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Extracellular deoxyribonuclease production by periodontal bacteria

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Background and Objective: Whilst certain bacteria have long been known to secrete extracellular deoxyribonuclease (DNase), the purpose in microbial physiology was unclear. Recently, however, this enzyme has been demonstrated to confer enhanced virulence, enabling bacteria to evade the host's immune defence of extruded DNA/chromatin filaments, termed neutrophil extracellular traps (NETs). As NETs have recently been identified in infected periodontal tissue, the aim of this study was to screen periodontal bacteria for extracellular DNase activity.

Material and Methods: To determine whether DNase activity was membrane bound or secreted, 34 periodontal bacteria were cultured in broth and on agar plates. Pelleted bacteria and supernatants from broth cultures were analysed for their ability to degrade DNA, with relative activity levels determined using an agarose gel electrophoresis assay. Following culture on DNA-supplemented agar, expression was determined by the presence of a zone of hydrolysis and DNase activity related to colony size.

Results: Twenty-seven bacteria, including red and orange complex members *Porphyromonas gingivalis*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia*, *Streptococcus constellatus*, *Campylobacter rectus* and *Prevotella nigrescens*, were observed to express extracellular DNase activity. Differences in DNase activity were noted, however, when bacteria were assayed in different culture states. Analysis of the activity of secreted DNase from bacterial broth cultures confirmed their ability to degrade NETs.

Conclusion: The present study demonstrates, for the first time, that DNase activity is a relatively common property of bacteria associated with advanced periodontal disease. Further work is required to determine the importance of this bacterial DNase activity in the pathogenesis of periodontitis.

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Deoxyribonucleases (DNases) are enzymes that hydrolyse nucleic acids to yield oligonucleotides, and bacterial extracellular DNase activity has been reported within the literature for several decades, with studies focusing in particular on secretion by haemolytic streptococci (1,2). Interestingly, work by Porschen & Sonntag (3) reported that a significant number of gram-negative anaerobes (19 of the 57 strains tested), including the periodontal bacteria *Fusobacterium nucleatum*, also exhibited extracellular DNase activity. An array of isoforms of bacterial DNases exist, which work at a range of pHs, exhibit varying specificities for DNA and even RNA, and display diverse cleavage capabilities, yielding differently sized oligonucleotide products. Indeed, in group A streptococci,

on which the majority of the work on extracellular bacterial DNases has concentrated, four enzymes (A, B, C and D) are described, which are distinguished by their distinct antigenicity and electrophoretic mobility (4,5).

Whilst extracellular DNase expression is a distinguishing characteristic of certain bacteria, such as *Staphylococcus aureus*, the significance and purpose of DNase production by bacteria has thus far remained largely unclear. Proposed physiological roles for bacterial DNase production include liberation of nucleotides that can subsequently be used to confer growth advantage (6). Other studies have demonstrated that bacterial DNases can reduce the viscosity of purulent exudate (7) and hence potentially enable bacterial dissemination within the tissue and throughout the host (8,9). More recently, however, a re-emergence of research into DNase production has arisen following the discovery of neutrophil extracellular traps (NETs), which are extruded DNA filaments that immobilize and kill bacteria. Indeed it has now been shown that the expression of extracellular DNase enables bacteria to evade this important host antimicrobial mechanism, leading to increased pathogenicity (10,11).

The periodontal disease state is characterized by the presence of a high proportion of neutrophils within the inflammatory exudates that follow microbial colonization (12). Recently, a potential role for NETs in periodontal disease pathogenesis was reported (13). We therefore hypothesize that certain periodontal pathogens may exhibit significant DNase activity, which may co-operatively contribute to biofilm pathogenicity. This study therefore aimed to determine, for the first time, the prevalence of extracellular DNase expression in 34 of the principal bacterial species associated with periodontal health and disease (14).

Material and methods

Bacterial strains

All periodontal organisms were originally purchased lyophilized from the American Type Culture Collection (ATCC, Rockville, MD, USA). Once reconstituted and grown on agar plates, samples of each bacteria were stored at −80°C in tryptone soya broth (TSB; Oxoid, Cambridge, UK) supplemented with 5% dimethyl sulphoxide. The 34 bacterial stains were grouped into the microbial colour complexes, as described by Socransky *et al*. (14).

