

# Extracellular proteolytic activities expressed by *Bacillus pumilus* isolated from endodontic and periodontal lesions

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The purpose of the present study was to identify 12 *Bacillus* isolates that had been obtained from root canals of teeth requiring endodontic therapy and from periodontal pockets in severe marginal periodontitis, and to determine whether these isolates exhibited extracellular proteolytic activity and, using *in vitro* assays, whether any such activity could degrade substrates that would be pathophysiologically relevant with regard to the production of endodontic and periodontal lesions. Biochemical and carbohydrate fermentation patterns were used in the identification of all strains, which was confirmed by determination of the 16S rRNA gene sequence for strain BJ0055. Screening for production of extracellular proteolytic activity by all strains was done with a general proteinase substrate. All isolates were identified as representing *Bacillus pumilus* and all exhibited extracellular proteolytic activity. The putative pathophysiological relevance of extracellular proteinase production in strain BJ0055 was assessed using fluorophore-labelled elastin and collagen and several chromogenic peptides. Probable classes of proteinases acting on each substrate were investigated using class-specific inhibitors. Activity–pH profiles were determined in buffers at different pH values. Extracellular activities that were caseinolytic, elastinolytic, collagenolytic, glutamyl endopeptidase-like, and alanyl tripeptidyl peptidase-like were observed. No trypsin-like activities were detected. Serine- and chymotrypsin-like serine proteinase activities were detected, with activity observed at neutral and alkaline, but not acidic, pH. *B. pumilus* strains isolated from endodontic and periodontal lesions exhibited extracellular activities that degrade elastin, collagen and other substrates. These activities may be virulence factors that contribute to tissue damage in apical periodontitis and severe marginal periodontitis.

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**Abbreviations:** AAPV, alanyl-alanyl-prolyl-valyl; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid; DFP, di-isopropylfluorophosphate; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane; FL, phenylalanyl-leucyl; FLE, phenylalanyl-leucyl-glutamyl; FPA, phenylalanyl-prolyl-alanyl; GGL, glycyl-glycyl-leucyl; HPA, Hide Powder Azure; K, lysyl; L, leucyl; LTR, leucyl-threonyl-arginyl; MMP-1, matrix metalloproteinase 1 (tissue collagenase); OP, o-phenanthroline; pNA, p-nitroanilide; R, arginyl; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone; TPPI, tripeptidyl-peptidase I; VLK, valyl-leucyl-lysyl.

## INTRODUCTION

Apical and marginal periodontitis are diseases caused by a small subset of the approximately 700 microbial species that constitute the human oral microbiota (Paster *et al.*, 2006). The predominant pathogens are *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola*, although other organisms have been implicated as contributing or candidate pathogens (Socransky & Haffajee, 1997; Holt & Ebersole, 2005). Moreover, unusual micro-organisms may

contribute to the aetiology of periodontitis (Helgason *et al.*, 2000), especially if they elaborate virulence factors relevant to the disease process (Socransky, 1979).

Previously, we reported the isolation under aseptic surgical conditions of *Bacillus* spp. from periodontal pockets associated with severe marginal periodontitis and from root canals of teeth with necrotic pulps/apical periodontitis (Johnson *et al.*, 1999). *Bacillus* species isolated from human oral samples have often been regarded as transient microflora (Marsh & Martin, 1992) or as external contaminants due to careless handling of endodontic samples (Dahlen & Moller, 1992). For these reasons, the genus *Bacillus* has received relatively little attention in oral microbiology. However, *Bacillus* species as suspected pathogens in dental plaque or oral lesions have been reported in a survey of collagenolytic activity of bacteria in dental plaque clinically associated with gingivitis or periodontitis (Soderling & Paunio, 1981), from cases of apical periodontitis (Molander *et al.*, 1998) and from marginal and apical periodontitis (Helgason *et al.*, 2000). In the latter report it was suggested that virulent clones of *Bacillus cereus* and *Bacillus thuringiensis* might play a role in oral disease (Helgason *et al.*, 2000).

It is apparent that *Bacillus* spp. are emerging opportunistic pathogens in oral disease. Therefore, the purpose of this study was (i) to identify the previously isolated *Bacillus* strains to the species level, and (ii) to determine whether these strains exhibited extracellular proteolytic activities capable of degrading components of periodontal tissues. Such activities may function as virulence factors in endodontic and periodontal infections.

## METHODS

### Identification, growth and characterization of *Bacillus* isolates.

The selection of subjects, collection of clinical samples and bacterial isolation and initial characterization have all been described previously (Johnson *et al.*, 1999), together with Institutional Review Board Approval and subject informed consent.

