Analysis of a Lys-specific serine endopeptidase secreted via the type IX secretion system in Porphyromonas gingivalis

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**Abstract**

*Porphyromonas gingivalis*, a significant causative agent of adult periodontitis, possesses a novel secretion system called the type IX secretion system (T9SS). A number of virulence factors, such as Arg-gingipain (Rgp), are translocated onto the cell surface and into the extracellular milieu via the T9SS. In this study, we found that the PGN_1416 90 to 120 kDa diffuse protein bands were located in the outer membrane fraction and that the presence of the bands was dependent on genes involved in the T9SS and the formation of anionic lipopolysaccharide (A-LPS). These data strongly suggest that the PGN_1416 protein is secreted by the T9SS and anchored onto the cell surface by binding to A-LPS. Enzymatic analysis using outer membrane fractions suggested that the PGN_1416 protein has a Lys-specific serine endopeptidase activity and that its activation requires processing by Rgp. Homologues of the gene encoding PGN_1416, which is referred to as *pepK*, were found in bacteria belonging to the phyla *Bacteroidetes* and *Proteobacteria*, while homologues encoding the C-terminal domain, which is essential for T9SS-mediated secretion, and the catalytic domain were only observed in bacteria belonging to the *Bacteroidetes* phylum.
Introduction

The periodontal diseases periodontitis and gingivitis, which are defined as inflammatory diseases of the periodontal tissues, are induced by bacterial infection. *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium, is closely associated with the aetiology of periodontitis; the microorganism is detected in 85.7% of patients suffering from the disease (Yang *et al.*, 2004). *P. gingivalis* possesses a number of virulence factors, such as proteinases, haemagglutinins, lipopolysaccharide (LPS), capsule and fimbriae (Nakayama *et al.*, 1996; Lamont & Jenkinson, 1998).

The majority of the proteolytic activity of the culture supernatants and the bacterial surfaces is derived from gingipains, which are categorised into two major classes of extracellular cysteine proteinases specific for Arg-X and Lys-X peptide bonds [Arg-gingipains (Rgps) and Lys-gingipain (Kgp)] (Curtis *et al.*, 1999). Rgps are encoded by *rgpA* and *rgpB*, and Kgp is encoded by *kgp* (Curtis *et al.*, 1999). The physiological functions of gingipains involve metabolism and virulence, such as acquisition of energy sources, degradation of host defence proteins and processing of bacterial proteins, including fimbrial subunits (Kadowaki *et al.*, 2000).

We previously found that the gene products of *rgpA*, *rgpB* and *kgp* were translocated across the outer membrane by the Por secretion system, which is now referred to as the type IX secretion system (T9SS) and which involves the *porK*, *porL*, *porM*, *porN*, *porP*, *porQ*, *porT*, *porU*, *porV*, *porW* and *sov* gene products (Sato *et al.*, 2010; McBride & Zhu, 2013). Homologues of these genes are found in a number of bacteria belonging to the phylum *Bacteroidetes* and associated with *Bacteroidetes* gliding motility (McBride & Zhu, 2013; Nakane *et al.*, 2013). Comparison of the protein profiles from
particle-free culture supernatants between T9SS-proficient and T9SS-deficient strains revealed that 10 proteins, including the PGN_1416 protein, were detected only in the culture supernatant derived from the T9SS-proficient strain (Sato et al., 2013). The PGN_1416 protein contains the C-terminal domain (CTD) that is commonly observed in proteins secreted via the T9SS (Sato et al., 2013). These findings suggest that the PGN_1416 protein is secreted by the T9SS. The PGN_1416 protein is predicted to be a lysyl endopeptidase based on a bioinformatic resource analysis (Kyoto Encyclopedia of Genes and Genomes; KEGG). The gene encoding the PGN_1416 protein was tentatively designated pepK (gene for Lys (K)-specific endopeptidase) in this study. However, the biological and biochemical properties of the PGN_1416 protein in P. gingivalis are unknown.

