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(54) Title: NOVEL NUCLEASE FROM HAEMOPHILUS INFLUENZAE DEMONSTRATING THERMOSTABILITY AND USES THEREOF IN INDUSTRY AND SCIENTIFIC RESEARCH

(57) Abstract: Disclosed is a novel polypeptide of *Haemophilus influenzae*. The disclosed polypeptide exhibits nuclease activity that is significantly higher than currently known nucleases. The disclosed polypeptide also exhibits thermostability in regard to its nuclease activity.



**NOVEL NUCLEASE FROM HAEMOPHILUS INFLUENZAE
DEMONSTRATING THERMOSTABILITY AND USES THEREOF IN
INDUSTRY AND SCIENTIFIC RESEARCH**

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT**

[0001] This invention was made with government support under grant number AI024616 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0002] The present application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62,120,655, filed on February 25, 2015, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND

[0003] The field of the invention relates to nucleases. In particular, the field of the invention relates to DNA nucleases isolated from bacteria such as *Haemophilus influenzae*, which exhibit thermostability.

[0004] A nuclease is an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of a polynucleotide such as DNA and/or RNA and release nucleotides from the polynucleotide (*e.g.*, guanosine monophosphate (GMP) or adenosine monophosphate (AMP)). Nuclease activity may be exploiting for a number of potential industrial uses including production of nucleotide flavor enhancers (*e.g.*, GMP or inosine monophosphate (IMP), which is produced by deaminating AMP), biomedical research, and digestion of DNA in pulmonary secretions (*e.g.*, such as in cystic fibrosis).

[0005] Recently, two new flavor enhancers have been discovered that are composed of nucleotides – guanosine 5'-phosphate (GMP) and inosine 5'-monophosphate disodium salt

(IMP). These flavonoids heighten the the sensitivity of the umami (*i.e.*, savory) flavor in foods containing monosodium glutamate (MSG).

[0006] Cystic Fibrosis is an autosomal recessive genetic disorder that primarily affects the lungs. It is caused by a genetic mutation in the gene for cystic fibrosis transmembrane conductance regulator (CFTR) protein. This protein is required to regulate sweat, digestive fluids, and mucus. Cystic fibrosis often leads to lung disease in which the lungs produce a thick, viscous fluid often leading to lung infections. These lung infections lead to the accumulation of leukocytes. Leukocytes release extracellular DNA in large quantities which amount to approximately 10.2% of the dry weight of secretions. The large size of DNA causes is a primary reason for the viscosity of mucus in cystic fibrosis. (*See* Chernick, Warren S., and Giulio J. Barbero. "Composition of tracheobronchial secretions in cystic fibrosis of the pancreas and bronchiectasis." *Pediatrics* 24.5 (1959): 739-745, which is incorporated herein by reference in its entirety). One drug (*i.e.*, Pulmozyme®) treats the viscosity of mucus in cystic fibrosis through the use of recombinant human DNase – which cleaves DNA in mucus, decreasing the viscosity of mucus and promoting clearance of the mucus from lungs.

[0007] Many industrial and medical applications require the degradation of nucleic acid. Current nucleases accomplish this act rather slowly, limiting production and/or the efficiency of potential medical treatments. Also, many current nucleases do not exhibit prolonged stability at ambient or elevated temperatures. Therefore, new nucleases that exhibit relative high nuclease activity and thermostability are desired.

SUMMARY

[0008] Disclosed is a novel polypeptide from *Haemophilus influenza* and uses thereof in industry, medicine, and science. The disclosed polypeptide exhibits nuclease activity and is several orders of magnitude (22-1400 ×) more effective than current nucleases used in the production of nucleotide substrates. The disclosed nuclease also exhibits thermostability.

[0009] In one embodiment, the disclosed polypeptide comprises the amino acid sequence of SEQ ID NO:1 or comprises an amino sequence having at least about 80% sequence identity to SEQ ID NO:1 (or having at least about 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:1), where the polypeptide has nuclease activity and is thermostable. The isolated polypeptide typically has DNase activity and preferably the isolated polypeptide is thermostable, for example, where the polypeptide is thermostable at 55°C and does not lose more than about 50% of nuclease activity (or no more than 40%, 30%, 20% 10% of nuclease activity) when heated to 55°C for 10 minutes. Preferably, the peptide does not lose more than about 50% of nuclease activity (or no more than 40%, 30%, 20% 10% of nuclease activity) when stored at 4 °C for at least six months. Also disclosed, are enzyme compositions and reaction mixtures comprising the disclosed polypeptides.

[0010] Also disclosed are isolated polynucleotides encoding the disclosed polypeptides having nuclease activity. The isolated polynucleotides may comprise the nucleotide sequence of SEQ ID NO:2 or may comprise a nucleotide sequence having at least about 80% sequence identity to SEQ ID NO:2 (or having at least about 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:2), where the encoded polypeptide has nuclease activity and is thermostable.

[0011] Also disclosed are vectors comprising the isolated polynucleotides. In some embodiments, the vectors comprise a promoter operably linked to the isolated polynucleotide. The disclosed vectors may be utilized to transform a host cell. As such, also disclosed are recombinant host cells comprising the disclosed vectors. Also disclosed are methods for preparing the isolated polypeptides. The disclosed methods may include culturing a recombinant host cell comprising the vector that expresses the polypeptide of and isolating the polypeptide thus expressed.

[0012] Also disclosed are pharmaceutical compositions comprising the isolated polypeptide having nuclease activity and a pharmaceutical carrier. The disclosed pharmaceutical compositions may be administered in methods for treating a patient having a

disease or disorder characterized by secretions comprising nucleic acid, where the method comprises administering the pharmaceutical composition comprising the polypeptide having nuclease activity to the patient. Suitable patients may include patients having cystic fibrosis and suitable routes of administration for the pharmaceutical composition may include pulmonary routes.

[0013] Also disclosed are methods for performing enzymatic hydrolysis of DNA. The disclosed methods may be utilized to prepare flavor enhancers such as guanosine monophosphate (GMP) and inosine monophosphate (IMP). In the methods for preparing GMP, the methods may include treating DNA with the isolated polypeptide having nuclease activity to prepare GMP. In the methods for preparing IMP, the methods may include treating DNA with the isolated polypeptide having nuclease activity to prepare AMP and deaminating the AMP to prepare IMP.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Figure 1 provides the amino acid sequence of isolated polypeptide from *Haemophilus influenzae* (SEQ ID NO:1) and the nucleotide sequence of gene encoding the isolated polypeptide from *Haemophilus influenzae* (SEQ ID NO:2).

[0015] Figure 2 illustrates that the *Haemophilus influenza* (NTHi) nuclease is active when bound to a solid phase substrate.

[0016] Figure 3 provides a comparison of *Pasteurellaceae* thermonuclease with NTHi nuclease and other nucleases.

[0017] Figure 4 provides results of light scattering, demonstrating effect of pH on size of NTHi nuclease.

[0018] Figure 5 Genomic arrangement and sequence of the NTHi *nuc* in the *H. influenzae* 2019 genome. The boldface letters indicate the 13-amino-acid signal sequence. The homologous sequence in the *H. influenzae* RD KW20 genome is HI1296.

[0019] Figure 6 weight markers; lane 2, herring sperm DNA alone; lane 3, DNase I (1 U) and herring sperm DNA; lane 4, Nuc (1 μ g) and herring sperm DNA. (B) Results of digestion of 25 mM FRET-labeled nucleotide at 25°C with 0.035 nM nuclease (●) or 6.00 nM DNase I (*). The differences in the velocity of the enzymes can be readily seen. The graph also demonstrates the loss of nuclease activity in the presence of 4 mM EDTA (○) due to chelation of the divalent cations Mg^{2+} and Ca^{2+} .

[0020] Figure 7 Evidence that the nuclease is the factor responsible for dispersal of NTHI biofilms. (A) “Comet tails” caused by the release of organisms from NTHI 2019 microcolonies/nascent biofilms as organisms transition from the biofilm to planktonic phase over a 24-h period. (B) Study performed on 2019 Δ *nuc*, which had no evidence of microcolony formation or dispersal of organisms. (C) Partial complementation in *cis* of 2019 Δ *nuc* because the expression of *nuc* is unregulated in the complemented strain. Small microcolonies were seen with the comet tail configurations similar to those seen in panel A.

[0021] Figure 8 A 50-image stacked z-series at $\times 20$ magnification of 24-h biofilms grown in continuous flow chambers. The samples were stained with propidium iodide (red) and MAb 6E4 (green) prior to visualization. At 24 h, the NTHI 2019 Δ *nuc* biofilm contains increased amounts of eDNA and large aggregates of organisms (B). This compares to lesser amounts of eDNA and diffuse arrangement of organisms within the biofilm of NTHI 2019 (A) and NTHI 2019 Δ *nuc*::*nuc* (C).

[0022] Figure 9 Cryo sections through 24-h biofilms from NTHI 2019 wild type (A), NTHI 2019 Δ *nuc* (B), and NTHI 2019 Δ *nuc*::*nuc* (C). The DNA matrix is stained with DAPI, and the NTHI strains are stained with MAb 6E4. Scale bar, 20 μ m. The organisms in the parent strain and in the complemented mutant are clearly dispersed throughout the biofilm, whereas they are clustered in the Δ *nuc* mutant.

[0023] Figure 10 Lateral views and stacked z-series of 48-h biofilms stained with Live/Dead stain (green-red) and DRAQ-5 (blue). (A and C) Biofilm images of NTHI 2019 demonstrating a predominance of live organisms (green), while the images of NTHI

2019 Δ *nuc* biofilm (B and D) demonstrate that the majority of organisms were dead (red) by 48 h.

[0024] Figure 11 Confocal microscopy analysis of 10- μ m-thick sections of biofilm formation at day 5 in the chinchilla middle ear after infection with NTHI 2019, NTHI 2019 Δ *nuc*, and NTHI 2019 Δ *nuc::nuc*. The NTHI are stained with MAb 6E4 (green), and DNA is stained with DRAQ5 (blue). There is an aggregation of organisms staining with MAb 6E4 in NTHI 2019 Δ *nuc* (B) compared to the more diffuse display of organisms in the wild type and the complemented mutant (A and C).

[0025] Figure 12 Results of cultural studies at day 4 of quantitative nasopharyngeal cultures (panel A), middle ear taps at day 4 (panel B), and biofilm/mucosa cultures (panel C). The black bar shows results of wildtype infection, the hatched bar shows the results of *nuc* infection, and the grey bar shows infection due to the complemented mutant. Using ANOVA analysis, none of the cultures showed significant differences.

DETAILED DESCRIPTION

[0026] The present invention is described herein using several definitions, as set forth below and throughout the application.

[0027] Unless otherwise specified or indicated by context, the terms “a”, “an”, and “the” mean “one or more.” For example, “a polypeptide” should be interpreted to mean “one or more polypeptides.”

[0028] As used herein, “about,” “approximately,” “substantially,” and “significantly” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of these terms which are not clear to persons of ordinary skill in the art given the context in which they are used, “about” and “approximately” will mean plus or minus $\leq 10\%$ of the particular term and “substantially” and “significantly” will mean plus or minus $>10\%$ of the particular term.

[0029] As used herein, the terms “include” and “including” have the same meaning as the terms “comprise” and “comprising.” The terms “comprise” and “comprising” should be interpreted as being “open” transitional terms that permit the inclusion of additional components further to those components recited in the claims. The terms “consist” and “consisting of” should be interpreted as being “closed” transitional terms that do not permit the inclusion of additional components other than the components recited in the claims. The term “consisting essentially of” should be interpreted to be partially closed and allowing the inclusion only of additional components that do not fundamentally alter the nature of the claimed subject matter.

[0030] As used herein, a “patient” may be interchangeable with “subject” or “individual” and means an animal, which may be a human or non-human animal, in need of treatment. Non-human animals may include dogs, cats, horses, cows, pigs, sheep, and the like. A “patient in need thereof” may include a patient having a disease, disorder, or condition that is associated with DNA-containing secretions (*e.g.*, cystic fibrosis).