Putative *Bacillus* spp. isolated previously were endospore-forming Gram-positive rods (Johnson *et al.*, 1999). In the present study, *Bacillus* isolates were identified by confirmation of Gram stain, cellular morphology and endospore formation, and by biochemical and carbohydrate fermentation patterns using API 20E and API 50 CHB kits used according to the manufacturer's instructions (bioMérieux). In addition, the identity of one isolate (BJ0055) was confirmed by sequencing the 16S rRNA gene. Genomic DNA was prepared using a Qiagen DNeasy kit according to the manufacturer's instructions, with lysozyme serving as the lytic agent. This was then used as template for an AccumPrime PFX (Invitrogen) PCR reaction with the primer pair OL398 (5'-TCCTACGGGAGGCAGCAGT-3') and OL399 (5'-GGACTACCAGGGTATCTAATCCTGTT-3'), described originally by Nadkarni *et al.* (2002), resulting in a product of approximately 550 bp. This fragment was purified by gel extraction using a Qiaquick gel extraction kit (Qiagen), according to the manufacturer's protocol, before being cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen), creating construct pLES226. The cloned insert was then sequenced (University of Georgia Integrated Biotech Laboratories) using the M13 forward and reverse primers, the

target sequences of which are located immediately upstream and downstream, respectively, of the pCR2.1 multiple cloning site. The sequence was subjected to BLAST analysis ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

Cultures were grown in tryptic soy broth with glucose (TSBG; Difco) or in a semi-defined medium (modified FMC; MFMC) based on that of Terleckyj *et al.* (1975) and containing (per litre): 20 g D-glucose, 6 g Na<sub>2</sub>H<sub>3</sub>O<sub>2</sub>·3H<sub>2</sub>O, 600 mg ammonium sulfate, 10 mg NaCl, 440 mg KH<sub>2</sub>PO<sub>4</sub>, 300 mg K<sub>2</sub>HPO<sub>4</sub>, 3.15 g Na<sub>2</sub>HPO<sub>4</sub>, 2.05 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 225 mg NaC<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O, 200 mg L-cysteine, 2.5 g Na<sub>2</sub>CO<sub>3</sub>, 5.0 g acid-hydrolysed casein, 1.0 g enzymic digest of casein, 1.0 g yeast extract, 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 mg MnSO<sub>4</sub>, 35 mg adenine, 27 mg guanine, and 30 mg uracil. The pH was adjusted to 7.8 and the medium was filter-sterilized. In preliminary experiments, cultures were grown for 24–48 h at 37 °C aerobically with shaking, and in 5–10% CO<sub>2</sub> in air (static) or anaerobically (static).

**Detection and preliminary characterization of extracellular proteolytic activities.** Growth and the presence of extracellular proteolytic activity were observed in both TSBG and MFMC and under all growth conditions, with the greatest proteolytic activity in shaken aerobic cultures (data not shown). Subsequent experiments were all done with shaken aerobic cultures. Unconcentrated culture supernatants of all strains (grown in TSBG) were screened for the presence of extracellular proteolytic activity essentially as described by Makinen & Makinen (1987), using the general proteinase substrate Hide Powder Azure (HPA; Calbiochem) at 10 mg ml<sup>-1</sup> (Rinderknecht *et al.*, 1968). Complete digestion of HPA (1 mg ml<sup>-1</sup>) yielded an A<sub>595</sub> of 0.30 (data not shown). Strain BJ0055 showed the greatest proteolytic activity and was selected for further characterization. *Bacillus pumilus* BJ0055 was grown in MFMC and culture fluids were collected by centrifugation. The culture fluids were then passed through membrane filters (0.45 µm pore size) and concentrated 300-fold by ultrafiltration (3 kD cut-off).

Concentrated culture fluids were screened for proteolytic activity against a variety of fluorogenic and chromogenic substrates. Fluorescein-labelled type IV collagen and BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid)-conjugated casein and elastin were obtained from Molecular Probes and were assayed according to the manufacturer's instructions ([www.probes.invitrogen.com](http://www.probes.invitrogen.com)) using a Gemini EM microplate fluorometer (Molecular Devices) at 37 °C. Briefly, enzyme and buffer (50 mM Tris/10 mM NaCl, pH 7.9) were pre-incubated in microplate wells for 10 min at 37 °C, after which the reaction was started by the addition of substrate. Data were collected in the kinetics mode. Instrument settings were excitation 495 nm and emission 515 nm for collagen and excitation 485 nm, emission 514 nm and cut-off 495 nm for casein and elastin.

Chromogenic substrates used were amino acid- or peptide-p-nitroanilides (pNAs) obtained from Bachem or Sigma. Substrates with blocked amino-termini included R (arginyl), FL (phenylalanyl-leucyl), FLE (phenylalanyl-leucyl-glutamyl), GGL (glycyl-glycyl-leucyl) and AAPV (alanyl-alanyl-prolyl-valyl). Substrates with free amino-termini included K (lysyl), L (leucyl), FPA (phenylalanyl-prolyl-alanyl), VLK (valyl-leucyl-lysyl), and LTR (leucyl-threonyl-arginyl).

