

Nucleases from *Prevotella intermedia* can degrade neutrophil extracellular traps

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SUMMARY

Periodontitis is an inflammatory disease caused by periodontal bacteria in subgingival plaque. These bacteria are able to colonize the periodontal region by evading the host immune response. Neutrophils, the host's first line of defense against infection, use various strategies to kill invading pathogens, including neutrophil extracellular traps (NETs). These are extracellular net-like fibers comprising DNA and antimicrobial components such as histones, LL-37, defensins, myeloperoxidase, and neutrophil elastase from neutrophils that disarm and kill bacteria extracellularly. Bacterial nuclease degrades the NETs to escape NET killing. It has now been shown that extracellular nucleases enable bacteria to evade this host antimicrobial mechanism, leading to increased pathogenicity. Here, we compared the DNA degradation activity of major Gram-negative periodontopathogenic bacteria, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*. We found that *Pr. intermedia* showed the highest DNA degradation activity. A genome search of *Pr. intermedia* revealed the presence of two genes, *nucA* and *nucD*, putatively encoding secreted nucleases, although their enzymatic and biological activities are unknown. We cloned *nucA*- and *nucD*-encoding nucleases from *Pr. intermedia* ATCC 25611 and characterized their gene products. Recombinant NucA and NucD

digested DNA and RNA, which required both Mg²⁺ and Ca²⁺ for optimal activity. In addition, NucA and NucD were able to degrade the DNA matrix comprising NETs.

INTRODUCTION

Pathogenic bacteria are able to colonize the mucosal region by evading the host immune response. Neutrophils are a first line of defense and a central part of the human innate immunity and are recruited to the site of infection by chemokines secreted from macrophages or local cells upon contact with microbial pathogens (Nauseef & Borregaard, 2014). In addition to phagocytic activity, neutrophils possess a novel neutrophil-mediated defense mechanism, termed neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004). NETs are extracellular fibrous structures of decondensed nuclear chromatin associated with an array of antimicrobial compounds, including histones, LL-37, defensins, myeloperoxidase, and neutrophil elastase, that disarm and kill bacteria extracellularly (Brinkmann *et al.*, 2004). They are released by viable neutrophils or following a unique form of programmed cell death known as NETosis, which is dependent on the production of reactive oxygen species and decondensation of nuclear DNA catalyzed by peptidyl arginine deiminase-4 (Li *et al.*, 2010). NET release is likely governed by the magnitude and chronicity of

the stimulus. Currently recognized stimuli for NET release include both Gram-positive and Gram-negative bacteria (Brinkmann *et al.*, 2004), lipopolysaccharide (Clark *et al.*, 2007), fungi such as *Candida albicans* (Byrd *et al.*, 2013), and host-derived inflammatory mediators such as proinflammatory cytokines tumor necrosis factor- α , interleukin-1 β (IL-1 β), and IL-8 (Keshari *et al.*, 2012).

Periodontitis is an inflammatory disease caused by periodontal bacteria in subgingival plaque. It is a chronic, polymicrobial, dysbiotic inflammatory disease of the periodontal tissues and results in decreased tooth support. Several studies have reported the role of NETs in the pathogenesis of periodontitis. Vitkov *et al.* (2009) demonstrated that NETs were abundant in pus exudate harvested from periodontal pockets of patients with chronic periodontitis, and they postulated that NETs may act as a protective shield, preventing bacteria from adhering to and colonizing the gingival epithelium. A NET-like structure was reported to increase in patients with gingivitis compared with healthy controls, suggesting that increased NETs may be a feature of periodontitis (White *et al.*, 2016). Hirschfeld *et al.* (2015) reported that neutrophils are attracted to dental biofilms, in which they are stimulated by microbes to release NETs, suggesting that neutrophils and NETs may be involved in host biofilm control. These studies suggest a role for NETs as a defense mechanism within periodontal pockets.

Nucleases hydrolyze nucleic acids to yield oligonucleotides. Intracellular bacterial nucleases participate in replication, recombination, or DNA repair to maintain the chromosome. In contrast, the presence of extracellular nucleases has been reported in Gram-positive pathogens, including *Staphylococcus aureus* (Berends *et al.*, 2010), *Streptococcus pyogenes* (Sumbly *et al.*, 2005; Buchanan *et al.*, 2006; Chang *et al.*, 2011), *Streptococcus agalactiae* (Derre-Bobillot *et al.*, 2013), *Streptococcus suis* (de Buhr *et al.*, 2014, 2015), *Streptococcus sanguinis* (Morita *et al.*, 2014), and *Streptococcus pneumoniae* (Beiter *et al.*, 2006). Extracellular nucleases produced by these bacteria are reported to degrade NETs, allowing escape from NET killing via degradation of NET scaffold DNA. It has now been shown that extracellular nucleases enable bacteria to evade this host antimicrobial mechanism, leading to increased pathogenicity. Periodontal disease results from polymicrobial synergy among Gram-negative anaerobic periodontal bacteria in

subgingival biofilms. These periodontopathogenic bacteria may colonize the periodontal region via NET degradation. It is reasonable to speculate that, based on data from other diseases involving NET–bacteria interactions, NET degradation may contribute to periodontitis pathogenesis.

Regarding extracellular nucleases of oral bacteria, Morita *et al.* (2014) reported that *S. sanguinis*, an initial colonizer of the tooth surface, has been shown to produce a cell-wall-anchored nuclease termed SWAN. Moreover, Palmer *et al.* (2012) assessed the deoxyribonuclease (DNase) activity of principal bacterial species associated with periodontal disease and found that 27 of the 34 periodontal bacteria exhibited membrane-bound and secreted bacterial DNase activity. In addition, they also revealed differences in DNase expression by these bacteria under specific growth conditions (plate culture versus planktonic). However, the enzymatic properties and biological role of nucleases produced by periodontal bacteria remain unclear.

In this study, we compared the DNA degradation activity of major Gram-negative periodontopathogenic bacteria, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*, using plate culture. In addition, we evaluated NET degradation by nuclease secreted into the culture supernatant. *Prevotella intermedia* demonstrated the highest DNA degradation activity among the Gram-negative periodontopathogenic bacteria tested. Consequently, we cloned genes *nucA* and *nucD*, which encode nucleases in *Pr. intermedia* ATCC 25611, characterized their gene products, and confirmed their ability to degrade NETs.