In this study, we examined various P. gingivalis strains for the presence of PGN_1416 proteins using anti-PGN_1416 antiserum. In addition, we constructed PGN_1416-deficient mutants and performed biological and biochemical analyses comparing these mutants with their parent strains. The results presented here strongly suggest that the PGN_1416 protein is secreted via the T9SS and is a serine endopeptidase with the ability to digest a Lys-X peptide bond.
Materials and Methods

Bacterial strains and plasmids
The bacterial strains and plasmids used in this study are listed in Tables S1 and S2, respectively.

Media and bacterial growth conditions
Media and conditions for growth of *P. gingivalis* and *Escherichia coli* strains used in this study have been previously described (Shoji et al., 2013).

Chemicals
The proteinase inhibitors *Nα*-p-tosyl-L-lysine chloromethyl ketone (TLCK) and EDTA-Na2 were purchased from Wako. Other inhibitors and synthetic substrates were obtained from the Peptide Institute.

Polyclonal antibody
To prepare recombinant PGN_1416 protein for use as an antigen, the DNA fragment between bases 1,381 and 2,580, encoding 400 amino acids, was amplified from the PGN_1416-encoding gene of *P. gingivalis* ATCC 33277 with primers 1416DOFOR and 1416DOREV (Table S3). The resulting fragment was inserted into the *Neo*-HindIII site of plasmid pET32b, generating pKD601. *E. coli* BL21(DE3) cells harbouring pKD601 were grown in LB broth and induced with isopropyl β-D-thiogalactoside at a final concentration of 1 mM, followed by incubation for 2 h to overproduce the recombinant protein. The induced recombinant protein was purified using a
Ni-nitrilotriacetic acid purification system (Clontech). To obtain antiserum against the PGN_1416 protein, mice were immunised with 10 µg of recombinant protein in conjunction with Freund’s incomplete adjuvant (Sigma). The injections were performed at 2-week intervals. The blood was collected after four injections. Mouse polyclonal antiserum was harvested from the blood by centrifugation at 3,000 x g for 10 min at 4°C after an overnight incubation at 4°C and stored at -20°C until the immunoblot analyses.

Construction of bacterial strains

The DNA regions upstream and downstream of a gene were PCR amplified from the chromosomal DNA of P. gingivalis ATCC 33277 using primer pairs N1416-UR and N1416-UR and N1416-DF and N1416-DR, respectively (U indicates upstream, F indicates forward, D indicates downstream, and R indicates reverse). The primers used in this study are listed in Table S3. The amplified upstream DNA fragment was digested with HindIII and BamHI. The amplified downstream DNA was digested with NotI and BamHI. Both digested products were ligated together with pBluescript II SK(−), which had been digested with HindIII and NotI. The 1.5-kb BamHI ermF DNA fragment was inserted into the BamHI site of the resulting plasmids to yield pKD600 for mutagenesis. The plasmid was digested with NotI and introduced into P. gingivalis ATCC 33277 and KDP129 (kgp::Cm') by electroporation to produce KDP600 (pepK::Em') and KDP601 (kgp::Cm' pepK::Em'), respectively.

Sample preparation

Membrane fraction. P. gingivalis grown to mid-log phase was subjected to the
following fractionation as previously described (Murakami et al., 2002). Briefly, the cells were harvested by centrifugation at 10,000 x g for 20 min, washed twice with 10 mM HEPES-NaOH buffer (pH 7.4) containing 150 mM NaCl, suspended in 10 mM HEPES buffer (pH 7.4) with 10 µM E-64 and disrupted in a French pressure cell at 100 MPa. Unbroken cells and large debris were removed by centrifugation at 1,000 x g for 10 min, and the supernatants (whole-cell lysates) were subjected to ultracentrifugation at 100,000 x g for 1 h. The precipitates were suspended in 10 mM HEPES buffer (pH 7.4) containing 20 mM MgCl₂ supplemented with Triton X-100 at a final concentration of 1% and mixed gently at room temperature (RT) for 30 min. The solution was subjected to ultracentrifugation at 100,000 x g for 1 h to yield the bacterial outer membrane fraction as the precipitate.

**Sucrose density gradient centrifugation.** Sucrose density gradient centrifugation was performed as previously described (Shoji et al., 2013).

