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Porphyromonas gingivalis mutant defective in a putative extracytoplasmic function sigma factor shows a mutator phenotype

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Porphyromonas gingivalis *mutant defective in a putative ECF sigma factor shows a mutator phenotype.*
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Introduction: *Porphyromonas gingivalis* is implicated as a major pathogen in the development and progression of chronic periodontitis. *P. gingivalis* must possess the ability to tolerate stress signals outside the cytoplasmic membrane by transcriptional activation of genes encoding proteins involved in defense or repair processes. Some bacteria utilize a distinct subfamily of sigma factors to regulate extracytoplasmic function (hence termed the ECF subfamily).
Methods: To elucidate their role in *P. gingivalis*, a chromosomal mutant carrying a disruption of an ECF sigma factor PG1318-encoding gene was constructed. Hemagglutination and proteolytic activities were measured in the PG1318-defective mutant. Reverse transcription-polymerase chain reaction (RT-PCR) analysis and southern blot analysis were used to assess transcription of *kgp* in the PG1318-defective mutant. Frequency of spontaneous mutation that conferred resistance to L-trifluoromethionine was measured in the PG1318-defective mutant.
Results: The PG1318-defective mutant formed non-pigmented colonies on blood agar plates at a relatively high frequency. Arginine-specific and lysine-specific proteinase activities of the non-pigmented variants were remarkably decreased compared with those of the parent strain and the pigmented variants. RT-PCR analysis showed that *kgp* was not transcribed in some non-pigmented variants and southern blot analysis revealed that there was a deletion in their *kgp* region. Frequency of mutation conferring resistance to L-trifluoromethionine was significantly higher in the PG1318-defective mutant than in the wild-type.
Conclusion: These results suggest that PG1318 plays a role in the regulation of mutation frequency in the bacterium.

Key words: ECF sigma factor; mutator; *Porphyromonas gingivalis*

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Bacteria are affected by constantly changing nutrient availability and by exposure to various forms of physical stress, including osmotic, oxidative, and temperature shock. To adapt to these changes, bacteria use multiple regulatory pathways. A quite simple and effective mechanism employed by bacteria that results in a major alteration in gene expression is the use of alternative sigma factors that alter RNA polymerase core specificity (7, 10, 11, 32). One such class of sigma factors appears to control envelope stress responses, including protein folding, cell wall biosynthesis, and pathogenesis. Many of these responses are controlled by extracytoplasmic function (ECF) sigma factors, which belong to a subfamily of the sigma 70 class (18).

The anaerobic gram-negative bacterium *Porphyromonas gingivalis* is considered to be one of the etiologically important

agents for periodontal disease (13). This bacterium expresses numerous potential virulence factors, such as fimbriae, hemagglutinins, lipopolysaccharides, and various proteases that are capable of hydrolysing collagen, immunoglobulins, iron-binding proteins, and complement factors (12, 15). To colonize and survive at the gingival crevice, *P. gingivalis* must be capable of sensing and responding to the prevailing environmental conditions, including variations in temperature, oxygen tension, pH, nutrient availability, and the presence of other bacterial cells. However, little is known about changes in gene expression that are involved in responses to extracytoplasmic stress.

According to The Institute for Genomic Research (TIGR) genome sequence database (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org_search=&org=gpg), the genome of *P. gingivalis* possesses a total of eight sigma factors: one RpoD homologue (sigma-70 factor, PG0594), one RpoN homologue (sigma-54 factor, PG1105) and six sigma factors belonging to the ECF subfamily (PG0162, PG0214, PG0985, PG1318, PG1660, and PG1827). In this study, we constructed a *P. gingivalis* mutant that was defective in one of the ECF sigma factors, PG1318, and analysed the functions of this protein in the organism. The PG1318-defective mutant formed less-pigmented or non-pigmented colonies on blood agar plates at a relatively high frequency compared with the wild-type parent strain. Frequency of mutation conferring resistance to L-trifluoromethionine (TFM) was higher in the PG1318-defective mutant than in the wild-type parent. These results suggest that the PG1318 ECF sigma factor is involved in regulation of mutation frequency in the bacterium.

Materials and methods

Bacterial culture conditions

Cells of *P. gingivalis* were grown anaerobically (10% CO_2, 10% H_2, and 80% N_2) in enriched brain–heart infusion (BHI) broth and on enriched tryptic soy (TS) agar (20). For blood agar plates, defibrinated laked sheep blood was added to enriched TS agar at 5%.

Chemicals

N-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt was obtained from Sigma-Aldrich Japan (Tokyo, Japan), and benzoyl-L-arginine *p*-nitroanilide hydrochloride was obtained from Peptide Institute, Inc., (Osaka, Japan).