[0031] Disclosed herein are polypeptides from *Haemophilus influenza* or mutants or variants thereof that exhibit nuclease activity. An exemplary polypeptide may comprises the amino acid sequence of SEQ ID NO:1, or may comprises an amino acid sequence having at least about 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:1. Mutant or variant may include mutants having one or more amino acid substitutions, deletions, additions and/or amino acid insertions, where preferably the mutant or variant maintains nuclease activity. Also disclosed are nucleic acid molecules that encode the disclosed polypeptide (*e.g.*, polynucleotides that encode the polypeptide of SEQ ID NO:1 or mutants or variants thereof). For example, contemplated are polynucleotides (*e.g.*, DNA or RNA) comprising the nucleotide sequence of SEQ ID NO:2 or mutants or variants thereof, for example polynucleotides having at least about 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:2).

[0032] The disclosed polypeptides from *Haemophilus influenza* or variant or mutants thereof preferably exhibit nuclease activity, which may include DNase and/or RNase activity on double-stranded and/or single-stranded DNA and/or RNA. The nuclease activity of the polypeptide, which may be assessed by methods known in the art, preferably is greater than known nucleases (*e.g.*, by at least 5-fold greater, 10-fold greater, 20-fold greater, 100-fold greater, 1000-fold greater, or more).

[0033] Also contemplated are bacterial, plant, fungal, insect, or animal host cell expression vectors that express the disclosed polypeptides or variants or mutants thereof. Vectors may be used to transform appropriate host cells (*e.g.*, *E. coli*). The transformed host cell may be cultivated or fermented such that the polypeptide is expressed constitutively or after adding a reagent that induces expression (*e.g.*, via an inducible promoter). Expression vectors as contemplated herein may include control sequences that modulate expression of the encoded polypeptide. Expression control sequences may include constitutive or inducible promoters (*e.g.*, T3, T7, Lac, trp, or phoA), ribosome binding sites, or transcription terminators.

[0034] The vectors disclosed herein may be utilized to transform host cells. Suitable host cells include bacterial, plant, fungal, insect, or animal host cell. Suitable bacteria include, but are not limited to: Gram-negative bacteria such as *Escherichia* species (*e.g.*, *E. coli*), other Gram-negative bacteria, (*e.g.*, *Pseudomonas* sp., such as *Pseudomonas aeruginosa*, or *Caulobacter* sp., such as *Caulobacter crescentus*), or Gram-positive bacteria (*e.g.*, *Bacillus* sp., in particular *Bacillus subtilis*).

[0035] Also disclosed are methods for expressing, preparing, isolating, separating, or purifying the disclosed polypeptides or variants or mutants thereof. In some embodiments, the methods may be utilized to produce the polypeptides as disclosed herein. The steps of the methods may include: (i) cultivating or fermenting a transformed host cell (*e.g.*, a bacterial host cell as contemplated herein) which comprises an expression vector (as contemplated herein) which in turn comprises a nucleic acid molecule encoding the disclosed polypeptides

or variants or mutants thereof (as contemplated herein), wherein cultivation occurs under conditions which cause expression of the polypeptides; and (ii) isolating, separating, or purifying the polypeptide. The transformed bacteria may be cultivated or fermented using methods known in the art in order to express the polypeptide. An exemplary isolation, separation, or purification method may include one or more of the following steps: a cell disruption step, a clarification step (*e.g.*, via centrifugation or filtration), a chromatographic separation step, a dialysis step, and a precipitation step.

[0036] The terms “nucleic acid” and “nucleic acid sequence” refer to a nucleotide, oligonucleotide, polynucleotide (which terms may be used interchangeably), or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand).

[0037] The terms “amino acid” and “amino acid sequence” refer to an oligopeptide, peptide, polypeptide, or protein sequence (which terms may be used interchangeably), or a fragment of any of these, and to naturally occurring or synthetic molecules. Where “amino acid sequence” is recited to refer to a sequence of a naturally occurring protein molecule, “amino acid sequence” and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

[0038] The amino acid sequences contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, a variant, mutant, or derivative polypeptide may include conservative amino acid substitutions relative to a reference polypeptide. “Conservative amino acid substitutions” are those substitutions that are predicted to interfere least with the properties of the reference polypeptide. In other words, conservative amino acid substitutions substantially conserve the structure and the function of the reference protein. The following table provides a list of exemplary conservative amino acid substitutions.

Original Residue	Conservative Substitution
Ala	Gly, Ser
Arg	His, Lys
Asn	Asp, Gln, His
Asp	Asn, Glu
Cys	Ala, Ser
Gln	Asn, Glu, His
Glu	Asp, Gln, His
Gly	Ala
His	Asn, Arg, Gln, Glu
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	His, Met, Leu, Trp, Tyr
Ser	Cys, Thr
Thr	Ser, Val
Trp	Phe, Tyr
Tyr	His, Phe, Trp
Val	Ile, Leu, Thr

Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

[0039] A “deletion” refers to a change in the amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides. A deletion removes at least 1, 2, 3, 4, 5, 10, 20, 50, 100, or 200 amino acids residues or nucleotides. A deletion may include an internal deletion or a terminal deletion (*e.g.*, an N-terminal truncation or a C-terminal truncation of a reference polypeptide or a 5'-terminal or 3'-terminal truncation of a reference polynucleotide).

[0040] A “fragment” is a portion of an amino acid sequence or a polynucleotide which is identical in sequence to but shorter in length than a reference sequence. A fragment may comprise up to the entire length of the reference sequence, minus at least one nucleotide/amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous nucleotides or contiguous amino acid residues of a reference polynucleotide or reference polypeptide, respectively. In some embodiments, a fragment may comprise at least 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, 250, or 500 contiguous nucleotides or contiguous amino acid residues of a reference polynucleotide or reference polypeptide, respectively. Fragments may be preferentially selected from certain regions of a molecule. The term “at least a fragment” encompasses the full length polynucleotide or full length polypeptide.

[0041] A “full length” polynucleotide sequence is one containing at least a translation initiation codon (*e.g.*, methionine) followed by an open reading frame and a translation termination codon. A “full length” polynucleotide sequence encodes a “full length” polypeptide sequence.

[0042] “Homology” refers to sequence similarity or, interchangeably, sequence identity, between two or more polynucleotide sequences or two or more polypeptide sequences. Homology, sequence similarity, and percentage sequence identity may be determined using methods in the art and described herein.

[0043] The terms “percent identity” and “% identity,” as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences. Percent identity for a nucleic acid sequence may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely

available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including “blastn,” that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called “BLAST 2 Sequences” that is used for direct pairwise comparison of two nucleotide sequences. “BLAST 2 Sequences” can be accessed and used interactively at the NCBI website. The “BLAST 2 Sequences” tool can be used for both blastn and blastp (discussed below).

[0044] Percent identity may be measured over the length of an entire defined polynucleotide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures, or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0045] A “variant,” “mutant,” or “derivative” of a particular nucleic acid sequence may be defined as a nucleic acid sequence having at least 50% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the “BLAST 2 Sequences” tool available at the National Center for Biotechnology Information’s website. (See Tatiana A. Tatusova, Thomas L. Madden (1999), “Blast 2 sequences - a new tool for comparing protein and nucleotide sequences”, FEMS Microbiol Lett. 174:247-250). Such a pair of nucleic acids may show, for example, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length.

[0046] Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

[0047] The phrases “percent identity” and “% identity,” as applied to polypeptide sequences, refer to the percentage of residue matches between at least two polypeptide sequences aligned using a standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail above, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide. Percent identity for amino acid sequences may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including “blastp,” that is used to align a known amino acid sequence with other amino acids sequences from a variety of databases.

[0048] Percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0049] A “variant,” “mutant,” or “derivative” of a particular polypeptide sequence is defined as a polypeptide sequence having at least 50% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the “BLAST 2 Sequences” tool available at the National Center for Biotechnology Information’s website. (See Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250). Such a pair of polypeptides may show, for example, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length of one of the polypeptides. A “variant” or a “derivative” may have substantially the same functional activity as a reference polypeptide. For example, a variant or derivative of a cysteine protease may have cysteine protease activity (*e.g.*, autoproteolytic cysteine protease activity).

[0050] The disclosed polypeptides may be modified so as to comprise an amino acid sequence or modified amino acids, such that the disclosed polypeptides cannot be said to be naturally occurring. In some embodiments, the disclosed polypeptides are modified and the modification is selected from the group consisting of acylation, acetylation, formylation, lipoylation, myristoylation, palmitoylation, alkylation, isoprenylation, prenylation, and amidation. An amino acid in the disclosed polypeptides may be thusly modified, but in particular, the modifications may be present at the N-terminus and/or C-terminus of the polypeptides (*e.g.*, N-terminal acylation or acetylation, and/or C-terminal amidation). The modifications may enhance the stability of the polypeptides and/or make the polypeptides resistant to proteolysis.

[0051] The words “insertion” and “addition” refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively. An insertion or addition may refer to 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, or 200 amino acid residues or nucleotides.

[0052] “Operably linked” refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with a second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame.

[0053] A “recombinant nucleic acid” is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques such as those described in Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1 3, Cold Spring Harbor Press, Plainview N.Y. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

[0054] “Substantially isolated or purified” nucleic acid or amino acid sequences are contemplated herein. The term “substantially isolated or purified” refers to nucleic acid or amino acid sequences that are removed from their natural environment, and are at least 60% free, preferably at least 75% free, and more preferably at least 90% free, even more preferably at least 95% free from other components with which they are naturally associated.

[0055] “Transformation” describes a process by which exogenous DNA is introduced into a recipient cell. Transformation may occur under natural or artificial conditions according to various methods well known in the art, and may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method for transformation is selected based on the type of host cell being transformed and

may include, but is not limited to, bacteriophage or viral infection, electroporation, heat shock, lipofection, and particle bombardment. The term “transformed cells” includes stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transformed cells which express the inserted DNA or RNA for limited periods of time.

[0056] A “composition comprising a given amino acid sequence” and a “composition comprising a given polynucleotide sequence” refer broadly to any composition containing the given polynucleotide or amino acid sequence. The composition may comprise a dry formulation or an aqueous solution. The compositions may be stored in any suitable form including, but not limited to, freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. The compositions may be aqueous solution containing salts (*e.g.*, NaCl), detergents (*e.g.*, sodium dodecyl sulfate; SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, and the like).

[0057] Novel Nuclease from *Haemophilus Influenzae*

[0058] Disclosed are isolated polypeptides comprising the amino acid sequence of SEQ ID NO:1 or comprising an amino sequence having at least about 80% sequence identity to SEQ ID NO:1 (*e.g.*, having at least about 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:1). The disclosed polypeptides typically have nuclease activity, and DNase activity in particular. In some embodiments, the disclosed polypeptides have DNase activity and RNase activity. In other embodiments, the disclosed polypeptides have DNase activity but do not have RNase activity. The disclosed polypeptides may exhibit a relatively high specific activity in regard to nuclease activity (*e.g.*, in regarding to DNase activity, RNase activity, or both). In some embodiments, the disclosed polypeptides have a specific activity of at least about 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 units/μg.

[0059] The disclosed polypeptides typically maintain nuclease activity after being exposed to elevated temperatures and may be characterized as thermostable. In some embodiments, the disclosed polypeptides may be exposed to an elevated temperature of

greater than about 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C, for at least about 10, 20, 30, 40, 50, or 60 minutes, and the disclosed polypeptides will retain at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of their nuclease activity prior to exposure to the elevated temperature. For example, the disclosed polypeptides may exhibit thermostability at a temperature of 55°C and may not lose more than about 50% of nuclease activity when heated to 55°C for 10 minutes. Preferably, the isolated polypeptide does not lose more than about 50% of nuclease activity when stored at 4°C for at least six months in a suitable storage buffer. The nuclease activity of the disclosed polypeptides may be measured as “units.” A unit of nuclease activity may be defined as the amount of the polypeptide which will completely degrade 1 µg of DNA in X minutes (for example, where X = 10 minutes) at temperature Y (for example, where Y = 37°C) in a suitable reaction buffer.

[0060] The isolated polypeptide may comprise heterologous amino acid sequences. For example, the isolated peptide may comprise one or more heterologous amino acid sequences fused to the amino acid sequence of SEQ ID NO:1 or fused to an amino sequence having at least about 80% sequence identity to SEQ ID NO:1. As would be understood in the art, a “heterologous” amino acid sequence is an amino acid sequence to which the isolated polypeptide is not fused in the context of *Haemophilus influenzae* and/or an amino acid sequence that does not naturally occur in *Haemophilus influenzae*.

