Determination of active site of Lysine-specific cysteine proteinase (Lys-gingipain) by use of a *Porphyromonas gingivalis* plasmid system

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The abbreviations used: Amp; ampicillin, Boc; *t*-butyloxycarbonyl; *E. coli*; *Escherichia coli*, Gm; gentamicin, Kgp; Lys-gingipain, MCA; 4-methyl-7-coumaryl-amide, PAGE; polyacrylamide gel electrophoresis, PCR; polymerase chain reaction, *P. gingivalis*; *Porphyromonas gingivalis*, Rgp; Arg-gingipain, SDS; sodium dodecyl sulfate, Tc; tetracycline, Z; Benzyloxycarbonyl

ABSTRACT

Porphyromonas gingivalis, a major etiological bacterium of periodontal diseases, produces a unique lysine-specific cysteine proteinase (Lys-gingipain, Kgp) implicated in the virulence of this organism. Our observations show the expression of a catalytically active recombinant Kgp in a *P. gingivalis* Kgp-null mutant and the restoration of its functions by use of a shuttle plasmid vector stable in *P. gingivalis*. The Kgp-expressing mutant exhibited a similar catalytic activity to that of the wild-type strain. This mutant also restored the ability to form black-pigmented colonies on blood agar plates and to generate a 19-kDa hemoglobin receptor protein responsible for hemoglobin binding. In order to establish the importance of the active-site Cys residue and elucidate its role in bacterial black pigmentation we constructed three Kgp mutants with changed potential active-site Cys residues. The cells expressing a single mutation (C476A) showed the high Kgp activity and the black pigmentation. In contrast, the cells expressing the single mutant (C477A) and the double mutant (C476A/C477A) exhibited neither Kgp activity nor black pigmentation. These results indicate that the 477th Cys residue is essential for both the Kgp activity and the black pigmentation of *P. gingivalis*.

Key words: Arg-gingipain, Lys-gingipain, Periodontal diseases, *Porphyromonas gingivalis*, recombinant expression

1. Introduction

Porphyromonas gingivalis is one of the most important pathogenic bacteria in progressive periodontal disease[1-7] and it produces a unique class of cysteine proteinases in both cell-associated and secretory forms, now termed Arg-gingipain (Rgp) and Lys-gingipain (Kgp).[6, 8-17] Rgp is encoded by two *rgp* genes (*rgpA* and *rgpB*),[18-23] whereas Kgp is encoded by a single *kgp* gene.[25-27] The *kgp* gene encodes a polyprotein with 1732 amino acid residues, which consists of four domains: a signal peptide, a prosequence, a proteinase, and C-terminal adhesin domains.[26, 24, 27] The C-terminal adhesin domain of *kgp* is highly homologous to that of *rgpA*, although the prosequence and proteinase domains have no sequence similarity. The C-terminal adhesin domain, which is thought to be involved in hemagglutination, comprises three subdomains. A 19-kDa subdomain, termed HbR (also Hgp15), is thought to be a hemoglobin-binding receptor and thereby participates in heme accumulation. On the other hand, the *rgpA* gene encodes a polyprotein with 1704 amino acid residues, while it also consists of four domains. The *rgpB* gene is almost identical to the *rgpA* gene, except for the fact that it lacks most of the C-terminal adhesin domain of *rgpA*.[21]

We previously found the Kgp-null mutant to form less black-pigmented colonies on blood agar plates and failed to express a 19-kDa HbR protein, probably due to a disruption of the processing of its precursor proteins, such as RgpA, Kgp, and HagA, to generate HbR in the Kgp-null mutant.[25] Given that colonial black pigmentation is caused by the accumulation of heme derivatives on the bacterial cell surface,[29] the disruption of HbR generation is likely to account for the non-pigmentation of the Kgp-null mutant. In addition to a disruption of the generation of HbR, we also showed this Kgp-null mutant to decrease the ability to degrade fibrinogen.[25] These results provide evidence suggesting that Kgp is associated with hemagglutination, hemoglobin binding, heme accumulation, and bleeding tendency in the periodontal pockets.