Assays for chromogenic substrates were performed as described for fluorogenic substrates but using a SpectraMax Plus microplate spectrophotometer (Molecular Devices) at 37 °C. Reactions were started by addition of substrate (0.2 mM final concentration), and absorbance at 405 nm (A<sub>405</sub>) was recorded.

Types of proteolytic activities were assessed by use of class-specific inhibitors (Beynon & Salvesen, 1989). Inhibitors were obtained from Bachem, Boehringer Mannheim or Sigma. The inhibitors and their target proteinase classes are shown in Tables 1 and 2. Assays were run

**Table 1.** Extracellular proteolytic activity of *B. pumilus* BJ0055: digestion of fluorophore-conjugated proteins and its inhibition by class-specific inhibitors

Results are representative of several assays run on different days and with different batches of concentrated culture fluids. Abbreviations: DFP, di-isopropylfluorophosphate; E-64, *L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane; OP, *o*-phenanthroline; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone. NAD, No activity detected in the presence of inhibitor; ND, not done.

Inhibitor	Target proteinase	Relative activity on:		
		Casein	Elastin	Collagen
None	—	1.000*	1.000	1.000
DFP				
0.5 mM	Serine	NAD	0.202	NAD
5 mM		NAD	0.148	NAD
TLCK				
10 µM	Trypsin-like serine	0.989	1.061	ND
100 µM		1.035	1.066	ND
Chymostatin				
100 µM	Chymotrypsin-like serine	0.796	0.239	NAD
500 µM		0.922	0.208	NAD
TPCK				
10 µM	Chymotrypsin-like serine	1.127	0.908	ND
100 µM		1.196	0.561	ND
OP				
2 mM	Metallo-, metal-activated	1.056	0.865	0.742
10 mM		1.032	0.750	1.151
EDTA				
2.5 mM	Metallo-, metal-activated	1.041	0.875	1.081
10 mM		1.004	0.735	0.904
E-64				
1 µM	Cysteine	1.015	0.935	1.240
10 µM		1.001	0.860	1.413
Pepstatin				
1 µM	Aspartic	0.970	0.840	1.093
10 µM		1.272	1.125	0.940
Bestatin				
5 µM	Alanyl aminopeptidase	1.244	1.165	1.109
50 µM		1.301	1.190	1.004

\*Values corresponding to relative activity=1.000, in RFU (relative fluorescence units)  $\text{min}^{-1}$ : casein,  $1.87 \times 10^5$ ; elastin,  $2.00 \times 10^6$ ; collagen,  $2.42 \times 10^6$ .

as described for fluorogenic and chromogenic substrates, with enzyme and inhibitor pre-incubated in buffer before addition of substrate. In preliminary experiments it was observed that methanol, the solvent for *o*-phenanthroline (OP), by itself inhibited proteolytic activity (data not shown). All OP results were corrected for this effect by use of appropriate controls.

**Table 2.** Extracellular proteolytic activity of *B. pumilus* BJ0055: hydrolysis of peptide-pNA substrates and its inhibition by class inhibitors

Results are representative of several assays run on different days and with different batches of concentrated culture fluids. Abbreviations: AAPV, elastase-like; DFP, di-isopropylfluorophosphate; GGL, chymotrypsin-like; FLE, glutamyl endopeptidase; FPA, alanyl tripeptidyl peptidase; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone; E-64, *L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane. NAD, No activity detected in the presence of inhibitor.

Inhibitor	Target proteinase	Relative activity on:			
		AAPV	GGL	FLE	FPA
None	—	1.000*	1.000	1.000	1.000
DFP					
0.5 mM	Serine	NAD	NAD	0.024	0.570
5 mM		NAD	NAD	NAD	0.569
TLCK					
10 µM	Trypsin-like serine	0.990	0.996	1.010	1.037
100 µM		0.949	0.991	1.007	1.030
Chymostatin					
100 µM	Chymotrypsin-like serine	0.025	NAD	0.742	0.518
500 µM		0.040	0.192	0.631	0.382
TPCK					
10 µM	Chymotrypsin-like serine	0.934	0.899	1.012	0.884
100 µM		0.017	NAD	0.852	0.464
OP					
2 mM	Metallo-, metal-activated	0.710	1.000	0.988	0.650
10 mM		0.784	0.477	1.000	0.488
EDTA					
2.5 mM	Metallo-, metal-activated	0.964	1.036	0.735	1.080
10 mM		0.969	1.154	0.618	1.035
E-64					
1 µM	Cysteine	1.096	1.178	0.909	1.117
10 µM		1.168	1.070	0.957	1.125
Pepstatin					
1 µM	Aspartic	1.101	1.141	0.896	1.117
10 µM		0.963	1.059	1.037	1.184
Bestatin					
5 µM	Alanyl aminopeptidase	1.101	0.974	0.992	1.133
50 µM		1.090	1.162	1.021	1.176

\*Values corresponding to relative activity=1.000, in  $\text{mA}_{405} \text{min}^{-1}$ : AAPV, 29.8; GGL, 25.0; FLE, 71.8; FPA, 36.5.

