discussion.

Claims

Applicant has amended claim 7 to recite "[a]n isolated Dispersin B (DspB) polypeptide comprising a polypeptide encoded by a nucleic acid sequence of at least 95% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1, wherein the DspB polypeptide cleaves a 1→4 glycosidic bond of β-substituted N-acetylglucosaminide." Sequence identity is discussed in the specification at page 10, line 34 to page 12, line 2. In particular, the specification recites a sequence identity of 95% at page 11, lines 20-25. Further, claim 7 recites nucleotides 61 to 1143, which represents the coding sequence for the mature DspB polypeptide. A signal sequence is discussed at page 9, lines 7-9. At Example 3 at page 38, lines 1-9, the coding sequence for the mature DspB polypeptide (amino acids 21-281) is exemplified. Since the first 20 amino acids are the signal sequence, then the signal sequence is encoded in the nucleic acid at
nucleotides 1-60 of SEQ ID NO:1. Hence the coding sequence of the mature DspB polypeptide begins at nucleotide 61. The three 3’ terminal nucleotides of SEQ ID NO:1 are TGA, a well known stop codon. Thus, the coding sequence for the mature DspB polypeptide is nucleotides 61 to 1143. Additionally, the cleavage activity of the DspB polypeptide is supported at page 22, lines 16-20.

Claim 10 has been amended to depend on claim 7 and is supported at page 21, lines 3-28. Claim 11 has been amended to depend on claim 7 and is supported at page 29, line 31 to page 33, line 2.

Claim 15 has been amended to depend on claim 7 and is supported at page 34, lines 6-22. New claim 35 has been added. New claim 35 depends on claim 7 and is supported at page 33, lines 3-10.

New claim 36 has been added. New claim 36 depends on claim 7 and is supported at page 31, lines 12-35.

Claims 21 and 26 have been amended to depend on claim 7. Thus, these claims incorporate all of the limitations of claim 7. Support for claims 21 and 26 can be found throughout the specification, including at page 4, lines 24-28; page 22, line 32 to page 23, line 27; page 24, line 4 to page 27, line 11; page 28, lines 14-23; and page 29, lines 15-30. Following the Amendment, method claims 21 and 26 are linked to elected polypeptide claim 7. Applicant hereby requests rejoinder of non-elected claims 21 and 26 under MPEP § 821.04(b) upon the allowability of claim 7.

Objections to the Claims

The Examiner objects to claim 7 as it is dependent on non-elected claims 1-4. As currently amended, claim 7 is an independent claim. This objection is now moot.

The Examiner objects to claims 7-11 and 15 due to the comma following the term "soluble". This has been deleted.

In view of the foregoing, Applicant respectfully requests removal of the objections to the claims.
Rejection under 35 U.S.C. § 112, first paragraph

The Examiner rejects claims 7-11 and 15 under 35 U.S.C. § 112, first paragraph, for allegedly lacking written description and enablement. Both rejections are directed to the recitation of "an active fragment or variant of β-N-acetylglucosaminidase". The recitation of "an active fragment or variant of β-N-acetylglucosaminidase" has been deleted from the pending claims. In view of amended claims 7-11 and 15, Applicant respectfully requests removal of the rejections under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner rejects claims 7 and 10 under 35 U.S.C. § 112, second paragraph, for alleged indefiniteness. The Examiner asserts that "an isolated amino acid sequence" in claim 7 and "the amino acid sequence of claim 8 or 9" in claim 10 are indefinite terms. Applicant has amended the claims and these terms are not recited in the currently amended claims 7 and 10. In view of the amendments, the rejections of claim 7 and claim 10 are now moot, and Applicant respectfully requests removal of these rejections.

Rejections under 35 U.S.C. § 102(b)

The Examiner rejects claims 7-11 and 15 under 35 U.S.C. § 102(b) as allegedly anticipated by each of Clarke et al. (2005), Graham et al. (2005), and Somerville et al. (2005). The Examiner asserts that the cited references disclose a polypeptide sequence that has 11.6%, 12.5%, and 11.6% identity to SEQ ID NO:2. In view of amended claim 7, the DspB polypeptide comprises "a polypeptide encoded by a nucleic acid sequence of at least 95% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1, wherein the DspB polypeptide cleaves β-substituted N-acetylglucosaminide." The cited polypeptide sequences are not encoded by a nucleic acid of at least 95% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1. Thus, the cited art does not disclose each and every element of any of claims 7-11 and 15. In view of the foregoing, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b).
In view of the above amendments and remarks, Applicant respectfully requests a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,

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Date: August 16, 2007

[Signature]

Brian R. Dorn
Reg. No. 57, 395

BRD:RAD:sab
Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.
Office Action Summary

Application No. | Applicant(s)
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10/538,902 | KAPLAN, JEFFREY B.
Examiner | Elizabeth Slobodyansky, PhD
Art Unit | 1652

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).

Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) □ Responsive to communication(s) filed on 25 June 2007.
2a) □ This action is FINAL. 2b) □ This action is non-final.
3) □ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) □ Claim(s) 1-34 is/are pending in the application.
   4a) Of the above claim(s) 1-6,12-14 and 16-34 is/are withdrawn from consideration.
5) □ Claim(s) ____ is/are allowed.
6) □ Claim(s) 7-11 and 15 is/are rejected.
7) □ Claim(s) ____ is/are objected to.
8) □ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

9) □ The specification is objected to by the Examiner.
10) □ The drawing(s) filed on 14 June 2005 is/are: a) □ accepted or b) □ objected to by the Examiner.
    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
11) □ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) □ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) □ All  b) □ Some * c) □ None of:
   1. □ Certified copies of the priority documents have been received.
   2. □ Certified copies of the priority documents have been received in Application No. ____.
   3. □ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

   * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) □ Notice of References Cited (PTO-892)
2) □ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) □ Information Disclosure Statement(s) (PTO/SB/08)
   Paper No(s)/Mail Date 6/14/05.
4) □ Interview Summary (PTO-413)
   Paper No(s)/Mail Date. _____.
5) □ Notice of Informal Patent Application
6) □ Other: ____. 

U.S. Patent and Trademark Office
PTOL-326 (Rev. 06-06)
DETAILED ACTION

The amendment filed June 25, 2007 amending claims 20-22 and 26 has been entered.

Claims 1-34 are pending.

Election/Restrictions

Applicant's election with traverse of Group II, claims 7-11 and 15, SEQ ID NO:2, in the reply filed on June 25, 2007 is acknowledged. The traversal is on the ground(s) “that sufficient reasons and/or examples to justify a Restriction Requirement have not been provided. Applicants submit that it would not be unduly burdensome to examine the nucleic acids that encode the elected polypeptides. Applicants submit that it would not be unduly burdensome to search claims drawn to methods using the polypeptide of claims 8-9 or a composition of claim 11. A search of the polypeptide or composition will likely yield the same results as searching the methods themselves. Therefore, Applicants respectfully submit that the Examiner has not established an undue burden in examining Groups I to IV and VIII to X in the same application” (Remarks, page 6).

This is not found persuasive because the examination of Groups I-IV and VIII-X would require additional search of patent and non-patent documents that are not required for Group II and would require additional considerations. Furthermore, Applicants are reminded that “Process claims that depend from or otherwise include all the limitations of the patentable product will be entered as a matter of right if the amendment is presented prior to final rejection or allowance, whichever is earlier”
(Office action mailed May 24, 2007, pages 6-7). Applicants further argue “that it would not be unduly burdensome to examine all of the polypeptides (A) to (E) (SEQ ID NOs: 2, 4, 6, 8, and 10). These sequences are all highly identical to one another. The sequences only differ by several amino acid residues from one another. For example, SEQ ID NO:2 only differs from SEQ ID NO:6 at amino acid residues 103 and 168 (See Figure 1). A search of one sequence would likely yield the other sequences. Therefore, Applicants respectfully submit that the Examiner has not established an undue burden in examining Groups (A) to (E) in the same application” (ibid). This is not found persuasive because the identity among sequences is in the range of 48.7%-30.5%. Each sequence should be searched separately. It is noted that Applicants do not state that the sequences are obvious over each other. Therefore, each sequence should be searched and considered separately.

The requirement is still deemed proper and is therefore made FINAL.

Claims 1-6, 12-14 and 16-34 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected Groups I, III-, there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in the reply filed on June 25, 2007.

**Claim Objections**

Claim 7 is objected to as dependent from non-elected claims 1-4. In the interests of the compact prosecution claim 7 was construed as if it were properly written, i.e. included all limitations of claims 1-4.
Claims 7-11 and 15 are objected to because of the following informalities: a comma is not needed after "soluble".

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 7-11 and 15 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claims 7-11 and 15 are drawn to or depend from "an active fragment or variant of β-N-acetylglucosaminidase". "Active fragment" as "a portion of the amino acid sequence of SEQ ID NO:2, 4, 6, 8 or 10 with similarities to the consensus sequence of the family 20 glycosyl hydrolase" (page 13, lines 4-10). Neither the consensus sequence nor the active fragments of SEQ ID NO:2 are disclosed. However, a consensus sequence is usually a small sequence that dies not exhibit enzymatic properties. Therefore, the genus of active fragments of β-N-acetylglucosaminidase is defined by neither structure nor function. the specification defined “functionally equivalent variants” as “polypeptide sequences structurally different from the dispersin B protein, but having no significant
functional difference from the protein" (page 13, lines 11-15). Therefore, the genus of variants of β-N-acetylglucosaminidase” is defined by function only.

The specification discloses β-N-acetylglucosaminidase from Actinobacillus actinomycetemcomitans strain CU1000N having the amino acid sequence of SEQ ID NO: 2 encoded by SEQ ID NO: 1 and β-N-acetylglucosaminidases from different sources having the amino acid sequence of SEQ ID NOs: 4, 6, 8 or 10 that are 48.7%-30.5% homologous to SEQ ID NO:2. The specification fails to describe any other representative species by any identifying characteristics or properties other than the functionality of being β-N-acetylglucosaminidase and does not disclose the structure: function correlation common to all members of the genus. Given this lack of description of representative species encompassed by the genus of the claim, the specification fails to sufficiently describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize that applicants were in possession of the claimed invention.

Claims 7-11 and 15 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for β-N-acetylglucosaminidase having the amino acid sequence of SEQ ID NO:2, fusion protein, a pharmaceutical composition and an antiseptic solution comprising thereof, does not reasonably provide enablement for β-N-acetylglucosaminidase comprising “an active fragment” or variant of β-N-acetylglucosaminidase having an undefined homology to SEQ ID NO:2. The specification does not enable any person skilled in the art to which it pertains, or with
which it is most nearly connected, to make the invention commensurate in scope with these claims.

Factors to be considered in determining whether undue experimentation is required, are summarized in In re Wands 858 F.2d 731, 8 USPQ2nd 1400 (Fed. Cir, 1988). They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Factors pertinent to this discussion include predictability of the art, guidance in the specification, breadth of claims, and the amount of experimentation that would be necessary to use the invention.

While recombinant and mutagenesis techniques are known, it is not routine in the art to screen for multiple substitutions or multiple modifications, as encompassed by the instant claims, and the positions within a protein's sequence where amino acid modifications can be made with a reasonable expectation of success in obtaining the desired activity/utility are limited in any protein and the result of such modifications is unpredictable. In addition, one skilled in the art would expect any tolerance to modification for a given protein to diminish with each further and additional modification, e.g. multiple substitutions.

The specification does not support the broad scope of the claims which encompass active fragments or variants of β-N-acetylglicosaminidases having an
undefined structure because the specification does not establish: (A) regions of the protein structure which may be modified without affecting the β-N-acetylglucosaminidases enzymatic activity; (B) the general tolerance of β-N-acetylglucosaminidases to modification and extent of such tolerance; (C) a rational and predictable scheme for modifying any of an enzyme residues with an expectation of obtaining the desired biological function; and (D) the specification provides insufficient guidance as to which of the essentially infinite possible choices is likely to be successful.

Without sufficient guidance, beyond that provided, making β-N-acetylglucosaminidase comprising an active fragment of SEQ ID NO:2 or a variant thereof having no defined homology to SEQ ID NO:2 is unpredictable and the experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue. The scope of the claims must bear a reasonable correlation with the scope of enablement (In re Fisher, 166 USPQ 19 24 (CCPA 1970)).

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 7 and 10 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.
Claim 7 recites “An isolated amino acid sequence”. “Sequence” is not a chemical compound and cannot be isolated. Amending the claim to recite a protein, a polypeptide or β-N-acetylglucosaminidase, for example, would obviate this rejection.

Claim 10 recites “the amino acid sequence of claim 8 or 9”. There is no antecedent basis for this phrase as claims 8 and 9 are drawn to β-N-acetylglucosaminidase protein.

**Claim Rejections - 35 USC § 102**

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 7-11 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by Clarke et al.

Clarke et al (form PTO-1449 filed June 14, 2005, reference AA) teach β-N-acetylglucosaminidase having the amino acid that is 11.6% identical to SEQ ID NO:2 of the instant invention and a fusion protein comprising thereof (page 8806, 1st column; page 8811, Table 3). Therefore, it is comprising an active fragment thereof and is a variant thereof.

Claims 7-11 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by Graham et al.
Graham et al (form PTO-1449 filed June 14, 2005, reference AB) teach \( \beta \)-N-acetylhexoaminidase having the amino acid that is 12.5% identical to SEQ ID NO:2 of the instant invention and a fusion protein comprising thereof (page 16823, 1st column; page 16824, 1st column). Therefore, it is comprising an active fragment thereof and is a variant thereof.

Claims 7-11 and 15 are rejected under 35 U.S.C. 102(b) as being anticipated by Somerville et al.