General genetic procedures

Unless otherwise stated, DNA cloning, sequencing, southern blot ting, *Escherichia coli* plasmid purification, and other common molecular biology techniques were carried out using standard procedures (25).

Construction of the PG1318-defective mutant

P. gingivalis W83 genome sequence data were obtained from the TIGR website (http://www.tigr.org). To disrupt the PG1318 ECF sigma factor encoding gene, the genome region including the PG1318 gene was amplified by polymerase chain reaction (PCR) from the chromosomal DNA of *P. gingivalis* W83 using *Takara Ex Taq* (Takara Bio, Otsu, Japan) and gene-specific primers. A DNA fragment containing part of the 5′ end of the PG1318 gene and upstream region of the ATG initiation codon and a DNA fragment containing the 3′ end of the PG1318 gene and downstream region of its stop codon were amplified using primer sets 1318NFOR (5′-AGAGAGCCGATTGCTTGAAT-3′) and 1318NREV (5′-GGGATCCCTTCGTACATGGCTATCCGAT-3′), 1318CFOR (5′-GGGATCCCTCACTCAATTTCCTGAAG-3′) and 1318CREV (5′-GCAATTTAGGCCACAATGCT-3′), respectively. The *Bam*HI restriction site (underlined) was incorporated into these primers to facilitate subcloning of the PCR product. Both fragments were ligated into the multiple cloning site of the T-vector (pGEM-T Easy, Promega, Tokyo, Japan). A *Bam*HI–*Sac*I fragment containing the 3′ end of the PG1318 gene was extracted from the resulting plasmid and ligated into the *Bam*HI–*Sac*I site of the plasmid containing the 5′ end of the PG1318 gene to yield pKD820 (PG1318). The *ermF-ermAM* cassette of pKD355 (29) was inserted into the *Bam*HI site within PG1318 of pKD820 to yield pKD825. This plasmid DNA was linearized by *Not*I digestion and introduced into *P. gingivalis* W83 cells by electroporation as described previously (19), resulting in KDP310 (*PG1318*::*ermF ermAM*). To rule out the possibility of a polar effect of *ermF-ermAM*, we constructed the PG1318 mutant using the *ermF* cassette of pKD355F, which contained neither promoter nor terminator of *ermF*. The *ermF* cassette of pKD355F was inserted into the *Bam*HI site within PG1318 of pKD820 to yield pKD828. The plasmid DNA was linearized by *Not*I digestion and introduced into *P. gingivalis* W83 cells by electroporation, resulting in KDP313 (*PG1318*::*ermF*). Correct gene replacement of these strains that had been generated by double cross-over recombination events was verified by PCR and southern blot analysis (data not shown).

Appearance of colonial variants of PG1318-deficient mutant

One hundred microliters of the culture of *P. gingivalis* W83 and the PG1318 mutant KDP313 (*PG1318*::*ermF*), which were diluted $1 : 10^7$ in fresh BHI broth, were spread on enriched TS agar plates supplemented with 5% defibrinated sheep blood and incubated anaerobically at 37°C. Pigmentation of colonies on blood agar plates after 14 days was examined and the number of pigmented colonies was counted.

Hemagglutination assay

The hemagglutination assay was performed as described previously (27). Briefly, stationary-phase cultures of *P. gingivalis* strains in enriched BHI medium were centrifuged, washed with phosphate-buffered saline (PBS), and resuspended in PBS at an optical density at 600 nm of 0.85. The bacterial suspensions were then diluted in a twofold series with PBS. A 100-μl aliquot of each of the dilutions was mixed with an equal volume of sheep erythrocyte suspension (1% in PBS) and incubated in a round-bottom microtiter plate at room temperature for 3 h.

Enzymatic assays

Lys-X-specific and Arg-X-specific cysteine proteinase activities were determined using the synthetic substrates *N*-(*p*-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt and benzoyl-L-arginine *p*-nitroanilide hydrochloride, respectively. Samples were prepared using the method of Hasegawa et al. (9) with some modification. In brief, the absorbance at 595 nm was measured after 48-h incubation in anaerobic conditions at 37°C. An aliquot (3.0 ml) of the culture was subjected to centrifugation, and the resulting supernatant was used as 'the culture supernatant' for the assay. Pelleted cells were washed several times with 10 mM HEPES/NaOH (pH 7.4) and then suspended in the same buffer to adjust the absorbance at 595 nm (A_{595}) to 1.0 and used as 'whole cells'. Gingipain activities were determined using a 200-μl reaction buffer containing 0.2 mM substrate, 50 mM Tris–HCl (pH 8.0), and 10 mM

dithiothreitol. The reaction was started by adding 4.0 μl of the culture supernatant or the whole cells. The reaction mixtures were incubated at 37°C for 30 min. After 40 μl of 50% acetic acid had been added, release of the cleaved product, *p*-nitroanilide, was determined by measuring absorbance at 405 nm. Proteinase activities were divided by the A_{595} values of cell density to normalize all the values per A_{595} unit.