[0061] In some embodiments, the heterologous amino acid sequences are fused at the N-terminus and/or the C-terminus of the amino acid sequence of the disclosed polypeptides. Heterologous amino acid sequences may include protein tags, for example, protein tags selected from the group consisting of affinity tags, solubilization tags, chromatography tags, epitope tags, and fluorescence tags. Affinity tags may include, but are not limited to chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), and poly(His) tag. Solubilization tags may include, but are not limited to thioredoxin (TRX and poly (NANP). Chromatography tags may include, but are not limited to polyanionic amino acids such as FLAG-tag. Epitope tags are short peptide sequence which form an epitope for a high-affinity antibody. Suitable epitopes may include, but are not limited to V5-tag, Myc-tag,

and HA-tag. Fluorescence tags may include, but are not limited to green fluorescence protein (GFP) and its derivatives.

[0062] The heterologous amino acid sequences may be fused directly to the amino acid sequence of the disclosed polypeptides. Alternatively, the heterologous amino acid sequences may be fused to the amino acid sequence of the disclosed polypeptides via a peptide linker. For example, a peptide linker may be utilized where the peptide linker comprises a recognition sequence for a protease and can be cleaved by the protease in order to separate the disclosed polypeptides from the heterologous amino acid sequences fused via the peptide linker to the disclosed polypeptides (*e.g.*, N-polypeptide → cleavable linker → heterologous peptide tag-C; or N-heterologous peptide tag → cleavable linker → polypeptide-C).

[0063] Also disclosed are enzyme compositions. The enzyme compositions typically exhibit nuclease activity and DNase activity in particular. The enzyme compositions may include (a) the isolated polypeptides as disclosed herein; and (b) a storage buffer. The isolated polypeptides may be present at any suitable unit concentration in the enzyme compositions, for example, with respect to DNase activity. In some embodiments, the enzyme compositions comprise the disclosed polypeptides at a unit concentration of at least about 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 units/μl in the enzyme composition. The storage buffer optionally comprises a pH buffering system (*e.g.*, Tris-HCl) providing a pH of about 6.0 – 9.0, preferably, 6.5 – 8.5, more preferably 7.0 – 8.0. The storage buffer may include a stabilizing agent such as glycerol. For example, the storage buffer may include glycerol at a concentration of at least about 5, 10, 20, 30, 40, or 50%, preferably 30-70%, more preferably 40-60%.

[0064] Also disclosed are reaction mixtures, for example, reaction mixtures for performing nucleic acid hydrolysis (*e.g.*, DNA hydrolysis, RNA hydrolysis, or both). The reaction mixtures may include: (a) the isolated polypeptides as disclosed herein; (b) heterologous nucleic acid such as DNA and/or RNA (*e.g.*, nucleic acid that is not naturally

present in *Haemophilus influenzae*); and (c) an aqueous solution wherein the isolated polypeptide hydrolyzes the heterologous nucleic acid. The isolated polypeptides may be present at a suitable concentration in the reaction mixtures (*e.g.*, at a concentration of at least about 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, or 1.0 units/ μ l in the reaction mixtures). The reaction mixture optionally comprises a pH buffering system (*e.g.*, Tris-HCl) providing a pH of about 6.0 – 9.0, preferably, 6.5 – 8.5, more preferably 7.0 – 8.0. The reaction mixture optionally comprises a divalent cation, such as Ca^{2+} and/or Mg^{2+} , present at a suitable concentration, such as at a concentration of at least about 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 mM (preferably within a range of about 0.5 – 5.0 mM).

[0065] Also disclosed herein are isolated polynucleotides encoding the isolated polypeptides disclosed herein. In some embodiments, the disclosed polynucleotides comprise the nucleotide sequence of SEQ ID NO:2 or comprise a nucleotide sequence having at least about 80% sequence identity to SEQ ID NO:2, wherein the encoded polypeptide preferably has nuclease activity and preferably exhibits thermostability.

[0066] Also disclosed are vectors comprising the disclosed isolated polynucleotides. The vectors typically are heterologous vectors (*e.g.*, vectors that do not naturally exist in *Haemophilus influenzae*), such as bacterial vectors (*e.g.*, plasmids) or yeast vectors. The disclosed vectors optionally may include a promoter, preferably a heterologous promoter and optionally an inducible promoter, operably linked to the isolated polynucleotide. The promoter preferably promotes transcription of the operably linked polynucleotide in a suitable cell (*e.g.*, a bacterial cell, a yeast cell, or a eukaryotic cell).

[0067] Also disclosed are recombinant host cells that comprise the vectors disclosed herein. The recombinant host cells may be utilized in methods for expressing the isolated polypeptide disclosed herein. The methods may include: (a) culturing the recombinant host cell comprising a vector as disclosed herein (*e.g.*, in which the vector comprises a promoter operably linked to an isolated polynucleotide encoding the isolated polypeptide), wherein the

recombinant host cell expresses the polypeptide; and (b) isolating the polypeptide thus expressed.

[0068] Also disclosed are pharmaceutical compositions. The pharmaceutical compositions may include: (a) the isolated polypeptides disclosed herein at a suitable concentration as disclosed herein (*e.g.*, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 units/ μ l); and (b) a pharmaceutical carrier. The pharmaceutical compositions may be administered to a patient in need thereof, including a patient having a disease that is characterized by secretions comprising nucleic acid. As such, the pharmaceutical compositions may be administering to the patient in order to hydrolyze nucleic acid in the secretions. In some embodiments, the patient has cystic fibrosis.

[0069] The disclosed polypeptides may be utilized in methods for performing enzymatic hydrolysis of nucleic acid, either *in vivo* and/or *in vivo*. For example, the disclosed polypeptides may be utilized in an enzymatic hydrolysis method comprising contacting DNA with the isolated polypeptide in a reaction mixture *in vitro* and enzymatically hydrolyzing the DNA to produce nucleotides. Because the disclosed polypeptides exhibit thermostability, the hydrolysis methods may be performed at relatively high temperatures (*e.g.* at a temperature greater than about 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C). Alternatively, the hydrolysis methods may be performed after the isolated polypeptide has been exposed to a relatively high temperature (*e.g.* to a temperature greater than about 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C). The disclosed enzymatic hydrolysis methods may be utilized to prepare GMP nucleotides (*e.g.*, wherein the DNA hydrolyzed in the method comprises GMP and the method includes isolating the GMP from nucleotides produced in the enzymatic hydrolysis). The disclosed methods also may be utilized to prepare AMP nucleotides (*e.g.*, wherein the DNA hydrolyzed in the method comprises GMP and the method includes isolating the GMP from nucleotides produced in the enzymatic hydrolysis). Optionally, the AMP nucleotides produced in the disclosed methods may be deaminated to prepare IMP from the AMP nucleotides.

EXAMPLES

[0070] The following Examples are illustrative and are not intended to limit the scope of the claimed subject matter.

[0071] Example 1

[0072] This invention relates to a thermonuclease from *Haemophilus influenza* (Nontypeable *Haemophilus influenzae* 2019 strain thermonuclease). It is stable over a wide range of temperature and has been found to be significantly more potent at degrading all forms of genetic material than current nucleases. It has 22-fold greater activity than Staphylococcal thermonuclease and Benzonase, and 1400-fold greater activity than DNase I. The nuclease enzyme can digest double stranded, single stranded and plasmid DNA. It can be purified recombinantly in *E. coli* with high yields. Purification is straight-forward and a highly purified product can be obtained. The nuclease can be heated to 65 °C with minimal loss of activity and is stable at 4 °C for prolonged periods of storage (over 6 months). It has application for industrial processes in which the removal of nucleic acids is critical, preparation of phosphorylated nucleotides in the food industry, biomedical potential use in an aerosol for digestion of DNA in pulmonary secretions and in research laboratories performing medical and molecular biology research.

[0073] We have been studying biofilms produced by *Haemophilus influenzae*. These structures are composed of DNA. Our studies have shown that the organism possesses a thermonuclease which is involved in remodeling the biofilm structures.

[0074] Example 2

[0075] Reference is made to Cho *et al.*, “Role of the Nuclease of Nontypeable *Haemophilus influenzae* in Dispersal of Organisms from Biofilms,” *Infect Immun.* 2015 Mar; 83(3):950-957, published online 2014 Dec 29, the contents of which are incorporated herein by reference in their entirety.

[0076] Abstract

[0077] Nontypeable *Haemophilus influenzae* (NTHI) forms biofilms in the middle ear during human infection. The biofilm matrix of NTHI contains extracellular DNA. We show that NTHI possesses a potent nuclease, which is a homolog of the thermonuclease of *Staphylococcus aureus*. Using a biofilm dispersal assay, studies showed a biofilm dispersal pattern in the parent strain, no evidence of dispersal in the nuclease mutant, and a partial return of dispersion in the complemented mutant. Quantitative PCR of mRNA from biofilms from a 24-h continuous flow system demonstrated a significantly increased expression of the nuclease from planktonic organisms compared to those in the biofilm phase of growth ($P < 0.042$). Microscopic analysis of biofilms grown in vitro showed that in the nuclease mutant the nucleic acid matrix was increased compared to the wild-type and complemented strains. Organisms were typically found in large aggregates, unlike the wild-type and complement biofilms in which the organisms were evenly dispersed throughout the biofilm. At 48 h, the majority of the organisms in the mutant biofilm were dead. The nuclease mutant formed a biofilm in the chinchilla model of otitis media and demonstrated a propensity to also form similar large aggregates of organisms. These studies indicate that NTHI nuclease is involved in biofilm remodeling and organism dispersal.

[0078] Introduction

[0079] Nontypeable *Haemophilus influenzae* (NTHI) is frequently found as a component of the normal upper respiratory tract bacterial flora (1). This species is a cause of airway infections, including otitis media in children, sinusitis, and acute exacerbations of chronic bronchitis in adults (1). NTHI has been shown to be capable of forming biofilms both in vitro and in the upper and lower human respiratory tract during human disease (2,–8).

Bacterial biofilm matrices are an elaborate network of molecules, which can include pili, polysaccharides, extracellular DNA (eDNA), and bacterial and host-derived substances that help shape and secure the biofilm to an inanimate or host surface (9). The matrix protects the underlying bacteria from assault by the host immune response and antibiotic treatment, thus contributing to the recalcitrance of biofilm infections to antimicrobial treatment (10). The matrix of NTHI biofilms has been shown to contain double-stranded eDNA (11). Our laboratory has been interested in studying possible mechanisms controlling the matrix eDNA in an NTHI biofilm. Studies of the sequenced genome of *H. influenzae* strains KW20 Rd (HI1296) and 86-028NP (NTHI1828) indicated that an open reading frame (ORF) with high homology to the *Staphylococcus aureus* thermonuclease was present. A number of studies have shown that mechanisms are present in bacteria to degrade biofilm matrix and release organisms from the biofilm to planktonic phase of growth (12,–14). Studies by Steichen et al. demonstrated that *Neisseria gonorrhoeae* expressed a thermonuclease, which was involved in biofilm eDNA matrix remodeling (15). In *S. aureus*, it has been shown that its thermonuclease is regulated by the SaeRS two-component system (16) and that this nuclease is involved in facilitating the escape of *S. aureus* from neutrophil extracellular traps (NETs) (17). We investigate in the present study whether NTHI expresses a nuclease and, if so, whether it plays a role in biofilm remodeling and organism dispersal.

[0080] Materials and Methods

[0081] Bacteria and culture conditions. Bacterial strains used in the study are shown in Table 1.

[0082] Table 1

Nontypeable <i>H. influenzae</i> strain or plasmid	Genotype, relevant characteristic(s), and/or sequence (5'-3') ^a	Source or reference
NTHI strains		
2019	Wild type	Our laboratory
2019Δ <i>hmr</i>	Δ <i>hmr</i> ; Kan	This study
2019Δ <i>hmr::hmr</i>	Δ <i>hmr::hmr</i> ; Kan Sp	This study
Plasmids		
pGEM-T		Promega
pET151/D-TOPO		Life Technologies
pUC18K3	Amp Kan	This laboratory
p60L1-Sp2	Sp	10
pCEC#27	pUC18K3::Δ <i>hmr::kan</i> ; Kan	This study
pCEC#29	pET151::hmr; Amp	This study
pCEC#31	p60L1Sp2::hmr; Sp	This study

[0083] Nontypeable *H. influenzae* 2019 (NTHI 2019) is a clinical isolate described in previous studies (18). NTHI 2019 was grown from frozen stock cultures at 37°C in 5% CO₂ in brain heart infusion agar (Difco) supplemented with 10 µg of hemin/ml and 10 µg of NAD/ml (sBHI). *Escherichia coli* K-12 was grown in Luria-Bertani medium with or without agar and supplemented with antibiotics as needed.