Somerville et al (form PTO-1449 filed June 14, 2005, reference AD) teach \( \beta \)-N-acetylglucosaminidase having the amino acid that is 11.6% identical to SEQ ID NO:2 of the instant invention and a fusion protein comprising thereof (page 6751; page 6753). Therefore, it is comprising an active fragment thereof and is a variant thereof.

**Conclusion**

The art made of record and not relied upon is considered pertinent to applicant's disclosure.

Kaplan et al. J. Bacteriology (August 2003) Vol. 185, No. 16, pages 4693-4698 (form PTO-1449 filed June 14, 2005, reference AC). This is the work of the inventor's Group published after the effective filing date of this application of December 20, 2002. The disclosed sequence of 361 amino acids is 95.3% identical to SEQ ID NO:2, 100% identical to residues 21-381 of SEQ ID NO:2 (381 amino acids).
Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth Slobodyansky, PhD whose telephone number is 571-272-0941. The examiner can normally be reached on M-F 10:00 - 6:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ponnathapura Achutamurthy, PhD can be reached on 571-272-0928. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Elizabeth Slobodyansky, PhD
Primary Examiner
Art Unit 1652

July 6, 2007
This listing of claims will replace all prior versions and listings of claims in the application:

**Listing of Claims:**

1. (original) An isolated nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

2. (Previously Presented) The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence with 50% sequence identity to at least 30 contiguous nucleotides of SEQ ID NO:1, 3, 5, 7 or 9.

3. (Previously Presented) The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence of SEQ ID NO:1, 3, 5, 7 or 9.

4. (Previously Presented) A nucleic acid sequence encoding a fusion polypeptide comprising the isolated nucleic acid sequence of claim 1 and a second nucleic acid sequence encoding a second polypeptide.

5. (original) A vector comprising the nucleic acid sequence of claim 1, 2, 3 or 4.

6. (original) A host cell comprising the vector of claim 5.

7. (original) An isolated amino acid sequence encoded by the nucleic acid sequence of claim 1, 2, 3 or 4.

8. (original) An isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

9. (original) The isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 comprising SEQ ID NO:2, 4, 6, 8 or 10.
10. (original) A fusion protein comprising the amino acid sequence of claim 8 or 9 and a second polypeptide.

11. (original) A pharmaceutical composition comprising an effective amount of the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9 and a pharmaceutically acceptable carrier.

12. (original) A method for enhancing efficacy of an antibiotic against a bacterial infection comprising administering the pharmaceutical composition of claim 11 in combination with or prior to administration of the antibiotic.

13. (original) A medical device coated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

14. (original) A wound healing device impregnated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

15. (original) A liquid antiseptic solution comprising the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

16. (original) A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising mutating a dspB gene of bacterial cells to inhibit detachment of bacterial or fungal cells from biofilms.

17. (original) A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising decreasing expression or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in the bacterial cells so that detachment of bacterial or fungal cells from the biofilm is decreased.

18. (original) An isolated mutant of Actinobacillus actinomycetemcomitans which forms biofilm colonies which tightly adhere to surfaces but which are unable to release cells into the medium or spread over the surface.

19. (original) The mutant of claim 18 wherein the dspB gene is mutated.
20. (Currently Amended) A method for identifying an agent which modulates detachment of bacterial or fungal cells from biofilms comprising assessing an agent’s ability to modulate activity or expression or levels of soluble, β-N-acetylglucosaminidase a protein of claim 9.

21. (Currently Amended) A method for promoting detachment of bacterial or fungal cells from a biofilm comprising contacting bacterial cells with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof a protein of claim 9, so that detachment of bacterial or fungal cells from a biofilm is promoted.

22. (Currently Amended) A method for reducing risk of infection of an organism by bacteria or fungi on a medical device or surgical instrument comprising contacting the medical device or surgical instrument with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof a protein of claim 9 prior to contacting the organism with the medical device or surgical instrument.

23. (original) The method of claim 22 wherein the medical device is coated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

24. (original) The method of claim 23 wherein the coating of soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is dried on the medical device.

25. (original) The method of claim 22 wherein the medical device is a catheter and the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is in a catheter lock solution in the catheter.

26. (Currently Amended) A method for inhibiting, preventing or treating bacterial or fungal infections comprising administering to an organism soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof a protein of claim 9 so that detachment of bacterial or fungal cells from a biofilm is promoted.
27. (original) The method of claim 26 wherein the bacterial or fungal infection is from a bacterium or fungus that produces a N-acetylglucosaminidase containing biofilm polysaccharide that can be degraded by soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

28. (original) The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a coating on a medical device implanted in the organism.

29. (original) The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a pharmaceutical composition.

30. (original) The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is incorporated into a liquid disinfecting solution and applied topically to the subject prior to insertion of an implantable medical device.

31. (original) The method of claim 26 wherein a wound dressing applied to the subject is impregnated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

32. (original) A primer pair which identifies bacteria with DspB homologs.

33. (original) The primer pair of claim 32 comprising SEQ ID NO:12 and SEQ ID NO:13.

34. (original) A kit for identifying bacteria with DspB homologs comprising the primer pair of claim 32 or 33 and instructions for use of the primer pair to identify bacteria with DspB homologs.
REMARKS

In the Office Action of May 24, 2007, the Examiner requires restriction to one of eleven claim groups and one of five sequence identifiers (A) - (E).

Without acquiescing to the statements made in the Restriction Requirement, Applicants hereby elect Group II, claims 7-11 and 15, with traverse, for further prosecution in the instant application. In addition, Applicants elect Group (A) sequence of SEQ ID NO:2, with traverse.

The traversal is on the ground that sufficient reasons and/or examples to justify a Restriction Requirement have not been provided. Applicants submit that it would not be unduly burdensome to examine the nucleic acids that encode the elected polypeptides. Applicants submit that it would not be unduly burdensome to search claims drawn to methods using the polypeptide of claims 8-9 or a composition of claim 11. A search of the polypeptide or composition will likely yield the same results as searching the methods themselves. Therefore, Applicants respectfully submit that the Examiner has not established an undue burden in examining Groups I to IV and VIII to X in the same application.

Additionally, Applicants submit that it would not be unduly burdensome to examine all of the polypeptides (A) to (E) (SEQ ID NOs: 2, 4, 6, 8, and 10). These sequence are all highly identical to one another. The sequences only differ by several amino acid residues from one another. For example, SEQ ID NO:2 only differs from SEQ ID NO:6 at amino acid residues 103 and 168 (See Figure 1). A search of one sequence would likely yield the other sequences. Therefore, Applicants respectfully submit that the Examiner has not established an undue burden in examining Groups (A) to (E) in the same application.

Applicants have amended claims 20-22 and 26 to link these claims to product claims. Support for the amendments can be found at page 4, line 24 to page 5, line 4 and at page 5, lines 22-28. No new matter has been added by the amendment.

Applicants would like to note that when product claims are elected and subsequently found allowable, withdrawn process claims that depend from or otherwise include all the limitations of the allowable product claim(s) should be rejoined in accordance with MPEP § 821.04. In view of the current amendments, claims 12-15 and 20-31 link to the elected Group II claims.
In view of the foregoing, Applicants believe that the claims are in condition for allowance and such action is respectfully requested. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,

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Date: June 25, 2007

Brian R. Dorn, Ph.D.
Reg. No. 57,395
BRD:RAD:sab
What is Claimed is:

1. An isolated nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

2. The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence with 50% sequence identity to at least 30 contiguous nucleotides of SEQ ID NO:1, 3, 5 7 or 9.

3. The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence of SEQ ID NO:1, 3, 5 7 or 9.

4. A nucleic acid sequence encoding a fusion polypeptide comprising the isolated nucleic acid sequence of claim 1, 2 or 3 and a second nucleic acid sequence encoding a second polypeptide.

5. A vector comprising the nucleic acid sequence of claim 1, 2, 3 or 4.

6. A host cell comprising the vector of claim 5.

7. An isolated amino acid sequence encoded by the nucleic acid sequence of claim 1, 2, 3 or 4.

8. An isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

9. The isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8.
comprising SEQ ID NO: 2, 4, 6, 8 or 10.

10. A fusion protein comprising the amino acid sequence of claim 8 or 9 and a second polypeptide.

11. A pharmaceutical composition comprising an effective amount of the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9 and a pharmaceutically acceptable carrier.

12. A method for enhancing efficacy of an antibiotic against a bacterial infection comprising administering the pharmaceutical composition of claim 11 in combination with or prior to administration of the antibiotic.

13. A medical device coated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

14. A wound healing device impregnated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

15. A liquid antiseptic solution comprising the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

16. A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising mutating a dspB gene of bacterial cells to inhibit detachment of bacterial or fungal cells from biofilms.

17. A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising decreasing
expression or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in the bacterial cells so that detachment of bacterial or fungal cells from the biofilm is decreased.

18. An isolated mutant of Actinobacillus actinomycetemcomitans which forms biofilm colonies which tightly adhere to surfaces but which are unable to release cells into the medium or spread over the surface.

19. The mutant of claim 18 wherein the dspB gene is mutated.

20. A method for identifying an agent which modulates detachment of bacterial or fungal cells from biofilms comprising assessing an agent's ability to modulate activity or expression or levels of soluble, β-N-acetylglucosaminidase.

21. A method for promoting detachment of bacterial or fungal cells from a biofilm comprising contacting bacterial cells with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof so that detachment of bacterial or fungal cells from a biofilm is promoted.

22. A method for reducing risk of infection of an organism by bacteria or fungi on a medical device or surgical instrument comprising contacting the medical device or surgical instrument with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof prior to contacting the organism with the medical device or surgical instrument.
23. The method of claim 22 wherein the medical device is coated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

24. The method of claim 23 wherein the coating of soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is dried on the medical device.

25. The method of claim 22 wherein the medical device is a catheter and the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is in a catheter lock solution in the catheter.

26. A method for inhibiting, preventing or treating bacterial or fungal infections comprising administering to an organism soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof so that detachment of bacterial or fungal cells from a biofilm is promoted.

27. The method of claim 26 wherein the bacterial or fungal infection is from a bacterium or fungus that produces a N-acetylglucosaminidase containing biofilm polysaccharide that can be degraded by soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

28. The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a coating on a medical device implanted in the organism.
29. The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a pharmaceutical composition.

30. The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is incorporated into a liquid disinfecting solution and applied topically to the subject prior to insertion of an implantable medical device.

31. The method of claim 26 wherein a wound dressing applied to the subject is impregnated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

32. A primer pair which identifies bacteria with DspB homologs.

33. The primer pair of claim 32 comprising SEQ ID NO:12 and SEQ ID NO:13.

34. A kit for identifying bacteria with DspB homologs comprising the primer pair of claim 32 or 33 and instructions for use of the primer pair to identify bacteria with DspB homologs.
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Inventors: Jeffrey B. Kaplan
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Filing Date: Herewith
Examiner: Not yet assigned
Group Art Unit: Not yet assigned
Title: Compositions and Methods for Enzymatic Detachment of Bacterial and Fungal Biofilms

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By Kathleen A. Tyrrell, Reg. No. 39,350

Mail Stop Commissioner for Patents
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Dear Sir:

Preliminary Amendment

Please enter the following amendments and remarks in the record.

Amendments to the claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks begin on page 6.
Listing of the claims:

Claim 1 (original): An isolated nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

Claim 2 (currently amended): The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence with 50% sequence identity to at least 30 contiguous nucleotides of SEQ ID NO: 1, 3, 5, 7 or 9.

Claim 3 (currently amended): The isolated nucleic acid sequence of claim 1 comprising a nucleic acid sequence of SEQ ID NO: 1, 3, 5, 7 or 9.

Claim 4 (currently amended): A nucleic acid sequence encoding a fusion polypeptide comprising the isolated nucleic acid sequence of claim 1, 2 or 3 and a second nucleic acid sequence encoding a second polypeptide.

Claim 5 (original): A vector comprising the nucleic acid sequence of claim 1, 2, 3 or 4.

Claim 6 (original): A host cell comprising the vector of claim 5.

Claim 7 (original): An isolated amino acid sequence encoded by the nucleic acid sequence of claim 1, 2, 3 or 4.
Claim 8 (original): An isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm.

Claim 9 (original): The isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 comprising SEQ ID NO:2, 4, 6, 8 or 10.

Claim 10 (original): A fusion protein comprising the amino acid sequence of claim 8 or 9 and a second polypeptide.

Claim 11 (original): A pharmaceutical composition comprising an effective amount of the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9 and a pharmaceutically acceptable carrier.

Claim 12 (original): A method for enhancing efficacy of an antibiotic against a bacterial infection comprising administering the pharmaceutical composition of claim 11 in combination with or prior to administration of the antibiotic.

Claim 13 (original): A medical device coated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

Claim 14 (original): A wound healing device impregnated with the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.
Claim 15 (original): A liquid antiseptic solution comprising the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof of claim 8 or 9.

Claim 16 (original): A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising mutating a dspB gene of bacterial cells to inhibit detachment of bacterial or fungal cells from biofilms.

Claim 17 (original): A method for inhibiting detachment of bacterial or fungal cells from biofilm comprising decreasing expression or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in the bacterial cells so that detachment of bacterial or fungal cells from the biofilm is decreased.

Claim 18 (original): An isolated mutant of Actinobacillus actinomycetemcomitans which forms biofilm colonies which tightly adhere to surfaces but which are unable to release cells into the medium or spread over the surface.

Claim 19 (original): The mutant of claim 18 wherein the dspB gene is mutated.