- 'Red' complex: *Porphyromonas gingivalis* ATCC 33277 and *Tannerella forsythia* ATCC 43037.
- 'Orange' complex: *Fusobacterium nucleatum* ssp. *polymorphum* ATCC 10953, *Fusobacterium nucleatum* ssp. *nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611, *Streptococcus constellatus* ATCC 27823, *Parvimonas micra* ATCC 33270, *Campylobacter rectus* ATCC 33238, *Porphyromonas nigrescens* ATCC 33563, *Campylobacter showae* ATCC 51146 and *Campylobacter gracilis* ATCC 33236.
- 'Yellow' complex: *Streptococcus sanguis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, *Streptococcus intermedius* ATCC 27335, *Streptococcus oralis* ATCC 35037 and *Streptococcus mitis* ATCC 49456.
- 'Green' complex: *Capnocytopha sputigena* ATCC 33612, *Aggregatibacter actinomycetemcomitans* serotype a ATCC 29523 and *Capnocytophaga gingivalis* ATCC 33624.
- 'Purple' complex: *Veillonella parvula* ATCC 10790, *Actinomyces odontolyticus* ATCC 17929 and *Actinomyces oris* ATCC 43146.
- 'Noncomplexed' bacteria: *Propionibacterium acnes* ATCC 11827 and 11828, *Actinomyces naeslundii* ATCC 12104, *Leptotrichia buccalis* ATCC 14201, *Neisseria mucosa* ATCC 19696, *Streptococcus mutans* ATCC 25175, *Prevotella melaninogenica* ATCC 25845, *Gemella morbillorum* ATCC 27824, *Streptococcus anginosus* ATCC 33397, *Prevotella denticola* ATCC 35308, *Actinomyces israelii* ATCC 12102 and *Aggregatibacter actinomycetemcomitans* serotype b ATCC 43718.

Culture techniques

All strains were cultured on plates and within broth according to previously described methodologies (15). Each strain was maintained on tryptone soya agar (TSA; Oxoid) with 5% horse blood (Oxoid) in anaerobic conditions (MiniMacs anaerobic chamber; Don Whitley Scientific, Shipley, UK; 80% nitrogen, 10% carbon dioxide and 10% hydrogen) at 37°C, with the exception of *P. gingivalis* and *T. forsythia*, which were cultured on blood agar plates with the addition of hemin (5 μg/mL), *N*-acetylmuramic acid (0.1 mg/mL) and vitamin K (0.5 mg/mL). For broth culture of bacteria, TSB was prepared according to the manufacturer's instructions. Enriched TSB was prepared by the addition of glucose (1 mg/mL), hemin (5 μg/mL) and either vitamin K (0.3 μg/mL) for *P. gingivalis* or *N*-acetylmuramic acid (10 μg/mL) for *T. forsythia*. Ten millilitres of sterile broth was inoculated from plate cultures and incubated anaerobically for 6 d with agitation. Although a large panel of bacteria were used, each with varying rates of growth, this duration of incubation allowed growth to reach the stationary phase, a time point at which DNase activity has been reported to be maximal (6). Molecular, biochemical and morphological analysis of each strain was also employed to confirm identity and culture purity where appropriate (data not shown). Additionally, *Staphylococcus aureus* ATCC 9144 was grown aerobically on TSA without horse blood and used as a positive control for DNase assays.

Determination of DNase activity using an agar plate assay

DNase test agar plates (Sigma, Poole, UK) consisting of TSA supplemented with mammalian DNA (2 mg/mL) were prepared according to the manufacturer's instructions. For the assay of *P. gingivalis* and *T. forsythia*, agar was supplemented with hemin, *N*-acetylmuramic acid and vitamin K. Plates were spot inoculated prior to anaerobic incubation for 6 d to allow sufficient growth of all bacteria, each of which had differing rates of growth. Plates were subsequently flooded with 0.05% Toluidine Blue O (Hopkin and Williams, Chadwell

Heath, UK) for 3 min and rinsed. The DNase activity was visualized as zones of purple staining, demonstrating hydrolysed DNA surrounding bacterial colonies. Images of the DNase test plates were captured using a FinePix digital camera (Fujifilm, Bedford, UK) and binarized using ImageJ software (http://rsb-web.nih. gov/ij/; Fig. 1A). A 1 cm^2 black opaque square was included on all plates to enable determination of area from all images. Subsequent analysis enabled the area of DNA hydrolysis to be calculated and normalized to the area of bacterial colony growth.