**Proteolytic activity as a function of pH.** The dependence of BODIPY-elastin- and FPA-pNA-digesting activities on pH was assessed by running assays as described above in buffers with various pH values. Citrate/phosphate buffer was used for pH 3–6, Tris for pH 7–9 and CAPS for pH 10–11.

## RESULTS AND DISCUSSION

### Identification and characterization of *Bacillus* isolates

In the present study, all 12 isolates were confirmed as belonging to the genus *Bacillus* (Gram-positive, facultatively anaerobic, endospore-forming rods) and were identified as representing *B. pumilus* using biochemical and carbohydrate fermentation patterns. Strains were identified based on hydrolysis of gelatin and the  $\beta$ -galactosidase substrate ONPG, the production of acetoin and use of citrate, the inability to hydrolyse starch or to reduce nitrate to nitrite, and fermentation of tagatose and gentiobiose but not turanose, sorbitol or galactose (data not shown). These characteristics are consistent with *B. pumilus* (Logan & Berkeley, 1984). Furthermore, BLAST sequence analysis revealed that the 536 bp fragment contained within pLES226 and representing the 16S rRNA gene of strain BJ0055 matched exactly, without mistake, the sequence of the 16S rRNA gene of the type strain of *B. pumilus*, ATCC 7601<sup>T</sup> ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) (data not shown).

*Bacillus anthracis* has long been known to be a major human pathogen (Xu & Frucht, 2007). Other members of the genus are regarded as opportunistic pathogens causing endophthalmitis after penetrating eye injuries, food poisoning and, in compromised patients, bacteraemia, septicemia, endocarditis, and central nervous system or respiratory infections (Callegan *et al.*, 1999; Ewald *et al.*, 2006; Granum & Lund, 1997; Banerjee *et al.*, 1988; Drobniewski, 1993; Tuazon *et al.*, 1979). *B. pumilus* can also be a systemic pathogen, causing bacteraemia in neutropenic patients and producing an allergen involved in allergic pneumonitis in machinists (Banerjee *et al.*, 1988; Bernstein *et al.*, 1995; Tuazon *et al.*, 1979). Additionally, *B. pumilus* has been found in the intestinal flora of humans (Vann *et al.*, 1976) and *B. pumilus* strains have been shown to produce exotoxins that are cytotoxic for cultured mammalian cells (Brophy & Knoop, 1982; Hoult & Tuxford, 1991). Furthermore, this organism can produce clindamycin-associated enterocolitis in guinea-pigs (Brophy & Knoop, 1982).

Members of the genus *Bacillus* as oral pathogens have received little attention, having been regarded as contaminants when isolated from endodontic or other oral samples (Dahlen & Moller, 1992; Marsh & Martin, 1992). However, in more recent reports of *Bacillus* isolates from marginal and periapical sites, carefully controlled clinical and sampling procedures made such contamination unlikely (Johnson *et al.*, 1999; Sunde *et al.*, 2000, 2002). Thus it is probable that such *Bacillus* isolates are in fact part of the microbiota of the lesion. It has been postulated that the presence of unusual organisms in such lesions suggests an aetiological role (Helgason *et al.*, 2000), and a number of *Bacillus* isolates from periodontal and endodontic lesions have been reported. These include 12

*Bacillus* isolates from marginal and periapical periodontitis (Johnson *et al.*, 1999), one *Bacillus* sp. isolated from a series of endodontic samples (Molander *et al.*, 1998), seven *Bacillus* spp. including *B. pumilus* from another series (Sunde *et al.*, 2002), and 20 *B. cereus* and *B. thuringiensis* isolates from periodontal and periapical lesions (Helgason *et al.*, 2000). In an earlier study, 15% of apical periodontitis samples contained *B. pumilus*, with numbers as high as approximately 0.7% of the total viable count (Johnson *et al.*, 1999). We propose that members of the genus *Bacillus* that were isolated carefully from oral disease sites are legitimate isolates and potential oral pathogens.