Claim 20 (original): A method for identifying an agent which modulates detachment of bacterial or fungal cells from biofilms comprising assessing an agent’s ability to modulate activity or expression or levels of soluble, β-N-acetylglucosaminidase.
Claim 21 (original): A method for promoting detachment of bacterial or fungal cells from a biofilm comprising contacting bacterial cells with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof so that detachment of bacterial or fungal cells from a biofilm is promoted.

Claim 22 (original): A method for reducing risk of infection of an organism by bacteria or fungi on a medical device or surgical instrument comprising contacting the medical device or surgical instrument with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof prior to contacting the organism with the medical device or surgical instrument.

Claim 23 (original): The method of claim 22 wherein the medical device is coated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

Claim 24 (original): The method of claim 23 wherein the coating of soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is dried on the medical device.

Claim 25 (original): The method of claim 22 wherein the medical device is a catheter and the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is in a catheter lock solution in the catheter.

Claim 26 (original): A method for inhibiting, preventing or treating bacterial or fungal infections comprising
administering to an organism soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof so that detachment of bacterial or fungal cells from a biofilm is promoted.

Claim 27 (original): The method of claim 26 wherein the bacterial or fungal infection is from a bacterium or fungus that produces a N-acetylglucosaminidase containing biofilm polysaccharide that can be degraded by soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

Claim 28 (original): The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a coating on a medical device implanted in the organism.

Claim 29 (original): The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is administered as a pharmaceutical composition.

Claim 30 (original): The method of claim 26 wherein the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof is incorporated into a liquid disinfecting solution and applied
topically to the subject prior to insertion of an implantable medical device.

Claim 31 (original): The method of claim 26 wherein a wound dressing applied to the subject is impregnated with the soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof.

Claim 32 (original): A primer pair which identifies bacteria with DspB homologs.

Claim 33 (original): The primer pair of claim 32 comprising SEQ ID NO:12 and SEQ ID NO:13.

Claim 34 (original): A kit for identifying bacteria with DspB homologs comprising the primer pair of claim 32 or 33 and instructions for use of the primer pair to identify bacteria with DspB homologs.
REMARKS

Claims 1-34 are pending in the instant application. Claims 2 and 3 have been amended to correct the punctuation and claim 4 has been amended to place all multiple claims in U.S. format.

No new matter is added by these amendments. Entry of these amendments is respectfully requested.

Respectfully submitted,

Kathleen A. Tyrrell
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Date: June 14, 2005

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Compositions and Methods for Enzymatic Detachment of Bacterial and Fungal Biofilms

Introduction

This application claims the benefit of priority from U.S. Provisional Application Serial No. 60/435,817, filed December 20, 2002, which is herein incorporated by reference in its entirety.

Field of the Invention

The present invention provides isolated nucleic acid sequences and amino acid sequences encoded thereby for the protein, soluble, β-N-acetylglucosaminidase or dispersin B, and active fragments and variants thereof, which promote detachment of bacterial cells from biofilms. Vectors comprising the nucleic acid sequences as well as host cells expressing the dispersin B protein or active fragments or variants thereof are also provided. A biofilm detachment mutant of A. actinomycetemcomitans is also described. The nucleic acid and amino acid sequences of the present invention are useful in methods for modulating detachment of bacterial or fungal cells from biofilms as well as in methods for identifying agents which modulate detachment of bacterial or fungal cells from biofilms. Thus, these nucleic acid and amino acid sequences and agents are expected to be useful in the prevention and treatment of bacterial or fungal infections and in disinfectant and antiseptic solutions.

Background of the Invention

Biofilms are populations of bacteria or fungi growing attached to an inert or living surface. Mounting evidence
has shown that biofilms constitute a significant threat to human health. The Public Health Service estimates that biofilms are responsible for more than 80% of bacterial infections in humans (National Institutes of Health, 1998 RFA# DE-98-006). Examples of diseases caused by biofilms include dental caries, periodontitis, cystic fibrosis pneumonia, native valve endocarditis, and otitis media (Costerton et al. Science 1999 284:1318-1322), as well as infection of various medical devices such as urinary catheters, mechanical heart valves, cardiac pacemakers, prosthetic joints, and contact lenses (Donlan, R. M. 2001 Emerging Infect. Dis. 7:277-281). Fungi also form biofilms of clinical significance, for example Candida infections. Biofilm infections afflict tens of millions of patients in the U.S. annually and require a significant expenditure of health care dollars (Costerton et al. Science 1999 284:1318-1322). Bacteria growing in biofilms exhibit increased resistance to antimicrobial agents and are nearly impossible to eradicate. New methods for treating biofilm infections are needed.

Bacteria in a biofilm are enmeshed in an extracellular polysaccharide (EPS) substance that holds the bacteria together in a mass, and firmly attaches the bacterial mass to the underlying surface. Previous studies have demonstrated that enzymes that degrade EPS are capable of causing the detachment of cells from biofilms. For example, over expression of alginate lyase, an enzyme that catalyzes the degradation of the EPS alginate, causes colonies of Pseudomonas aeruginosa to become less adherent to surfaces (Boyd, A. and Chakrabarty, A. M. Appl. Environ. Microbiol. 1994 60:2355-2359). Alginate lyase has been suggested for use in treating P. aeruginosa infections in the lungs of cystic fibrosis patients (Mrsny et al. Pulm. Pharmacol. 1994 7:357-366). A similar polysaccharide lyase has been shown to be produced by P. fluorescens (Allison et
Two other EPS-degrading enzymes, endo-β-1,4-mannanase from the plant pathogen *Xanthomonas campestris* (Dow et al. Proc. Nat. Acad. Sci. USA 2003 100:10995-11000) and disaggretase from the methanogenic archaebacterium *Methanosarcina mazei* (Liu et al. Appl. Environ. Microbiol. 1985 49:608-613), have also been shown to cause biofilm cell detachment. In the case of *X. campestris*, production of the EPS-degrading enzyme was required for full virulence of the bacteria in plants. Detachment of cells from biofilm colonies of the dental pathogen *Streptococcus mutans* was shown to be caused by an unidentified endogenous enzymatic activity (Lee et al. Infect. Immun. 1996 64:1035-1038). A complex mixture of polysaccharide-hydrolyzing enzymes was shown to remove biofilms from steel and polypropylene substrata (Johansen et al. Appl. Environ. Microbiol. 1997 63:3724-3728). These findings indicate that EPS-degrading enzymes can potentially be used as agents to remove biofilms from surfaces.

Although enzymes are commonly used to remove biofilms in industrial environments, no studies have investigated the potential use of enzymes as agents for the removal of biofilms in clinical environments. Of particular concern in the clinic are biofilm infections of indwelling medical devices, especially intravascular catheters. Catheter infections are common in hospitalized patients and are associated with high levels of morbidity and mortality. A promising new approach to treating these infections is the use of catheters that are coated or impregnated with antimicrobial agents such as antibiotics (Schierholz et al. J. Antimicrobial. Chemother. 2000 46:45-50), silver (Bechert et al. Infection 1999 27:S24-S29), and peptide quorum-sensing inhibitors (Balaban et al. J. Infect. Dis. 2003 187:625-630). Numerous studies have demonstrated that medical devices with antimicrobial activity decrease the

The present invention provides isolated proteins and active fragments and variants thereof and nucleic acid sequences encoding such proteins and active fragments and variants thereof involved in detachment of bacterial cells.

Methods for modulating detachment of biofilm cells of bacteria or fungi and identifying agents which modulate bacterial or fungal detachment via these proteins and active fragments and variants thereof and/or nucleic acid sequences are also provided.

Summary of the Invention

In one embodiment the present invention provides isolated proteins and active fragments and variants thereof which promote detachment of bacterial or fungal cells from a biofilm. The isolated proteins are referred to herein as soluble, β-N-acetylglucosaminidase or dispersin B.

In another embodiment the present invention provides isolated nucleic acid sequences encoding soluble, β-N-acetylglucosaminidase and active fragments and variants thereof as well as vectors comprising these sequences and host cells expressing the vectors.

In another embodiment the present invention provides methods for modulating detachment of bacterial or fungal cells from biofilms. In one embodiment the method comprises mutating the bacterial cells to inhibit detachment of bacterial cells from biofilms. In another embodiment, the method comprises increasing expression and/or levels of soluble, β-N-acetylglucosaminidase or active fragments or variants thereof in the bacterial or fungal cells so that detachment is increased. In yet another embodiment, the method comprises decreasing expression and/or levels of soluble, β-N-acetylglucosaminidase or active fragments or variants thereof or inhibiting activity of soluble, β-N-acetylglucosaminidase or active fragments or variants thereof so that detachment of bacterial cells is decreased.

In another embodiment the present invention provides an isolated mutant of Actinobacillus actinomycetemcomitans which forms biofilm colonies which tightly adhere to surface but which are unable to release cells into the medium or spread over the surface.

In another embodiment the present invention provides a method for identifying agents which modulate detachment of bacterial or fungal cells from biofilms which
comprises assessing the ability of an agent to modulate activity and/or levels and/or expression of soluble, β-N-acetylglucosaminidase.

In another embodiment the present invention provides compositions and methods for using these compositions to prevent the dissemination of infectious bacteria via administration of an agent which inhibits soluble, β-N-acetylglucosaminidase expression and/or activity in the bacterial cells.

In another embodiment the present invention provides compositions and methods for preventing or inhibiting attachment of infectious bacteria or fungi to a surface or removing infectious bacteria or fungi from a surface which comprises treating the surface with soluble, β-N-acetylglucosaminidase, or an active fragment or variant thereof.

In yet another embodiment the present invention provides PCR primer pairs and kits comprising such primer pairs that can be used to identify additional bacterial species with homologues of soluble, β-N-acetylglucosaminidase.

According to the invention there is also provided an isolated nucleic acid comprising a polynucleotide that is amplifiable by polymerase chain reaction with a forward primer of SEQ ID NO:12 and a reverse primer of SEQ ID NO:13 and said polynucleotide encodes a polypeptide that cleaves β-substituted N-acetyl glucosaminide; or complement thereof.

According to the invention there is also provided a nucleic acid encoding a fusion polypeptide comprising the isolated nucleic acid according to the invention and a second nucleic acid encoding a second polypeptide.

According to the invention there is also provided a vector comprising the nucleic acid sequence according to the invention.

According to the invention there is also provided a host cell comprising the vector according to the invention.

According to the invention there is also provided a method of producing a recombinant polypeptide comprising culturing the host cell according to the invention.

According to the invention there is also provided an isolated polypeptide comprising a polypeptide encoded by the polynucleotide according to the invention.

According to the invention there is also provided a composition comprising:

(a) the polypeptide according to the invention; and

b) an antibiotic.

According to the invention there is also provided a composition comprising:

(a) the polypeptide according to the invention; and
(b) an antimicrobial or antifungal.

According to the invention there is also provided a fusion protein comprising the polypeptide according to the invention and a second polypeptide.

According to the invention there is also provided a pharmaceutical composition comprising:

a) the polypeptide according to the invention; and

b) a pharmaceutically acceptable carrier.

According to the invention there is also provided use of the pharmaceutical composition according to the invention to inhibit a biofilm associated with bacterial infection.

According to the invention there is also provided a medical device coated with the polypeptide according to the invention.

According to the invention there is also provided a wound dressing impregnated with the polypeptide according to the invention.

According to the invention there is also provided a transdermal patch comprising the polypeptide according to the invention.

According to the invention there is also provided use of the polypeptide according to the invention in the manufacture of a medicament to promote detachment of bacterial or fungal cells from a biofilm.

According to the invention there is also provided a method of inhibiting infection on a medical device or surgical instrument by bacteria or fungi comprising contacting the medical device or surgical instrument with the polypeptide according to the invention.

According to the invention there is also provided a method of inhibiting infection on a medical device or surgical instrument by bacteria or fungi comprising coating the medical device or surgical instrument with the polypeptide according to the invention.

According to the invention there is also provided use of the polypeptide according to the invention in the manufacture of a medicament to inhibit or treat bacterial and/or fungal infections.

According to the invention there is also provided a method of inhibiting infection on a wound dressing by bacteria or fungi comprising impregnating the wound dressing the polypeptide according to the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated
element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.
Brief Description of the Figure

Figure 1 provides a clustal alignment of exemplary dispersin B orthologs of the present invention from *A. actinomycetemcomitans* strain CU1000N (SEQ ID NO:2), *A. actinomycetemcomitans* strain IDH781 (SEQ ID NO:6), *Haemophilus aphrophilus* strain NJ8700 (SEQ ID NO:8), *A. ligniersii* strain 19393 (SEQ ID NO:4), and *A. pleuropneumoniae* strain IA5 (SEQ ID NO:10).

Detailed Description of the Invention


Tight adherence has been shown to play an important role in the ability of A. actinomycetemcomitans to colonize the mouths of rats (Fine et al. Arch. Oral Biol. 2001 46:1065-1078.), and is believed to have an equally important role in its ability to colonize humans. The tight adherence to surfaces is dependent on the presence of long, bundled pili (fimbriae) that form on the surface of the cell (Inouye et al. FEBS Microbiol. Lett. 1990 69:13-18; Rosan et al. Oral. Microbiol. Immunol. 1988 3:58-63). Mutations in flp-1, which encodes the major pilin protein subunit, result in cells that fail to produce fimbriae or adhere to surfaces (Kachlany et al. Mol. Microbiol. 2001 40:542-554).

Biofilm colonies of A. actinomycetemcomitans have been shown to release cells into liquid medium which then attach to the surface of the culture vessel and form new colonies, enabling the biofilm to spread (Kaplan, J. B. and Fine D. H. Appl. Environ. Microbiol. 2002 68: 4943-4950.).