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from *P. gingivalis* cells grown to the exponential phase (OD_{600} = 0.7) by using an SV Total RNA Isolation system (Promega) according to the manufacturer's instructions. The RNA samples were then incubated with DNase I (Nippon Gene, Tokyo, Japan) at 37°C for 15 min. The quality of RNA was assessed by measurement of the ratio of absorbance of the RNA at 260 nm to that at 280 nm and by electrophoresis of total RNA followed by staining with ethidium bromide. An aliquot of RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) with a random hexamer primer (Toyobo, Osaka, Japan) and ReverTra Ace (Toyobo). The cDNA were then used for PCR amplifications with *Tfi* DNA polymerase (Invitrogen, Carlsbad, CA). Reactions were prepared according to the manufacturer's instructions with *kgp*-specific primers (kgpRTFOR; 5′-CAGGTAGGTCAGCCA ACCAT-3′ and kgpRTREV; 5′-AGC ATGAGTAGCGGCAAGAT-3′), *PG1318*-specific primers (PG1318RTFOR; 5′-TGG CTGATAAGCTATTGATTTCG-3′ and PG1318RTREV; 5′-TAATCATCGGGC GACAAAA-3′), and *16S rRNA*-specific primers (16SrRNARTFOR; 5′-GCGA CGATGGGTAGGGGAACTGAGAGG-3′ and 16SrRNARTREV; 5′-TTCAGTGTCA GTCGCAGTATGGCAAG-3′). The PCR thermal profile consisted of 2 min at 94°C, 25 cycles of 15 s at 94°C, 20 s at 55°C, and 1 min at 72°C.

Southern blot hybridization

Chromosomal DNAs of W83 (wild-type) and KDP313 (*PG1318*::*ermF*) were digested with *Bam*HI (for *kgp*-specific probe) or *Eco*RV (for *PG1318*-specific probe), separated on a 1.0% agarose gel, and transferred to a nylon membrane. Labeling of the probes and detection of hybridization signals were carried out using an AlkPhos Direct™ system (GE Healthcare Bio-sciences Ltd, Little Chalfont, Bucks, UK) and CDP-star™ detection reagent (GE Healthcare Biosciences Ltd). DNA fragments, used for a probe, were obtained by PCR conducted with the set of specific primers. A DNA fragment (522 bp) comprising the catalytic domain of *kgp* was amplified using the oligonucleotide primers kgpRTFOR and kgpRTREV. A DNA fragment (531 bp) comprising the 3′-terminal region of *PG1318* and its downstream region was amplified using the oligonucleotide primers 1318CFOR and 1318CREV.

Determination of mutation frequency

For the assay, 10 parallel cultures per *P. gingivalis* strain were first inoculated with a small number (OD_{600} = 0.1) of bacterial cells. To do this, a number of colonies on the agar plate were inoculated into liquid BHI medium, allowed to grow anaerobically to a steady state at 37°C for 1 day, and diluted in prewarmed medium. Cultures were again grown anaerobically to a steady state for 2 days to obtain parallel, independent cultures. The number of resistant mutants that emerged in each culture was determined by plating 5.0 μl of each of these cultures on TS agar plates containing 1.0 mM TFM. Yoshimura et al. (33) reported that growth of *P. gingivalis* W83 was completely inhibited by 1.0 mM TFM. The total number of cells was determined by plating a 10^6 dilution of these cultures on non-selective TS agar plates. Colonies on both selective and non-selective plates were counted after anaerobic incubation for 7 days. The frequency of resistant mutants was expressed as the number of resistant cells divided by the total number of viable cells per culture.

Statistical analysis

Student's *t*-test was used to evaluate the difference in mutation frequencies between W83 and KDP313 by using SPSS for Windows software (version 15.0J, SPSS Japan Inc., Tokyo, Japan).

Results

Construction of PG1318-defective mutant of *P. gingivalis*

To analyse the biological significance of ECF sigma factors in *P. gingivalis* cells, the PG1318-deficient mutant was constructed. The PG1318 mutant (KDP310) yielded three types of colonies. The first group displayed a phenotype similar to that of the W83 wild-type strain: hemolytic and black-pigmented [94.5% of the total colony-forming units (CFU)]. The second group was non-black-pigmented and displayed no detectable hemolysis on blood agar plates (1.9% of the total CFU). The third group was weakly pigmented (3.6% of the total CFU). To confirm changes in the PG1318 gene in the three types of mutants, southern blot hybridization and RT-PCR analyses were performed and revealed proper construction in all of the three phenotypes of the PG1318 mutants (data not shown).