[0084] Construction of the nuclease (*nuc*) deletion mutant. The whole gene, except for the first and the last codon of the *nuc* ORF, was replaced with a kanamycin resistance cassette. Approximately 500 bp upstream and downstream, the arms of *nuc* were PCR amplified. The upstream homology arm contained EcoRI at the 5' end and KpnI at the 3' end, and the downstream homology arm contained XbaI at the 5' end and HindIII at the 3' end. The homology arms were ligated into pUC18K3, flanking the kanamycin resistance cassette in the multiple cloning site. The resulting plasmid, pCEC#27, is shown in Table 1. The plasmid was transformed into NTHI 2019, and transformants were screened on sBHI plates containing 15 µg of ribostamycin/ml. The sequences of mutant transformants (NTHI 2019Δ*nuc*) were confirmed by PCR and DNA sequencing.

[0085] Complementation of the *nuc* deletion. Previous studies in our laboratory used p601.1-Sp2 plasmid for chromosomal complementation in NTHI 2019 (18). Appropriate primers to clone the entire *nuc* ORF were designed in which both the 5' end of the forward primer and the 3' end of the reverse primer contained SmaI restriction enzyme sites. The PCR-amplified product was cloned into p601.1-Sp2 using the SmaI restriction enzyme site. The final plasmid was pCEC#31 (Table 1). This plasmid was transformed into NTHI 2019Δ*nuc*. The transformants were screened on BHI agar plate supplemented with 15 µg of ribostamycin/ml and 25 µg of spectinomycin/ml. The complemented strain was designated NTHI 2019Δ*nuc*::*nuc* (Table 1). PCR and DNA sequencing were used to confirm the sequence fidelity and the correct orientation of the complementation.

[0086] Cloning, expression, and purification of Nuc. Nuc, without the signal sequence, was expressed in pET151/D-TOPO (Life Technologies) with cleavable 6×His tag

in BL21(DE3) *E. coli* cells and induced with 1.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside; Invitrogen) at an optical density of 0.6 at 18°C. Cell cultures were pelleted and frozen at -20°C in preparation for lysis. The frozen cells were thawed in lysis buffer (100 mM Tris [pH 9.1], 5 mM CaCl₂, 200 mM NaCl, 50 mM imidazole) plus a Mini-Complete protease inhibitor cocktail tablet (Roche) and lysed with an Emulsiflex C3. Subsequently, His-tagged Nuc was added to Ni-nitrilotriacetic acid (Ni-NTA) affinity resin (Qiagen). The resin was then washed with the lysis buffer, and the protein was eluted with lysis buffer containing 250 mM imidazole. The eluted protein was concentrated and mixed 1:200 with Tobacco etch virus protease at 4°C for 16 h to cleave the His tag. The cleaved products were passed over a Ni-Sepharose column (HisTrap FF; GE Healthcare) and the flowthrough was subjected to gel filtration (Superdex 75 25/60; GE Healthcare) to separate Nuc from contaminants and to exchange it into the final purification and reaction buffer (100 mM Tris [pH 9.1], 5 mM CaCl₂, 200 mM NaCl, 5 mM dithiothreitol [DTT]).

[0087] DNase assays. Two different assays were used to assess the enzymatic activity of Nuc. The first assay involved combining either Nuc or DNase I (New England BioLabs, Inc.) with DNA template in DNase I buffer at 37°C. After 30 min, DNA running buffer was added to the samples, and they were immediately run on a 1% agarose gel. The gel was stained with 0.5 g of ethidium bromide/ml, destained with double-distilled H₂O, and then viewed using UV light.

[0088] Nuclease enzyme activity was also measured by a fluorescence resonance energy transfer activity study (FRET) assay using a Tecan Infinite M200 Pro. The FRET substrate was a single-stranded 30-mer oligonucleotide with the 5' end modified with Cy3 fluorophore and the 3' end modified with Black Hole Quencher 2 (19). The substrate was incubated with either Nuc or DNase I (positive control) at 26°C, and then the absolute fluorescence was measured every 10 s for 5 min. Each study shown was performed a minimum of six times on multiple Nuc samples.

[0089] Biofilm dispersal studies. Biofilm dispersal was analyzed according to the method of Kaplan and Fine (14), which takes advantage of the microcurrents caused by edge evaporation in an open 100-mm culture dish in which small numbers of organisms (102 to 103 CFU/ml) are incubated overnight at 37°C under 85% humidity and 5% CO₂ for 24 h. After the incubation, the medium was cultured and carefully decanted, and the plate was stained with gentian violet in 20% ethanol for 2 min and then washed vigorously with distilled water. After 20 min, the plate was washed again with distilled water, dried, and viewed with a Nikon SMZ800 scope with a Diagnostic Instruments, Inc., digital camera module at ×5, ×20, and ×35. The images were then processed using SPOT software 5.1 (SPOT Imaging Solutions, Inc.). These experiments were performed in triplicate on separate occasions studying NTHI 2019, NTHI 2019 Δ nuc, and NTHI 2019 Δ nuc::nuc.

[0090] Biofilm growth over glass using continuous flow chambers. NTHI strains NTHI 2019, NTHI 2019 Δ nuc, and NTHI 2019 Δ nuc::nuc were grown in continuous flow chambers for in vitro biofilm growth by the method of Schwartz et al. (20). Chambers were made with the same dimension and material as previously described (20), except the outflow fitting material was changed from copper to plastic. Glass coverslips (22 mm by 50 mm) were placed in the chambers and were stabilized with silicone. The chambers with the coverslips and influent and effluent tubing were sterilized by autoclaving. NTHI strains were grown to mid-log phase in RPMI 1640 medium (Life Technologies) supplemented with protoporphyrin IX (1 µg/ml), hypoxanthine (0.1 mg/ml), uracil (0.1 mg/ml), β-NAD (10 µg/ml), sodium pyruvate (0.8 mM), and Neu5Ac (100 µM). The chambers with clamped effluent tubing on a flat surface were inoculated with 6-ml cultures diluted to an A₆₀₀ of 0.25. The chambers were incubated at 37°C for 2 h to allow for bacteria to adhere to the coverslip. The influent tubing was aseptically connected to the flask containing supplemented RPMI medium, diluted 1:4 in phosphate-buffered saline (PBS), and then to the chambers via a 23-gauge needle (1 in.), which was aseptically inserted through the inlet stopper. Both inflow and outflow tubing were fed through the same pump to maintain constant volume in the chambers. The chambers, on a flat surface, were incubated at 37°C for 48 h at a flow rate of 100 µl/min. When indicated, biofilms were stained with Live/Dead stain according to the manufacturer's

instructions (Life Technologies) prior to fixation. DRAQ-5 was included in the embedment in these experiments. In other experiments, at 48 h the medium was carefully replaced with 4% paraformaldehyde in PBS to fix the bacteria in situ. Fixed cells were labeled with monoclonal antibody (MAb) 6E4 (anti-KDO) and a fluorescein isothiocyanate-conjugated secondary antibody (GAM-IgG/FITC; Jackson ImmunoResearch, West Grove, PA), taking care to avoid disruption of the biofilm on the coverslips during the procedures. The use of a mounting medium, Fluoro-Gel III with propidium iodide (Electron Microscopy Sciences), allowed labeling of the eDNA matrix in the specimens. All samples were viewed by confocal laser-scanning microscopy using a Nikon Digital Eclipse C1 laser scanning confocal microscope (Nikon Instruments, Inc.) at a magnification of $\times 20$.

[0091] Chinchilla model of NTHI biofilm formation. NTHI strains including NTHI 2019, NTHI 2019 Δ nuc, and NTHI 2019 Δ nuc::*nuc* were studied for their ability to form biofilms in the middle ears of adult chinchillas (*Chinchilla lanigera*; mean weight, 400 to 600 g; Rauscher's Chinchilla Ranch, LaRue, OH). The animals were acclimated to the vivarium for a period of 7 to 10 days. Both middle ears were inoculated with 300 μ l of sterile pyrogen-free saline containing 1,500 to 2,000 CFU of NTHI via transbullar inoculation as previously described (21, 22), with the actual inoculum received confirmed by plate count. Middle ears were then monitored daily for signs of otitis media via video otoscopy and tympanometry. Mature biofilms were formed by NTHI in the chinchilla middle ear 5 days after challenge (21, 22) and in the present study chinchillas were also sacrificed at this time point. The bullae were dissected away from the skull, opened to visualize the inferior bulla, and any fluid present was aseptically collected. Images were taken of all bullae. The mucosa, along with any biofilm present, were collected from the right bulla and placed in a preweighed tube. These were homogenized, serially diluted, and plated to determine CFU of NTHI strain/mg (wet weight) of tissue. The left bulla from each chinchilla was filled with optimal cutting temperature (OCT) compound and snap-frozen for histological analysis. Recovered effusions were serially diluted and plated for semiquantitative determination of CFU of NTHI/ml of middle ear fluid. All studies involving chinchillas were performed under an Institutional

Animal Care and Use Committee-approved protocol in compliance with all relevant federal guidelines and institutional policies.

[0092] Embedding in OCT for cryosectioning. To preserve the architecture of biofilms that had formed in vitro and in vivo, we embedded the biofilm sample in an OCT compound (Fisher Scientific, Pittsburgh, PA). The chinchilla biofilms were processed immediately after dissection for later cryosectioning. Briefly, the inferior and superior portions of iced, dissected bullae were separated, and any effusion present was retrieved by aspiration. The inferior bulla was then rinsed several times and drained via wicking onto absorbent paper. OCT was slowly added via 18-gauge needle. Bullae were then snap-frozen over liquid nitrogen and placed on a bed of dry ice. External bone was carefully chipped away, thereby eliminating the need for decalcification, as well as achieving our goal of leaving the middle ear mucosa and any attached biofilm intact. The resulting block was split in a plane perpendicular to the tympanic membrane. Serial sections (4- μ m thickness) were cut on a Leica CM3050S cryotome (Leica Microsystems, Inc., Bannockburn, IL). Sections were placed on Superfrost slides (Fisher Scientific) and stored at -80°C. Prior to staining, sections were fixed in 4% (wt/vol) paraformaldehyde (in 0.1 M phosphate buffer [pH 7.4]). The in vitro-grown biofilms were embedded in OCT, cryosectioned, fixed in situ, and stained with MAb 6E4 and secondary antibody goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch). The DNA matrix in the specimens was stained with DAPI (4',6'-diamidino-2-phenylindole).

[0093] COMSTAT analysis of confocal z-series. Quantitative analysis of each z-series was performed using COMSTAT (23). COMSTAT is a mathematical script written for MATLAB 5.3 (The Mathworks, Inc., Natick, MA) that quantifies three-dimensional biofilm structures by evaluating confocal image stacks, so that pixels may be converted into relevant measurements of biofilm, including total biomass and average thickness. To complete COMSTAT analysis, an information file was created for each z-series to adjust for the pixel sizes of the x, y and z axes and number of images in each z-series. COMSTAT was then used

to threshold the images to reduce background. Biomass and average and maximum thickness in each z-series were calculated by COMSTAT from the threshold images.