**Supernatant fractions.** A vesicle fraction (v.f.) was obtained by ultracentrifugation of the supernatants at 100,000 x g for 1 h at 4°C. Proteins in the supernatant fraction without vesicles were precipitated with trichloroacetic acid and used for SDS–PAGE and immunoblot analysis as a particle free fraction (p.f.f.).

**Gel electrophoresis and immunoblot analysis**

SDS-PAGE and immunoblot analysis were performed as previously described (Shoji et al. 2010, 2011).

**Dot blot analysis**

Dot blot analysis was performed as described previously (Shoji et al., 2011), with some
modifications. Briefly, *P. gingivalis* cells that had fully grown in enriched BHI medium were harvested, washed with PBS and suspended with PBS. The washed cells were adjusted to an OD595 of 1.0. Three microliters of the adjusted cells was blotted directly onto a nitrocellulose membrane and left to dry.

**Fluorometric assay**

A Triton X-100-insoluble outer membrane fraction from each strain was used for all enzymatic assays. Sample protein concentration was measured by the Bradford method (Bio-Rad) with bovine serum albumin as the standard. The proteolytic activities were determined with the synthetic substrates Boc-Val-Leu-Lys-MCA, Boc-Phe-Ser-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Gly-Pro-MCA and Suc(OMe)-Ala-Ala-Pro-Val-MCA. The reaction mixture (450 μl) contained equal protein amounts from the outer membrane fractions of ATCC 33277, KDP129, KDP601 and KDP981 and 10 μM of each synthetic substrate in 150 mM Tris-HCl (pH 9.2). For the fluorometric enzyme assays, the reaction mixture without the synthetic substrates was incubated at 37°C for 5 min, placed on ice and then, the synthetic substrates were added to the reaction mixture. After incubation at 40°C for 10 min, the reaction was terminated by the addition of 45% acetic acid, and the released MCA was then measured at 465 nm (excitation at 365 nm) on a fluorescence spectrophotometer.
**Results**

**Immunoblot analysis of various *P. gingivalis* strains with anti-PGN_1416 antiserum**

Cell lysates of *P. gingivalis* ATCC 33277, W83, TDC60, TDC117, TDC275, GAI7802, SU63 and HG66 were subjected to immunoblot analysis with anti-PGN_1416 antiserum. As shown in Figure 1a, all of the strains except HG66 exhibited diffuse protein bands with molecular masses of 50 to 120 kDa, and all the strains, including HG66, had a discrete protein band with a molecular mass of 95 kDa. We constructed a PGN_1416-deficient (pepK) mutant, which showed no reactivity to anti-PGN_1416 antiserum (Fig. 1b), verifying that the reactive protein bands were derived from PGN_1416. The results suggest that the PGN_1416 protein is generally produced in *P. gingivalis*.

Previous studies indicated that T9SS-dependent secretion proteins, such as gingipains and HBP35, are located at the outer membrane by attaching to A-LPS, which results in the formation of diffuse protein bands (Nakayama *et al*., 1995; Shoji *et al*., 2010). To determine whether the generation of the diffuse PGN_1416 protein bands is dependent on T9SS and A-LPS, immunoblot analysis of T9SS-deficient and A-LPS-deficient mutants with anti-PGN_1416 antiserum was performed (Fig. 1b and c).

We previously reported that *P. gingivalis* porK (PGN_1676), porL (PGN_1675), porM (PGN_1674), porN (PGN_1673), porP (PGN_1677), porQ (PGN_0645), porT (PGN_0778), porU (PGN_0022), porV (PGN_0023), porW (PGN_1877) and sov (PGN_0832) mutants were defective in the T9SS (Sato *et al*., 2010; Shoji *et al*., 2011). These mutants showed no diffuse PGN_1416 protein bands, suggesting that the PGN_1416 protein is secreted via T9SS (Fig. 1b).