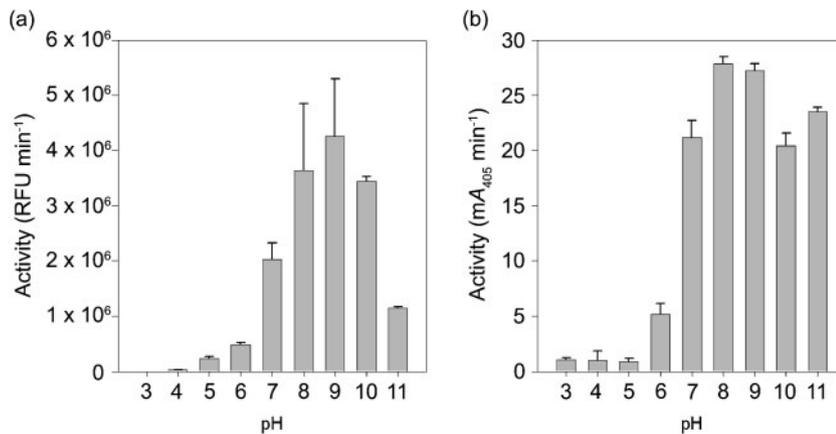
### Detection and characterization of extracellular proteolytic activities

Cell-free culture fluids from all 12 oral strains of *B. pumilus* exhibited proteolytic activity that degraded the general proteinase substrate HPA. The mean rate was 3.88 mg HPA ml<sup>-1</sup> h<sup>-1</sup> (range 1.03–5.30) (data not shown). The highest activity was seen with strain BJ0055, which was used for all subsequent experiments.

Extracellular proteolytic activities in concentrated culture fluids of *B. pumilus* BJ0055 degraded various protein (Table 1) and peptide (Table 2) substrates. In Table 1, caseinolytic activity was inhibited by di-isopropylfluorophosphate (DFP) and therefore is a serine proteinase, whereas elastinolytic and collagenolytic activities involve chymotrypsin-like serine proteinases that are inhibited by both DFP and chymostatin. This was confirmed for elastinolytic activity, which was also inhibited by tosyl phenylalanyl chloromethyl ketone (TPCK; Table 1); collagenolytic activity was not tested with TPCK. No other classes of caseinolytic, elastinolytic or collagenolytic activity were observed.

Table 2 shows the hydrolysis of various pNA-conjugated peptides that were used as convenient proteinase or peptidase substrates. Activities representing elastase-like (AAPV-pNA-hydrolysing) and chymotrypsin-like (GGL-pNA-hydrolysing) enzymes, glutamyl endopeptidase (FLE-pNA-hydrolysing), and alanyl tripeptidyl peptidase (FPA-pNA-hydrolysing) were observed. No leucine peptidase or trypsin-like activities were detected, as indicated by the failure to hydrolyse the following relevant peptide-pNA substrates: L (leucine aminopeptidase-like); FL (leucine endopeptidase-like); K, VLK, R and LTR (trypsin-like enzymes). The absence of trypsin-like activities was further indicated by the lack of inhibition by tosyl lysyl chloromethyl ketone (TLCK; Tables 1 and 2). In addition, no aminopeptidases other than alanyl tripeptidyl peptidase were detected. No pNA substrates with a free amino-terminus, other than FPA, were hydrolysed.

Glutamyl endopeptidase activity was inhibited by DFP and therefore is a serine proteinase, whereas elastase- and GGL-pNA-hydrolysing activities are chymotrypsin-like serine



**Fig. 1.** Effect of pH on extracellular elastolytic (a) and alanyl tripeptidyl peptidase (b) activities. Assays were run in triplicate and results are expressed as means  $\pm$  SD.

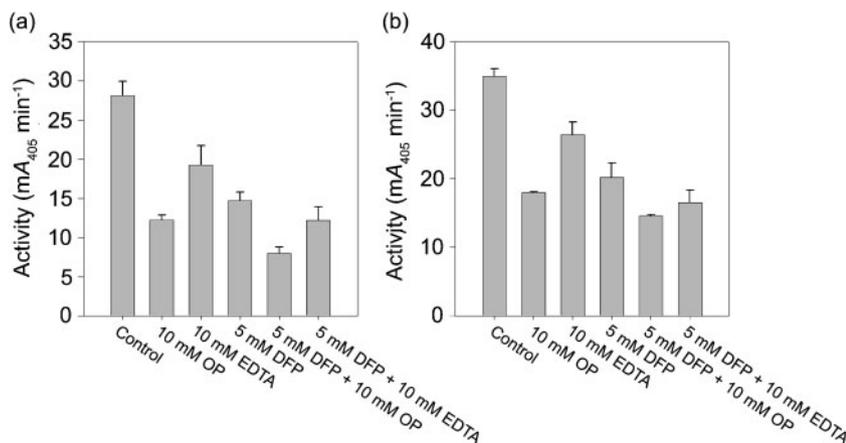
proteinases that are inhibited by DFP, chymostatin and TPCK. This inhibition by DFP, chymostatin and TPCK was virtually complete for both activities, as was the DFP inhibition of glutamyl endopeptidase. The GGL-*p*NA-hydrolysing chymotrypsin-like activity may be metal activated or stabilized as it was slightly inhibited by 10 mM OP. No other classes of elastase-like, chymotrypsin-like- or glutamyl endopeptidase activities were observed. In contrast to results for elastase- and chymotrypsin-like activities and glutamyl endopeptidase, inhibition of alanyl tripeptidyl peptidase showed both chymotrypsin-like serine- and metallo-proteinase characteristics with incomplete inhibition by DFP, chymostatin, TPCK or OP.