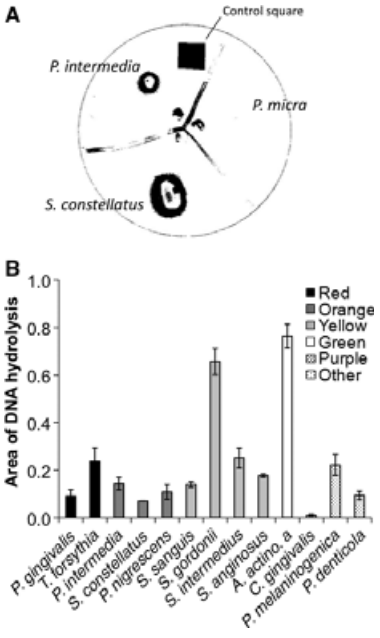

Fig. 1. Bacterial DNase activity detected after 6 d growth on DNase test agar plates. (A) Example binarized image of Toluidine Blue O-stained zones of DNA hydrolysis around colonies for three periodontal bacteria that exhibit different relative levels of DNase activity. Control square represents 1 cm^2. Delineation of the *P. micra* colony is not visible as no zone of DNA hydrolysis was present. (B) Bacteria are grouped according to colour-coded microbial complexes associated with periodontal disease (14), and only bacteria demonstrating positive activity are presented. The area of DNA hydrolysis by secreted DNases (in millimetres squared) was normalized according to the size of bacterial colony. Results are the means of three independent experiments ± SEM.

DNase activity assay by agarose gel electrophoresis

Day 6 bacterial broth cultures were pelleted by centrifugation at 1000 *g* and 4°C for 40 min. The supernatant was filter sterilized through a 0.22 μm cellulose acetate membrane (Corning, Lowell, MA, USA). The bacterial pellet was rinsed using sterile phosphate-buffered saline prior to suspension in 1 mL phosphate-buffered saline and estimation of bacterial density by spectrophotometry at 600 nm (Jenway 6300; Bibby Scientific Ltd, Stone, UK; 16). Using previously determined values for the density of bacterial species based on absorbance at 600 nm (Forsyth Institute, Boston, MA, USA), the bacterial pellet was adjusted to a concentration of 1×10^9 cells/mL and the bacterial supernatant was diluted by the same factor. Based on the method previously described by Sumby *et al.* (9), 10 μL of each sample was incubated with calf thymus DNA (0.4 μg/μL; Sigma) and DNase reaction buffer (New England Biolabs, Ipswich, MA, USA) in a total volume of 50 μL at 37°C for 2 h. A 2 μL sample of the reaction mixture was separated on a 0.8% agarose gel, stained with 0.5 mg/mL ethidium bromide and illuminated under UV light (G:box; SynGene, Cambridge, UK) to visualize the extent of DNA degradation (17,18). An image was captured using GeneSnap software (SynGene), and ImageJ software (http://rsbweb.nih.gov/ij/) was used to determine the migration distance of the degraded DNA front. A range of bovine pancreatic DNase standards (0–500 Kunitz/mL; Sigma) were also assayed to enable estimation of DNase concentration in each of the bacterial samples. The minimal detection limit was 8 Kunitz/L for samples in phosphate-buffered saline, i.e. the bacterial pellet, and 1 Kunitz/L for samples in TSB, i.e. supernatant samples. These were the lowest concentrations to produce a visible and quantifiable shift in the migration distance of the DNA during agarose gel electrophoresis.

Neutrophil extracellular trap degradation analysis

Human neutrophils were isolated from venous blood by discontinuous Percoll density gradient followed by ammonium chloride lysis of red blood cells as previously described (19). Subsequently, 1×10^5 neutrophils suspended in 500 μL RPMI were seeded into wells of a 24-well plate (Corning) and stimulated with 25 nM phorbol myristate acetate for 3 h at 37°C (20). The NETs were stained using 1 μM SYTOX green (Invitrogen, Paisley, UK) prior to addition of 250 μL of bacterial supernatant per well for 15 min at room temperature (11). The NETs and cells were observed under a fluorescence microscope (Nikon Eclipse TE300, Kingston upon Thames, UK) and images captured using a CoolPix digital camera (Nikon).