Recently, Sztukowska *et al.*[30] reported the C-terminal domain of Kgp to be necessary for the assembly and expression of catalytically active Kgp. They also found that the expression of the Kgp proteinase domain alone resulted in the expression of Kgp with a weak activity. In the present study, we constructed a shuttle plasmid vector harboring the *kgp* gene lacking its C-terminal adhesin domain and successively expressed in the Kgp-null mutant. The mutant expressing recombinant Kgp restored the enzymatic activity comparable to that of the wild-type strain and the ability to form black-pigmented colonies on blood agar plates. Using this shuttle plasmid vector system, we also constructed three Kgp mutants with changed potential active-site Cys residues and demonstrate that Cys residue (477th Cys) at its potential active site is therefore essential for both the Kgp activity and the black pigmentation of *P. gingivalis*.

2. Materials and methods

2.1. Reagents

Benzyloxycarbonyl (Z)-Phe-Arg-4-methyl-7-counmarylamide (MCA) and *t*-butyloxycarbonyl (Boc)-Val-Leu-Lys-MCA as synthetic substrates were purchased from the Peptide Institute (Osaka, Japan).

2.2. Bacteria and culture conditions

P. gingivalis ATCC33277 was used as a wild-type strain. The Kgp-deficient mutant (KDP129) has been described previously.[25] Both the wild-type strain and the KDP129 were grown under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) at 37˚C in enriched BHI broth (containing, per liter, 37 g of brain heart infusion (Difco), 5 g of yeast extract (Difco), 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K1), on tryptic soy agar (TS) (containing, per liter, 40 g of Trypto-Soya agar (Nissui, Tokyo, Japan), 5 g of brain

heart infusion (Difco), 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K1) and blood agar plate (TS plate containing 5% sheep blood). L broth (containing, per liter, 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of sodium chloride) was used to grow the *E. coli* cells. Tetracycline (Tc) (1 μg/ml), gentamicin (Gm) (100 μg/ml), and ampicillin (Amp) (200 μg/ml) were added to the medium when necessary.

2.3. Plasmid construction

A shuttle vector stably existing in *P. gingivalis*, pYKP028, was a kind gift from Dr. Yumi Kumagai in Nippon Dental University, Japan.[31] All plasmids were amplified by polymerase chain reaction (PCR) and pNKV encoding the partial *kgp* gene was used as a template.[24] Briefly, the *Kpn*I restriction enzyme sites were designated as the 5'- and 3'-region and the Kgp gene including the promoter region, signal-peptide, pro-peptide, and mature proteinase domain were amplified. After being digested by *Kpn*I, these fragments were collected and subcloned into the *Kpn*I site of pYKP028 dephosphorylated by bovine alkaline phosphatase. These plasmids were then introduced into *P. gingivalis*. A mutation occurred by site-directed mutagenesis using the primers noted below: The primer used: Kgp-s; 5'-AATGGTACCTATCATATCAG-3' and Kgp-as; 5'-GCGGTACCTTAACGTACATCGTTTGCAGGT-3' for NOP003 (pNOP003), 5'-GCCTGTGTTACAGCTCAATTCGATT-3' and DM-as; 5'-GTTCCCAATAGCTAAGAAGT-3' for C476A (pNOP031), 5'-TGCGCTGTTACAGCTCAATTCGATT-3' and DM-as for C477A (pNOP021), 5'-GCCGCTGTTACAGCTCAATTCGATT-3' and DM-as for C476A C477A (pNOP011).

Mobilization from E. coli to P. gingivalis ---- The procedure for the mobilization of plasmids from *E. coli* to *P. gingivalis* has been previously described.[32] One tenth volume of an early exponential phase culture of *E. coli* SM10λpir (1 × 10^8 cells/ml) harboring only pYKP028 as a control, pNOP003, pNOP011, pNOP021, and pNOP031 was mixed with one tenth volume of the early exponential phase culture of *P. gingivalis* KDP129 (Kgp-deficient mutant) (1 × 10^9 cells/ml), and the mixed cells were then collected by centrifugation. The mixed pellet was suspended in 1 ml of pre-warmed enriched BHI broth, spotted on enriched TS plates and then incubated anaerobically at 37°C for 1 day. The cells on the plates were collected, suspended in 1 ml of pre-warmed BHI broth, spread on the TS plates containing Tc and Gm and incubated anaerobically at 37°C for 7 days. After selection, we obtained about 50-100 colonies in all mutants.