Gingival crevicular fluid is approximately neutral in pH in good oral health and becomes more alkaline (pH 8.5) as periodontitis develops (Bickel & Cimasoni, 1985). Accordingly, it was of interest to examine the modulation of selected activities by pH. The effects of pH on elastolytic and alanyl tripeptidyl peptidase activities are shown in Fig. 1. It is important to note that both substrates were stable under all conditions of pH, temperature, time and assay buffer used in these experiments (data not shown). Furthermore, fluorescence of the elastin substrate is insensitive to pH as the BODIPY reporter contains no

ionizable groups (Jones *et al.*, 1997). Thus, the results shown in Figs 1 and 2 are not attributable to pH effects on substrates.

Elastinolytic activity (Fig. 1a) peaked at pH 9 with substantial activities at pH 8 and 10, consistent with neutral/alkaline proteinase activity. The pH profile for alanyl tripeptidyl peptidase activity (Fig. 1b) was broad, peaking at pH 8 and 9 with substantial activity from pH 7 to 11. This may represent a mixture of activities. Acid proteinase activities were not detected for either substrate. These results suggest that the elastinolytic and alanyl tripeptidyl peptidase activities could be active *in situ* at the elevated pH of periodontitis lesions, as high as 8.5 (Bickel & Cimasoni, 1985). Activity at pH 11 is unlikely to be pathophysiologically important in periodontitis. However, it probably does represent part of the array of proteinases exhibited by *B. pumilus*, some of which retain activity even at pH 12 and 13 (Kumar, 2002; Miyaji *et al.*, 2006).

The broad pH activity profile of alanyl tripeptidyl peptidase (FPA-*p*NA hydrolysing) and its partial inhibition by serine- and metallo-class inhibitors suggested a mixture of activities. This concept was investigated using mixed inhibitor studies at pH 9 and 11. The results for inhibition of alanyl tripeptidyl peptidase activity by mixtures of DFP



**Fig. 2.** Effects of mixed inhibitors on alanyl tripeptidyl peptidase activity at pH 9 (a) and 11 (b). Assays were run in triplicate, and results are expressed as means  $\pm$  SD. DFP, Di-isopropylfluorophosphate; OP, o-phenanthroline.

and either OP or EDTA at pH 9 and 11 are shown in Fig. 2a and b, respectively. Inhibition by mixtures of DFP and either OP or EDTA was greater than that for any of these inhibitors alone, at both pH values. However, 30–40 % of total activity was still resistant even to these combinations of inhibitors.

Bacterial proteinases, lipases and haemolysins are considered to be virulence factors (Drobniewski, 1993; Travis *et al.*, 1995, 1997), and members of the genus *Bacillus* are noted for the production of extracellular forms of such activities (Gupta *et al.*, 2002; Logan & Berkeley, 1984; Priest, 1985). In view of the proteolytic properties of the *B. pumilus* strains as described in the present study, proteinases are of particular interest. The potential of proteinases as virulence factors in periodontitis and in systemic disease has been recognized (Travis *et al.*, 1995, 1997; Xu & Frucht, 2007), with sites of action in the host including cytokines, collagen, clotting factors, immunoglobulins, extracellular matrix proteins, cell-surface receptors and disruption of signal transduction.

The *B. pumilus* strains isolated in this study exhibited an extracellular activity that extensively degraded the general proteinase substrate HPA (data not shown). An extracellular proteinase from an oral strain of *B. cereus* has been purified and characterized as a true metalloenzyme collagenase similar to that produced by *Clostridium histolyticum* (Makinen & Makinen, 1987; Soderling & Paunio, 1981). Such activities have been implicated as virulence factors contributing to the initiation and progression of endodontic and periodontal lesions (Johnson *et al.*, 1999; Travis *et al.*, 1995, 1997).