One aspect of the present invention relates to a mutant of A. actinomycetemcomitans that forms biofilm colonies which are tightly adherent to surfaces but which are unable to release cells into the medium or spread over the surface. The biofilm detachment mutant of A.
actinomycetemcomitans is referred to herein as mutant JK1023. To produce the A. actinomycetemcomitans biofilm detachment mutant JK1023, the A. actinomycetemcomitans strain CU1000N was mutagenized with transposon IS903\kan. The mutant strain (designated JK1023) was then isolated. This mutant strain displays a colony morphology on agar that is rougher than the wild-type A. actinomycetemcomitans rough-colony phenotype (Fine et al. Microbiol. 1999 145:1335-1347; Haase et al. Infect. Immun. 1999 67:2901-2908; Inouye et al. FEMS Microbiol. Lett. 1990 69:13-18). JK1023 colonies had a hard texture and were extremely difficult to remove from the agar surface. When cultured in broth, strain JK1023 produced biofilm colonies which were similar in size and shape to those of the wild-type strain, but which failed to produce satellite colonies on the surface of the culture vessel. Adherence of JK1023 cells to polystyrene was equal to that of wild-type strain CU1000N as measured by a 96-well microtiter plate binding assay.

To demonstrate that biofilm colonies of mutant strain JK1023 of the present invention were deficient in biofilm cell detachment, biofilm colonies were grown for 24 hours on polystyrene rods suspended in broth in the wells of a 24-well microtiter plate. The amount of biofilm cell detachment was then quantified by staining the bacteria growing on the bottom of the well with crystal violet. Colonization at the bottom of the well results from cells that detach from the biofilm colonies growing on the polystyrene rod and fall to the bottom of the well. In this assay, biofilm colonies of strain JK1023 produced significantly less growth on the bottom of the well than the wild-type strain (P < 0.01, unpaired two-tailed t test). These data indicate that mutant strain JK1023 exhibited a wild-type surface attachment phenotype but a decreased biofilm cell detachment phenotype when compared to the wild-type strain CU1000N.
DNA sequence analysis of the region surrounding the transposon insertion site of this mutant strain revealed the insertion to be in a 1,143 bp open reading frame designated herein as \textit{dspB}. The \textit{dspB} gene from strain CU1000 was predicted to encode a protein, referred to herein as dispersin B, having 381 amino acid residues with a molecular mass of 43.3 kDa. The 5' end of \textit{dspB} contained a predicted signal peptide, suggesting that dispersin B may be a secreted protein.

In addition to \textit{A. actinomycetemcomitans}, \textit{dspB} nucleic acid sequences or fragments have also been isolated from \textit{Actinobacillus pleuropneumoniae}, \textit{Haemophilus aphrophilus} and \textit{Actinobacillus ligniersii}. \textit{DspB} is not present in the genomes of \textit{Haemophilus influenzae}, \textit{Pasteurella multicaido}, \textit{Mannheimia haemolytica}, \textit{Actinobacillus equuli} and \textit{Haemophilus ducreyi} among the strains that were tested. Accordingly, another aspect of the present invention relates to nucleic acid sequences encoding dispersin B or active fragments and variants thereof as well as amino acid sequences of dispersin B and active fragments and variants thereof. Also encompassed by the present invention are vectors comprising these nucleic acid sequences as well as host cells comprising the vectors which express dispersin B or an active fragment thereof.

By the term "nucleic acid sequence" as used herein it is meant to include, but is not limited to, unmodified RNA or DNA or modified RNA or DNA. Thus, by nucleic acid sequence it is meant to be inclusive of single- and double-stranded DNA, DNA that is a mixture of single-and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules containing DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. Further, the DNA or RNA sequences of the present invention may comprise a
modified backbone and/or modified bases. A variety of modifications to DNA and RNA are known in the art for multiple useful purposes. The term "nucleic acid sequence" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acid sequences, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The DNA sequence of dspB from strain CU1000 was deposited into GenBank under accession no. AY228551 and released on August 4, 2003. The nucleic acid sequence for this DNA is SEQ ID NO:1. Nucleic acid sequences encoding orthologs of dispersin B protein have been identified in A. ligniersii strain 19393, A. actinomycetemcomitans strain IDH781, Haemophilus aphrophilus strain NJ8700 and A. pleuropneumoniae strain IA5 and are depicted in SEQ ID NO:3, 5, 7 and 9, respectively. Accordingly, preferred isolated nucleic acid sequences of the present invention comprise SEQ ID NO:1, 3, 5, 7 or 9.

Also included within the present invention are allelic variants of the exemplified dspB nucleic acid sequences of SEQ ID NO:1, 3, 5 7 or 9 encoding proteins with similar enzymatic activities to dispersin B and nucleic acid sequences with substantial percent sequence identity to the exemplified dspB nucleic acid sequences of SEQ ID NO: 1, 3, 5, 7 or 9 encoding proteins with similar enzymatic activities.

By the term "allelic variant" as used herein it is meant one of two or more alternative naturally occurring forms of a gene, each of which comprises a unique nucleic acid sequence. Allelic variants encompassed by the present invention encode proteins with similar or identical enzymatic activities.

The term "percent sequence identity" as used herein with respect to nucleic acid sequences refers to the
residues in two nucleic acid sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison is preferably over a length of at least about 9 contiguous nucleotides, more preferably about 18 contiguous nucleotides, and even more preferably at least about 30 to 50 contiguous nucleotides or more. Various algorithms well known in the art are available for measuring nucleic acid sequence identity. Examples include, but are not limited to, FASTA (including FASTA2 and FASTA3), Gap and Bestfit, which are programs in Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisconsin.

By “substantial percent sequence identity” when referring to a nucleic acid sequence or fragment thereof, of the present invention, it is meant that when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), at least about 50% of the nucleotide bases as measured by any well known algorithm of sequence identity, such as FASTA, BLAST or Gap are the same. For purposes of the present invention, more preferably, at least about 60% to 70%, even more preferably 80% to 90%, and most preferably at least about 95-98% of the nucleotide bases, as measured by any well known algorithm of sequence identity, such as FASTA, BLAST or Gap, are identical.

Nucleic acid sequences sharing substantial percent sequence identity and encoding proteins with similar functional activity are referred to herein as orthologues.

Deduced amino acid sequences of dispersin B and exemplary orthologues thereof are shown in Figure 1. Specifically, the amino acid sequence of dispersin B of A. actinomycetemcomitans strain CU1000N (SEQ ID NO:2), and orthologs of dispersin B from A. actinomycetemcomitans strain IDH781 (SEQ ID NO:6), Haemophilus aphrophilus strain NJ8700 (SEQ ID NO:8), A. ligniersii strain 19393 (SEQ ID
NO:4), and A. pleuropneumoniae strain IA5 (SEQ ID NO:10) are shown.


Similarity between dispersin B and lacto-N-biosidases is high in the regions surrounding Arg47 and the acidic amino acid pair Asp202 and Glu203. These residues have been shown to participate in substrate binding and catalysis in other family 20 glycosyl hydrolases (Mark et al. J. Biol. Chem. 2001 276:10330-10337; Mark et al. J. Biol. Chem. 1998 273:19618-19624; Prag et al. J. Mol. Biol.2000 300:611-617). The C-terminal half of dispersin B contained three Trp residues that were conserved in L. lactis lacto-N-biosidase (positions 236, 279, and 353). Multiple Trp residues are present in the C-terminal regions of the catalytic domains of all family 20 glycosyl hydrolases (Graham et al. J. Biol. Chem. 1988 263:16823-16829; Tews et al. Gene 1996 170:63-67). These Trp residues line the part of the substrate binding pocket that is complementary to the hydrophobic surfaces of the hexosamine sugar ring (Tews

In a preferred embodiment an isolated amino acid sequence of the present invention comprises SEQ ID NO:2, 4, 6, 8 or 10 or an active fragment or variants thereof. Preferred active fragments are those comprising a portion of the amino acid sequence of SEQ ID NO:2, 4, 6, 8 or 10 with similarities to the consensus sequence of the family 20 glycosyl hydrolase.

"Active variants" or "functionally equivalent variants" as used herein are polypeptide sequences structurally different from the dispersin B protein, but having no significant functional difference from the protein. For example, when orthologous polypeptide sequences from various strains of A. actinomycetemcomitans are aligned, divergence in amino acid sequence is observed, usually 0 to 10 percent (Kaplan et al. Oral Microbiol. Immunol. December 2002 17:354-359; Kaplan et al. Infect. Immun. 2001 69:5375-5384). Proteins displaying this amount of divergence are considered functionally equivalent variants because of the fact that mixing of genetic alleles that encode these variants is often observed in populations (Kaplan et al. Oral Microbiol. Immunol. December 2002 17:354-359). The dispersin B sequence from A. actinomycetemcomitans strain IDH781 (SEQ ID NO:6), therefore, is expected to be a functionally equivalent or active variant of SEQ ID NO:2, and is included in the scope of the present invention. Similarly, dispersin B sequences from other strains of A. actinomycetemcomitans, such as those that exhibit different serotypes, restriction fragment length polymorphism genotypes, 16S ribosomal RNA genotypes, or arbitrarily-primed PCR genotypes that are commonly observed among phylogenetically diverse strains isolated from different subjects (Kaplan et al. J. Clin.
Similarly, orthologous proteins from phylogenetically diverse species of bacteria are usually functionally equivalent or active variants, as evidenced by the fact that a common method for cloning genes of interest into plasmids is to screen a plasmid library for plasmids that complement a genetic mutation in a different species of bacteria (Kaplan et al. J. Mol. Biol. 1985 183:327-340). This is especially true of bacterial enzymes. Orthologous enzymes of different bacterial species can exhibit up to 50% divergence or greater, yet still utilize the identical substrate, catalyze the same chemical reaction, and produce the same product. This sequence divergence results from genetic drift coupled with fixation of selected genetic changes in the population. The genetic changes that are selected and fixed are those that alter characteristics of the enzyme other than substrate, reaction, and product, as for example, reaction rate, pH optimum, temperature optimum, level of expression, and interactions with other enzymes, such that these genetic changes confer upon a bacterial cell a selective advantage in its environment.

Since A. actinomycetemcomitans is genetically closely related to A. pleuropneumoniae (Dewhirst et al. J. Bacteriol. 1992 174:2002-2013) and produces a biofilm similar to that produced by A. actinomycetemcomitans, which as demonstrated herein detaches upon contact with A. actinomycetemcomitans dispersin B, it is expected that the A. pleuropneumoniae DspB homologue identified in SEQ ID NO:10 is a functionally equivalent or active variant of SEQ ID NO:2, and is included in the scope of the present invention. Similarly, since Actinobacillus ligniersii is
genetically closely related to *Actinobacillus pleuropneumoniae* (Dewhirst et al. *J. Bacteriol.* 1992 174:2002-2013) and *Haemophilus aphrophilus* is genetically closely related to *A. actinomycetemcomitans* (Dewhirst et al. *J. Bacteriol.* 1992 174:2002-2013; Kaplan et al. *J. Clin. Microbiol.* 2002 40:1181-1187), and since both *Actinobacillus ligniersii* and *Haemophilus aphrophilus* produce biofilms similar to that produced by *A. actinomycetemcomitans*, it is expected that the *Actinobacillus ligniersii* and *Haemophilus aphrophilus* dispersin homologues identified in SEQ ID NO:4 and SEQ ID NO:8, respectively, are functionally equivalent or active variants of SEQ ID NO:2, and are included in the scope of the present invention.

The above mentioned examples demonstrate functionally equivalent or active variants of *A. actinomycetemcomitans* dispersin B that occur in nature. As will be understood by those of skill in the art upon reading this disclosure, however, artificially produced genes that encode functionally equivalent or active variants of *A. actinomycetemcomitans* dispersin B can also be produced routinely in accordance with the teachings herein using various well known genetic engineering techniques. For example, a genetically engineered dispersin B enzyme that lacks 20 N-terminal amino acid residues, and also contained a 32 amino acid residue C-terminal tail, which if functionally equivalent to the natural dispersin B enzyme has been produced. It has also been shown that the methionine residue at the N-terminus of this genetically engineered dispersin B enzyme, when expressed in *E. coli*, was removed by the action of methionine aminopeptidase, yet the absence of the methionine did not affect enzyme activity. It has also been shown that cleavage of the C-terminal 28 amino acid residues from this genetically engineered dispersin B enzyme has no affect on enzyme
activity. These examples demonstrate that artificial genes can be produced that encode functionally equivalent variants of *A. actinomycetemcomitans* dispersin B. These artificially produced functionally equivalent variants of *A. actinomycetemcomitans* dispersin B are included in the scope of the present invention.