Next, we determined whether the diffuse PGN_1416 protein bands were present in
A-LPS-deficient mutants. We used PGN_0242, PGN_0663, PGN_1056 (VimA), PGN_1236 (PorR), PGN_1242 (Wzy), PGN_1251 (GtfB), PGN_1255 (Rfa), PGN_1302 (WaaI) and PGN_2005 (WzzP) mutants as A-LPS-deficient mutants. None of the A-LPS-deficient mutants examined had the diffuse protein bands, which suggests that the diffuse PGN_1416 protein bands were generated by attaching to A-LPS (Fig. 1c).

Subcellular location of the various PGN_1416 protein forms

Subcellular fractionation analysis revealed that the diffuse 50-120 kDa protein bands were located in the total membrane fraction (Fig. 2a). A 46-kDa protein reactive with anti-PGN_1416 was detected in the cytoplasmic/periplasmic and total membrane fractions. Next, the total membrane fraction was separated into inner and outer membrane fractions using sucrose density gradient centrifugation (Fig. 2b). The inner and outer membrane fractions were determined by the presence of the highest NADH-dependent ferricyanide reductase activity and LPS, respectively. The 95-kDa protein was found in the inner membrane fraction, whereas the diffuse protein bands were in the outer membrane fraction, which suggests that the PGN_1416 protein was located at the outer membrane as a diffuse form with molecular masses of 90-120 kDa (Fig. 2b). The diffuse form of the PGN_1416 protein was also observed in the vesicle fraction, whereas the PGN_1416 protein with a molecular mass of 65 kDa was located in the particle-free fraction of the culture supernatants (Fig. 2c).

T9SS-dependent localization of the PGN_1416 protein on the cell surface

Dot blot analysis revealed that the PGN_1416 protein was detected on the cell surface
of the wild-type strain, whereas it was not detected on that of the \textit{porK} or \textit{porT} mutant (Fig. 2d).

**Endopeptidase activity of a Triton X-100 insoluble fraction of the total membrane fraction**

Bioinformatic resource analysis (Kyoto Encyclopedia of Genes and Genomes KEGG) suggested that the PGN\_1416 protein is a lysyl endopeptidase. We constructed a PGN\_1416-deficient (\textit{pepK}) mutant from the \textit{kgp} strain to exclude the enzymatic activity derived from Kgp. We then compared endopeptidase activities of Triton X-100-insoluble fractions of the total membrane fractions from the \textit{kgp pepK} mutant and \textit{kgp} parent strain because the PGN\_1416 protein was located at the outer membrane by attaching to A-LPS. Using the synthetic substrate Boc-Val-Leu-Lys-MCA, lysyl endopeptidase activity was detected in the Triton X-100-insoluble fraction of the \textit{kgp} strain in a concentration-dependent manner, whereas the activity of the \textit{kgp pepK} mutant was significantly attenuated and not dependent on a protein concentration (Fig. 3a). Conversely, the ability to digest an Arg-X peptide bond was detected in both the \textit{kgp} and \textit{kgp pepK} mutants (Fig. 3b). The ability to digest Pro-X, Val-X or Phe-X peptide bond was not detected (data not shown). These results strongly suggest that \textit{P. gingivalis} possess lysyl endopeptidase activity other than Kgp at the outer membrane and that the Lys-specific activity was derived from the \textit{pepK} gene. Comparison of Lys-specific activities of the Triton X-100-insoluble fractions of the wild type, \textit{kgp} and \textit{kgp pepK} strains suggested that Lys-specific activity of PepK per cell was approximately one fortieth of that of Kgp per cell (data not shown).

We examined the effects of various proteinase inhibitors on the endopeptidase...
activity specific for the Lys-X peptide bond (Table 1). The Lys-specific activity disappeared completely in the presence of leupeptin and antipain, which are serine proteinase inhibitors. Tosyllysine chloromethyl ketone (TLCK) and chymostatin, which are also serine proteinase inhibitors, significantly suppressed the activity. Neither ethylene diaminotetraacetic acid (EDTA) as a metalloproteinase inhibitor nor pepstatin as an aspartic acid proteinase inhibitor inhibited the activity. Taken together, these results indicate that the activity detected in the outer membrane fraction is due to the presence of a Lys-specific serine endopeptidase.