METHODS

Bacterial strains and culture conditions

All strains were maintained on sheep blood agar plates under anaerobic condition (10% CO₂, 10% H₂, and 80% N₂). *Prevotella intermedia* ATCC 25611 (JCM 12248) was obtained from the Japan Collection of Microorganisms; *Pr. intermedia* ATCC 25611, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586, and *A. actinomycetemcomitans* Y4 were cultured anaerobically at 37°C in brain–heart infusion (BHI) broth supplemented with hemin (5 $\mu\text{g ml}^{-1}$) and

menadione ($1 \mu\text{g ml}^{-1}$). The *S. sanguinis* ATCC 10556 was cultured anaerobically at 37°C in BHI broth. *Escherichia coli* BL21 cells were grown aerobically in Luria–Bertani medium. When required, the medium was supplemented with $50 \mu\text{g ml}^{-1}$ ampicillin.

Evaluation of extracellular DNA degradation activity using an agar plate assay

Extracellular DNA degradation activity of periodontal bacteria was examined using a previously described method (Morita *et al.*, 2014). In brief, a $5\text{-}\mu\text{l}$ aliquot of an overnight culture was spotted onto BHI agar plates containing 2 mg ml^{-1} of salmon sperm DNA, 1 mM MgCl_2 , and 1 mM CaCl_2 (nuclease test agar). The plates were incubated at 37°C for 2 days under anaerobic conditions. For visualization, plates were flooded with 1 M HCl , and the appearance of a clear zone around bacterial growth indicated degradation of DNA.

Preparation of crude nuclease prepared from bacterial culture supernatant

Bacterial culture supernatant was separated by centrifugation at $12,000 \text{ g}$ for 30 min after 24 h of culture. The centrifuged culture supernatant was concentrated using solid ammonium sulfate to yield 80% saturation. The precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer at 10-fold concentration. The crude nuclease preparation was used to determine nuclease activity.

Purification of human neutrophils and NET induction

Neutrophils were isolated from freshly drawn blood of healthy donors as previously described (Morita *et al.*, 2014). Isolated neutrophils were seeded on coverslips (18 mm) coated with poly-L-lysine (0.01% solution for 5 min; Sigma-Aldrich, St Louis, MO) at a concentration of 5×10^5 cells per well in a 12-well plate and incubated for 30 min to allow the cells to attach to the well bottom. For quantification of NET DNA, 1×10^5 cells were seeded in a poly-L-lysine coated 96-well plate and incubated for 30 min to allow the cells to attach to the well bottom. For NET

induction, cells were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) (Wako, Osaka, Japan) and incubated for 2 h.

Quantification and visualization of NET degradation by crude nucleases of oral bacteria

After NET induction, the medium was replaced with nuclease assay buffer containing 20 mM HEPES (pH 7.0), 150 mM NaCl , 1 mM CaCl_2 , and 1 mM MgCl_2 . Then, *P. gingivalis*, *Pr. intermedia*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. sanguinis* crude nucleases were added at a ratio of 1 : 100. For quantification, 96-well plates were washed with phosphate-buffered saline (PBS) after 30-, 60-, 90-, or 180-min incubation. Then, Sytox Orange (Life Technologies, Carlsbad, CA) was added at a final concentration of $0.1 \mu\text{M}$, and, after 10-min incubation, fluorescence was measured (excitation 525 nm , emission $580\text{--}640 \text{ nm}$) using a fluorescence microplate reader (GloMax Multi detection system; Promega Corporation, Madison, WI). Five replicates per treatment were performed. For visualization, after 90-min incubation, coverslips were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Then, the coverslips were stained with DAPI. NETs were observed under a fluorescence microscope (Zeiss Axio Observer.Z1; Carl Zeiss, Jena, Germany).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) nuclease assay

The DNA degradation activity of crude extracellular nuclease preparations of *Pr. intermedia* was analyzed using an in-gel nuclease assay with some modification (Rosenthal & Lacks, 1977). Non-boiled crude nuclease samples were applied to SDS–PAGE using 10% polyacrylamide gels containing 0.2 mg ml^{-1} salmon sperm DNA. After electrophoresis, proteins were renatured by repeated washing in renaturation buffer [50 mM Tris-HCl (pH 7.5) and 0.04% mercaptoethanol], followed by incubation in activation buffer containing 1 mM MgCl_2 and 1 mM CaCl_2 for 3 h at 37°C . To visualize DNA degradation, gels were stained with ethidium bromide and examined under ultraviolet light. Nuclease activity was observed by a lack of staining around protein bands due to DNA degradation.

Biochemical analysis of *Pr. intermedia* extracellular nuclease

To examine the nuclease activity of *Pr. intermedia* crude nuclease, 0.25 µg of λ DNA (Takara Bio, Shiga, Japan) was incubated with 0.5 µg of crude nuclease in reaction buffer containing 50 mM Tris-HCl (pH 7.0), 150 mM NaCl with or without 1 mM CaCl₂, and 1 mM MgCl₂ at 37°C for 10 min. The reaction was stopped with EDTA at a final concentration of 10 mM, followed by electrophoresis in 0.8% agarose gel. The gel was then stained with ethidium bromide and visualized under ultraviolet light.

Cloning, expression, and purification of recombinant nucleases

Prevotella intermedia ATCC 25611 revealed the presence of two genes, *nucA* and *nucD*, putatively encoding secreted nucleases [GenBank Accession numbers LC127052 (*nucA*) and LC127053 (*nucD*)]. Recombinant N-terminally GST-tagged proteins were prepared using the *E. coli* expression vector pGEX6P-1 (GE Healthcare, Chalfont St Giles, UK). Oligonucleotide primers are summarized in the Table S1. The *nucA* gene was amplified by polymerase chain reaction (PCR) with primers rec-nucA-Fw and rec-nucA-Rv and cloned into the *Bam*HI-*Sal*I site of pGEX6P-1; the resulting plasmid was designated as pGEX-nucA. The *nucD* gene was amplified by PCR with primers rec-nucD-Fw and rec-nucD-Rv and cloned into the *Bam*HI-*Eco*RI site of pGEX6P-1; the resulting plasmid was designated as pGEX-nucD. These resultant plasmids were then transformed into *E. coli* BL21 cells and expressed. The recombinant proteins were purified using glutathione-Sepharose 4B medium (GE Healthcare). The fusion protein was then cleaved by PreScission protease, and products were eluted according to the manufacturer's instructions.

Preparation of rNucA-E104G and rNucD-N229G

The plasmids pGEX-nucA and pGEX-nucD were used to prepare recombinant protein variants constructed by mutagenesis. Replacement of amino acids was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA). The nucleotide sequences of the

mutagenic PCR primers and their complementary oligonucleotide primers (rNucA-E104G and rNucD-N229G) are listed in the Table S1. Mutations were confirmed by nucleotide sequencing.