As exemplified by strain BJ0055, oral isolates of *B. pumilus* also exhibit extracellular proteolytic activities that result in the degradation of a number of protein and peptide substrates. The results shown in Fig. 1 illustrate neutral and alkaline activities degrading elastin and FPA-pNA. Collagen and elastin, as well as the general substrate casein, were digested at neutral pH (Table 1) and collagenolysis and elastinolysis are part of the tissue destruction seen in marginal and periapical periodontitis. In addition to these activities, chymotrypsin-, glutamyl endopeptidase- and alanyl tripeptidyl peptidase-like activities were found (Table 2). While extracellular subtilisin- and glutamyl endopeptidase-like activities are typical of members of the genus *Bacillus* (Gupta *et al.*, 2002; Kakudo *et al.*, 1992; Leshchinskaya *et al.*, 1997; Priest, 1985), chymotrypsin-like activity in the genus *Bacillus* has been observed infrequently (Aoyama *et al.*, 2000; Kato *et al.*, 1992). Furthermore, when observed it has been described as subtilisin-like rather than chymotrypsin-like. For example, Aoyama *et al.* (2000) mischaracterized a soy-milk-coagulating enzyme from *B. pumilus* as 'subtilisin-like' despite its complete inhibition by chymostatin. Similarly, a chymostatin-inhibited serine proteinase from *Bacillus subtilis* was also described as subtilisin-like (Kato *et al.*, 1992). Chymostatin is not known to be an inhibitor of subtilisin (Ballinger & Wells, 1998), but does

target chymotrypsin-like serine proteinases (Beynon & Salvesen, 1989).

Furthermore, alanyl tripeptidyl peptidase-like activity is an unexpected finding. This activity, also known as tripeptidyl-peptidase I (TPPI) (Golabek & Kida, 2006; McDonald, 1998), is brought about by an aminopeptidase that can act on tripeptides with free amino-termini including FPA-pNA (Rawlings *et al.*, 2006). It is found in mammalian neuronal tissue, where enzyme deficiency leads to neurodegenerative disease (Golabek & Kida, 2006). Whereas the mammalian activity is due to an acidic peptidase (Golabek & Kida, 2006; McDonald, 1998), the *B. pumilus* activity has neutral/alkaline characteristics (Figs 1 and 2). With the exception of the alanyl tripeptidyl peptidase-like activity, the activities reported in Tables 1 and 2 were serine- or chymotrypsin-like serine-class proteolytic activities. Such extracellular proteinases have been observed previously in the genus *Bacillus* (Gupta *et al.*, 2002; Kato *et al.*, 1992; Leshchinskaya *et al.*, 1997; Priest, 1985), including *B. pumilus* (Aoyama *et al.*, 2000; Fabian, 1970; Kumar, 2002; Miyaji *et al.*, 2006; Tran-Chau & Urbaneck, 1974).

In contrast to these typical *Bacillus* extracellular proteinases, approximately 40 % of the alanyl tripeptidyl peptidase-like activity was resistant to high concentrations of serine or metallo-proteinase inhibitors, alone (Table 2) or in combination (Fig. 2). Furthermore, this activity was totally resistant to class inhibitors of cysteine and aspartic proteinases and alanyl aminopeptidases (Table 2). Thus, this activity is partially or totally resistant to inhibitors of the four major classes of proteinases and to an inhibitor of a highly relevant group of aminopeptidases.

This is a puzzling result as typical *Bacillus* extracellular neutral and alkaline proteinases belong to serine or metallo classes (Gupta *et al.*, 2002; Miyaji *et al.*, 2006; Priest, 1985). Mammalian TPPI is also resistant to inhibition by DFP and belongs to the sedolisin family of serine-carboxypeptidases with greatest activity at acid pH (Golabek & Kida, 2006; Kakudo *et al.*, 1992). However, the *B. pumilus* activity was virtually devoid of activity at pH values below 6 (Fig. 1b). Furthermore, the *B. pumilus* activity was not inhibited by bestatin, a classical inhibitor of alanyl aminopeptidases (Table 2; Beynon & Salvesen, 1989). Thus, the bestatin-DFP-EDTA-OP-resistant portion of the *B. pumilus* extracellular alanyl tripeptidyl peptidase activity could represent a novel enzyme, although the total activity probably consists of a mixture of activities with differing pH optima and inhibitor sensitivities. However, it should be noted that all proteolytic activities described here were observed in crude culture fluid concentrates. Proper and complete characterization of these activities will require that the individual enzymes be purified.

To summarize, these results suggest that *B. pumilus* BJ0055 exhibits at least three extracellular proteolytic activities, a caseinolytic serine proteinase, an elastin-degrading chymotrypsin-like enzyme and an alanyl tripeptidyl peptidase-like activity. Other activities may be related to these. The