Involvement of Rgp in activation of the Lys-specific serine endopeptidase

Interestingly, a Triton X-100-insoluble fraction of the total membrane fraction from the kgp rgpA rgpB mutant showed no Lys-specific activity, whereas that of the kgp mutant did show Lys-specific activity (Fig. 3c). To investigate whether the Rgp proteinase influences activation of the Lys-specific serine endopeptidase, the kgp rgpA rgpB and kgp pepK mutants were co-cultured, and the Lys-specific activity of the Triton X-100-insoluble fraction of the total membrane fraction from the co-culture was determined. The Lys-specific activity of the co-culture was dependent on protein concentration and was clearly higher than that of the kgp pepK mutant. The activity was approximately 60-80% of that of the kgp mutant (Fig. 3c).

To investigate the effects of gingipains on the molecular masses of various forms of PepK, various gingipain-deficient mutants were subjected to immunoblot analysis with anti-PepK antiserum (Fig. 4a and b). The diffuse protein bands in the gingipain-null mutant (kgp rgpA rgpB) migrated at higher molecular masses than that of the wild-type. In connection with the change of molecular masses of diffuse protein bands, the 46-kDa
PepK protein band, which was observed in the wild-type, kgp, rgpA, rgpB, kgp rgpA and kgp rgpB mutants, was not observed in the rgpA rgpB and kgp rgpA rgpB strains. Next, the co-culture of the kgp rgpA rgpB mutant with the kgp pepK mutant was subjected to immunoblot analysis with anti-PepK (Fig. 4c). The diffuse PepK protein bands of the co-culture had lower molecular masses than those of the kgp rgpA rgpB mutant, and the 46-kDa PepK protein band appeared in the co-culture. These results suggest that Rgp processes and activates the PepK protein.
Discussion

Our recent study (Sato et al., 2013) found that the PGN_1416 protein is detected in the particle-free culture supernatant of a T9SS-proficient strain (\textit{kgp rgpA rgpB}) but not in the particle-free culture supernatant of a T9SS-deficient strain (\textit{kgp rgpA rgpB porK}). Additional analysis revealed that the PGN_1416 protein possesses a CTD-like sequence at the C-terminus. Several lines of evidence indicate that PepK is secreted via the T9SS. First, the 50-120 kDa diffuse PepK proteins were located at the outer membrane. Second, A-LPS-deficient strains showed no diffuse protein bands immunoreactive to anti-PepK. Third, the presence of the 50-120 kDa diffuse PepK proteins was dependent on T9SS-related genes.

According to a bioinformatic database analysis, PepK may be a lysyl endopeptidase. In \textit{P. gingivalis}, the Lys-specific endopeptidase with the highest activity is Kgp. Therefore, in this study, we compared Lys-specific endopeptidase of the \textit{kgp pepK} mutant strain with that of the parent \textit{kgp} mutant strain and found Lys-specific endopeptidase activity in the Triton X-100-insoluble membrane fraction of the \textit{kgp} strain but not in the \textit{kgp pepK} strain. The PepK endopeptidase activity was sensitive to serine proteinase inhibitors. Chohnan et al. (2004) reported lysyl endopeptidases from \textit{Lysobacter} sp. strain IB-9374. The putative catalytic triad of the \textit{Lysobacter} peptidases was conserved in the amino acid sequence of PepK (Fig. 5a). These results strongly suggest that PepK is a Lys-specific serine endopeptidase.

Interestingly, catalytic activity of PepK was not detected in the outer membrane fraction of the \textit{kgp rgpA rgpB} mutant, and the activity was recovered by co-culture with the \textit{kgp pepK} mutant, suggesting that PepK is activated by Rgp. The results obtained
using immunoblot analysis were consistent with the co-culture activity analysis. Rgp-processed proteins have been previously reported. Fimbrillins, such as FimA and Mfa1, are processed on the cell surface by Rgp, resulting in the assembly of Fim and Mfa fimbriae, respectively (Nakayama et al., 1996). Nelson et al. (1999) suggested that the pro-form of periodontain, which is capable of inactivating human serpin, is processed by both Rgp and Kgp. Gingipains were also found to cleave and process the products encoded by rgpA, rgpB, kgp, hagA and ragA (Kadowaki et al. 1998; Murakami et al., 2002; Veith et al., 2002). These proteins, including PepK, are activated by gingipains, but gingipain-sensitive ligand A (GslA), which is able to induce cell signalling, is degraded and inactivated by gingipains (Haruyama et al., 2009). All of the gingipain-processed P. gingivalis proteins found thus far are located on the cell surface.