Biochemical analysis of recombinant enzymes

To examine the nuclease activity of recombinant enzymes, 0.25 µg of λ DNA was incubated with 0.1 µg of recombinant enzymes in reaction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl with or without 1 mM CaCl₂, and 1 mM MgCl₂ at 37°C for 10 min. To determine the optimal pH, 0.1 µg of recombinant enzymes was incubated in 50 mM sodium acetate buffer (pH 5.0–6.5) or 50 mM Tris-HCl buffer (pH 6.5–8.0) with 0.25 µg of λ DNA with 1 mM CaCl₂ and 1 mM MgCl₂ at 37°C for 5 min. To determine the recombinant protein cleavage preference of DNA forms, M13mp18 (single-stranded circular DNA), λ DNA (double-stranded linear DNA), and pUC18 (double-stranded circular DNA) were incubated with 0.1 µg of recombinant enzymes. To examine the ribonuclease activity of recombinant enzymes, total RNA purified from the murine macrophage RAW 264.7 cell line with the Qiagen RNeasy Mini Kit (QIAGEN, Hilden, Germany) was incubated with 0.1 µg of recombinant enzymes. The reaction was stopped with EDTA at a final concentration of 10 mM, followed by electrophoresis in 0.8% (for DNA degradation) or 1.5% (for RNA degradation) agarose gels. The gels were then stained with ethidium bromide and visualized under ultraviolet light.

Determination of NET degradation by recombinant enzymes

After NET induction, the medium was replaced with buffer containing 20 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂. Cells were then incubated with *Pr. intermedia* crude nuclease at a final concentration of 1 mg ml⁻¹, recombinant enzymes, and recombinant bovine pancreas DNase I (BP DNase I; Roche Diagnostics, Mannheim, Germany) at a final concentration of 1 µg ml⁻¹. After 1 h of incubation, cells were fixed with 4% paraformaldehyde in PBS for 30 min and washed again with PBS. Cells were permeabilized with PBS containing 0.05% Triton X-100 for 30 min and blocked overnight with 1% bovine serum albumin in PBS. Cells were then

incubated with a rabbit anti-human neutrophil elastase antibody (1 : 1000; Abcam, Cambridge, UK) for 1 h, followed by incubation with Cy3-conjugated AffiniPure donkey anti-rabbit IgG (1 : 1000; Jackson ImmunoResearch, West Grove, PA). Finally, DAPI was added and the slides were analyzed using a fluorescence microscope. To quantify NET degradation, neutrophil elastase released in the reaction buffer in accordance with NET degradation was quantified using the Neutrophil Elastase Activity Assay kit (Cayman, Ann Arbor, MI).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been deposited in the DNA Data Bank of Japan nucleotide sequence database under Accession numbers LC127052 (*nucA*) and LC127053 (*nucD*).

Ethics statement

Human venous blood was obtained from healthy volunteers after obtaining written informed consent according to a protocol approved by the Institutional Review Board of Showa University School of Dentistry.

Statistical analysis

Significant differences between the means of groups were evaluated using Student's *t*-test with *P* values of < 0.05 considered statistically significant.

RESULTS

Extracellular DNA degradation activity of periodontal bacteria

We first examined whether viable periodontal bacteria exhibit extracellular DNA degradation activity. The tested periodontal bacterial strains, *Pr. intermedia*, *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*, were cultured on nuclease test agar for 2 days. Extracellular nuclease diffused from the bacterial colonies to create a zone of DNA hydrolysis. The *S. sanguinis* was used as a positive control (Morita et al., 2014). A clear zone around growing colonies was observed for *P. gingivalis*, *Pr. intermedia*, *F. nucleatum*, and *S. sanguinis*, but no such

zone was seen for *A. actinomycetemcomitans* (Fig. 1). The DNA degradation activity was particularly notable for *Pr. intermedia*, whereas *P. gingivalis* and *F. nucleatum* expressed slight activity. Compared with the clear zone produced by *S. sanguinis*, which secretes a cell-wall-anchored nuclease, the zone produced by *Pr. intermedia* expanded broadly around the colony.

To evaluate the pathogenic role of the DNA degradation activity, crude nucleases prepared from bacterial culture supernatants were used in a NET degradation assay, which can detect extracellular nucleases secreted by bacteria. A time-course NET degradation profile by crude nuclease is shown in Fig. 2(A). The *Pr. intermedia* crude nuclease showed strong NET degradation activity, resulting in about 70% NET degradation 30 min post-incubation (Fig. 2A). When incubated with *P. gingivalis* crude nuclease, NETs decreased by approximately 30% after 90 min and 50% after 180 min post-incubation. Representative images of NET degradation exposed for 90 min with crude nuclease are shown in Fig. 2(B). NETs released from neutrophils were no longer visible following exposure to *Pr. intermedia* crude nuclease, indicating degradation of NET DNA. Following exposure to *P. gingivalis*, NETs were partially degraded. In comparison, exposure to *F. nucleatum*, *A. actinomycetemcomitans*, and *S. sanguinis* crude nucleases did not induce significant NET degradation under similar conditions.

Characterization of *Pr. intermedia* nuclease

To determine the molecular size of secreted *Pr. intermedia* nuclease, crude extracellular nuclease preparations were subjected to the SDS-PAGE nuclease assay. The crude nucleases separated by SDS-PAGE are shown in Fig. 3(A). Two clear bands were detected in the crude nuclease. The electrophoretic pattern indicated that the major molecular size of *Pr. intermedia* nuclease was approximately 35–39 kDa, with a minor molecular size of approximately 42 kDa. Nucleases generally require divalent cations for activity (Campbell & Jackson, 1980). Therefore, the effect of cations on *Pr. intermedia* nuclease activity was evaluated. In the absence of added cations, λ DNA remained almost intact. The addition of Mg^{2+} and Ca^{2+} led to DNA degradation, which was reversed by the addition of chelator EDTA to the

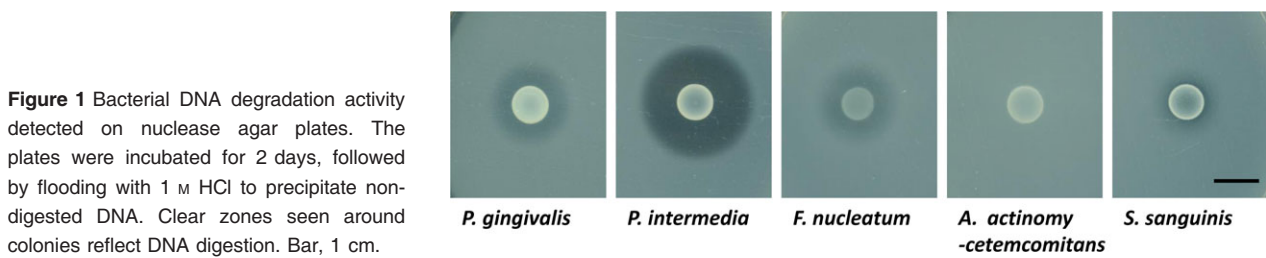


Figure 1 Bacterial DNA degradation activity detected on nuclease agar plates. The plates were incubated for 2 days, followed by flooding with 1 M HCl to precipitate non-digested DNA. Clear zones seen around colonies reflect DNA digestion. Bar, 1 cm.