The above mentioned examples demonstrate genetically-engineered, functionally equivalent variants of *A. actinomycetemcomitans* dispersin B that contain either a deletion of amino acid residues at the N-terminus of the protein, or the fusion of an additional polypeptide at the C-terminus of the protein. It is expected that other genetically-engineered alterations, such as the fusion of an additional polypeptide at the N-terminus of the protein, a deletion of amino acid residues at the C-terminus of the protein, internal deletions and insertions of amino acid residues, and amino acid substitutions, would also result in functionally equivalent variants of *A. actinomycetemcomitans* dispersin B. Information about which deletions, insertions, and amino acid substitutions would produce functionally equivalent variants of *A. actinomycetemcomitans* dispersin B can be obtained from amino acid sequence alignments, and from commonly available computer software that predicts polypeptide secondary structures based on both primary amino acid sequences and on amino acid sequence alignments with homologous proteins having known three-dimensional structures. *A. actinomycetemcomitans* dispersin B, for example, is a member of the family 20 glycosyl hydrolases, a family that includes several well-studied enzymes, and a family represented by numerous homologous primary amino acid sequences in the public databases. In some cases, three-dimensional structures of family 20 glycosyl hydrolases are known (Tews et al. Nature Struct. Biol. 1996 3:638-648).
All family 20 glycosyl hydrolases exhibit a \((\beta\alpha)_8\)-barrel motif (also known as a TIM-barrel motif; Tews et al. Nature Struct. Biol. 1996 3:638-648; Prag et al. J. Mol. Biol. 2000 300:611-617), which is by far the most common enzyme fold in the Protein Data Bank (PDB) database of known protein structures. It is estimated that 10% of all known enzymes have this domain (Wierenge, R. K., FEBS Lett. 2001 492:193-198). The \((\beta\alpha)_8\)-barrel motif is seen in many different enzyme families, catalyzing completely unrelated reactions. The availability of numerous homologous primary amino acid sequences, combined with the availability of the three-dimensional structures of several A. actinomycetemcomitans dispersin B homologues, forms the basis of these sequence alignments and secondary structure predictions. For example, the \((\beta\alpha)_8\)-barrel motif consists of eight \(\alpha\)-helices and eight \(\beta\)-strands such that eight parallel \(\beta\)-strands form a barrel on the inside of the protein, which are covered by eight \(\alpha\)-helices on the outside of the protein. Based on the above mentioned protein sequence alignments and structural predictions, it is expected that the eight \(\beta\)-strands in A. actinomycetemcomitans DspB comprise the amino acid residues surrounding positions 41-44, 69-81, 130-134, 169-171, 189-200, 253-256, 288-300, and 348-350 of SEQ ID NO:2. Any alteration in the amino acid sequence that disrupts the \(\beta\)-strand architecture of these eight regions would be expected to result in a decrease in enzyme activity because of a concomitant disruption in the three-dimensional structure of the \((\beta\alpha)_8\)-barrel of the enzyme. Similarly, based on the above mentioned protein sequence alignments and structural predictions, it is expected that the eight \(\alpha\)-helices in A. actinomycetemcomitans DspB comprise the amino acid residues surrounding positions 52-63, 89-93, 143-149, 176-183, 214-228, 269-284, 309-321, and 361-374 of SEQ ID NO:2. Any alteration in the amino acid sequence
that disrupts the α-helical architecture of these eight regions would be expected to result in a decrease in enzyme activity because of a concomitant disruption in the three-dimensional structure of (βα)₈-barrel of the enzyme.

Similarly, because the β-strands consist of four inward pointing side chains (pointing into the β-barrel) and four outward pointing side chains (pointing towards the α-helices), it is expected that alterations in the inward-pointing amino acid residues will reduce enzyme activity because of concomitant alterations to the substrate binding pocket inside the (βα)₈-barrel, and that alterations in the outward-pointing amino acid residues will reduce enzyme activity when they interfere with the interactions between the β-strands and the α-helices. Similarly, the active site of family 20 glycosyl hydrolases is always located at the C-terminal end of the eight parallel β-strands of the barrel. It is expected that alterations in the homologous region of A. actinomycetemcomitans dispersin B will affect enzyme activity. Similarly, it is predicted that the introduction of insertions and deletions into the regions between the α-helices and the β-strands, namely in positions 45-51, 64-68, 82-88, 94-129, 135-142, 150-168, 172-175, 182-188, 201-213, 229-252, 257-268, 285-287, 301-308, 322-347, and 351-360, in SEQ ID NO:2, will not effect enzyme activity. Similarly, it is expected that almost any alteration of residues 47 (Arginine), 203 (Aspartate) and 204 (Glutamate) will result in complete loss of enzyme activity, because these three residues have been shown to participate directly in substrate binding and catalysis in all family 20 glycosyl hydrolases (Mark et al. J. Biol. Chem. 1998 273: 19618-19624; Prag et al. J. Mol. Biol. 2000 300:611-617; Mark et al. J. Biol. Chem. 2001 276"10330-10337). Similarly, it is expected that alteration of the three tryptophan residues at positions 236, 257 and 350, to any non-aromatic amino acid residue will result in a
decrease in enzyme activity because these three tryptophan residues have been shown to line part of the substrate-binding pocket that is complementary to the hydrophobic surfaces of the substrate hexosamine sugar ring (Tews et al. Nature Struct. Biol. 1996 3:638-648). As a result of the locations of these essential amino acid residues in A. actinomycetemcomitans dispersin B, it is predicted that no more than 46 amino acid residues can be deleted from the N-terminus, and no more that 31 amino acids can be deleted from the C-terminus, without loss of enzyme activity. All of these genetic alterations that result in functionally equivalent variants are included in the scope of the present invention.

Genes encoding functionally different variants of A. actinomycetemcomitans dispersin B can also be produced in accordance with the teachings of the instant application using well known genetic engineering techniques. For example, as mentioned above, it is expected that almost any alteration of residues 47 (Arginine), 203 (Aspartate) and 204 (Glutamate) in SEQ ID NO:2 will result in complete loss of enzyme activity. Alternatively, variants of A. actinomycetemcomitans dispersin B that exhibit characteristics that may be useful in a clinical setting could also be artificially produced. For example, the temperature optimum of A. actinomycetemcomitans dispersin B is 30°C. It may be desirable to produce a genetically-engineered variant of dispersin B that exhibits a temperature optimum of 37°C, thereby resulting in an increased effectiveness of the enzyme or decreased cost of treatment. Such variants can be artificially produced by first creating random mutations in the A. actinomycetemcomitans dspB gene sequence, for example by using UV light or a chemical mutagen like nitrosoguanidine, and then screening large numbers of these random variants,
for example in a quantitative 96-well microtiter plate assay (Kaplan et al. J. Bacteriol. 2003 185:4693-4698), for ones that exhibit higher temperature optima. An alternative method is to utilize directed evolution of sequences by DNA shuffling (Christians et al. Nature Biotechnol. 1999 17:259-264; Dichek et al. J. Lipid Res. 1993 34:1393-1340), combined with a high-throughput robotic screen based upon a quantitative 96-well microtiter plate assay (Kaplan et al. J. Bacteriol. 2003 185:4693-4698) to identify variants with increased temperature optima. The aforementioned methods can also be used to produce variants of A. actinomycetemcomitans dispersin B that exhibit increased substantivity to biomaterials, increased pH optima, increased stability in aqueous solutions, increased reaction rate, increased stability upon desiccation, and other characteristics that could result in increased effectiveness of the enzyme or decreased cost of treatment. An alternative method that can be used to produce useful variants is site-directed mutagenesis. For example, it is expected that the eight α-helices of the (βα)s-barrel in A. actinomycetemcomitans dispersin B contain many amino acid residues that are exposed on the outer surface of the enzyme, and that altering the outward-pointing amino acid residues of the eight α-helices will alter the outer surface properties of the enzyme, thereby potentially increasing the substantivity of the enzyme for biomaterials without affecting enzyme activity. Accordingly, these outward pointing amino acid residues can be systematically mutated, for example from polar residues to charged residues, and the resulting mutants screened to identify variants with increased substantivity to biomaterials. Functionally different variants of A. actinomycetemcomitans dispersin B that are intended to improve the clinical efficiency or cost effectiveness of the enzyme, when applied to detaching bacterial or fungal cells from
biofilms, are included in the scope of the present invention.

Also provided in the present invention are fusion proteins and nucleic acid sequences encoding fusion proteins. Fusion proteins of the present invention comprise an amino acid sequence for an isolated soluble, β-N-acetylglucosaminidase protein which promotes detachment of bacterial cells from a biofilm and a second polypeptide. Exemplary second polypeptides of these fusion proteins include, but are not limited to, those which facilitate purification such as a His tag, those which facilitate attachment to a surface such as an antibody or a protein such as albumin, fibronectin or thrombin, and/or those which target the enzyme to the surface of bacterial or fungal cell such as a specific bacterial or fungal receptor. Nucleic acid sequences encoding such fusion proteins comprise an isolated nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof which promotes detachment of bacterial or fungal cells from a biofilm and a second nucleic acid sequence encoding a second polypeptide. In a preferred embodiment, the second nucleic acid sequence encodes a polypeptide such as a His tag which facilitates purification, an antibody or protein such as albumin, fibronectin or thrombin which facilitates attachment of the fusion protein to a surface, or a bacterial or fungal receptor which specifically targets the fusion protein to the surface of a bacterial or fungal cell, respectively.

The dispersin B protein engineered to contain an octahistidine metal binding site at its C-terminus was expressed in *E. coli*. The protein was purified by Ni-affinity chromatography and the dispersin B portion was cleaved from the hybrid protein using thrombin. Analysis of the purified cleaved dispersin B protein by SDS-PAGE revealed the protein to migrate with an apparent molecular
mass of 41 kDa. The N-terminal sequence of dispersin B was XCVKGNSIYPQK (SEQ ID NO:11) (where X is an unidentified residue). This matched codons 22 to 33 of CU1000 dspB, thus indicating that the dipeptide Met-Asn was cleaved from the N-terminus of the dispersin B fusion protein when expressed in E. coli. Analysis of purified, cleaved dispersin B protein by mass spectrophotometry resulted in a single major peak with an apparent molecular mass of 41.5 kDa, consistent with the predicted molecular mass of 41.4 kDa for the cleaved and processed dispersin B protein. The yield of dispersin B expressed in E. coli was 30 mg of protein per liter of culture.

The ability of dispersin B to cleave the glycosidic linkages of various 4-nitrophenyl-labeled synthetic hexosamine substrates was tested in an in vitro enzyme assay. Dispersin B showed specificity for the 1-4 glycosidic bond of β-substituted N-acetylglucosaminide, consistent with the known functions of other family 20 glycosyl hydrolases (Tews et al. Nature Struct. Biol. 1996 3:638-648). Dispersin B showed no activity against α-substituted N-acetylglucosaminide, or against α- or β-substituted N-acetylgalactosamine.

The glycosyl hydrolase activity of dispersin B was optimal at pH 5.0, which is similar to the pH optima of other family 20 glycosyl hydrolases. Dispersin B displayed maximum activity at 30°C. Dispersin B glycosyl hydrolase activity was inhibited by quinacrine (Kovacs, P. and Csaba, G. Cell Biochem. Funct. 2001 19:287-290) and NAG-thiazoline (Mark et al. J. Biol. Chem. 2001 276:10330-10337), two small molecule inhibitors of family 20 β-N-acetylglucosaminidases.

The effects of dispersin B protein on biofilm cell detachment of A. actinomycetemcomitans mutant strain JK1023 were then examined. In these experiments, dispersin B
protein was added to growth medium of mutant strain JK1023 to determine if addition of this protein restored release of cells into the medium and dispersion. Polystyrene rods containing biofilm colonies of strain JK1023 were suspended in broth containing various amount of dispersin B, and the amount of biofilm cell detachment was measured by staining the bacteria growing on the bottom of the well with crystal violet. Purified dispersin B restored the ability of mutant strain JK1023 to release cells into the medium and colonize the bottom of the microtiter plate well in a dose-dependent manner. Heat-inactivated dispersin B had no effect on biofilm cell detachment of strain JK1023.

The effects of dispersin B protein on detachment of preformed biofilm colonies of A. actinomycetemcomitans and other bacteria were also examined. In these experiments, addition of dispersin B caused the detachment of preformed biofilm colonies of wild-type strain CU1000. Dispersin B (50 µg/ml) caused a 90% reduction in the amount of surface-associated bacteria after 6 hours. Further, analysis by light micrography showed that the surface of treated colonies became grainy and flocculent when compared to the smooth-textured biofilm colonies observed with mock-treated cells. Also, the surface of the culture vessel became covered with a similar grainy material which had a fibrous appearance under higher power. These findings are consistent with the observed reduction in adherence of preformed biofilm colonies treated with dispersin B.

Dispersin B caused a similar reduction in biofilm density when tested against biofilm colonies of four phylogenetically diverse strains of A. actinomycetemcomitans representing four different serotypes, a strain of the closely related bacterium Haemophilus aphrophilus, and two strains of the swine pathogen Actinobacillus pleuropneumoniae. Dispersin B did not cause the detachment of biofilm colonies of Neisseria
subflava, Cardiobacterium hominis or Streptococcus mitis, bacteria which do not have biofilms comprising N-acetyl glucosamine residues.

Dispersin B also causes the detachment of

5 Staphylococcus epidermidis from surfaces. The Gram-positive bacterium S. epidermidis is the most common cause of infection associated with catheters and other indwelling medical devices. S. epidermidis produces an extracellular slime composed of a polysaccharide containing primarily N-acetylglucosamine residues (Mack et al. J. Bacteriol. 1996 178:175; Baldassarri et al. Infect. Immun. 1996 64:3410) which enables it to form adherent films on plastic surfaces. Biofilm bacteria such as S. epidermidis are highly resistant to antibiotics and host defenses and nearly impossible to irradicate (Costerton et al. Annu. Rev. Microbiol. 1995 49:711). Thus, attachment of this bacteria to indwelling devices such as catheters can lead to osteomyelitis, acute sepsis and death, particularly in immunocompromised patients, and is a leading cause of nosocomial bloodstream and cardiovascular infections as well as morbidity in hospitalized patients (Vuong, C. and Otto, M. Microbes, Infect. 2002 4:481).