Genes homologous to pepK are mainly found in bacteria in genera belonging to the phylum Bacteroidetes, such as Porphyromonas, Parabacteroides, and Tannerella. Proteins encoded by these homologous genes, which are composed of more than 800 amino acid residues, appear to have a CTD at the C-terminus (Fig. 5b). Interestingly, amino acid sequences homologous to the catalytic domain of PepK are found not only in the CTD-containing proteins in Bacteroidetes bacteria but also in the proteins of bacteria in genera belonging to the phylum Proteobacteria, such as Acidovorax, Pseudomonas, Lysobacter and Xanthomonas (Fig. 5a). These Proteobacteria proteins are generally smaller than the PepK homologues found in Bacteroidetes bacteria, especially in the C-terminal region. This analysis suggests that a prototype of the PepK homologues in Bacteroidetes bacteria was likely generated by addition of a C-terminal region containing the CTD sequence to a catalytic domain with a propeptide.
The pepK mutant showed no changes in Rgp and Kgp activity compared with the wild-type parent strain, formed black-pigmented colonies on the blood agar and possessed wild-type levels of fimbriae on the cell (data not shown). These findings indicate that PepK has no effect on gingipain activities, colonial pigmentation or the formation of fimbriae. However, the wide distribution of pepK homologues beyond the phylum Bacteroidetes may be associated with the virulence potential of each bacterium, although further studies are needed to determine if the presence of pepK is influential in the pathogenesis of P. gingivalis.
Acknowledgements

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References


Figure Legends

**Figure 1.** Immunoblot analysis with various *P. gingivalis* strains using anti-PepK. Whole cell lysates of *P. gingivalis* wild-type strains, including the naturally non-pigmented strain HG66 (a), T9SS-deficient mutants and the pepK mutant (b) and A-LPS-deficient mutants (c) were subjected to immunoblot analysis with anti-PGN_1416 (PepK).

**Figure 2.** Subcellular location of the PepK protein using immunoblot analysis. (a) Whole cell lysates (W) of *P. gingivalis* ATCC 33277 were fractionated to the cytoplasmic/periplasmic fraction (C/P) and the total membrane fraction (TM). (b) The total membrane fraction was then fractionated by sucrose gradient centrifugation resulting in the inner (IM) and outer (OM) membrane fractions. (c) The culture supernatant (sup) was separated into the vesicle fraction (v.f.) and the particle-free culture supernatant (p.f.f) by centrifugation. (d) Whole cells of *P. gingivalis* ATCC 33277 (WT), KDP355 (*porK*), KDP117 (*porT*) and KDP600 (*pepK*) were blotted directly onto a nitrocellulose membrane. The blotted membrane was subjected to immunodetection with anti-PepK or anti-PtpA. PtpA is a surface protein secreted independently of T9SS.

**Figure 3.** Endopeptidase activity of the outer membrane fraction. The endopeptidase activities of the outer membrane fractions of bacterial cells were determined with the synthetic substrates Boc-Val-Leu-Lys-MCA (a) and Boc-Phe-Ser-Arg-MCA (b). Bar: 1, KDP129 (*kgp*); 2, KDP601 (*kgp pepK*). (c) Cells of KDP601 (*kgp pepK*) and KDP981
(kgp rgpA rgpB) were inoculated into the same culture medium and incubated overnight (co-culture). The endopeptidase activities of the outer membrane fractions of bacterial cells were determined with Boc-Val-Leu-Lys-MCA. Bar: 1, KDP129 (kgp); 2, KDP601 (kgp pepK); 3, KDP981 (kgp rgpA rgpB); 4, co-culture of KDP601 and KDP981.