[0094] Quantitative real-time PCR. SYBR green quantitative real-time PCR (qRT-PCR) was used to measure the expression of *nuc* in the 24-hour NTHI 2019 (24). For each target, qRT-PCR primer sets were selected using Primer Express software (Agilent Technologies) and obtained from Integrated DNA Technologies (Coralville, IA). RNA samples were converted into cDNA as follows: 2 µl of random hexamer primers (Invitrogen, Carlsbad, CA) was added to 2 µg of total RNA in a total volume of 12 µl, followed by incubation at room temperature for 10 min to allow primers to anneal, and then transitioned to 70°C to relax RNA secondary structure and finally cooled on ice for 2 min. Then, 2 µl of 0.1 M DTT, 4 µl of SuperScript II 10× reaction buffer, 1 µl of 10 mM deoxynucleoside triphosphates, and 1 µl of SuperScript II were added to each tube buffer (all reagents were obtained from Invitrogen), followed by incubation at 42°C for 4 h. At this point, RNA was degraded with 3.5 µl of 0.5 M EDTA at 65°C for 15 min, and reactions were neutralized with 5 µl of 1 M Tris and 21.5 µl of Tris-EDTA buffer (all reagents were obtained from Ambion/Applied Biosystems). cDNAs were purified using the Qiagen PCR cleanup kit, quantitated, and then diluted to 10 ng/µl and used as the templates for qRT-PCR. Relative RNA quantities were determined by standard curve (5-fold dilutions of purified genomic DNA [gDNA] ranging from 100 to 0.00032 ng/µl), and all values were normalized to the amount of outer membrane protein 6 (ompP6) RNA in each sample. OmpP6 is considered constitutively expressed in *H. influenzae*. Our data on more than 60 NTHI mRNA samples confirmed this fact. We performed 50-µl reactions in triplicate in 1× SYBR green master mix (Ambion/Applied Biosystems) with 3.5 mM magnesium chloride, 10 ng of template or gDNA standard, and 1 µM final concentrations of each primer. RT-PCR was performed on the ABI Prism 7000 sequence detection system (Quantum Analytics). Each RT-PCR assay was performed four times on three matched biofilm and planktonic samples, and genes were considered validated if results were consistent for all assays and the absolute fold change values were equivalent to or greater than the corresponding array fold change. The data were

normalized against NTHI P6 (25) and standardized against NTHI 2019 wild-type genomic DNA.

[0095] Statistical analysis. The statistical analyses for the qRT-PCR experiments were performed using the linear-mixed-modeling (LMM) framework. Estimation was performed using restricted maximum-likelihood (REML) estimation and the denominator degrees of freedom for tests of model coefficients were obtained using the containment method as implemented in SAS v9.3. For the qRT-PCR experiments, the outcome variable was the ratio of expression of HI1296 (*nuc*) and HI0501 (*p6*). A random intercept was included to adjust for the multiple measurements taken from each experiment. A model that included an intercept and an indicator for whether the observation came from the biofilm or planktonic phase growth was fit to the data. All significance tests based on LMM analyses were conducted at the $\alpha = 0.05$ level and were performed using SAS v9.3. The CFU studies were analyzed using Prism 6.0 with analysis of variance using nonparametric tests (the Kruskal-Wallis test).

[0096] Results

[0097] NTHI Nuc has homology to the *S. aureus* thermonuclease.

[0098] We provide here evidence that NTHI expresses a nuclease, which plays a role in biofilm remodeling and organism dispersal and appears to be under the control of quorum sensing. NTHI Nuc contains 154 amino acids, and the first 13 are a signal (SpII) peptidase sequence (Fig. 5). The nuclease has homology to the staphylococcal thermonuclease with conserved amino acids (35%) across 70% of the sequence and an E value of $4e-10$. There are 24 NTHI genomes in the public databases which have high homology (E values of less than 10^{-38}) to the NTHI 2019nuc amino acid sequence.

[0099] NTHI Nuc has nuclease activity. NTHI Nuc was expressed in *E. coli* without the signal sequence, purified by affinity and molecular sieve chromatography, and studied to determine whether it would digest DNA. Figure 6A shows the results of agarose gel studies

demonstrating that NTHI Nuc can digest double-stranded DNA. Studies using FRET-labeled nucleotides demonstrated that NTHI Nuc is a nuclease that is dependent upon a divalent cation, calcium, for activity (Fig. 6B) and can also cleave single-stranded DNA. NTHI Nuc shows markedly increased activity compared to bovine DNase 1 at 180 times the concentration of NTHI Nuc.

[00100] Expression of *nuc* in biofilms and the planktonic phase of growth. We measured the relative *nuc* expression and compared this to the constitutively expressed *H. influenzae* gene, *ompP6*. As shown in Table 2, the expression of *nuc* was significantly greater in the planktonic state than the biofilm state in NTHI 2019 ($P < 0.042$).

[00101] Table 2

Results of qRT-PCR studies of *muc* expression in biofilms and the planktonic phase of growth^a

State	<i>n</i>	Mean	SD	Range	Fold difference		<i>P</i>
					Minimum	Maximum	
Planktonic phase	12	407.2	116.1	252.5	648.17	1.52	0.042
Biofilms	12	267.3	93.3	141.0	430.5		

^a Assays were derived from four separate biofilm/planktonic studies, with three assays on each sample. Mean, standard deviation, and range values are expressed as pg of Nuc/pg of P6.

[00102] In other studies we have performed, we have demonstrated that the NTHI Nuc has 1,200 to 1,400 times greater activity than DNase 1; thus, small changes in gene expression can substantially augment enzymatic activity (Fig. 6B and data not shown). Some idea of these differences in the velocity of NTHI Nuc and bovine DNase 1 can be seen in Fig. 6B. These qRT-PCR experiments suggest that the expression of *nuc* is regulated during biofilm formation and may play a role in the control of biofilm dispersal.

[00103] Biofilm dispersal experiments. In order to study the effect of Nuc on NTHI biofilm dispersal, we adapted the method of Kaplan to study NTHI 2019, NTHI 2019 Δ *nuc*, and NTHI 2019 Δ *nuc::nuc*. The results are shown in Fig. 7. In this assay, microturbulence in the plates causes a comet-like tail to form as organisms disperse from the nascent biofilm or microcolony (14). This can be seen by the comet tail of satellite colonies formed by NTHI 2019 grown for 24 h in this system (Fig. 7A). No evidence of this was found in the plates containing NTHI 2019 Δ *nuc* (Fig. 7B). The phenotype has been partially restored in NTHI 2019 Δ *nuc::nuc*. The *nuc* expression in NTHI 2019 Δ *nuc::nuc* is unregulated as the complementing gene is driven from the spectinomycin promoter. This results in smaller biofilms (microcolonies), but the comet-like plumes can still be seen in NTHI 2019 Δ *nuc::nuc* (Fig. 7C). These data strongly suggest that Nuc is involved in biofilm dispersal in NTHI.

[00104] Deletion of *nuc* affects biofilm formation. In order to determine the role of the nuclease in the NTHI biofilm, NTHI 2019, NTHI 2019 Δ *nuc*, and NTHI 2019 Δ *nuc::nuc* were studied in a continuous flow biofilm chamber (Fig. 8). Confocal microscopic analysis of the continuous flow biofilms indicated the organisms in the Δ *nuc* biofilm were aggregated compared to the diffusely distributed organisms in the biofilm formed by the wild-type or Δ *nuc::nuc* strains. The intensity of propidium iodine staining was also increased in the NTHI 2019 Δ *nuc* mutant, suggesting an increase in the amount eDNA in the biofilm matrix. The biofilm formed by NTHI 2019 Δ *nuc::nuc* restored the wild-type biofilm phenotypes. Frozen sections of a separate set of wild-type, mutant, and complemented mutant biofilms showed a similar clustering of organisms in the mutant compared to the wild type and the complemented mutant (Fig. 9). COMSTAT analysis indicated that the height and mass of the

biofilms were not statistically different between the biofilms formed by the three strains. Our studies demonstrated that the majority of organisms in the NTHI 2019 Δ *nuc* biofilm were dead by 48 h (Fig. 10). All biofilm studies were performed in at least three separate experiments with similar results.

[00105] Chinchilla middle ear infection studies. To study the impact of the *nuc* mutation during in vivo infection, NTHI 2019, NTHI 2019 Δ *nuc*, and NTHI 2019 Δ *nuc::nuc* were studied in a chinchilla model of experimental otitis media over a 5-day period (21). Three animals were studied for each strain. Both ears were infected and, at sacrifice, one bulla was used for microscopy and the second bulla was used for microbiological studies. Gross examination of the middle ears at sacrifice indicated that each of the strains appeared to induce biofilms of similar size based on the wet weight of the biofilms. Moreover, semiquantitative cultures from nasopharyngeal lavage fluids and middle ear fluids showed no significant differences in relative CFU of NTHI/ml; however, despite variability among tissue samples recovered from these outbred animals, there was a notable trend to increased NTHI/mg of middle ear mucosa (including associated mucosal biofilm) in animals challenged with NTHI 2019 Δ *nuc* at sacrifice (see Fig. 12). Confocal analysis of 10- μ m-thick cryosections of the biofilm stained with DRAQ5 and MAb 6E4 showed evidence of inflammatory cell infiltration, eDNA, and organisms (Fig. 11) in biofilms from each of the animals. However, in the cohort of animals challenged with NTHI 2019 Δ *nuc*, there was increased density of NTHI with aggregation of organisms in the NTHI 2019 Δ *nuc* biofilm compared to the biofilms induced by either the wild-type strain or its the complemented mutant (Fig. 11). These data are similar to the aggregation seen in the in vitro studies conducted with NTHI 2019 Δ *nuc* and suggest that the dispersal of organisms from the NTHI 2019 Δ *nuc* mutant was also diminished in vivo.

[00106] Discussion

[00107] A number of bacterial species responsible for causing human disease, including *Neisseria gonorrhoeae*, *Campylobacter jejuni*, *Enterococcus faecalis*, and *Bacillus*

anthracis, encode a protein homologous to the *S. aureus* thermonuclease. The nuclease is present among many NTHI strains since 24 NTHI genomes contain a gene with high homology (<e10⁻³⁸) to NTHI 2019*nuc*. The functional properties and structure of the *S. aureus* thermonuclease have been studied for over 50 years (26). Recently, studies have shown that *S. aureus* and *N. gonorrhoeae* encode a thermonuclease, which plays a role in remodeling the eDNA matrix of their biofilms (16, 27). We provide evidence that the NTHI Nuc is a nuclease capable of digesting single- and double-stranded nucleic acids. It is an extremely potent thermonuclease and has ~1,400-fold greater activity than DNase I on a molar basis (unpublished data). NTHI makes a biofilm whose matrix is also comprised primarily of eDNA (11), and in the present study, we show that the nuclease produced by NTHI is involved in the remodeling of the biofilm eDNA matrix structure, as well as organism dispersal, and its absence results in aggregation of organism within biofilms in vitro and in vivo.

[00108] At the functional level, like the *S. aureus* and *N. gonorrhoeae* thermonucleases, the NTHI nuclease plays a role in remodeling the eDNA matrix of the biofilm (27, 28). The biofilm formed by the NTHI 2019 Δ *nuc* mutant shows aggregation of organisms within the biofilm and an increase in eDNA. Live/Dead studies of in vitro grown biofilms in flow chambers showed decreased viability of organisms within the 2019 Δ *nuc* biofilm (Fig. 10). This is most probably due to loss of remodeling of the matrix, failure to disperse organisms, and aberrant water channel formation, leading to nutrient limitation. Studies of the NTHI 2019, 2019 Δ *nuc*, and 2019 Δ *nuc::nuc* biofilms in vitro and in the chinchilla model of otitis media showed that loss of NTHI Nuc activity resulted in a substantial increase in the aggregation of organisms within the biofilm compared to the wild-type strain and the complemented mutant strain. Quantitative cultures of fluid from the middle ear chamber and mucosa/biofilm of the mutant strain showed wide variability but overall similar numbers of viable organisms compared to the wild-type and complemented mutant strains (see Fig. 12). The differences were not significant. Juneau et al. showed that NTHI induced polymorphonuclear neutrophil (PMN) NETs in murine bone marrow-derived macrophages (29). These NETs had no impact on NTHI viability; thus, it is doubtful that the

increased bacterial population seen in the NTHI 2019 Δ *nuc* biofilm in the chinchilla was due to loss of the effects of the NTHI nuclease on PMN nets. We would speculate that NTHI in biofilm in the middle ear environment are trapped within the biofilm, and it is possible that in this in vivo environment adequate nutrients are available to allow them to survive. Based on these results, we would surmise that the absence of the nuclease results in an increase in eDNA matrix and retention of organisms within restricted areas of the biofilm, supporting the hypothesis that the nuclease is involved in biofilm matrix remodeling and organism release, as has been described for *S. aureus* thermonuclease (28). This is supported by our dispersion studies, which showed no evidence of sustained organism dispersion in the NTHI 2019 Δ *nuc* mutant.