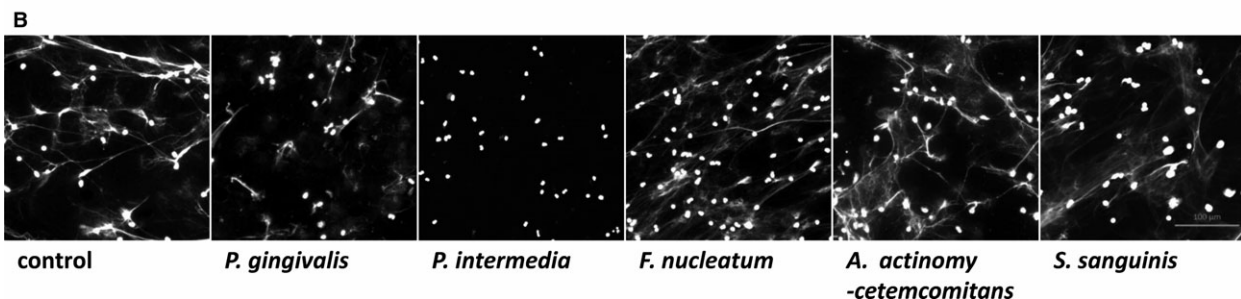
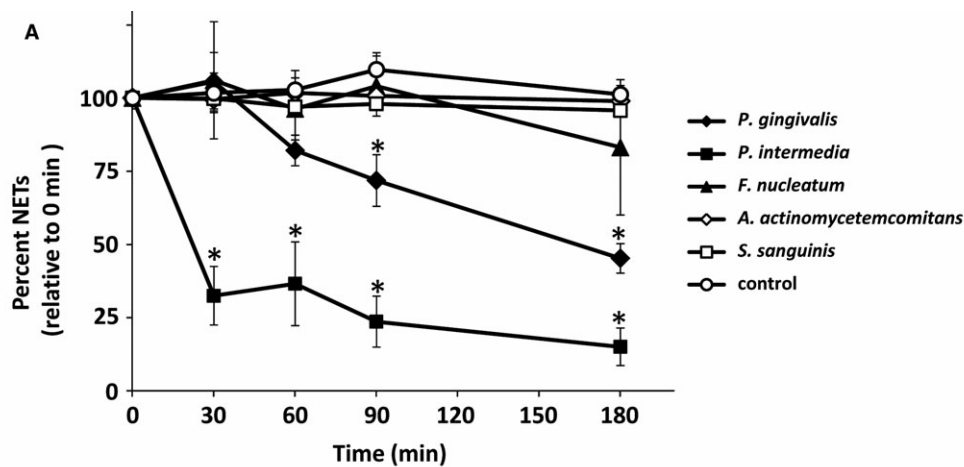


Figure 2 Neutrophil extracellular traps (NET) degradation by crude nuclease. (A) Quantification of NET degradation by staining using the extracellular DNA stain Sytox orange. Data are presented as percent NET levels relative to time-point zero. Controls were incubated with 50 mM Tris-HCl buffer (pH 7.5) rather than crude nuclease. Error bars represent the standard deviation of three independent experiments. Statistical significance ($*P < 0.05$) was determined using Student's *t*-test relative to the control. (B) Representative images of NET degradation by crude nuclease prepared from bacterial culture supernatant. Human neutrophils were stimulated with PMA to release NETs, which were then exposed to bacterial crude nucleases for 90 min.

reaction mixture, indicating that *Pr. intermedia* nuclease activity is cation dependent (Fig. 3B).

Identification of putative nucleases of *Pr. intermedia*

To identify the responsible nucleases of *Pr. intermedia*, we searched for putative secreted or surface-attached nuclease homologs with molecular sizes ranging from 35 to 42 kDa using the reference

Pr. intermedia strain 17 genome sequence. We identified two plausible candidates, *PIN17_A1415* and *PIN17_0064* genes. *PIN17_A1415* is a 1074-bp open reading frame that encodes a potential 357-amino-acid precursor protein with a deduced molecular mass of 41 kDa. The *PIN17_A1415* protein contains a putative signal peptidase cleavage site between positions 29 and 30, as shown using the SIGNALP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). An INTERPROSCAN (<http://www.ebi.ac.uk/interpro/search/se>

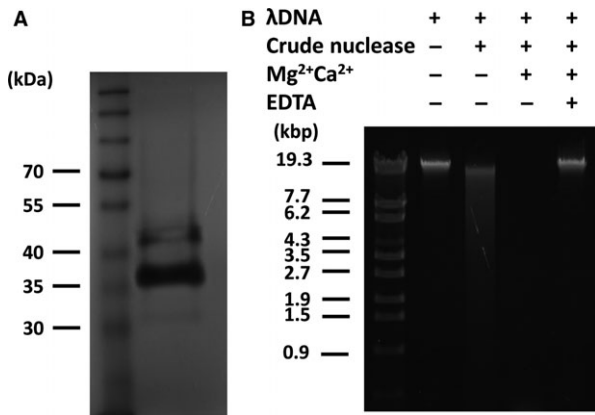


Figure 3 SDS-PAGE nuclease assay and biochemical analysis of *Prevotella intermedia* crude nuclease. (A) *Pr. intermedia* crude nuclease was detected in SDS-PAGE gels containing 0.2 mg ml⁻¹ salmon sperm DNA. After electrophoresis, proteins were re-natured. This was followed by incubation in activation buffer containing 1 mM MgCl₂ and 1 mM CaCl₂ for 3 h at 37°C. To visualize DNA degradation, gels were stained with ethidium bromide and examined under ultraviolet light. Two protein bands were observed to possess nuclease activity. (B) λ DNA was incubated with *Pr. intermedia* crude nuclease with or without 1 mM MgCl₂ and 1 mM CaCl₂ at 37°C for 10 min. Following electrophoresis, DNA was stained with ethidium bromide and visualized under ultraviolet light.