Four different strains of S. epidermidis isolated from infected intravenous catheters were used in these experiments. All four strains contained the ica genetic locus and produced dark red colonies on Congo red agar, both of which are indicative of slime production (Aricola et al. J. Clin. Microbiol. 2001 39:2151; Aricola et al. Biomaterials 2002 Biomaterials 23:4233). The ability of the four strains to form biofilms was measured by making serial dilutions of overnight cultures in fresh broth and then transferring the dilutions to the wells of a 96-well polystyrene microliter plate. After 16 hours of incubation, the wells were washed under running
tap water to remove loosely adherent cells and the bacteria remaining attached to the bottoms of the well were stained with crystal violet. As expected all four strains produced adherent biofilms as indicated by the presence of dark-staining material on the bottoms of the wells. The amount of dark-staining material was quantitated by measuring its optical density at 590 nm in a microliter plate reader. When dispersin B protein was added to the wells 30 minutes prior to washing (final concentration, 40 µg/ml) little or no biofilm material was evident. In contrast, heat inactivated dispersin B protein had no effect on *S. epidermidis* biofilms. Two other N-acetylglucosaminidase enzymes that are homologous to *A. actinomycetemcomitans* dispersin B, *Serratia marcescens* chitinase and jack bean β-hexosaminidase, also had no effect on *S. epidermidis* biofilms. Unlike the orthologs described herein, these homologous proteins share less than 25% identity with dispersin B and do not exhibit biofilm-releasing activity. Thus, these experiments are demonstrative of dispersin B enzymatic activity being responsible for removing *S. epidermidis* biofilm cells from the surfaces of the wells.

Dispersin B had no effect on viability of *S. epidermidis* cells.

The amount of dispersin B protein and the length of time needed to remove *S. epidermidis* biofilms from the microliter plate wells were also examined. In these experiments, multiple wells were inoculated with a 10^-4 dilution of a *S. epidermidis* culture and the plate was incubated for 16 hours. After washing away loosely adherent cells, the wells were filled with phosphate buffered saline (PBS) and then various amounts of dispersin B protein (200 pg to 120 µg per ml final concentrations) were added to the wells for various lengths of time (0 to 9 minutes). Dispersin B treatment at a concentration of 4.8 µg/ml
resulted in a decrease in absorbance to background levels (ca. 0.09 O.D. units) after 2 minutes. At a concentration of 40 ng/ml, dispersin B resulted in a greater than 50 percent reduction in optical density after 9 minutes (from 3.63 to 1.74 O.D. units. These data demonstrate that dispersin B causes detachment of *S. epidermidis* biofilms of clinically achievable concentrations of the enzyme.

Biofilm cell detachment was quantitated by growing *S. epidermidis* biofilms on polystyrene rods and then transferring the rods to tubes containing PBS (as a control) or PBS with 60 μg/ml of dispersin B. The tubes were incubated for 15 minutes, rinsed in PBS, and the bacteria remaining attached to the rods after treatment were removed by sonication and then quantitated by plating serial dilutions of the sonicates on agar. Mock-treated and dispersin B-treated rods were compared after staining with crystal violet. The mock-treated control rod contained a layer of dark-staining material corresponding to the thick biofilm that formed on its surface. The dispersin B-treated rod showed no trace of dark-staining material and was similar in appearance to a rod which was sonicated prior to staining and to an uninoculated rod. Quantitation of cells remaining attached to the rods revealed that dispersin B treatment resulted in a 5.8 log reduction in the number of surface-associated bacteria.

The ability of dispersin B to remove *S. epidermidis* biofilms grown attached to polyurethane and Teflon intravenous catheters was also examined. In these experiments, catheters were placed in tubes containing a 10⁻³ dilution of a *S. epidermidis* culture and incubated for 16 hours. The catheters were then rinsed with PBS and transferred to tubes containing PBS (as a control) or PBS with 60 μg/ml of dispersin B. After 5 minutes the catheters were rinsed with PBS and the biofilm bacteria
remaining attached to the surface were stained with methylene blue (for polyurethane catheters) or crystal violet (for Teflon catheters). The control catheters contained a layer of dark-staining material on their surfaces indicating the presence of a biofilm, whereas the dispersin B-treated catheters contained no dark-staining material and were similar in appearance to uninoculated catheters.

Thus, dispersin B of the present invention is capable of removing S. epidermidis biofilms from various plastic biomaterials.

The ability of precoating surfaces with dispersin B to prevent S. epidermidis biofilm formation was also demonstrated. In these experiments, polyurethane and Teflon catheters in tubes containing PBS or PBS with 40 \( \mu g/ml \) of dispersin B were incubated at 4°C for 24 hours. The catheters were then rinsed with PBS and transferred to tubes containing a 10\(^{-1}\) dilution of a S. epidermidis culture. After 6 hours, the catheters were rinsed with PBS to remove loosely adherent cells and then stained as described supra. The surfaces of control catheters were covered with a layer of dark-staining material indicating the presence of a biofilm, whereas the surfaces of dispersin B-coated catheters contained no dark-staining material and were similar in appearance to uninoculated catheters. As shown, precoating plastic catheters with dispersin B of the present invention significantly reduced S. epidermidis attachment or biofilm formation. Catheters that were precoated for 10 minutes, and catheters that were precoated for 24 hours and then dried, were also resistant to colonization and biofilm formation by S. epidermidis.

Thus, as demonstrated by these experiments, addition of an isolated dispersin B protein as well as mutation of the dspB gene modulates the detachment of cells from
biofilm colonies of various bacteria, particularly bacteria with a biofilm comprising a polysaccharide containing N-acetylglucosamine. Fungi also form biofilms of clinical significance which may compromise polysaccharide containing N-acetylglucosamine. It is believed that dispersin B will also be effective in degrading these fungal polysaccharides and modulating detachment of such fungal cells from their biofilms.

Accordingly, the present invention also relates to methods for modulating detachment of bacterial or fungal cells from biofilms, particularly bacteria or fungal with a biofilm comprising a polysaccharide containing N-acetylglucosamine.

By "modulating detachment" as used herein it is meant to be inclusive of increases as well as decreases in bacterial or fungal biofilm detachment or release of bacterial or fungal cells from the biofilm. Further, by "modulating detachment" it is also meant to be inclusive of changes in the ability of the bacteria or fungal to attach as a biofilm. For example, as demonstrated herein, dispersin B modulates detachment of S. epidermidis not only by promoting detachment but also by inhibiting the ability of the bacteria to attach to surfaces and form a biofilm.

In one embodiment of the present invention, the method comprises mutating dspB of bacterial cells to inhibit detachment of bacterial cells from biofilms such as in the JK1023 mutant of the present invention. In another embodiment, the method comprises decreasing expression and/or levels of soluble, β-N-acetylglucosaminidase or inhibiting activity of soluble, β-N-acetylglucosaminidase in bacterial cells so that detachment of bacterial cells is decreased.

The present invention also provides methods for promoting detachment of bacterial or fungal cells from a biofilm which comprises contacting bacterial or fungal
cells with soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof or a nucleic acid sequence encoding soluble, β-N-acetylglucosaminidase or an active fragment or variant thereof. For example, A. actinomycetemcomitans dispersin B was found to detach biofilms of Haemophilus aphrophilus, Actinobacillus pleuropneumoniae and S. epidermidis. It is believed that biofilm detachment of Actinobacillus ligniersii, as well as other bacteria or fungi with a biofilm comprising a polysaccharide containing N-acetylglucosamine including, but in no way limited to, Staphylococcus aureus and Yersinia pestis will also be promoted in the presence of soluble β-N-acetylglucosaminidase or an active fragment thereof of the present invention.

Accordingly, isolated dispersin B proteins and active fragments or variants thereof can be used to prevent or inhibit bacterial or fungal biofilm attachment and to treat infections by such bacteria or fungi.

In one embodiment, the isolated dispersin B protein or active fragment or variant thereof is used directly as a parenteral to treat biofilm infections such as mastitis in ewes, intramammary infections in cows or osteomyelitis and infective endocarditis in humans. In this embodiment, the isolated soluble, β-N-acetylglucosaminidase protein or active fragment or variant thereof is preferably administered as a pharmaceutical composition in a pharmaceutically acceptable carrier to an organism.

By "organism", as used herein it is meant to be inclusive of all animals including, but not limited to mammals, and most preferably humans.

Any pharmaceutically acceptable vehicle or carrier, as well as adjuvant, can be used in the manufacture, dissolution and administration of pharmaceutical preparations comprising dispersin B protein or active fragment or variant thereof. Such vehicles, carriers and
adjuvants are well known to those of skill in the art and described in text books such as *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985.

Appropriate concentrations of active composition to be incorporated into pharmaceutical compositions can be routinely determined by those skilled in the art and is dependent upon the form of administration as well as the severity of the condition being treated.

Pharmaceutical formulations suitable for oral administration may be provided in convenient unit forms including, but not limited to, capsules or tablets, each containing a predetermined amount of the dispersin B protein or active fragment or variant thereof; as a powder or granules; as a solution, a suspension or as an emulsion. The dispersin B protein or active fragment or variant thereof can also be presented as a bolus, electuary, or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets may be coated according to methods well known in the art. Timed release formulations, which are known in the art, may also be suitable. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may contain conventional additives such as suspending agents, non-aqueous vehicles, including edible oils, or preservatives.

Dispersin B protein or active fragments or variants thereof of the present invention may also be formulated for parenteral administration, such as by injection, for example bolus injection or continuous infusion, and may be provided in unit dose form in ampules, pre-filled syringes, small volume infusion or in multi-dose containers with an
added preservative. Pharmaceutically acceptable compositions comprising a dispersin B protein or active fragment or variant thereof for parenteral administration may be in the form of a suspension, solution or emulsion in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle such as sterile, pyrogen free water, before use.

For topical administration to the epidermis, dispersin B protein or an active fragment or variant thereof of the present invention may be formulated in an ointment, cream, or lotion, or as a transdermal patch. Ointments and creams, may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, suspending agents, thickening agents, or coloring agents. Formulations suitable for topical administration in the mouth include lozenges comprising dispersin B protein or an active fragment or variant thereof in a flavored base, usually sucrose and acacia or tragacan; pastilles comprising the active ingredient in an inert base such as gelatin and glycerin or sucrose and acacia; and mouth washes comprising the active ingredient in a suitable liquid carrier. For topical administration to the eye, the dispersin B protein or active fragment or variant thereof can be made up in solution or suspension in a suitable sterile aqueous or non-aqueous vehicle. Additives such as buffers (e.g. sodium metabisulphite or disodium edeate) and thickening agents such as hypromellose can also be included.
For intra-nasal administration, dispersin B protein or an active fragment or variant thereof of the present invention can be provide in a liquid spray or dispersible powder or in the form of drops. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents, or suspending agents. Liquid sprays are conveniently delivered from pressurized packs.

For administration by inhalation, dispersin B protein or active fragment or variant thereof of the present invention can be delivered by insufflator, nebulizer or a pressurized pack or other convenient means of delivering the aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the dispersin B protein or active fragment or variant thereof of the present invention can take the form of a dry powder composition, for example a powder mix of the active component and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules, cartridges or blister packs of gelatins, from which the powder can be administered with the aid of an inhalator or insufflator.

When desired, any of the above-described formulations may be adapted to provide sustained release of the dispersin B protein or active fragment or variant thereof. The amount of dispersin B protein or active fragment or variant thereof of the present invention required for use in treatment will of course vary not only with the particular protein or active fragment or variant selected but also with the route of administration, the nature of
the condition being treated, and the age and condition of the organism.

Increasing detachment of bacteria from a biofilm is also expected to decrease resistance of the bacteria to antibiotic therapy. Accordingly, the present invention also provide methods for enhancing efficacy of antibiotic therapy against bacterial infections by administration of a pharmaceutical composition of the present invention in combination with or prior to administration of an antibiotic.

In another embodiment of the present invention, wound dressings including but not limited to sponges or gauzes can be impregnated with the isolated dispersin B protein or active fragment or variant thereof to prevent or inhibit bacterial or fungal attachment and reduce the risk of wound infections. Similarly, catheter shields as well as other materials used to cover a catheter insertion sites can be coated or impregnated with a dispersin B protein or active fragment or variant thereof to inhibit bacterial or fungal biofilm attachment thereto. Adhesive drapes used to prevent wound infection during high risk surgeries can be impregnated with the isolated protein or active fragment or variant thereof as well. Additional medical devices which can be coated with a dispersin B protein or active fragment or variant thereof include, but are not limited, central venous catheters, intravascular catheters, urinary catheters, Hickman catheters, peritoneal dialysis catheters, endotracheal catheters, mechanical heart valves, cardiac pacemakers, arteriovenous shunts, scleral buckles, prosthetic joints, tympanostomy tubes, tracheostomy tubes, voice prosthetics, penile prosthetics, artificial urinary sphincters, synthetic pubovaginal slings, surgical sutures, bone anchors, bone screws, intraocular lenses, contact lenses, intrauterine devices, aortofemoral grafts and vascular grafts. Exemplary solutions for impregnating
gauzes or sponges, catheter shields and adhesive drapes or coating catheter shields and other medical devices include, but are not limited to, phosphate buffered saline (pH approximately 7.5) and bicarbonate buffer (pH approximately 9.0).

In yet another embodiment, an isolated dispersin B protein or active fragment or variant thereof can be incorporated in a liquid disinfecting solution. Such solutions may further comprise antimicrobials or antifungals such as alcohol, providone-iodine solution and antibiotics as well as preservatives. These solutions can be used, for example, as disinfectants of the skin or surrounding area prior to insertion or implantation of a device such as a catheter, as catheter lock and/or flush solutions, and as antiseptic rinses for any medical device including, but not limited to catheter components such as needles, Leur-Lok connectors, needleless connectors and hubs as well as other implantable devices. These solutions can also be used to coat or disinfect surgical instruments including, but not limited to, clamps, forceps, scissors, skin hooks, tubing, needles, retractors, scalers, drills, chisels, rasps and saws.

The nucleic acid and amino acid sequences of the present invention, as well as the mutant JK1023 strain can also be used to identify agents which modulate detachment of bacterial or fungal cells from biofilms. For example, the ability of an agent to modulate activity and/or expression of soluble, β-N-acetylglucosaminidase of the present invention can be assessed.