[00109] Nuc expression studies comparing 24-h biofilm mRNA with planktonic mRNA suggested that the NTHI *nuc* expression is significantly reduced in the organisms in the biofilm phase compared to those in the planktonic phase of growth. The NTHI nuclease is extremely potent and, while the differences in expression are not great (1.5-fold), they are significant between NTHI 2019 planktonic bacteria and biofilm (Table 2) samples. These studies implicate a potential role for an autoinducer in control of expression of nuclease activity in the biofilm but further studies are necessary. Due to potentially deleterious effects of the nuclease on the NTHI DNA and RNA, it is reasonable that there would be tight control of the NTHI *nuc* expression, and it would appear that Nuc is important to ensure the organism's survival and dispersal in the biofilm environment.

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- [00140] It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been

illustrated by specific embodiments and optional features, modification and/or variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00141] Citations to a number of patent and non-patent references are made herein. The cited references are incorporated by reference herein in their entireties. In the event that there is an inconsistency between a definition of a term in the specification as compared to a definition of the term in a cited reference, the term should be interpreted based on the definition in the specification.

CLAIMS

We claim:

1. An isolated polypeptide comprising the amino acid sequence of SEQ IQ NO:1 or comprising an amino sequence having at least about 80% sequence identity to SEQ ID NO:1, wherein the polypeptide has nuclease activity and is thermostable.
2. The isolated polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ IQ NO:1.
3. The isolated polypeptide of claim 1 or 2, wherein the polypeptide has DNase activity.
4. The isolated polypeptide of any of the foregoing claims, wherein the polypeptide is thermostable at 55°C and does not lose more than about 50% of nuclease activity when heated to 55°C for 10 minutes.
5. The isolated polypeptide of any of the foregoing claims, wherein the peptide does not lose more than about 50% of nuclease activity when stored at 4°C for at least six months.
6. The isolated polypeptide of any of the foregoing claims further comprising a heterologous peptide tag.
7. The isolated polypeptide of claim 6, wherein the tag is an affinity tag.
8. An enzyme composition comprising: (a) the isolated polypeptide of any of the foregoing claims; and (b) a storage buffer.
9. The composition of claim 8, wherein the storage buffer comprises a pH buffering system providing a pH of about 6.5 – 8.5.

10. The composition of claim 8 or 9, wherein the storage buffer comprises glycerol at a concentration of at least about 5%, 10%, 20%, 30%, 40%, or 50%.
11. A reaction mixture comprising:
 - (a) the isolated polypeptide of any of the foregoing claims;
 - (b) heterologous DNA; and
 - (c) an aqueous solution wherein the isolated polypeptide hydrolyzes the heterologous DNA.
12. The reaction mixture of claim 11, further comprising: (d) a buffering system providing a pH of between 6.5 – 8.5.
13. The reaction mixture of claim 11 or 12, further comprising: (e) a divalent cation at a concentration of 0.05 – 10 mM.
14. An isolated polynucleotide encoding the polypeptide of any of claims 1-7.
15. The isolated polynucleotide of claim 14 comprising the nucleotide sequence of SEQ ID NO:2 or comprising a nucleotide sequence having at least about 80% sequence identity to SEQ ID NO:2, wherein the encoded polypeptide has nuclease activity and is thermostable.
16. A heterologous vector comprising the isolated polynucleotide of claim 14 or claim 15.
17. The vector of claim 16, further comprising a promoter, preferably a heterologous promoter and optionally an inducible promoter, operably linked to the isolated polynucleotide.
18. A recombinant host cell comprising the vector of claim 17.

19. A method for expressing the isolated polypeptide of any of claims 1-7, the method comprising: (a) culturing the recombinant host cell of claim 18, wherein the recombinant host cell expresses the polypeptide; and (b) isolating the polypeptide thus expressed.

20. A pharmaceutical composition comprising the isolated polypeptide of any of claims 1-7 and a pharmaceutical carrier.

21. A method for treating a patient having a disease or disorder characterized by secretions comprising nucleic acid, the method comprising administering the composition of claim 20 to the patient.

22. The method of claim 21, wherein the patient has cystic fibrosis.

23. A method for performing enzymatic hydrolysis of DNA, the method comprising contacting the DNA with the isolated polypeptide in a reaction mixture *in vitro* and enzymatically hydrolyzing the DNA to produce nucleotides.

24. The method of claim 23, wherein the hydrolysis is performed at a temperature greater than about 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C.

25. The method of claim 23, wherein prior to contacting the DNA with the isolated polypeptide, the isolated polypeptide has been heated to a temperature greater than about 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C for at least about 1, 2, 5, 10, 20, 30, or 60 minutes.

26. The method of claim 23, wherein the nucleotides comprise GMP and the method further comprises isolating the GMP from the nucleotides produced in the enzymatic hydrolysis.

27. The method of claim 23, wherein the nucleotides comprise AMP and the method further comprises isolating the AMP from the nucleotides produced in the enzymatic hydrolysis.

28. The method of claim 27, further comprising deaminating the isolated AMP to prepare IMP.

Figure 1.**Nontypeable *Haemophilus influenzae* 2019 strain thermonuclease****Amino Acid Sequence:**

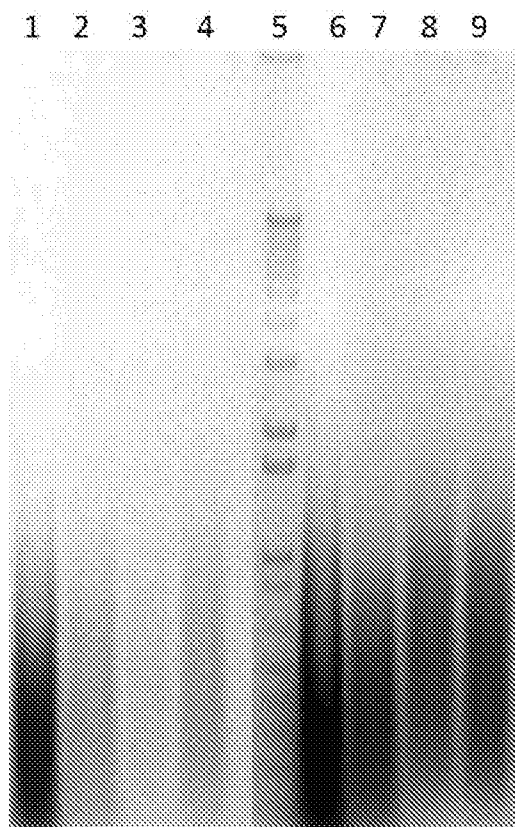
MTSLLLIFTVLSACSREKNTCRVVKISDGDTLTCLTKGNKSIKVRLAEIDAPEKSQAFGQ
KSKKTLSDLVYQKNVRLARKGKDRYQRTLAVVYYQKQININLEMVKQGMWAYKQYSHDPI
YLQAQENAQAKGIGLWADNNPIEPSQWRRQEK (SEQ ID NO:1)

Nucleotide sequence:

ttgacgagtttattacttattttttacogtactttctgcttggttctcgcgaaaaaaatacg
tgtcgtgtggttaaaattagtgatggcgatacgcttacttgcoctcactaaaggcaataaaa
tccattaaaagttcgtttagccgaaattgatgogccagaaaaatctcaagcctttgggcaa
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ggaaaagatcgctatcaacgcaccttagcgggtggtttattatcaaaaacaaaatattaat
ttagaaatgggttaaacaggggtatggcatgggcttataagcaatattcccatgatccgatt
tatcttcaagcgcaagaaaaatgcacaagccaaaggaattggtctttgggcagacaataat
ccgattgaaccaagtcaatggcgcgagacaagagaaaaattaa (SEQ ID NO:2)

Figure 2.

**NTHi Thermonuclease is active when bound to a
solid phase substrate**



1. Negative control (NTHi Nuc)
2. NTHi Nuc, 30min no shaking
3. NTHi Nuc, 20min with shaking
4. NTHi Nuc, 15min with shaking
5. Ladder
6. Negative control (Benzonase)
7. Benzonase, 30min no shaking
8. Benzonase, 20min with shaking
9. Benzonase, 15min with shaking

0.3mM protein added to each gel
8 micrograms herring sperm DNA
Dnase 1 buffer

Figure 3. Comparison of *Pasteurellaceae* thermonuclease with other nucleases

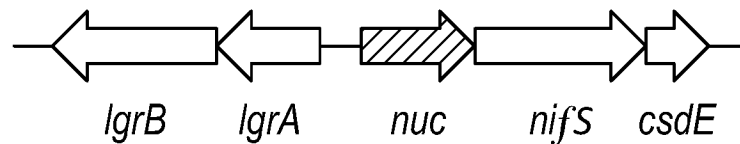
Sample	Velocity/nM of enzyme	Fold difference compared to DNAase 1*
NTHi Nuclease	141.3	1413
NTHi Nuclease (boiled)	1.95	19.5
<i>S. aureus</i> nuclease	6.8	68
<i>S. aureus</i> nuclease (boiled)	4.6	46
Benzonase	6.2	62
Benzonase (boiled)	4.9	49
NGO nuclease	1.37	13.7
NGO nuclease (boiled)	0	0
DNase I	0.1	1
DNase I (boiled)	0	0

*Compared to NEB Bovine DNase I

Figure 4. Light Scattering demonstrating effect of pH on size.

pH	Radius (nm)	Polydispersity (%)	SLS* MW (kDa)
7	2.19 ± 0.04	13.1 ± 0.3	19.3 ± 0.0
8	2.64 ± 0.04	32.5 ± 1.8	--
9	2.53 ± 0.01	20.3 ± 1.1	--

* SLS - Static Light Scattering



MTSLLLIFTVLSACSREKNTCRVVKISDGDTLTCLTKGNKSIKVRLAEID
 APEKSQAFGQKSKKTLSDLVYQKNVRLARKGKDRYQRTLAVVYYQKQNIN
 LEMVKQGMWAYKQYSHDPIYLLQAQENAQAKGIGLWADNNPIEPSQWRRQ
 EKN

154 amino acids pI=9.85
 Accession number* - NC - 0071467.2

* *Haemophilus influenzae* 86-028NP
 (SEQ ID NO:1)