2.4. Proteinase activity

The proteolytic activities of Rgp and Kgp were determined with Z-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA as described previously.[16, 17] Briefly, the reaction mixture (1 ml) contained various amounts of cell extracts or the culture supernatants of an overnight culture, 10 μM of each synthetic substrate, and 5 mM cysteine in 20 mM sodium phosphate buffer (pH 7.5). After incubation at 40°C for 10 min, the reaction was terminated by adding 1 ml of 10 mM iodoacetic acid (pH 5.0), and the released MCA was then measured at 460 nm (excitation at 380 nm) on a fluorescence spectrophotometer. As wild-type (ATCC33277) and KDP129 introduced only a vector (pYKP028) have Kgp and Rgp activities as strong as those of wild-type and KDP129, respectively (data not shown), the wild-type and KDP129 strains were therefore used in this study as a control.

2.5. Gel electrophoresis and immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli.[33] The proteinase inhibitors leupeptin and *p*-toluenesulfonyl-L-

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phenylalanine chloromethyl ketone were added to a solubilizing buffer to avoid proteolysis by endogeneous proteinases. For immunoblotting, protein on SDS gels was electrophoretically transferred to nitrocellulose membranes according to the method of Towbin *et al.*.[34] The blotted membranes were immunostained with the polyclonal antibodies immunoreacting to 40 amino acid sequences of the mature Kgp-N-terminus, which was both Rgp and Kgp purified[16, 17] or 19-kDa hemoglobin receptor protein purified.[35]

2.6. Sequencing

Nucleotide sequencing was determined by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster, CA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

2.7. Statistical analysis

The quantitative data are presented as the mean ± S.D. The statistical significance of differences between the mean values was assessed by Student's *t*-test. *p* values of <0.01 were considered statistically significant.

3. Results

3.1. Functional expression of KGP

To construct the Kgp expression system, a PCR-amplified DNA product containing the promoter region, signal peptide, propeptide and proteinase domain of *kgp* was inserted into the *P. gingivalis* shuttle plasmid vector pYKP028 which was able to be stably maintained in both *E. coli* and *P. gingivalis* (Fig. 1A). The resulting plasmid pNOP003 was mobilized from *E. coli* SM10λpir into the *P. gingivalis kgp* mutant (KDP129). KDP129 harboring pNOP003, which is designated as NOP003, was examined to determine the expression levels of both the Kgp and Rgp activities using the respective synthetic substrates (Fig. 1B and D). Although KDP129 had

no Kgp activity in either the cell lysate or the culture supernatant, NOP003 clearly restored its activity in both fractions. The expression of Kgp and Rgp at protein levels was also determined by a Western blot analysis using antibodies specific for Kgp and antibodies immunoreacting with the catalytic domains of both Rgp and Kgp (Fig. 1C and E). The 51-kDa proteinase domain of Kgp, like the 44-kDa proteinase domain of Rgp, was restored in NOP003.

3.2. Recovery of black pigmentation and processing of HbR

We previously demonstrated that Kgp deficiency resulted in the lack of colonial black pigmentation onto blood agar plates and the impaired production of HbR.[25] To determine whether NOP003 is able to form black pigmented colonies, it was incubated on the blood agar plate (Fig. 2A). Whereas KDP129 exhibited little black pigmentation, NOP003 formed more black pigmented colonies than those formed by the wild-type strain. Consistently, the ability to produce the 19-kDa HbR protein, which was not observed with KDP129, was restored in NOP003 to the level of that of the wild-type strain (Fig. 2B). These results indicate that the recombinant Kgp expressed in a form lacking the C-terminal adhesin domain has normal catalytic activity, thus resulting in the restoration of the bacterial functions caused by Kgp deficiency.