Examples of such agents include, but are not limited to antisense oligonucleotides or ribozymes targeted to the dspB gene, peptidomimetics of dispersin B, and small organic chemicals such as quinacrine and NAG-thiazoline which modulate dispersin B activity and/or levels and/or expression.
Agents which inhibit the ability of soluble, β-N-acetylglucosaminidase to promote detachment of bacterial cells from biofilms are expected to be useful in preventing the dissemination of infectious bacteria, particularly infectious bacteria of the oral cavity such as A. actinomycetemcomitans and closely related bacterium such as Haemophilus aphrophilus.

Agents which mimic dispersin B activity such as peptidomimetics and small organic molecules similar in structure and activity to dispersin B can be used in similar fashion to isolated dispersin B or an active fragment or variant thereof to prevent, inhibit or treat infection resulting from bacterial or fungal biofilm attachment to surfaces. Such uses are described herein in detail supra.

The present invention also provides primer pairs and kits comprising such primer pairs for use in identifying additional species of bacteria with dispersin B homologues. An exemplary degenerate primer pair useful in the kits of the present invention comprises 5'-GAYCAYGARAAYTAYCG-3' (SEQ ID NO:12) and 5'-TCNCCRTCRTARCTCCA-3' (SEQ ID NO:13), where Y is C or T, and R is A or G. Kits of the present invention preferably further comprise instructions for use of the kit and/or positive and negative control samples.

Bacteria identified by these kits as having a dispersin B homologue can be further examined to determine if the homolog is an ortholog exhibiting the same or similar enzymatic activity as dispersin B. The primers and kits of the present invention are thus useful in identifying additional bacteria, biofilm attachment of which can be modulated using the nucleic acid sequences, amino acid sequences, and agents described herein as well as additional orthologous nucleic acid sequences and amino acid of dispersin B.

The following nonlimiting examples are provided to
further illustrate the present invention.

**Examples**

**Example 1: Bacterial strains and growth conditions**

University, Ames, IL). Bacteria were grown in Trypticase soy broth (BD Biosystems) supplemented with 6 grams of yeast extract and 8 grams of glucose/liter. Inoculated culture vessels were incubated at 37°C in 10% CO₂, except for S. epidermidis cultures, which were incubated at 37°C in air.

Example 2: Cloning and sequencing dspB

The transposon insertion site in *A. actinomycetemcomitans* mutant strain JK1023 was cloned and sequenced by using an inverse PCR method in accordance with Kaplan et al. (Infect. Immun. 2001 69:5375-5384). The DNA sequence of the inverse PCR product was compared to the genome sequence of *A. actinomycetemcomitans* strain HK1651 from the Actinobacillus Genome Sequencing Project and the transposon was found to have inserted into a long open reading frame (ORF) which was designated *dspB*. Primers that hybridize to sequences upstream and downstream from HK1651 *dspB* were used to amplify by PCR the *dspB* coding region from *A. actinomycetemcomitans* strain CU1000 using methods in accordance with Kaplan et al. (Infect. Immun. 2001 69:5375-5384). The forward primer (5'-GGCGGCCATATGAAATTGTTGCGTAAAAGGCAATTCC-3' (SEQ ID NO:14)) introduced an NdeI restriction site (underlined) and an ATG initiation codon (lower case) at codon positions 19 to 20 of *dspB*, and the reverse primer (5'-GGCTTACCATCCCCATCTGTCTTATGATC-3' (SEQ ID NO:15)) replaced the *dspB* stop codon with a KpnI restriction site (underlined). The PCR product (1,106 bp) was digested with NdeI and KpnI and ligated into the NdeI/KpnI sites of plasmid pET29b (Novagen). The insert of the resulting plasmid (designated pRC1) was subjected to DNA sequence analysis in accordance with procedures described by Kaplan et al. (Infect. Immun. 2001 69:5375-5384).

Example 3: Expression and purification of recombinant
Dispersin B protein

Plasmid pRC1 carries a gene that encoded amino acids 21 to 381 of dspB fused to a 32 amino acid residue C-terminal tail containing an hexahistidine metal-binding site and a thrombin protease cleavage site which could be used to cleave the C-terminal tail from the hybrid protein. This gene was located downstream from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter.

Expression of DspB in E. coli

A one liter Erlenmeyer flask containing 500 ml of LB broth supplemented with 50 μg/ml of kanamycin was inoculated with 5 ml of an overnight culture of E. coli strain BL21(DE3) (Dubendorff, J. W. and Studier, F. W. J. Mol. Biol. 1991 219:61-68) transformed with pRC1. The flask was incubated at 37°C with agitation (200 rpm) until the optical density of the culture (measured at 280 nm) reached 0.6 (approximately 3 hours). IPTG was added to a final concentration of 0.2 mM and the flask was incubated for an additional 5 hours with agitation. The cells were harvested by centrifugation for 15 minutes at 6,000 x g and the cell pellet was stored at -80°C.

Protein purification

The cell pellet was thawed on ice and resuspended in 20 ml of lysis buffer [20 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate] containing 10 mg/ml lysozyme. The cell suspension was sonicated for 30 seconds at 50% capacity, 70% duty cycle in a Branson model 4550 sonicator equipped with a microprobe and then cooled on ice for 30 seconds. The sonication and cooling steps were repeated four additional times. The cells were pelleted by centrifugation as above and the supernatant was transferred to a new tube. The cell pellet was resuspended in 20 ml of lysis buffer without lysozyme and five additional cycles of sonication and cooling were performed. The cells were pelleted by centrifugation and the supernatant was removed and
transferred to a new tube. The two supernatants were combined and loaded onto a 3 ml bed volume Ni-affinity column (catalog no. 154-0990, Pharmacia) according to the instructions supplied by the manufacturer. The column was washed with 50 ml of wash buffer [50 mM MOPS (pH 8.5), 20 mM KCl] containing 5 mM imidazole, followed by 25 ml of wash buffer containing 50 mM imidazole and 25 ml of wash buffer containing 100 mM imidazole. Fractions (1.5 ml each) were collected during the final wash and assayed for the presence of the hybrid protein by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining in accordance with procedures described by Sambrook et al. (1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Fractions containing the protein were pooled and dialyzed overnight against water using a 10,000 MW cut-off dialysis membrane. The purified protein was digested with 5 units of thrombin (Novagen) per mg of protein for 1 hour at room temperature and the thrombin was removed using a Thrombin Cleavage Capture Kit (Novagen) according to instructions supplied with the kit. Undigested protein was removed by loading the sample onto a Ni-affinity column as described above and washing the column with 10 ml of wash buffer containing 5 mM imidazole. Fractions of the wash (1.5 ml each) were collected and analyzed for the presence of the protein by SDS-PAGE. Fractions containing the protein were pooled, dialyzed against water, and stored at -20°C.

N-terminal sequence analysis of the purified protein was carried out using the Edman degradation procedure on a Beckman model 2300 protein sequencer. Mass spectra were determined by using a Hitachi model 4414 mass spectrometer.

Example 4: Enzyme assays

Synthetic substrates (purchased from Sigma Chemical Co.) were 4-nitrophenyl-N-acetyl-β-D-galactosaminide, 4-nitrophenyl-N-acetyl-α-D-galactosaminide, 4-nitrophenyl-N-
acetyl-\(\beta\)-D-glucosaminide, and 4-nitrophenyl-N-acetyl-\(\alpha\)-D-glucosaminide. Enzyme reactions were carried out in a 10 ml volume containing 50 mM sodium phosphate buffer (pH 5.9), 100 mM NaCl, 5 mM substrate, and 3.7 \(\mu\)g/ml purified protein in a 15 ml polypropylene tube placed in a 37°C water bath. The reaction was terminated at various times by transferring 1 ml of the reaction mixture to a new tube containing 5 \(\mu\)l NaOH. The increase in absorption resulting from the release of p-nitrophenolate in each tube was measured in a Shimadzu UV-Mini spectrophotometer set to 405 nm.

**Example 5: Identification of dspB orthologues in other strains of A. actinomycetemcomitans and in other species of bacteria**

The microbial genome database www.ncbi.nlm.nih.gov was searched for homologues of *A. actinomycetemcomitans* dspB. dspB homologues were identified in the unfinished genomes of *A. pleuropneumoniae* serovars 1, 5 and 7. The *A. pleuropneumoniae* dspB homologues displayed approximately 60% identity at the amino acid level with the *A. actinomycetemcomitans* CU1000 DspB sequences. Additional searching was performed for DspB homologues in other members of the Pasteurellaceae family. The amino acid sequence of *A. actinomycetemcomitans* CU1000 DspB was aligned with the *A. pleuropneumoniae* DspB homologues and two regions of the sequence were identified that were highly conserved. Degenerate oligonucleotide primers were then synthesized that hybridized to DNA sequences encoding these conserved amino acids (5'-GAYCAYGARAAYTAYCG-3' (SEQ ID NO:12) and 5'-TCNCCRTCRTARCTCCA-3' (SEQ ID NO:13), where \(Y = \text{C or T}, \ R = \text{A or G}, \ \text{and } N = \text{A or C or G or T}) and these primers were used to amplify by PCR genomic DNAs purified from various species of Pasteurellaceae. A PCR product of the expected size was observed in genomic DNA from *A.*
actinomycetemcomitans strain IDH781 (Saarela et al. 1993. Oral Microbiol. Immunol. 8:111-115), A. pleuropneumoniae strain IA5 (obtained from the Veterinary Diagnostics Laboratory, Iowa State University, Ames, IA), Haemophilus aphrophilus strain NJ8700 (Kaplan et al. 2002 J. Clin. Microbiol. 40:1181-1187), and A. lignieresii strain 19393 (obtained from the American Type Culture Collection, Manassas, VA). No PCR product was observed with DNA from Haemophilus somnus, Actinobacillus equuli, Pasteurella multocida, and Mannheimia haemolytica.

The PCR products were cloned into multicopy plasmids and subjected to DNA sequence analysis. Figure 1 shows a comparison of the predicted DspB amino acid sequence of A. actinomycetemcomitans CU1000 DspB and the sequences of the DspB homologues from the other strain of A. actinomycetemcomitans and other Pasteurellaceae bacteria.

**Example 6: Overexpression of dspB in a wild-type strain of A. actinomycetemcomitans**

In order to determine the effects of overexpressing dspB in a wild-type strain of A. actinomycetemcomitans, a plasmid was constructed which contains dspB under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. This plasmid was introduced into wild-type strain CU1000, and the cells were grown in the presence of 1 mM IPTG. CU1000 cells harboring the dspB expressing plasmid exhibited a smooth-colony morphology on agar and produced biofilm colonies in broth that displayed a hyper-dispersing phenotype, as indicated by the presence of increased numbers of satellite colonies on the surface of the culture vessel. These findings confirm that dspB expression parallels the amount of biofilm dispersal.

**Example 7: Detachment of biofilm cells from polystyrene rods in microtiter plates**

An assay to measure the detachment of cells from
preformed biofilm colonies grown on polystyrene rods was carried out in 96-well microtiter plates. Biofilm colonies were grown on polystyrene rods suspended in broth in the 96-wells of a microtiter plate. Cells that detached from the biofilm fell to the bottom of the well where they attached to the surface and formed new biofilm colonies. The amount of biofilm growth on the bottom of the well, which was proportional to the number of cells that detached from the biofilm colonies on the rods, was measured by staining with crystal violet. The detachment assay was carried out as follows.

Construction of the apparatus

The lid of a 96-well polystyrene flat-bottomed tissue culture plate (Falcon No. 353072) was modified as follows:

First, 96 1.5-mm diameter holes were drilled in the lid, with each hole in a position corresponding to the center of one of the 96 wells. Then, an 11-mm long polystyrene rod (1.5-mm diameter, Plastruct Corp., City of Industry, CA) was placed in each hole (with one end of the rod flush against the top of the lid) and secured with trichloromethane plastic solvent. When this modified lid was placed on a 96-well microtiter plate bottom, the rods were suspended in the wells with the bottom of each rod approximately 2 mm above the bottom of the well. The modified lid was sterilized by soaking in 70% ethanol for 30 minutes and air drying in a biological safety cabinet.

Inoculation and incubation of polystyrene rods

The microtiter plate bottom was filled with medium (100 µl per well) and each well was inoculated with a single 2-3 day old colony from an agar plate using a sterile toothpick. The modified lid was then placed on the inoculated plate to submerge the polystyrene rods in the inoculated medium, and the plate was incubated at 37°C for 24 hours to allow that bacteria to adhere to the rods. The
lid was then transferred to a fresh microtiter plate containing prewarmed medium and incubated for an additional 24 hours to allow biofilm cells to detach from the rods. **Measuring detached cells**

The lid was removed and the plate was washed extensively under running tap water to remove loosely adherent cells. The wells were filled with 100 μl of Gram-staining reagent (2 grams crystal violet, 0.8 grams ammonium oxalate, 20 ml ethanol per 100 ml) and the plate was incubated at room temperature for 10 minutes. The plate was re-washed extensively under running tap water to remove unbound dye. The wells were than filled with 100 μl of ethanol and the plate was incubated at room temperature for 10 minutes to solubilize the dye. The optical density (at 590 nm) of the ethanol/dye solution in each well was measured using a Bio-Rad benchmark microplate reader.