**Figure 4.** Immunoblot analysis of *P. gingivalis* mutants with various combinations of *kgp, rgpA* and *rgpB* using anti-PepK. (a) Whole cell lysates of KDP129 (kgp), KDP131 (rgpA), KDP132 (rgpB), KDP134 (kgp rgpA), KDP135 (kgp rgpB), KDP133 (rgpA rgpB), KDP981 (kgp rgpA rgpB), KDP136 (kgp rgpA rgpB) and KDP600 (pepK) were subjected to immunoblot analysis with anti-PepK. (b) Another blot with whole cell lysates of KDP133 (rgpA rgpB), KDP981 (kgp rgpA rgpB) and KDP136 (kgp rgpA rgpB) was subjected to increased exposure times, resulting in appearance of the diffuse band in KDP133. (c) Whole cell lysates of co-cultures of KDP601 (kgp pepK) and KDP981 (kgp rgpA rgpB), KDP601, KDP981 and ATCC 33277 were subjected to immunoblot analysis with anti-PepK.

**Figure 5.** Amino acid sequence comparisons of PepK homologues. (a) The N-terminal regions including the catalytic domains. *Acidovorax citrulli* AAC00-1: Aave_4239, *Lysobacter* sp. IB-9374: BAC22111, *Parabacteroides distasonis* ATCC 8503: BDI_2829, *Porphyromonas gingivalis* ATCC 33277: PGN_1416, *Pseudomonas aeruginosa* PA7: PSPA7_0919, *Tannerella forsythia* ATCC 43037: BFO_3286, *Xanthomonas albilineans* GPE PC73: XALc_2516. Asterisks indicate the putative serine protease catalytic triad. (b) The C-terminal 80 amino acids of PepK homologues of *P. gingivalis, P. distasonis* and *T. forsythia*. The alignment was created using the
ClustalW and Boxshade programs hosted at the Swiss EMBnet web site
Fig. 1

(a) ATCC 33277, W83, TDC60, TDC117, TDC275, GA17802, SU63, HG66

(b) ATCC 33277, PGN-1676 (porL), PGN-1674 (porM), PGN-1673 (porP), PGN-0845 (porU), PGN-0778 (porP), PGN-0022 (porU), PGN-0023 (porM), PGN-0832 (sop), PGN-1416 (pepl)

(c) ATCC 33277, PGN-0242, PGN-0663, PGN-1236 (limA), PGN-1242 (wzy), PGN-1231 (gypB), PGN-1302 (lwaA), PGN-2005 (wzzD)
Fig.3

(a) Boc-Val-Leu-Lys-MCA

(b) Boc-Phe-Ser-Arg-MCA

(c) Boc-Val-Leu-Lys-MCA
ATCC 33277
KDP129 (kgp)
KDP131 (rgpA)  
KDP132 (rgpB)  
KDP134 (kgp rgpA)
KDP135 (kgp rgpB)
KDP133 (kgpA rgpB)
KDP981 (kgpA rgpB)
KDP136 (kgpA rgpB, pepK)
KDP600 (yard)  

210 140 100
70 55 45
30 35 40 45 50 55 60 65 70 75 80 85 90
kDa

ATCC 33277
KDP601 (kgp pepK)
KDP981 (kgpA rgpB)
KDP981 (kgpA rgpB)

210 140 100
70 55 45
30 35 40 45 50 55 60 65 70 75 80 85 90
kDa

ATCC 33277
KDP601 (kgp pepK)
KDP981 (kgpA rgpB)

cocultured, KDP601 and KDP981  

210 140 100
70 55 45
30 35 40 45 50 55 60 65 70 75 80 85 90
kDa
### (a)

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### (b)

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*Fig. 5*
Table 1. Effects of proteinase inhibitors on Lys-specific endopeptidase activity.

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<td>EDTA-Na₂</td>
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<tr>
<td>No inhibitor</td>
<td>100</td>
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Inhibitory effects of proteinase inhibitors on the Lys-specific endopeptidase were calculated using the following formula: (difference of the activities of KDP129 and KDP601 in the reaction mixture with inhibitors / difference of the activities of KDP129 and KDP601 in the reaction mixture without inhibitors) x 100 (%).