quence-search) and conserved domain sequence search showed significant similarities with a known protein domain, the Endonuclease/Exonuclease/Phosphatase domain (PF03372) at position 82–304 (Fig. 4A). The catalytic site and Mg²⁺-binding site residues of PF03372 are highly conserved in PIN17_A1415. Residues His-134, Asp-168, Asp-212, and His-252 have been shown to play a critical role in the catalytic mechanism of BP DNase I (Jones *et al.*, 1996), and these residues superimpose well with His-192, Asp-231, Asp-285, and His-345, respectively, in PIN17_A1415. The Mg²⁺-binding site in BP DNase I includes Glu-39 and Asp-251, which superimpose well with Glu-104 and Asp-344, respectively, in PIN17_A1415. PIN17_0064 is a 1086-bp open reading frame that encodes a potential 361-amino-acid precursor protein with a deduced molecular mass of 41.6 kDa. The PIN17_0064 protein contains a putative signal peptidase cleavage site between positions 19 and 20, as shown using the SIGNALP 4.1 server. An INTERPROSCAN and conserved domain sequence search showed significant similarities with a known protein domain, the Endonuclease_NS domain (PF01223) at position 128–344 (Fig. 4B). The Mg²⁺-binding site residue of PF01223 is conserved in

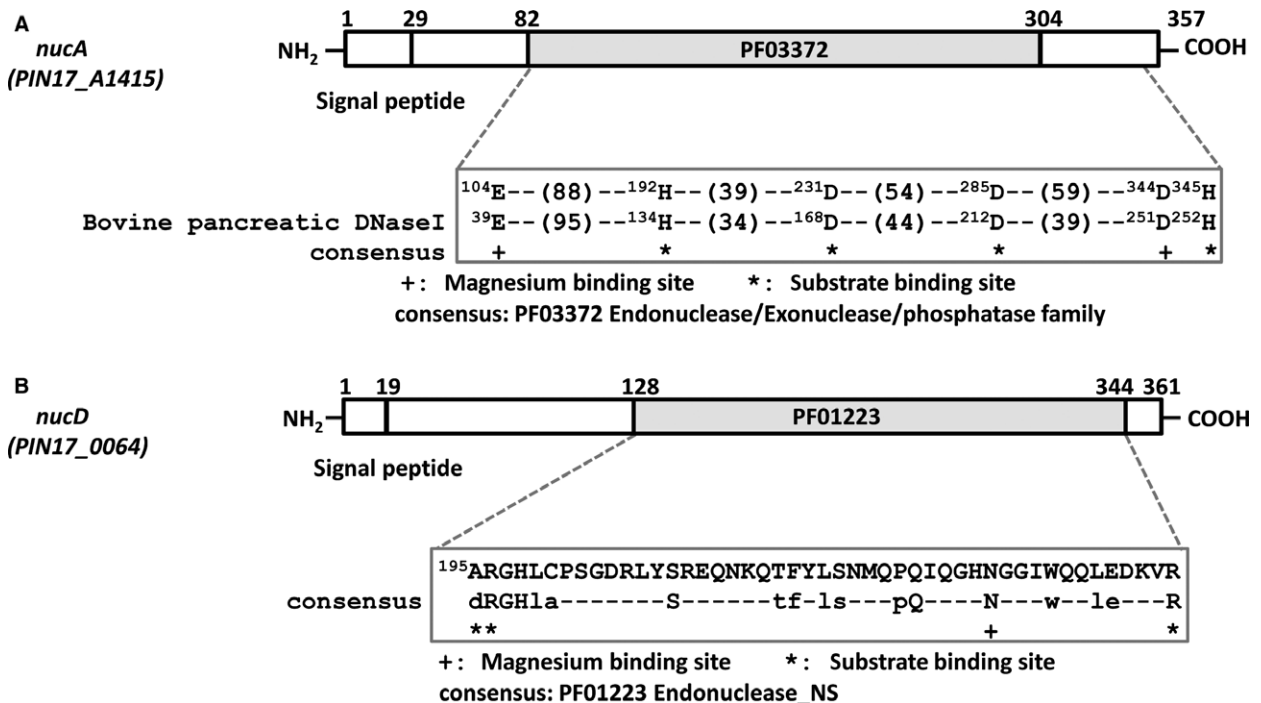


Figure 4 Schematic presentation of NucA and NucD protein domains. (A) NucA and (B) NucD.

PIN17_0064. PF01223 contains the strongly conserved DRGH sequence motif, which is a characteristic active site of members of this family; in the PIN17_0064 sequence, an arginine (Asn-195) replaces aspartic acid (Friedhoff *et al.*, 1994).

Based on these gene sequences from *Pr. intermedia* strain 17, we characterized the DNA sequence from *Pr. intermedia* ATCC 25611. *PIN17_A1415* and *PIN17_0064* were designated as *nucA* and *nucD*, respectively. The molecular sizes of the mature NucA and NucD proteins, excluding the signal peptide, were estimated to be 37.5 and 39.5 kDa, respectively. The mature NucA and NucD protein molecular sizes were congruent with the sizes of the nucleases estimated from the SDS-PAGE nuclease assay. However, the putative homolog of the minor nuclease (42 kDa) could not be predicted from the genome database search.

Characterization of recombinant NucA and NucD enzymatic activity

Full-length recombinant NucA and NucD (rNucA and rNucD) were expressed and purified. Purified rNucA and rNucD each produced a single band in SDS-

PAGE gels (see Fig. S1). To analyze the nuclease activities of rNucA and rNucD, the purified proteins were incubated with double-stranded linear λ DNA. DNA was completely digested by both rNucA and rNucD in the presence of Mg^{2+} and Ca^{2+} (Figs 5A and 6A). The substrate specificity for rNucA was then examined using circular double-stranded DNA (pUC18 vector), circular single-stranded DNA (M13mp18 virion DNA), and total RNA purified from RAW 264.7 cells. rNucA showed activity against all tested substrates (Fig. 5B). Similar substrate specificity experiments were performed using rNucD. DNA substrates were completely digested after 10 min, whereas RNA was not completely digested, indicating a propensity of NucD to efficiently digest DNA substrates (Fig. 6B). The nuclease activities of rNucA and rNucD against λ DNA at varying pH were subsequently analyzed. rNucA showed maximal activity at pH 6.0–7.0 (Fig. 5C), whereas rNucD showed broad nuclease activity (pH 6.0–8.0) (Fig. 6C).