Results

A panel of 34 periodontal bacteria were screened for extracellular DNase expression using two distinct methodologies. These approaches aimed to identify relative levels of bacterial DNase activity in plate and planktonic culture and also to determine whether activity was membrane associated or secreted. Assay of activity using DNase test agar plates demonstrated that 13 of the 34 bacteria analysed exhibited DNase activity, which diffused from the bacterial colony to create a zone of DNA hydrolysis (Fig. 1). Subsequently, analysis using the DNA agarose gel assay was employed using bacteria grown in broth culture and enabled membrane-bound activity within the bacterial pellet to be assayed separately from that of secreted activity present in the supernatant. Nineteen bacterial pellets and 15 culture supernatants of the 34 strains tested exhibited DNase activity (Fig. 2). All results regarding assay of DNase activity are summarized in Table 1. DNase expression appeared to be a relatively common trait amongst bacteria classified into the orange and yellow colour complexes. In addition,

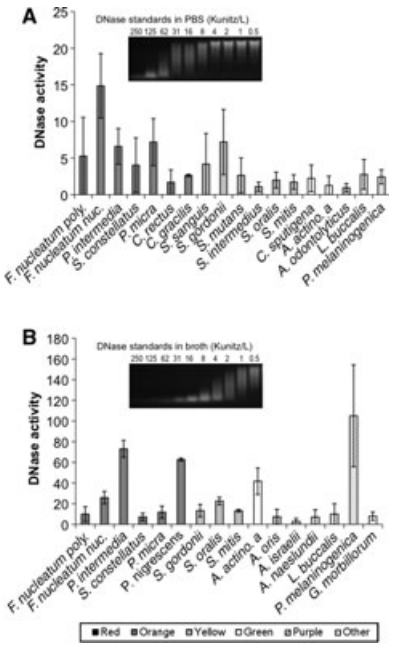

Fig. 2. Agarose gel assay of DNase activity. (A) Membrane-bound DNase activity detected by assay of bacterial pellets. (B) Secreted DNase activity detected by assay of bacterial supernatants. The DNase concentration was estimated by comparison with bovine pancreatic DNase standards (example of migration on gel shown in inset) and expressed in Kunitz per bacteria to allow normalization for bacterial growth. Bacteria are grouped according to microbial complexes associated with periodontal disease (14), and only DNase-positive bacteria are presented. Results are the means of three independent experiments ± SEM.

Table 1. Comparison of bacterial DNase activity in a panel of periodontal bacteria by two assay methods

ATCC number	Bacterial species	Plate-based assay	Gel-based assay	
			Pellet	Supernatant
Red complex				
33277	*P. gingivalis*	+	–	–
43037	*T. forsythia*	+	–	–
Orange complex				
10953	*F. nucleatum poly.*	–	+ +	+
25586	*F. nucleatum nuc.*	–	+ +	+ +
25611	*P. intermedia*	+	+ +	+ + +
27823	*S. constellatus*	+	+ +	+
33270	*P. micra*	–	+ +	+ +
33238	*C. rectus*	–	+	–
33563	*P. nigrescens*	+	+	+ + +
51146	*C. showae*	–	–	–
33236	*C. gracilis*	–	+	–
Yellow complex				
10556	*S. sanguis*	+	+ +	–
10558	*S. gordonii*	+ +	+ +	+ +
25175	*S. mutans*	–	+	–
27335	*S. intermedius*	+	+	–
33397	*S. anginosus*	+	–	–
35037	*S. oralis*	–	+	+ +
49456	*S. mitis*	–	+	+ +
Green complex				
33612	*C. sputigena*	–	+	–
29523	*A. actinomycetemcomitans* A	+ +	+	+ + +
33624	*C. gingivalis*	+	–	–
Purple complex				
10790	*V. parvula*	–	–	–
17929	*A. odontolyticus*	–	+	–
43146	*A. oris*	–	–	+
Other bacteria				
11827	*P. acnes*	–	–	–
11828	*P. acnes*	–	–	–
12102	*A. israelii*	–	–	–
12104	*A. naeslundii*	–	–	+
14201	*L. buccalis*	–	+	+
19696	*N. mucosa*	–	–	–
25845	*P. melanino*	+	+	+ + +
27824	*G. morbillorum*	–	–	+
35308	*P. denticola*	+	–	–
43718	*A. actinomycetemcomitans* B	–	–	–