**Example 8: Growth of biofilms on polystyrene rods**

Polystyrene rods (1.5 mm diam; Plastruct Corp., City of Industry, Calif.) were cut into 35 mm lengths, sterilized in 70% ethanol for 30 minutes, and air dried in a biological safety cabinet. Rods were placed into 1.5 ml microcentrifuge tubes containing 0.5 ml of broth inoculated with *S. epidermidis* and incubated for 16 hours. Rods were then rinsed under running tap water and then placed in fresh microcentrifuge tubes containing 0.5 ml of PBS or PBS plus dispersin B. Rods were rinsed with water and stained with crystal violet as previously described (Kaplan, J. B., and Fine, D. H. Appl. Environ. Microbiol. 2002 68:4943-4950). For sonication, rods were placed in 15 ml conical centrifuge tubes containing 3 ml of PBS at then sonicated for 30 seconds at 40% duty cycle and 70% capacity in Branson model 200 sonicator equipped with a cup horn. For quantitation of detached cells, sonicates were serially diluted and plated on medium solidified with 1.5% agar.

**Example 9: Growth of biofilms in polystyrene microtiter**
plates

The wells of a 96-well polystyrene microtiter plate (model 3595, Corning) were filled with 100 μl of broth inoculated with S. epidermidis and the plate was incubated for 16 hours. Microtiter plates were washed by aspirating the medium and washing the well three times with 200 μl of PBS, or by submerging the entire plate in a tub of cold, running tap water. Biofilms were stained with crystal violet as previously described (Kaplan, J. B., and Fine, D. H. Appl. Environ. Microbiol. 2002 68:4943-4950).

Example 10: 96-well microtiter plate biofilm cell detachment assay

The wells of a 96-well microtiter plate (Falcon no. 353072) were filled with 100 μl of medium containing 10^2 to 10^4 CFU of bacteria and incubated at 37°C in 10% CO₂ for 20 hours. Ten μl of enzyme solution [1 mg ml⁻¹ in phosphate buffered saline (PBS)], or 10 μl of PBS in the case of controls, was added to each well and the plates were incubated for an additional 6 hours. The wells were washed extensively under running tap water and the bacteria remaining attached to the surface were stained with crystal violet, rewashed, and destained with ethanol in accordance with procedures described by Kachlany et al. Mol. Microbiol. 2001 40:542-554). The optical density (O.D.) of the ethanol-dye solution was measured in a BioRad Benchmark microtiter plate reader set to 590 nm.

Example 11: Growth of biofilms on intravenous catheters

Polyurethane catheters (1.1 mm diam, model 381434, Becton-Dickinson) and Teflon catheters (1.2 mm diam, model 3055, Critikon) were employed. The tips of the catheters were plugged with sterile high vacuum grease to prevent media and dye from entering the lumen. Catheters were inoculated and treated as described above for polystyrene rods. Precoating of catheters with dispersin B was carried
out in PBS or in sodium phosphate buffer (pH 9) for 10 minutes to 24 hours. In some cases, coated catheters were air dried for 24 hours before use. Teflon catheters were stained with crystal violet as previously described (Kaplan, J. B., and Fine, D. H. Appl. Environ. Microbiol. 2002 68:4943-4950). Polyurethane catheters were stained with 1% methylene blue in water for 2 minutes.
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated nucleic acid comprising a polynucleotide that is amplifiable by polymerase chain reaction with a forward primer of SEQ ID NO:12 and a reverse primer of SEQ ID NO:13 and said polynucleotide encodes a polypeptide that cleaves β-substituted N-acetyl glucosaminide; or complement thereof.

2. The isolated nucleic acid of claim 1, wherein the polynucleotide has at least 90% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1.

3. The isolated nucleic acid of claim 1 or 2 comprising a nucleic acid of nucleotides 61 to 1143 of SEQ ID NO:1.

4. The isolated nucleic acid of claim 1, wherein the polynucleotide has at least 90% sequence identity to SEQ ID NO:3.

5. The isolated nucleic acid of claim 1, wherein the polynucleotide has at least 90% sequence identity to SEQ ID NO:5.

6. The isolated nucleic acid of claim 1, wherein the polynucleotide has at least 90% sequence identity to SEQ ID NO:7.

7. The isolated nucleic acid of claim 1, wherein the polynucleotide has at least 90% sequence identity to SEQ ID NO:9.

8. A nucleic acid encoding a fusion polypeptide comprising the isolated nucleic acid according to any one of claims 1 to 7 and a second nucleic acid encoding a second polypeptide.

9. The nucleic acid of claim 8, wherein the second nucleic acid encodes an antibody.
10. A vector comprising the nucleic acid sequence according to any one of claims 1 to 9.

11. A host cell comprising the vector of claim 10.

12. A method of producing a recombinant polypeptide comprising culturing the host cell of claim 11.

13. An isolated polypeptide comprising a polypeptide encoded by the polynucleotide according to any one of claims 1 to 9.

14. The isolated polypeptide of claim 13, wherein the polypeptide is encoded by a nucleic acid of at least 90% sequence identity to nucleotides 61 to 1143 of SEQ ID NO:1.

15. The isolated polypeptide of claim 13 or 14, wherein the polypeptide comprises amino acids 21 to 381 of SEQ ID NO:2.

16. The isolated polypeptide of claim 13, wherein the polypeptide is encoded by a nucleic acid of at least 90% sequence identity to SEQ ID NO:3.

17. The isolated polypeptide of claim 16, wherein the polypeptide comprises SEQ ID NO:4.

18. The isolated polypeptide of claim 16, wherein the polypeptide is encoded by a nucleic acid of at least 90% sequence identity to SEQ ID NO:5.

19. The isolated polypeptide of claim 18, wherein the polypeptide comprises SEQ ID NO:6.

20. The isolated polypeptide of claim 13, wherein the polypeptide is encoded by a nucleic acid of at least 90% sequence identity to SEQ ID NO:7.
21. The isolated polypeptide of claim 20, wherein the polypeptide comprises SEQ ID NO:8.

22. The isolated polypeptide of claim 13, wherein the polypeptide is encoded by a nucleic acid of at least 90% sequence identity to SEQ ID NO:9.

23. The isolated polypeptide of claim 22, wherein the polypeptide comprises SEQ ID NO:10.

24. A composition comprising:
   a) the polypeptide according to any one of claims 13 to 23; and
   b) an antibiotic.

25. A composition comprising:
   (a) the polypeptide according to any one of claims 13 to 23; and
   (b) an antimicrobial or antifungal.

26. A fusion protein comprising the polypeptide according to any one of claims 13 to 23 and a second polypeptide.

27. A pharmaceutical composition comprising:
   a) the polypeptide according to any one of claims 13 to 26; and
   b) a pharmaceutically acceptable carrier.

28. The pharmaceutical composition of claim 27, wherein the composition is formulated for oral administration.

29. The pharmaceutical composition of claim 27 or 28, wherein the composition is a lozenge or a mouth wash.

30. The pharmaceutical composition of claim 27, wherein the composition is formulated for topical administration.
31. The pharmaceutical composition of claim 30, wherein the composition is an ointment, cream, or lotion.

32. The pharmaceutical composition of claim 27, wherein the composition is formulated for intra-nasal administration.

33. Use of the pharmaceutical composition according to any one of claims 24 to 32 to inhibit a biofilm associated with bacterial infection.

34. A medical device coated with the polypeptide according to any one of claims 13 to 23 and 26.

35. The medical device of claim 34, wherein the medical device is an indwelling device.

36. The medical device of claim 34 or 35, wherein the medical device is selected from the group consisting of a central venous catheter, an intravascular catheter, an urinary catheter, a Hickman catheter, a peritoneal dialysis catheter, an endotracheal catheter, a mechanical heart valve, a cardiac pacemaker, an arteriovenous shunt, a schleral buckle, a prosthetic joint, a tympanostomy tube, a tracheostomy tube, a voice prosthetic, a penile prosthetic, an artificial urinary sphincter, a synthetic pubovaginal sling, a surgical suture, a bone anchor, a bone screw, an intraocular lens, a contact lens, an intrauterine device, an aortofemoral graft, and a vascular graft.

37. A wound dressing impregnated with the polypeptide according to any one of claims 13 to 23 and 26.

38. The wound dressing of claim 37, wherein the wound dressing is a sponge, gauze, or catheter shield.

39. A transdermal patch comprising the polypeptide according to any one of claims 13 to 23 and 26.
40. A method of inhibiting infection on a medical device or surgical instrument by bacteria or fungi comprising contacting the medical device or surgical instrument with the polypeptide according to any one of claims 13 to 23 and 26.

41. A method of inhibiting infection on a medical device or surgical instrument by bacteria or fungi comprising coating the medical device or surgical instrument with the polypeptide according to any one of claims 13 to 23 and 26.

42. The method of claim 40, wherein the medical device is a catheter and the polypeptide is in a catheter lock solution in the catheter.

43. Use of the polypeptide according to any one of claims 13 to 23 and 26 in the manufacture of a medicament to inhibit or treat bacterial and/or fungal infections.

44. A method of inhibiting infection on a wound dressing by bacteria or fungi comprising impregnating the wound dressing the polypeptide according to any one of claims 13 to 23 and 26.

45. An isolated nucleic acid according to any one of claims 1 - 9, a vector according to claim 10, a host cell according to claim 11, a polypeptide according to any one of claims 13 - 23, a composition according to any one of claims 24, 25, 27 - 32, a fusion protein according to claim 26, a medical device according to any one of claims 34 - 36, a method according to any one of claims 12, 40 - 42 and 44, a use according to claims 33 or 43, a wound dressing according to claim 37 and 38, a transdermal patch according to claim 39, substantially as hereinbefore described with reference to Figure 1 or the Examples, excluding comparative Examples.
FIGURE 1

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<110> University of Medicine and Dentistry of New Jersey  
Kaplan, Jeffrey B.

<120> Compositions and Methods for Enzymatic Detachment of Bacterial  
and Fungal Biofilms

<130> UMD-0015

<150> US 60/435,817  
<151> 2002-12-20

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agccagaattc atatgaaggg catctttgtat tttaaaacc ttaagcaggg taaggagtat 240
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35 40 45
Lys Glu Arg Asn Ile Glu Ile Val Pro Glu Val Asp Ser Pro Asn His
50 55 60
Met Thr Ala Ile Phe Asp Leu Leu Thr Leu Lys His Gly Lys Glu Tyr
65 70 75 80
Val Lys Gly Leu Lys Ser Pro Tyr Leu Ala Glu Glu Ile Asp Ile Asn
85 90 95
Asn Pro Glu Ala Val Glu Ile Ile Lys Thr Leu Ile Gly Glu Val Ile
100 105 110
Tyr Ile Phe Gly His Ser Ser Arg His Phe His Ile Gly Gly Asp Glu
115 120 125
Phe Ser Tyr Ala Val Glu Asn Asn His Glu Phe Ile Arg Tyr Val Asn
130 135 140
Thr Leu Asn Asp Phe Ile Asn Asn Lys Gly Leu Ile Thr Arg Ile Trp
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gattacggag aacatcgatctt aaacgggtgc aaaaagcag agggatcaag 240
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35 40 45

Ala Lys Ala Lys Gly Ile Glu Leu Ile Pro Glu Leu Asp Ser Pro Asn
50 55 60

His Met Thr Ala Ile Phe Lys Leu Val Gln Lys Asp Arg Gly Ile Lys
65 70 75 80

Tyr Leu Gln Gly Leu Lys Ser Arg Gln Val Asp Asp Glu Ile Asp Ile
85 90 95
Thr Asn Ala Asp Ser Ile Ala Phe Met Glu Ser Leu Met Ser Glu Val
100 105 110

Ile Asp Ile Phe Gly Asp Thr Ser Glu His Phe His Ile Gly Gly Asp
115 120 125

Glu Phe Gly Tyr Ser Val Glu Ser Asn His Glu Phe Ile Thr Tyr Ala
130 135 140

Asn Lys Leu Ser Tyr Phe Leu Glu Lys Lys Leu Lys Thr Arg Met
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Trp Asn Asp Gly Leu Ile Lys Ser Thr Phe Glu Gln Ile Asn Pro Asn
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35 40 45

Ala Lys Leu Lys Gly Ile Glu Leu Ile Pro Glu Leu Asp Ser Pro Asn
50 55 60

His Met Thr Ala Ile Phe Thr Leu Leu Lys Glu Lys Gly Lys Asn
65 70 75 80

Tyr Leu Gln Ser Leu Lys Ser Ser Pro Gln Asp Glu Glu Ile Ser Ile
85 90 95

Thr Asn Pro Asp Ser Ile Ala Phe Met Gln Ser Leu Leu Thr Glu Val
100 105 110

Ile His Thr Phe Gly Asp Ser Thr Lys His Phe His Ile Gly Gly Asp
115 120 125

Glu Phe Gly Tyr Asp Glu Asn Ser Asn His Glu Phe Ile Thr Tyr Ala
130 135 140

Asn Lys Leu Ala Asp Phe Leu Arg Glu Lys Gly Leu Lys Thr Arg Ile
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Trp Asn Asp Gly Leu Ile Lys Asn Thr Ile Asp Gln Leu Asn Pro Asn
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Ile Glu Ile Thr Tyr Trp Ser Tyr Asp Gly
180 185

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Lys Glu Arg Asn Ile Glu Ile Val Pro Glu Val Asp Ser Pro Asn His
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Met Thr Ala Ile Phe Asp Leu Leu Thr Leu Lys His Gly Lys Glu Tyr
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Val Lys Gly Leu Lys Ser Pro Tyr Ile Ala Glu Ile Asp Ile Asn
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Asn Pro Glu Ala Val Glu Val Ile Lys Thr Leu Ile Gly Glu Val Ile
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Tyr Ile Phe Gly His Ser Ser Arg His Phe His Ile Gly Gly Asp Glu
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Phe Ser Tyr Ala Val Glu Asn His Glu Phe Ile Arg Tyr Val Asn
130 135 140

Thr Leu Asn Asp Phe Ile Asn Ser Lys Gly Leu Ile Thr Arg Val Trp
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