Bioinformatics analysis suggested that several amino acid residues were essential for the nuclease activities of NucA and NucD (Fig. 4), as described above. Therefore, the amino acid residues E104 in NucA and N229 in NucD were converted to glycine

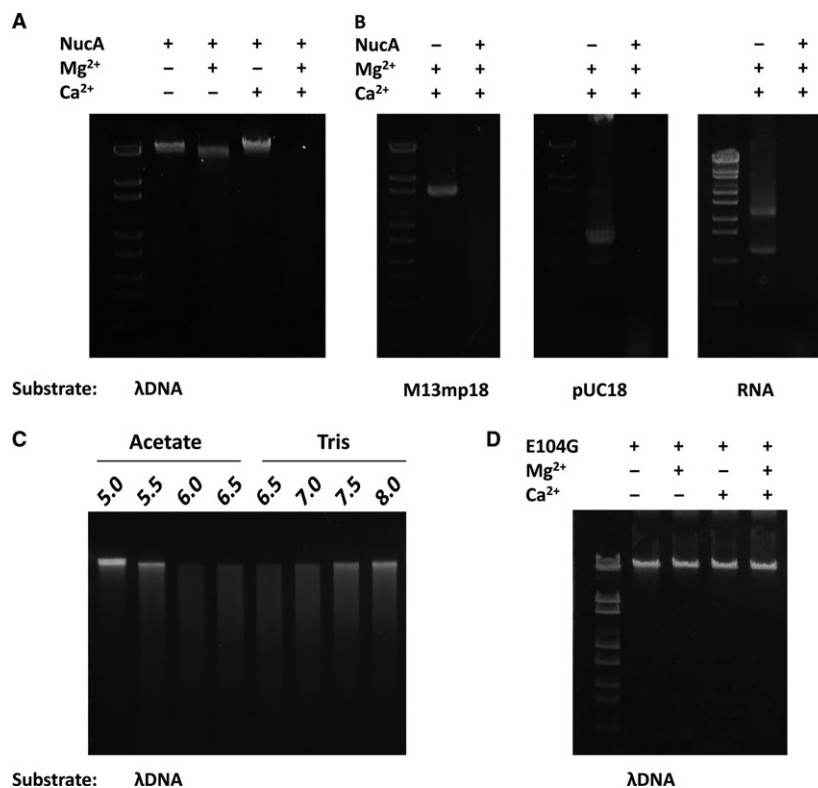


Figure 5 Enzymatic analysis of recombinant NucA and NucA-E104G (A) λ DNA was incubated with rNucA with or without 1 mM $MgCl_2$ and 1 mM $CaCl_2$. (B) The substrate preference of rNucA was examined using M13mp18 (single-stranded circular DNA), pUC18 (double-stranded circular DNA), and total RNA purified from murine macrophages. (C) λ DNA was incubated with rNucA with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at different pH. (D) λ DNA was incubated with rNucA-E104G with or without 1 mM $MgCl_2$ and 1 mM $CaCl_2$.

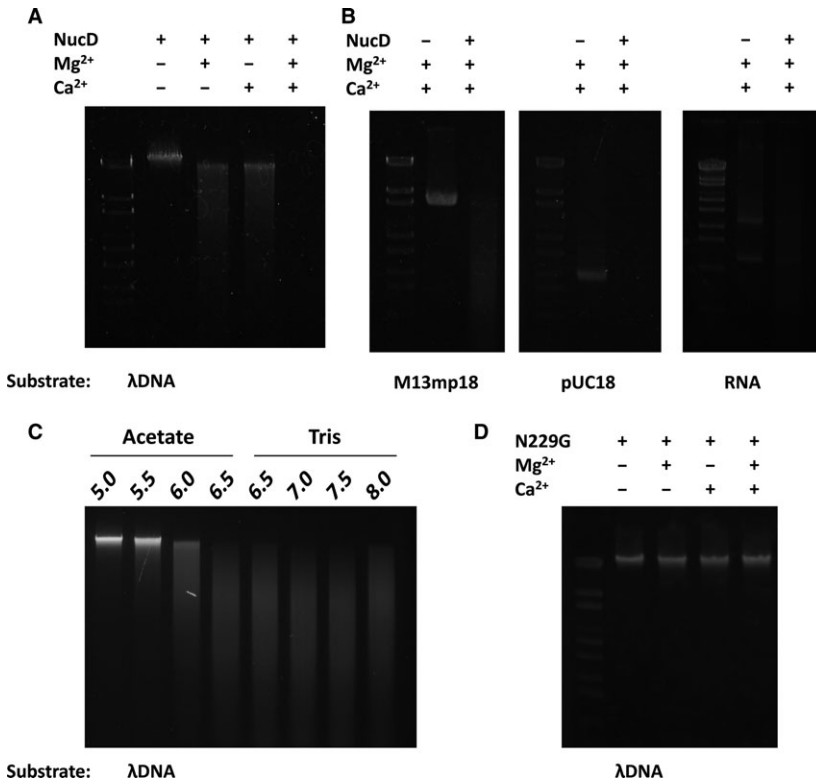


Figure 6 Enzymatic analysis of recombinant NucD and NucD-N229G. (A) λ DNA was incubated with rNucD with or without 1 mM MgCl₂ and 1 mM CaCl₂. (B) The substrate preference of rNucD was examined using M13mp18 (single-stranded circular DNA), pUC18 (double-stranded circular DNA), and total RNA purified from murine macrophages. (C) λ DNA was incubated with rNucD with 1 mM MgCl₂ and 1 mM CaCl₂ at different pH. (D) λ DNA was incubated with rNucD-N229G with or without 1 mM MgCl₂ and 1 mM CaCl₂.

by site-directed mutagenesis, generating rNucA-E104G and rNucD-N229G, respectively. These protein variants were expressed and purified, and their nuclease activities were characterized using λ DNA as the substrate. These proteins completely lost the ability to degrade DNA (Figs 5D and 6D).

Degradation of NETs by rNucA and rNucD

Degradation of NETs by bacterial nucleases has been shown for *S. pyogenes* Sda1 (Buchanan *et al.*, 2006), *S. pneumoniae* EndA (Beiter *et al.*, 2006), and *S. sanguinis* SWAN (Morita *et al.*, 2014) and is believed to be an important immune evasion mechanism. To analyze whether rNucA and rNucD are capable of cleaving NETs, we stimulated NET release by stimulating human neutrophils with PMA (Fig. 7). The addition of either rNucA or rNucD, crude nuclease of *Pr. intermedia*, or BP DNase I as a positive control (Beiter *et al.*, 2006; Chang *et al.*, 2011), to activated neutrophils caused extracellular structures to disappear, indicating that both rNucA and rNucD are capable of degrading NETs. In contrast, addition of rNucA-E104G and rNucD-N229G, inactive nuclease proteins, had no NET degradation effect (Fig. 7A).