3.3. Identification of active sites of KGP

We have previously suggested that an active-site Cys residue of Rgp is 471st Cys, based on the sequence similarity of the active site region to that of clostripain.[22] This notion is supported by the results obtained by active-site mapping experiments using Nα-[³H]acetyllysine chloromethyl ketone[36] and peptidyl chloromethanes.[37] Given that the sequence similarity of the active-site regions between Rgp and Kgp, the active site Cys residue of Kgp is assumed to be 476th and/or 477th Cys. To

determine which Cys residues are essential for the Kgp activity, we constructed and expressed Kgp mutants with changed these residues (C476A, C477A, and C476A/C477A, termed NOP031, NOP021, and NOP011, respectively) in KDP129 (Fig. 3A). A Western blot analysis with antibodies specific to Kgp revealed that all the three mutants were produced at an apparent molecular mass of 51 kDa, which is identical with that of the wild-type strain (Fig. 3B). One of the single mutants, NOP031, showed a significant Kgp activity in both the cell lysate and the culture supernatant, although their levels were lower than those of the wild-type strain (Fig. 3C). In contrast, the single mutant NOP021 and the double mutant NOP011 completely lost the Kgp activity. Consistent with these results, whereas the former single mutant formed black-pigmented colonies on a blood agar plate (Fig. 4) and generated the 19-kDa HbR protein (data not shown), the latter single mutant and the double mutant showed no colonial pigmentation and no production of HbR protein. On the other hand, the protein and activity levels of Rgp in all of these mutants were not significantly different from those of the wild-type strain (Fig. 5). These results indicate that the 477th Cys residue is required for the full activity of Kgp, but that the substitution of the active-site Cys residues of Kgp does not affect the expression of Rgp.

4. Discussion

The *P. gingivalis sod* gene encoding superoxide dismutase (SOD) has been shown to be expressed as an enzymatically active enzyme in *E. coli*.[38] Despite extensive efforts, however, attempts to generate recombinant Rgp and Kgp in *E. coli* or baculovirus have so far proven to be unsuccessful.[22, 39, 40] Given that SOD is a cytoplasmic protein, whereas Rgp and Kgp are cell surface and extracellular proteins, the success may depend on the cellular localization of individual proteins in *P. gingivalis*. Pavloff et al.[39]

successfully expressed *rgpB* in yeast and obtained the catalytically active enzyme, although its activity was lower than that of Rgp from the wild-type strain. These authors also demonstrated the importance of the truncation at the N-terminus for its activation. The ability of yeast to add carbohydrate moieties to proteins may serve for the correct folding of Rgp. More recently, the C-terminal adhesin domain of Kgp was shown to be necessary for the expression of the active enzyme.[30] They also suggested that the C-terminal domain is essential for the proper processing of this multiprotein complex and the assembly on the cell surface. In this study, we constructed wild-type Kgp and its mutants with changed active-site Cys residues using a shuttle plasmid containing *kgp* derivatives without replacement by homologous recombination. When the shuttle plasmid containing the *kgp* gene lacking the C-terminal adhesin domain was introduced into the Kgp-null mutant by electroporation, we could not obtain any transformants, probably due to the plasmid size. We therefore introduced the plasmid into the Kgp-null mutant by mobilization from *E. coli* to *P. gingivalis* with the aid of RP4 helper plasmid in the donor *E. coli*. The resulting strain NOP003, the *kgp* mutant harboring the plasmid pNOP003, exhibited 53-77% of the Kgp activity of the wild-type parent in the cell extract and culture supernatant, thus indicating that the C-terminal adhesin domain is not necessarily required for the correct folding of Kgp and the expression of its activity. In addition, the expression of Rgp at activity and the protein levels in the cells did not significantly change by the expression of the wild-type Kgp and its mutants, thus suggesting the generation of various bacterial proteins that are processed by Rgp to not be affected by whether Kgp is expressed or not.

Our previous study with Kgp-null mutants[25] indicated that Kgp plays an important role in heme accumulation and black pigmentation. As the C-terminal amino acid residue of HbR is Lys,[23-25, 39] it is likely that HbR

is processed by Kgp, thereby resulting in its binding to hemoglobin. Consequently, the bacterium acquires heme from hemoglobin, which is essential for its proliferation and growth. The *kgp* mutant harboring pNOP003 restored the ability to form black-pigmented colonies on the blood agar plate and to generate the normaly processed HbR, thus indicating that Kgp is directly involved in these events in the bacterium.