FIG. 5

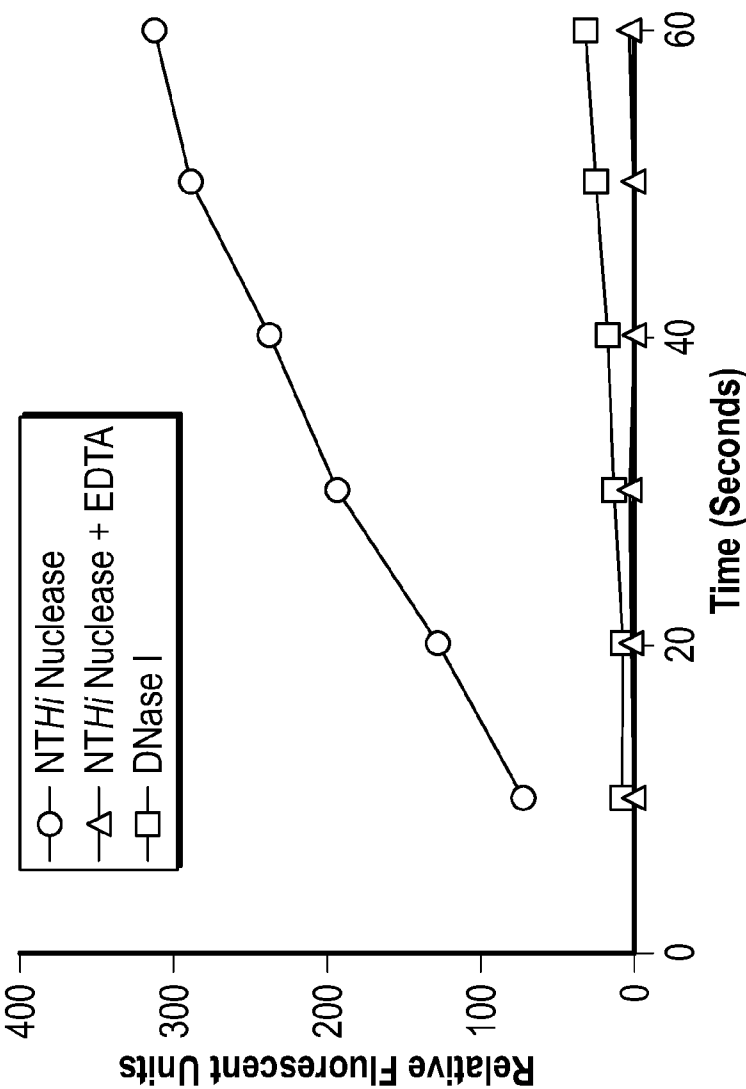


FIG. 6B

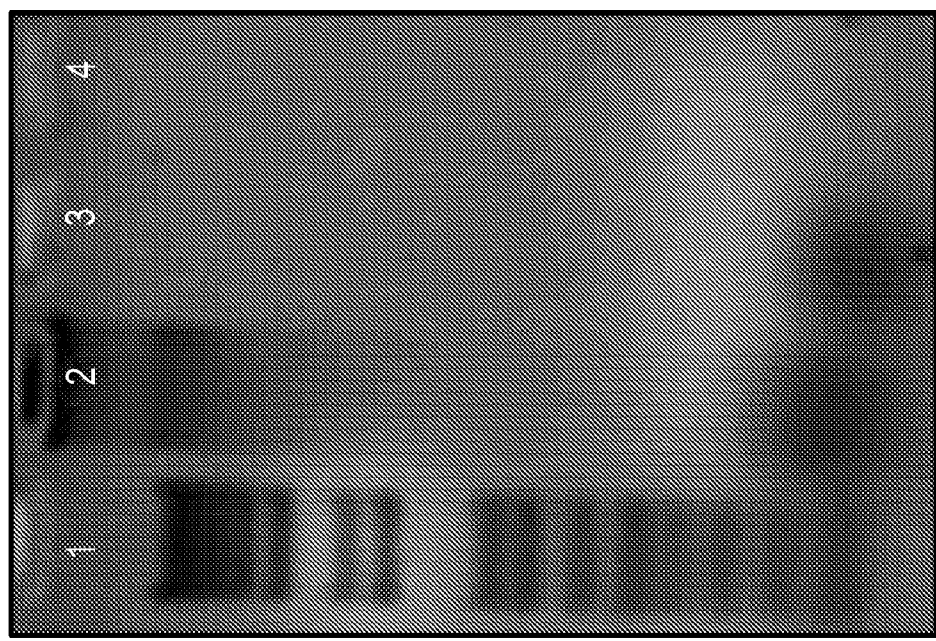


FIG. 6A

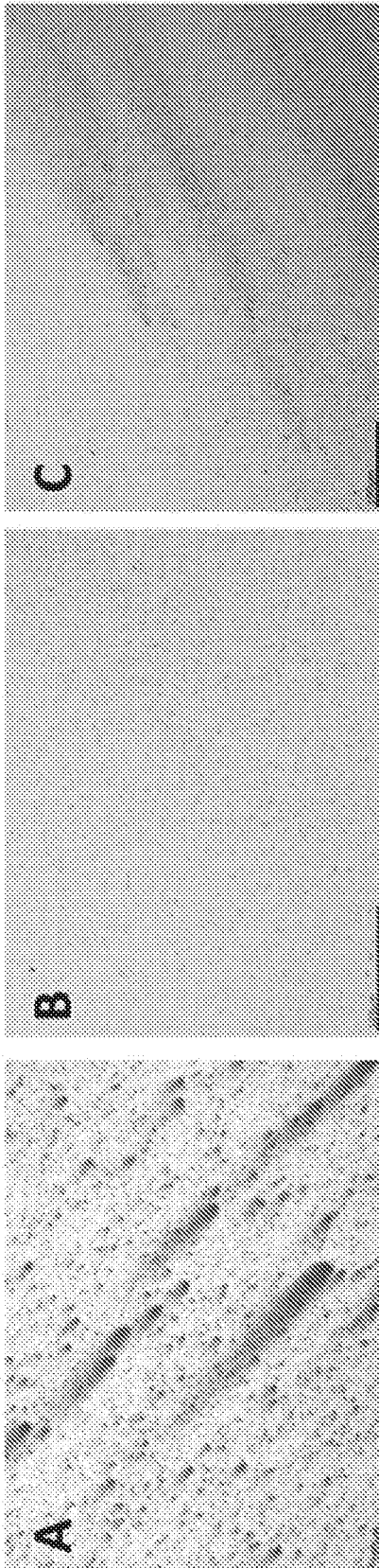


Figure 7.

Figure 8.

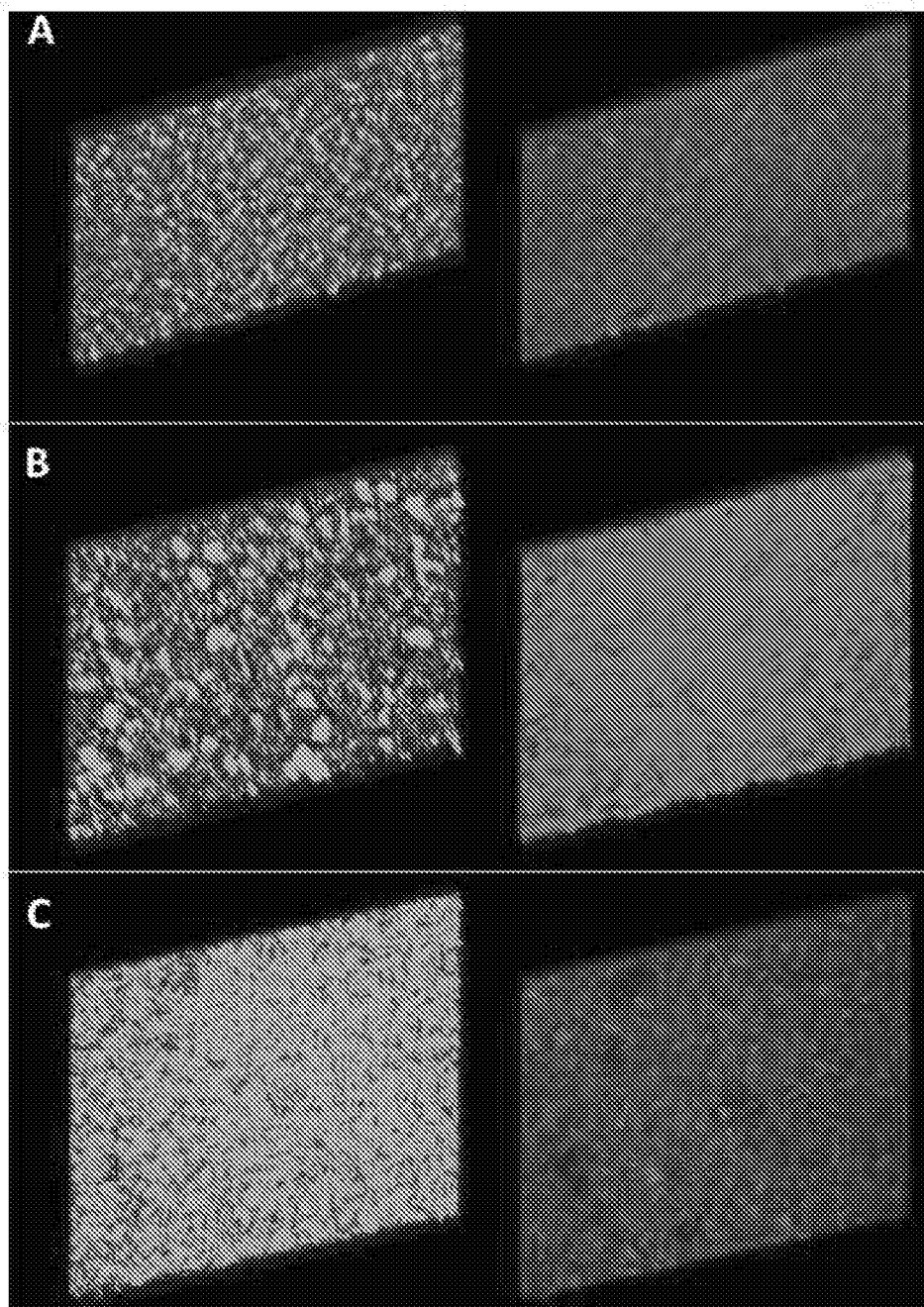
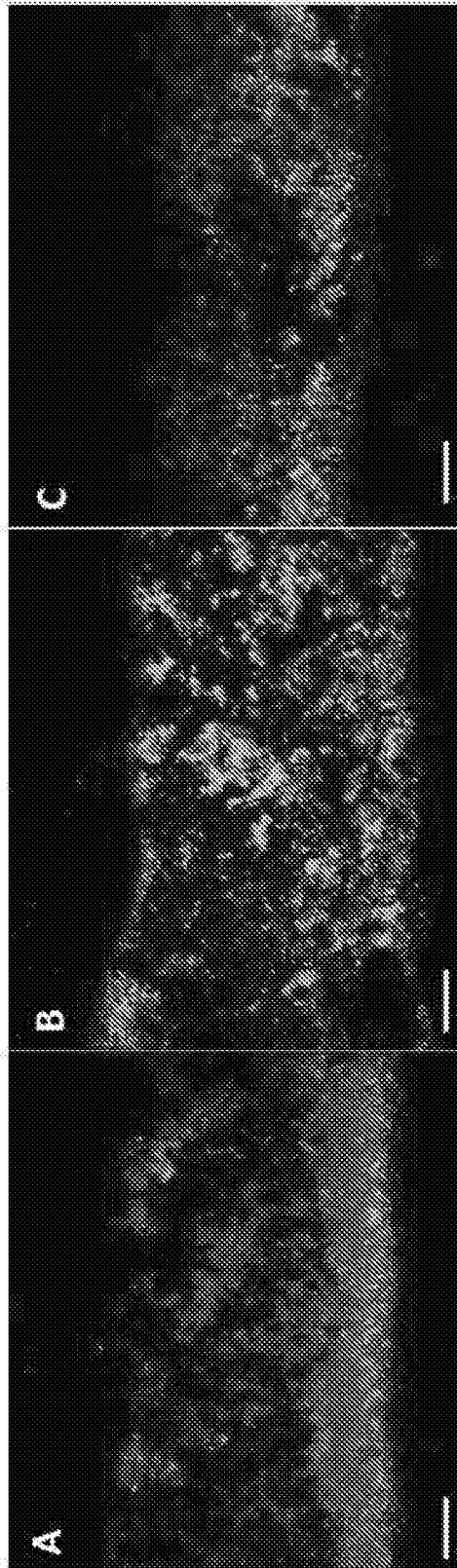


Figure 9.