We also quantified neutrophil elastase release in the reaction buffer in accordance with NET degradation (Fig. 7B). After incubation with either rNucA or rNucD, the amount of neutrophil elastase significantly increased, indicating NET degradation. In contrast, no significant change was observed when NETs were incubated with rNucA-E104G or rNucD-N229G (Fig. 7A,B). Although added in equal amounts, BP DNase I was more efficient in NET degradation than rNucA or rNucD, which correlates with its higher activity towards double-stranded DNA.

DISCUSSION

While numerous bacterial pathogens produce extracellular nucleases, their importance in virulence was uncovered relatively recently, with the discovery that nucleases can degrade NET scaffold DNA produced by neutrophils (Brinkmann *et al.*, 2004). Nuclease activity in anaerobic bacteria was first reported in 1974 (Porschen & Sonntag, 1974), and Palmer *et al.* (2012) recently reported that many periodontopathogenic bacteria produce extracellular DNases. However, the genetic diversity and functional roles of these nucleases are largely unknown. Here, we showed that

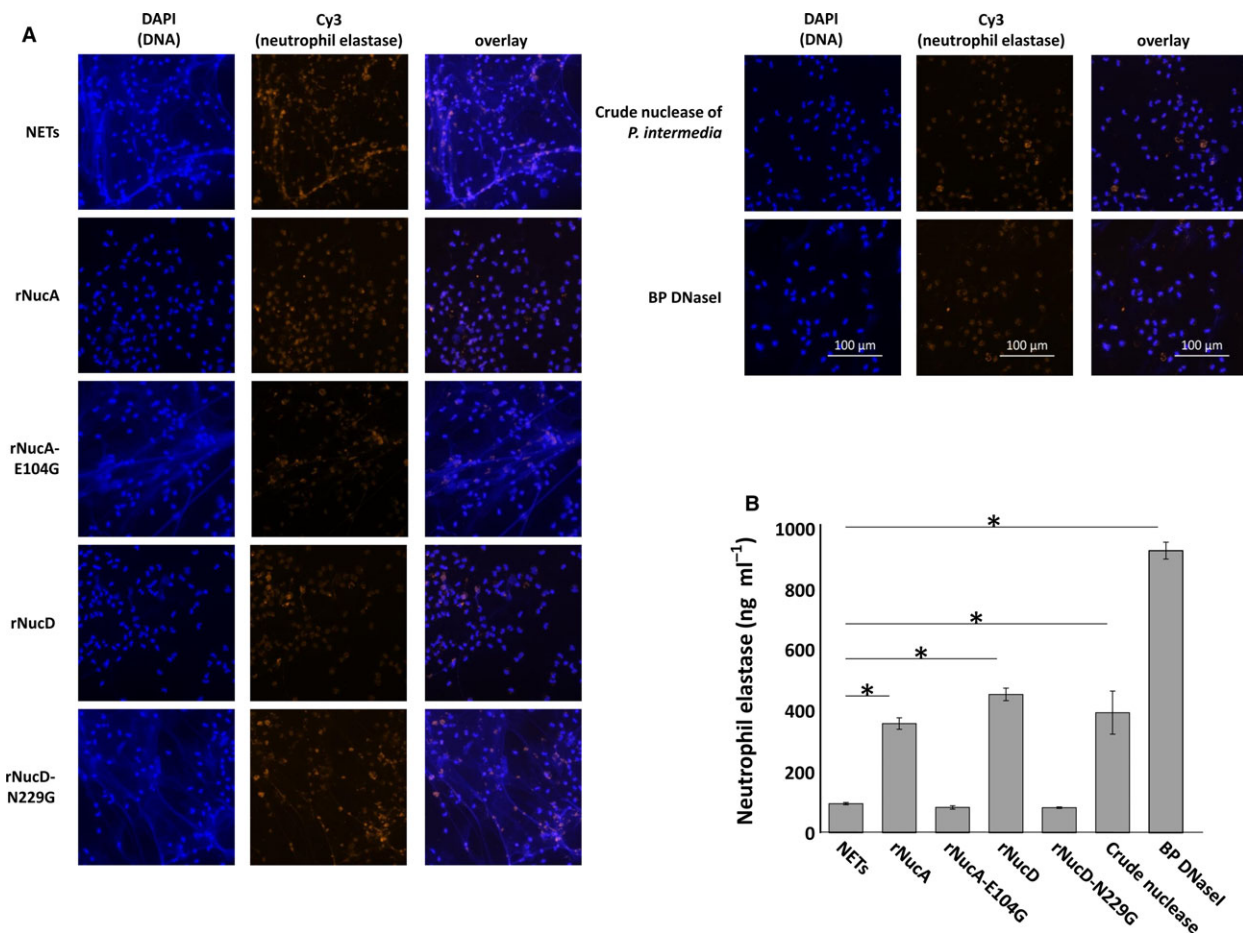


Figure 7 Determination of human neutrophil extracellular traps (NET) degradation. (A) Immunofluorescence microscopy of neutrophils stimulated with PMA. Nuclear DNA was stained with DAPI (blue) and neutrophil elastase was detected with an anti-human neutrophil elastase antibody and Cy3-labeled secondary antibody (orange). PMA-stimulated human neutrophils were incubated with rNucA, rNucA-E104G, rNucD, rNucD-N229G, or *Prevotella intermedia* crude nuclease. Recombinant bovine pancreas DNase I (BP DNase I) was used as a positive control. (B) Quantification of NET release. Neutrophil elastase released into the reaction buffer in accordance with NET degradation was quantified. Error bars represent the standard deviation of three independent experiments. Statistical significance ($*P < 0.05$) was determined by Student's *t*-test.

Pr. intermedia ATCC 25611 demonstrated the highest DNA degradation activity among Gram-negative periodontopathogenic bacteria tested. In addition, we identified NucA and NucD as nucleases in *Pr. intermedia*, characterized their gene products, and confirmed their ability to degrade NETs.