Bacteria are grouped according to microbial complexes associated with periodontal disease (14). For the plate-based assay, + 0–0.5 and + + > 0.5 mm^2/mm^2; for the gel-based assay of the pellet, + 1–4 and + + > 4 Kunitz/bacteria; and for the gel-based assay of supernatant, + 5–10, + + 10–20 and + + + > 20 Kunitz/bacteria.

the red complex bacteria *P. gingivalis* is recognized as an important periodontal pathogen and therefore to examine whether DNase expression varied within this species, six different strains of *P. gingivalis*, including five clinical isolates, were assayed using both DNase assay methods (Fig. 3). All strains analysed demonstrated DNase activity to varying degrees following analysis on DNase test agar (Fig. 3A), four strains secreted DNase when grown in broth, and five strains expressed membrane-bound DNase (Fig. 3B).

To demonstrate the potential *in vivo* functional role for the periodontal bacteria-derived DNases in degrading NET integrity, thereby indicating their potential for combating this innate immune defence mechanism, the supernatants of the three bacterial strains demonstrating the highest DNase activity by agarose gel assay were incubated with stimulated NET structures *in vitro*. Data clearly demonstrated that released NETs were no longer visible following exposure to previously determined DNase-positive culture supernatants, indicating breakdown of the NET DNA backbone. In comparison, exposure to culture supernatants from three bacterial strains which had previously not demonstrated DNase activity did not result in significant visible degradation of the released NETs in the assay conditions applied, thereby supporting the validity of this experimental approach (Fig. 4).

Discussion

Two distinct DNase activity assays were developed and used in this study. Both assays revealed that bacteria

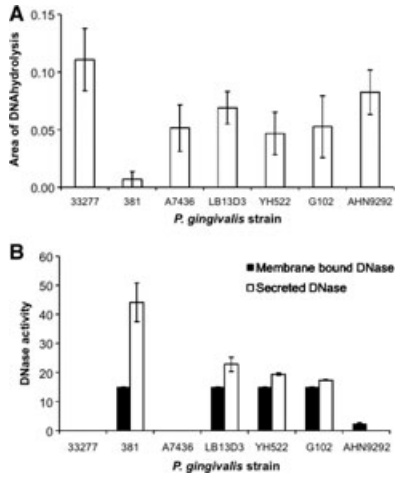

Fig. 3. DNase expression analysis in seven strains of *P. gingivalis*. (A) Detected by 6 d growth on DNase test agar. Results are the means of four independent experiments ± SEM. (B) Detected by agarose gel assay of 6 d broth cultures. Results are the means of three independent experiments ± SEM.

exhibit differential DNase activity in different growth conditions, a phenomenon consistent with that previously reported (21). Both the reported DNase test plate assay and the agarose gel assay of bacterial supernatant samples are capable of detecting DNase secreted or shed into the extracellular space by the bacteria. Interestingly, however, five bacterial strains were determined as DNase positive only when cultured on agar plates, while a further nine strains were only positive for secreted DNase when grown in broth culture (Table 1). Clearly, this difference in detection may be due to the growth state analysed, i.e. plate culture vs. planktonic, findings consistent with others who have previously demonstrated that culture conditions alter molecular and biochemical activity and subsequent bacterial phenotype (22,23). Alternatively, the bacteria may have differing susceptibility to lysis depending on the stage of growth, as all were cultured for 6 d regardless of growth rate. In addition, culture on DNase test agar plates resulted in the continuous exposure of the bacteria to the DNA substrate, which may also lead to induction of DNase expression. To test this hypothesis, we performed preliminary analysis using all five strains positive for DNase activity only when cultured on test agar plates along with a further two strains which did not exhibit DNase activity in either culture condition. None of these strains selected for culture in broth supplemented with DNA at a concentration equivalent to that present in the DNase test agar plates (2 mg/mL) demonstrated any statistically significant increases in DNase expression (data not shown). These data indicate that culture in the presence of DNA is not sufficient to induce DNase expression, and therefore other culture environmental conditions should be considered. It is interesting to note that certain extracellular DNases, similar to other virulence factors, are plasmid encoded in streptococci and are known to be inducible in appropriate stimulatory conditions, including host cell contact (9,24). Future studies could therefore investigate the induction of DNases in periodontal bacteria which coexist *in vivo* alongside other bacteria, epithelial and immune system cells.