Nishikata and Yoshimura determined the active site of Rgp by using a labeling method of active sites with N α-[3H]acetyllysine chloromethyl ketone.[36] The labeled active site peptide isolated by high performance liquid chromatography was [468]Asp-Val-Ala-Cys-Val-Asn-[474]Gly. The fact that the sequence resembled the active site structure of the arginine-specific cysteine proteinase clostripain from *C. histriticum* thus confirmed that the 471st Cys residue is the active site of Rgp. Based on the alignment with the sequence, an active site region of Kgp was therefore predicted to be [474]Gly-Asn-Cys-Cys-Val-Thr-[480]Ala. Since two Cys residues are present in this region, it appears to be impossible to determine which Cys is an active site of Kgp using the labeling method. Therefore, to identify which Cys is essential for the catalytic activity of Kgp, we constructed a mutated *kgp* gene with a base substitution at the Cys residues on the shuttle plasmid using site-directed mutagenesis and then introduced it into KDP129. The double mutant (C476A/C477A) and one of two single mutants (C477A) showed no Kgp activity, although a distinct immunoreactive protein band with antibodies to Kgp was detected at a similar molecular size. On the contrary, another single mutant (C476A) retained about 50% of the Kgp activity found in the cell lysate and the culture supernatant of the wild-type strain. These results indicate, for the first time, that the 477th Cys residue is essential for the expression of Kgp activity.

In conclusion, we herein observed the expression of an active Kgp from a plasmid possessing the *kgp* gene in *P. gingivalis*. The *P. gingivalis kgp* mutant expressing the recombinant Kgp showed colonial black pigmentation and a normal processing of HbR. Using this plasmid system, we demonstrated that [477]Cys of Kgp was able to function as an active site. This expression system is therefore considered to be useful to elucidate the mechanisms of transport for periplasm proteins, outer membrane proteins, and secretary proteins in *P. gingivalis*.

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Figure legends

Fig. 1 - Characterization of the recombinant Kgp expression mutant (NOP003) compared with wild-type *P. gingivalis* (ATCC33277). (A); A schematic drawing of the domain structures of NOP003 and ATCC33277. SP; Signal Peptide, Pro; Pro-region, nGP44 and cGP44; N-terminal and C-terminal region of Kgp hemagglutinin domain. K; restriction enzyme site for *Kpn*I. (B) and (C); The activity (B) and protein (C) levels of Kgp in the cell lysate and culture supernatant fractions of NOP003 were compared with those of ATCC33277 and Kgp-null mutant (KDP129) by activity measurements with Boc-Val-Leu-Lys-MCA as a substrate and a Western blot analysis with antibodies specific to Kgp, respectively. 2.5 µg of cell lysate and 5 µg of the supernatant were applied in these experiments. (D) and (E); The activity (D) and protein (E) levels of Rgp in the cell lysate and culture supernatant fractions of NOP003 were compared with those of ATCC33277 and Kgp-null mutant (KDP129) by activity measurements with Z-Phe-Arg-MCA as a substrate and a Western blot analysis with antibodies reacting with both Rgp and Kgp, respectively. 1 µg of cell lysate and 2 µg of the supernatant were applied in these experiments. The white and black bars indicate the cell lysate and the culture supernatant, respectively. The data are the mean ± S.D. of values from 5 independent experiments. *, $p<0.01$ for the indicated comparisons.

Fig. 2 - Restoration of the abilities to form black-pigmented colonies and to generate HbR protein in NOP003. (A) Colonial black pigmentation by ATCC33277, KDP129 and NOP003 that were anaerobically incubated at 37°C for 5 days onto a laked sheep blood agar plate. (B) Western blot analysis of the cell lysate and supernatant fractions from each bacterial strain with antibodies specific for HbR. 2 µg of cell lysate and the supernatant were applied in these experiments.

Fig. 3 - Characterization of Kgp mutants with changed active-site Cys residues in KDP129. (A) A sequence alignment of the active site regions of Kgp in ATCC33277, single mutants NOP031 (C476A) and NOP021 (C477A), and the double mutant NOP011 (C476A/C477A). (B) A Western blot analysis of the cell lysate and culture supernatant fractions from each mutant with antibodies specific Kgp. 2.5 µg of cell lysate and 5 µg of the supernatant were applied in these experiments. (C) The activity levels of Kgp in the cell lysate and culture supernatant fractions from each mutant. The white and black bars indicate the cell lysate and the culture supernatant, respectively. The data are the mean ± S.D. of values from 6 independent experiments. *, $p<0.01$ for the indicated comparisons.