The initial aim of this study was to characterize the DNA degradation activity of Gram-negative periodontopathogenic bacteria. Therefore, we evaluated the ability of Gram-negative periodontopathogenic bacteria to hydrolyze DNA using nuclease test agar. As shown in Fig. 1, compared with the clear zone produced by *S. sanguinis*, which secretes a cell wall-anchored nuclease (Morita *et al.*, 2014), the clear zone produced by *Pr. intermedia* clear zone was

broad, suggesting that *Pr. intermedia* nuclease is more active. *Prevotella intermedia* nuclease may aid in bacterial infiltration into the periodontal tissues, evoking a more widespread inflammatory response. In addition, the dental biofilm is composed of bacteria and an extracellular matrix. The matrix contains extracellular DNA derived from bacteria or host cells, playing an important role in biofilm structural integrity and nutrient storage (Jakubovics & Burgess, 2015). Nuclease may be used to degrade the biofilm matrix, so nuclease-producing oral bacteria could potentially use extracellular DNA as a source of phosphate, nitrogen, and carbon. However, it remains unclear whether bacterial nucleases in biofilms play a role in biofilm integrity.

We then demonstrated the potential role of these bacterial nucleases to degrade NETs, thereby indicating their potential for evading this innate immune defense system. The NET degradation assay using crude nuclease prepared from bacterial culture supernatant is capable of detecting nuclease secreted into the extracellular space by bacteria. Bacterial growth phase has been reported to affect the expression of extracellular nucleases. The production of nuclease by group A streptococcus is reported to increase in the early stationary phase (Sumbly *et al.*, 2005). Therefore, we measured the nuclease activity approximately in the late logarithmic growth phase. As shown in Fig. 2A, B, NET DNA was almost completely degraded by *Pr. intermedia* crude nuclease and partially degraded by *P. gingivalis* crude nuclease. The presence of DNA degradation activity in the culture supernatant suggested that nucleases of these bacteria are secretory enzymes. In addition, these data suggested that nuclease expression of both *P. gingivalis* and *Pr. intermedia* is not induced, but constantly expressed.

We further showed that *P. gingivalis* also degrades NETs through secreted extracellular nucleases. Palmer *et al.* (2012) showed that among six different strains of *P. gingivalis*, strain ATCC 33277 was the highest DNase producer on nuclease test agar plates, although it exhibited no detectable DNA degradation activity in the culture supernatant. In the present study, NET scaffold DNA was partially degraded by the culture supernatant of the same strain of *P. gingivalis*. We observed DNase activity using *P. gingivalis* cultured between the late logarithmic growth phase and early stationary growth phase. Because the DNase activity of *P. gingivalis* culture supernatant was not observed after 6 days of culture, we speculate that *P. gingivalis* nuclease may be easily inactivated by a bacterial protease, such as gingipains.

NETs have been identified as an important innate immune defense system. Several studies have shown that extracellular nuclease of pathogenic bacteria facilitates escape from NET killing through degradation of NET scaffold DNA, thereby allowing increased virulence. Because NETs have been identified in the gingival epithelium (Vitkov *et al.*, 2009), it is likely that nucleases of periodontopathogenic bacteria may contribute to their pathogenicity.

The enzymatic properties of nucleases produced by periodontal bacteria remain unclear. This study revealed that *Pr. intermedia* demonstrated the highest

DNA degradation activity among bacterial species tested. Therefore, we focused on *Pr. intermedia* nucleases, characterized their gene products, and confirmed their ability to degrade NETs.

In the present study, we found two plausible candidates, *PIN17_A1415* and *PIN17_0064* genes, which we designated *nucA* and *nucD*, respectively. NucA is homologous to SsnA of *S. suis* (de Buhr *et al.*, 2014), SWAN of *S. sanguinis* (Morita *et al.*, 2014), and SpnA of *S. pyogenes* (Sumbly *et al.*, 2005), while NucD is homologous to EndA of *S. pneumoniae* (Beiter *et al.*, 2006). Streptococcal nucleases typically possess a C-terminal cell-wall sorting signal and are anchored to the cell wall. The secretion and attachment to the cell surface of certain proteins in *Pr. intermedia* is dependent on a C-terminal domain (Veith *et al.*, 2013). Neither NucA nor NucD possessed such a C-terminal domain; therefore, they were predicted to be extracellular enzymes.

The biochemical properties of NucA and NucD were studied using full-length recombinant proteins. Biochemical analysis revealed that divalent cations, such as Mg²⁺ and Ca²⁺, were required for optimal enzymatic activity. The specificity and preference of the DNA substrate were examined using single-stranded circular DNA, double-stranded linear DNA, and double-stranded circular DNA. We also examined whether rNucA and rNucD could digest RNA. Consequently, rNucA and rNucD showed DNA and RNA degradation activity, confirming that rNucA and rNucD are nucleases that recognize a broad range of nucleic acid substrates. rNucA and rNucD also exhibited a broad pH range (pH 5.5–8.0), with maximal activity at pH 6.0–7.0. Hence, these nucleases are active in the gingival region.

Secreted streptococcal nucleases have been shown to digest the DNA scaffold of NETs, so we examined whether rNucA or rNucD digests NET DNA. We stimulated NET release by stimulating neutrophils with PMA. The extracellular fibrous structures positively stained for DNA (blue) and neutrophil elastase (orange). In contrast, such staining was not seen in NETs with rNucA and rNucD. Therefore, it is likely that rNucA and rNucD digested extracellular DNA released from activated neutrophils.

In summary, the present study demonstrated that the oral periodontopathogenic bacterium *Pr. intermedia* produces two nucleases, termed NucA and NucD. Biochemical analysis revealed that both nucleases

required Mg²⁺ and Ca²⁺ for nuclease activity and contribute to NET degradation. Periodontitis is an inflammatory disease caused by periodontal bacteria in subgingival plaque. These bacteria are able to colonize the periodontal region by evasion of the host immune response. NETs have been identified in the gingival epithelium and are thought to be the first line of defense against periodontal bacteria. Our results suggested that nuclease-producing periodontopathogenic bacteria evade the innate defense mechanism of NETs by their nuclease activity. NET degradation would result in bacterial infiltration into the periodontal region, evoking a more widespread inflammatory response and stimulating chronic inflammation. Nuclease activity may therefore play an important role in the survival and successful colonization of periodontal bacteria in the gingival region. In addition, periodontal bacteria exist within a biofilm *in vivo*; therefore, nuclease is likely to contribute to a mutually beneficial environment whereby other non-nuclease-producing bacteria are afforded protection from NETs. Moreover, nuclease expression is likely to confer NET-evasion ability to expressing bacteria, which may also be advantageous to the bacterial community. It is therefore conceivable that periodontopathogenic biofilms evade the bacterial-killing mechanism of NETs, stimulating chronic inflammation during periodontitis. The functional role of *Pr. intermedia* nucleases in the progression of periodontitis will be examined in a future study.

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