Phase of growth has been previously reported to affect the expression of extracellular bacterial DNases, and

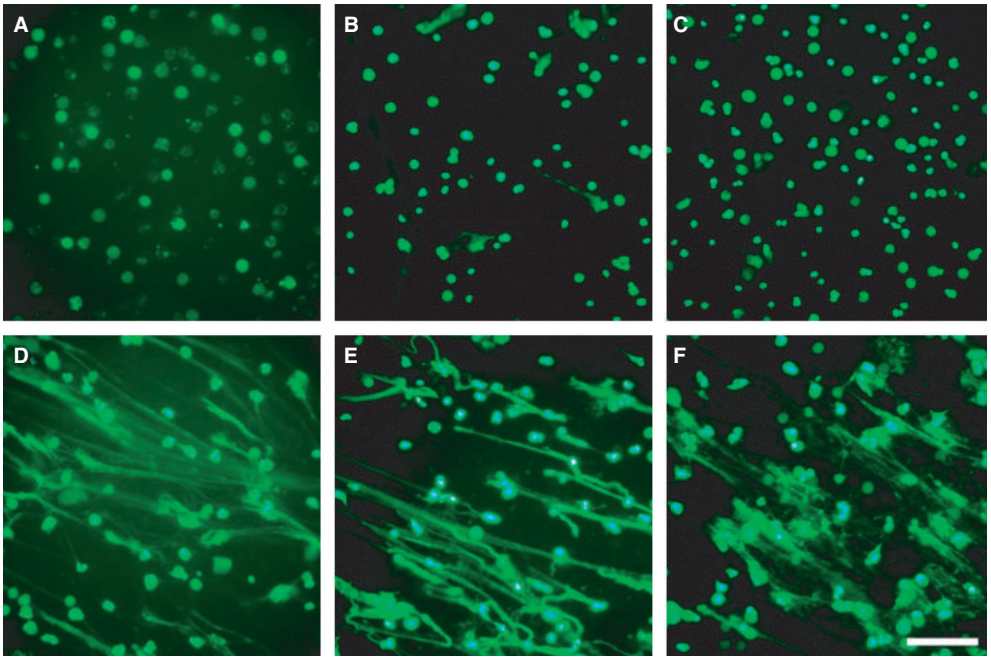

Fig. 4. Representative images of degradation of neutrophil extracellular traps (NETs) by secreted bacterial DNase. Human neutrophils were stimulated with 25 nM phorbol myristate acetate to release NETs, which were then exposed for 15 min with bacterial supernatant previously shown by agarose gel electrophoresis (Table 1) to be DNase positive. *P. intermedia* (A), *P. melaninogenica* (B) and *P. nigrescens* or (C) DNase negative, *P. gingivalis* (D), *P. denticola* (E) and *N. mucosa* (F). The NETs are visible in images D–F as strand-like structures. Results are representative of experiments performed in triplicate. Scale bar represents 100 μm.