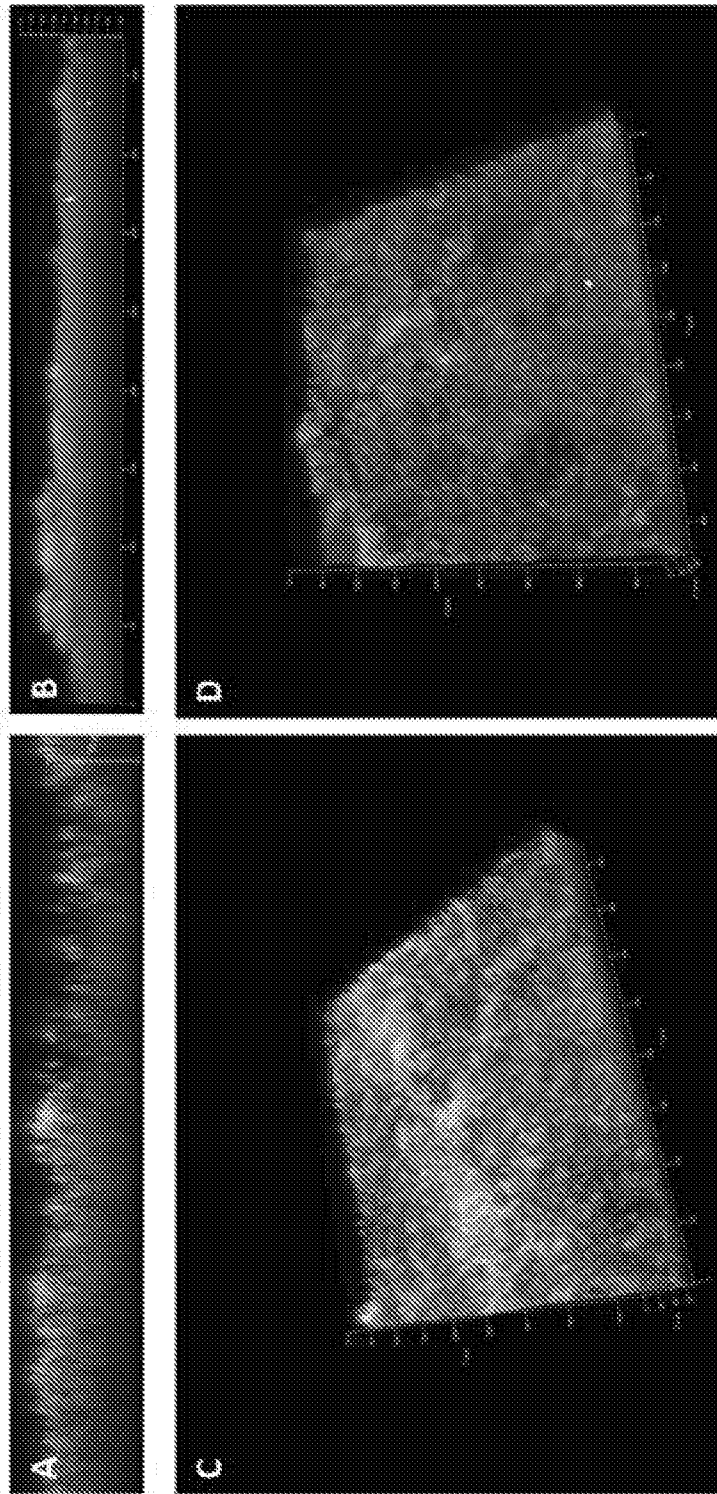
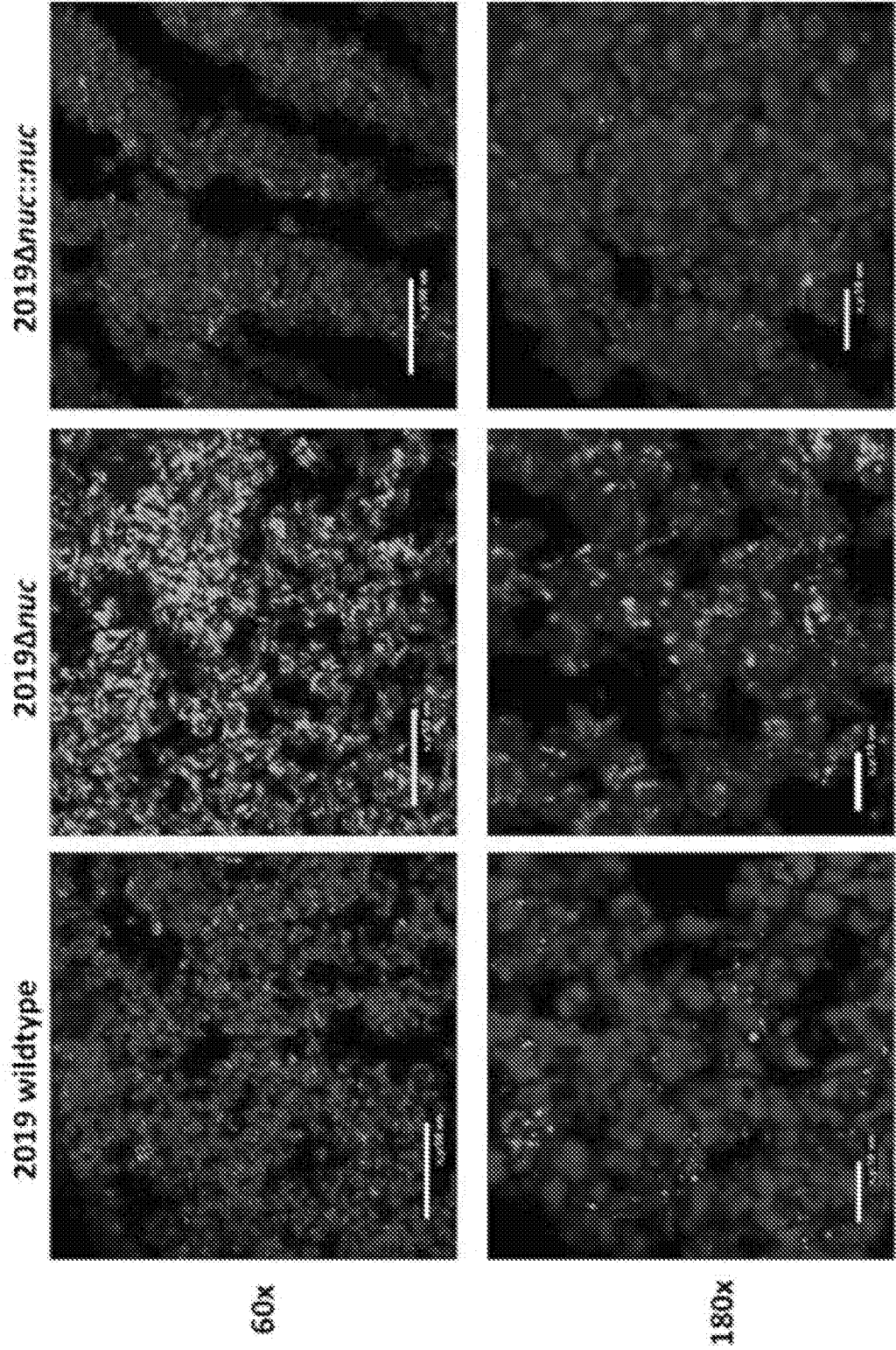


Figure 10.

Figure 11.



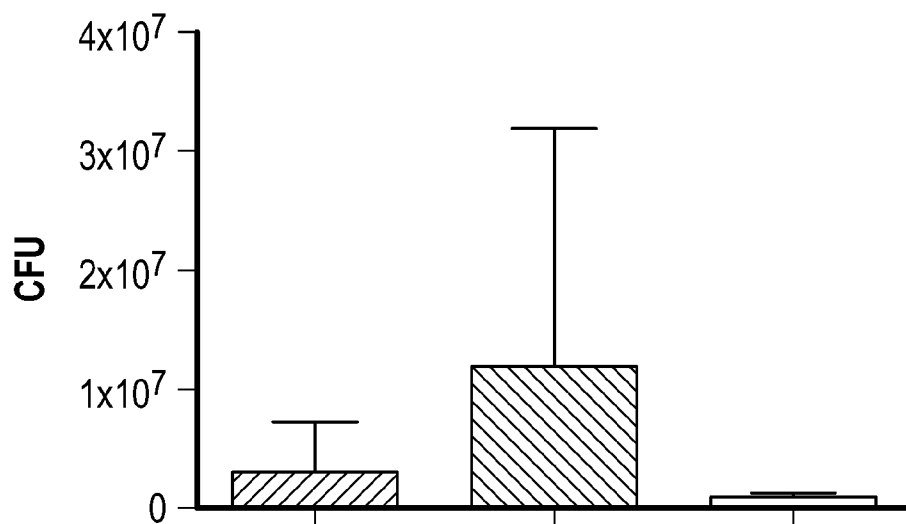


FIG. 12A

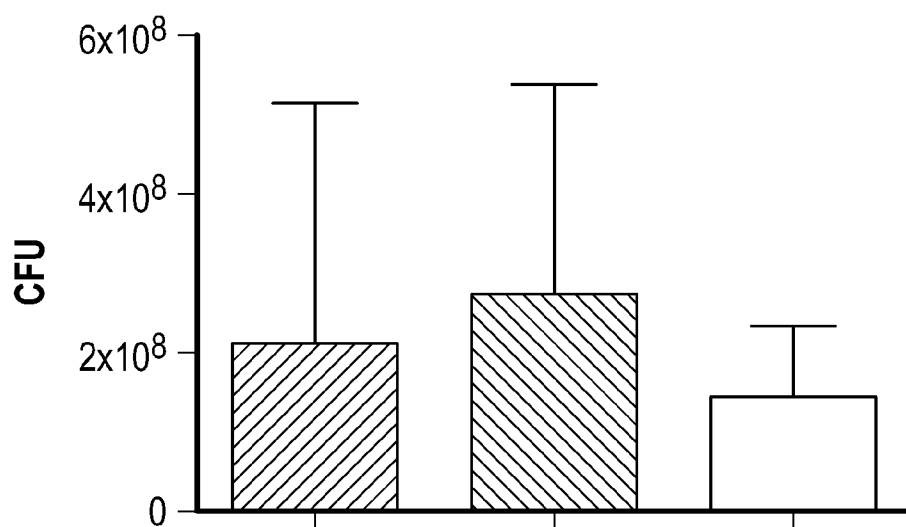


FIG. 12B

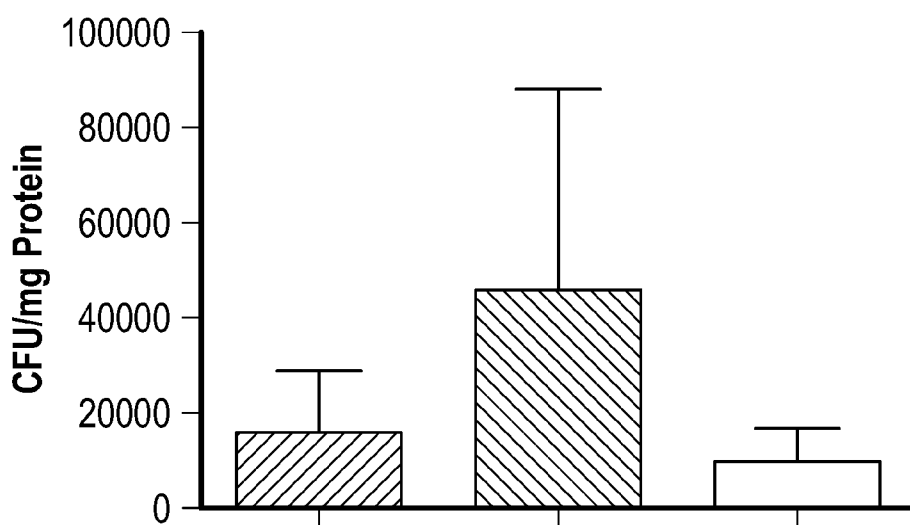


FIG. 12C

12/12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/019290

A. CLASSIFICATION OF SUBJECT MATTER	<i>C12N 9/22 (2006.01)</i> <i>C12N 15/63 (2006.01)</i> <i>A61K 38/46 (2006.01)</i> <i>A61P 11/00 (2006.01)</i>	
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
C12N 9/22, 15/63, A61K 38/46, A61P 11/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
PatSearch (RUTO internal), EMBL, NCBI, PAJ, Espacenet, DWPI, PCT Online, USPTO DP, CIPO (Canada PO), SIPO DB, Freepatentsonline, Protein BLAST, FASTA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	DATABASE GenBank, EDJ92667.1, 11.04.2007 POTTER BARRY V.L. et al. Stereochemical course of DNA hydrolysis by nuclease S1, The Journal of biological chemistry, 1983, Vol.258, no.3, pp.1758-1760	1-3, 14 8-13, 16-22, 28 15
X Y		23-27 8-13
Y	SALILIN K. et al. Adenine Nucleotide Depletion in Human Muscle During Exercise: Causality and Significance of AMP Deamination, Int. J. Sports Med., 1990, 11, pp.S62-S67, especially abstract	28
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 08 June 2016 (08.06.2016)	Date of mailing of the international search report 07 July 2016 (07.07.2016)	
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer U.Berezhnaya Telephone No. 495 531 65 15	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/019290

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NELSON Kristie L. et al. Potential for H-DNA in the Human MUC1 Mucin Gene Promoter, THE JOURNAL OF BIOLOGICAL CHEMISTRY, 1996, Vol. 271, no. 30, pp.18061-18067	16-22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/019290

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☒ on paper or in the form of an image file.
- b. ☒ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
- ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-7
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

Remark on Protest

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