Fig. 4 - The activity and protein levels of Rgp in Kgp mutants with changed active-site Cys residues expressed in KDP129. (A) A Western blot analysis of the cell lysate and culture supernatant fractions from each mutant with antibodies reacting with Rgp and Kgp. 1 µg of cell lysate and 2 µg of the supernatant were applied in these experiments. (B) The activity levels of Rgp in the cell lysate and culture supernatant fractions from each mutant. The white and black bars indicate the cell lysate and the culture supernatant, respectively. The data are the mean ± S.D. of values from 6 independent experiments.
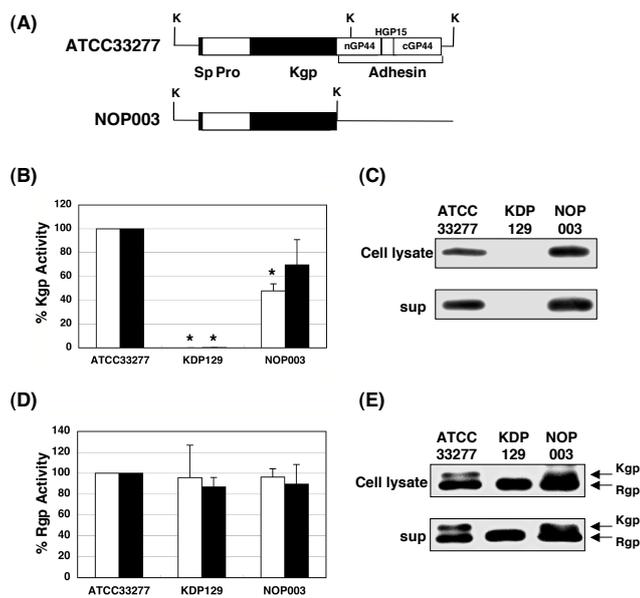
9

(A)

ATCC33277
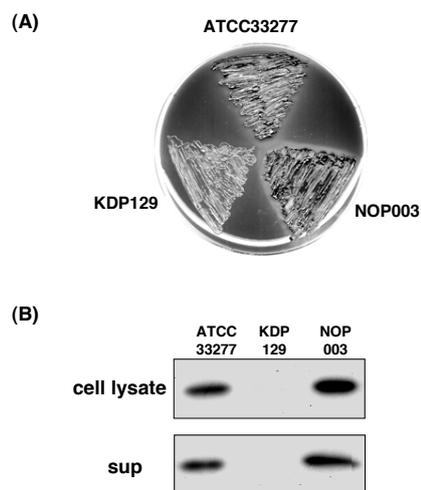
NOP003

(B)

% Kgp Activity

(C)

	ATCC 33277	KDP 129	NOP 003
Cell lysate			
sup			

(D)

% Rgp Activity

(E)

	ATCC 33277	KDP 129	NOP 003
Cell lysate			← Kgp / ← Rgp
sup			← Kgp / ← Rgp

Fig. 1 Ishida et al.

(A)

ATCC33277

KDP129 NOP003

(B)

	ATCC 33277	KDP 129	NOP 003
cell lysate			
sup			

Fig. 2 Ishida et al.

(A)

GN**CC**VTAQFD   **ATCC33277**
GN**A**CVTAQFD   **NOP031 (C476A)**
GNC**A**VTAQFD   **NOP021 (C477A)**
GN**AA**VTAQFD   **NOP011 (C476A/C477A)**

(B)

	ATCC 33277	KDP 129	NOP 031	NOP 021	NOP 011
Cell lysate					
sup					

(C)

% Kgp Activity

Fig. 3 Ishida et al.

(A)

	ATCC 33277	KDP 129	NOP 031	NOP 021	NOP 011
cell lysate					← Rgp
sup					← Rgp

(B)

% Rgp Activity

Fig. 4 Ishida et al.