data generated using an M1 serotype strain of group A *Streptococcus* indicated that DNase gene expression increases upon entry into the stationary phase (9). It is, however, unlikely that this factor contributed to the differences in bacterial activity observed here, as all experiments used bacteria grown to the stationary phase to enable detection of DNase activity generated at all stages of growth. For DNase test agar analysis, the DNA substrate was constantly available at all growth phases, and therefore activity could be detected throughout culture. Likewise, in broth culture it is likely that any extracellular DNase activity would accumulate within the media, and therefore would also be detectable, as our 'in-house' analyses demonstrated that commercial DNase incubated at 37°C remained stable for up to 6 d (data not shown). It is also difficult to compare absolute DNase activity between pellets and supernatants directly (Fig. 2) owing to differences in the sensitivities of the assays applied, and therefore this may also contribute to the differences in bacteria reported exhibiting activity in this study (Table 1). Notably, we found that the sensitivity of the gel-based assay was greater for samples suspended in bacterial broth, i.e. supernatant samples, compared with phosphate-buffered saline, i.e. bacterial pellet samples. This is illustrated in Fig. 2 (insets), where identical concentrations of bovine pancreatic DNase standards result in more complete DNA degradation and therefore greater migration on agarose gel when suspended in broth compared with the use of phosphate-buffered saline as reaction buffer. The difference in sensitivity may therefore be due to the presence in the bacterial broth of a greater abundance of divalent cations, which are known to be essential for the activity of DNases (25).

Of the previously hypothesized functions for bacterial extracellular DNase, the ability to reduce the viscosity of purulent exudate and enable bacterial dissemination may be of particular relevance to the periodontal bacteria examined here, which exist *in vivo* as a biofilm. Indeed, biofilms formed by bacteria such as *Pseudomonas aeruginosa* consist of relatively large quantities of bacterial DNA, and DNase activity is known to regulate biofilm formation (26). Whilst the biofilm confers protection to the bacteria, DNase activity may be required to allow specific bacteria to be released and disseminate throughout tissues. Recently, NETs have been identified as an important innate immune defence mechanism provided by neutrophils (20) and, clearly, bacterial DNase activity may also have evolved to act upon such extruded mammalian DNA. Studies have shown that bacterial DNase expression facilitates the escape, survival and increased virulence of *Streptococcus pneumoniae* (10) and the M1 serotype group A streptococci causing necrotizing fasciitis (11). As NETs have now been demonstrated in infected periodontal tissue (13), it is likely that bacterial DNase expression within the biofilm may contribute to pathogenicity, particularly as it has now been demonstrated here that DNases produced by the periodontal bacteria are capable of degrading the complex chromatin structure of NETs (27). Periodontal bacteria exist within a biofilm *in vivo*; therefore, DNase expression by certain species is also likely to contribute to a mutually beneficial environment whereby other non-DNase producing bacteria are afforded protection from NETs. Although no colour complex of periodontal bacteria appeared to have a greater incidence of DNase expression than any other colour complex, it was interesting to note that extracellular DNase production was more commonly detected in bacteria associated with colour complexes (91% of strains tested positive by one or more assay) compared with those not associated with a complex (55% of strains tested positive by one or more assay). Clearly, the bacterial strains analysed here were selected based on their reported association with the pathogenesis of periodontitis (14) and the ability to perform the DNase functional assays on them due to their culturability. Such bias in selection, however, may result in skewing of the data with regards to reporting of an association between DNase activity and disease phenotype. Indeed, a more thorough profile of the bacteria associated with different stages of periodontitis is now being obtained as a result of ongoing bacterial genome sequencing projects, and this is leading to both the identification and the molecular characterisation of significant numbers of nonculturable strains (28). Subsequently, these data will better inform us with regard to bacterial DNase expression and its association with the pathogenesis of periodontitis. Interestingly, our further characterization of seven strains of *P. gingivalis* (Fig. 3) demonstrated varying degrees of DNase expression within this one species, an observation previously made in *V. parvula* (3) and *S. intermedius* (29). However, strain A7436, which is considered to be a relatively invasive strain, exhibited only moderate DNase activity on DNase test agar plates, whilst no DNase expression in broth culture was detected. Interestingly, DNase expression again appeared to depend on the growth state of the bacteria, as the lowest DNase producer on DNase test agar plates (strain 381) secreted the highest level when grown in broth culture and, conversely, strain 33277 was the highest DNase producer on agar plates, although it exhibited no detectable DNase activity in broth culture.

This work clearly demonstrates that a significant number of bacteria involved in periodontal disease have the capacity for DNase expression and, as such, may contribute to disease pathogenesis. Future studies should therefore examine *in vivo* levels of DNase activity in disease and healthy periodontal sites to determine whether novel therapies based upon the modulation of DNase activity may provide alternative treatment approaches for periodontitis.

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