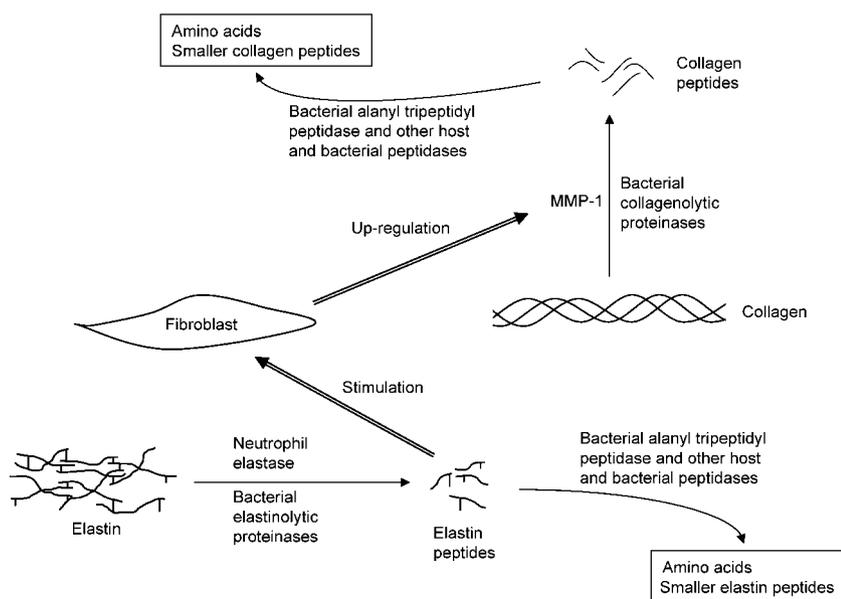
DFP-sensitive, chymostatin- and TPCK-resistant caseinolytic activity may be subtilisin-like (Ballinger & Wells, 1998; Nonaka *et al.*, 2004), as also may the glutamyl endopeptidase-like activity. Its inhibitor profile is similar to that of the caseinolytic activity, and an analogous enzyme from *Bacillus intermedius* uses both FLE-*p*NA and casein as substrates (Leshchinskaya *et al.*, 1997). The chymotrypsin-like elastinolytic activity has inhibitor sensitivities and a pH profile in common with a soybean-milk-coagulating activity from *B. pumilus* that was described by Aoyama *et al.* (2000); the AAPV- and GGL-*p*NA-degrading activities also share this inhibitor sensitivity profile. An alkaline protease purified from *B. pumilus* by Kumar (2002) had a broad pH profile with highest activity at pH 9–12, attacked di-, tri- and tetra-peptide-*p*NA substrates, required a free amino-terminus on the peptide, and preferred alanine or phenylalanine in the P1 position of the peptide. These properties are similar to those of the alanyl tripeptidyl peptidase-like activity in the present study.

### Possible interactions of proteinases and peptidases from oral bacteria and host in the pathogenesis of periodontitis

Collagen and elastin are major components of the structure of the periodontium (Chavrier, 1990; Crouch & Bornstein, 1978; Rosenbloom *et al.*, 1993), and destruction of periodontal tissue is one of the obvious manifestations of periodontitis. Fig. 3 shows a scheme in which the proteolytic activities of oral bacteria including *B. pumilus* might act in concert with host proteinases and peptidases to degrade collagen and elastin. The roles of tissue collagenase (matrix metalloproteinase 1; MMP-1) and neutrophil elastase in periodontal disease are well known

(Eley & Cox, 1992; Harrington, 1996; Loos & Tjoa, 2005). Furthermore, native collagen and elastase have non-fibrillar or less ordered regions that can be accessible to bacterial collagenolytic and elastinolytic activities that are not true collagenases or elastases (Harrington, 1996). These activities could produce nicks or gaps that might be access points, or larger peptides that might be substrates, for other bacterial activities such as the GGL-*p*NA-hydrolysing- and glutamyl endopeptidase-like activities observed here. Products of these reactions in turn could serve as substrates for alanyl tripeptidyl peptidase and other bacterial and host peptidases, ultimately reducing collagen and elastin to amino acids and small peptides.

This scheme is supported by several points. First, in fibroblast and gingival lamina propria models, elastin peptides upregulate the production of MMP-1 and down-regulate the production of tissue inhibitors of matrix proteinases (Cozlin *et al.*, 2006; Duca *et al.*, 2002). Thus, it is possible that elastinolysis by bacterial or host proteinases may have a double effect in the destruction of periodontal tissue, by directly degrading periodontal extracellular matrix material and by in effect enhancing or driving collagenolysis by host enzymes. Second, elastin (Debelle & Tamburro, 1999) and collagen (Crouch & Bornstein, 1978; Swann & Sotman, 1980) both contain substantial amounts of alanine, as much as 50–100 alanines per 1000 amino acid residues. Therefore, elastinolysis and collagenolysis are likely to yield alanyl peptide substrates for alanyl tripeptidyl peptidase. Third, a bone resorption study suggested that mammalian TPPI participated in bone resorption and that collagen was one of its natural substrates (Golabek & Kida, 2006). Thus, it is reasonable to suggest that bacterial and host proteinases, acting in concert, can play a key role in tissue destruction in apical and marginal periodontitis.



**Fig. 3.** Proposed scheme for combined actions of bacterial and host proteinases and peptidases in digestion of elastin and collagen in periodontal tissue. MMP-1, Matrix metalloproteinase 1.

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