Appearance of colonial variants of *P. gingivalis* PG1318 mutant

Previous studies have shown links between colonial pigmentation on blood agar plates, hemagglutination and Kgp/Rgp activity in *P. gingivalis* cells (4, 27). Modulation of bacterial virulence factors generally depends on environmental changes, so it is important to know the reason for the black pigmentation defects of some of the PG1318 mutants. To analyse the appearance of colonial variants of *P. gingivalis* PG1318 mutants, we counted the colonial variants after electroporation and during passage culture three times. As there is a possibility that colonial variants might be the result of a polar effect, we inserted the *ermF* cassette (no *ermF* promoter or *ermF* terminator) into the PG1318 gene. The PG1318 mutant (*PG1318*::*ermF*, KDP313) also yielded three types of colonies. The first group (B1, B2, B3, and B4) displayed a phenotype similar to that of the W83 wild-type strain: hemolytic and black-pigmented (88.8% of the total CFU). The second group (W2, W3, and W4) was non-black-pigmented and displayed no detectable hemolysis on blood agar plates (5.1% of the total CFU). The third group (W1) was weakly pigmented (6.1% of the total CFU). Although almost 95% of the colonies were pigmented or weakly pigmented during incubation for up to 14 days, about 5% of the colonies were non-pigmented. In contrast, the parent strain W83 formed all strongly pigmented colonies on blood agar plates. Next, the numbers of pigmented and non-pigmented colonies were counted during three serial passages. It was found that pigmented, non-pigmented, or weakly pigmented colonies appeared after inoculation of culture of a pigmented colony of the PG1318 mutant (Fig. 1A, Table 1). The results suggested that spontaneous mutation frequency might be enhanced by mutation in the PG1318 gene.

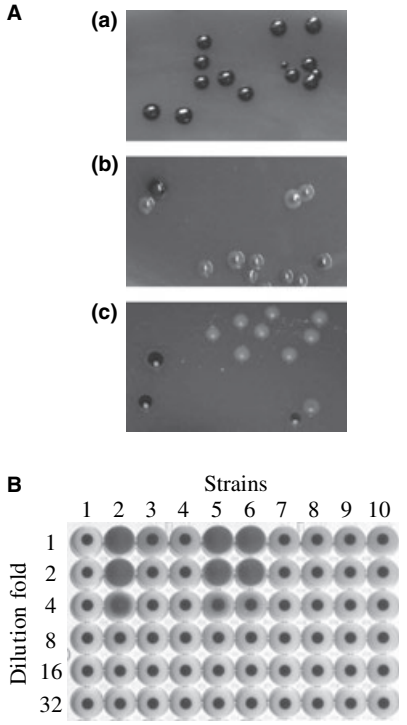

Fig. 1. (A) Morphology of *PG1318* insertion mutants. The strains were grown on enriched tryptic soy agar plates supplemented with 5% defibrinated sheep blood anaerobically at 37°C for 14 days. W83 (a), a pigmented variant of KDP313 (*PG1318*::*ermF*) (b), a white variant of KDP313 (c) were cultured and plated. (B) Hemagglutinin activity of colonial variants of the *Porphyromonas gingivalis* PG1318 mutant. *P. gingivalis* cells were grown in enriched brain–heart infusion broth, washed with phosphate-buffered saline (PBS), and resuspended in PBS at an optical density at 0.85. The suspension and its dilutions in a two-fold series were applied to the wells of a microtiter plate and mixed with sheep erythrocyte suspension. Lane 1, PBS control (without bacteria); lane 2, W83; lanes 3–6, black pigmented variants of KDP313 (B1–B4); lanes 7–10, no pigmented variants of KDP313 (W1–W4).

Hemagglutination and proteolytic activities in the *P. gingivalis* PG1318-defective colonial variants

We assessed the hemagglutination potential of *P. gingivalis* KDP313 colonial variants in comparison to that of *P. gingivalis* W83. Hemagglutinin activity was not detected in non-pigmented variants of KDP313 at the cell concentrations used (Fig. 1B). Even in black-pigmented variants of KDP313, various degrees of reduction in hemagglutinin activity were observed.

In a previous study (22), we found that a *kgp* mutant with no Kgp proteolytic activity showed reduced pigmentation. Some clones of KDP313 had reduced or no pigmentation, so we determined Rgp and Kgp activities of cell extracts and culture supernatants of the KDP313 colonial variants. As shown in Table 2, the non-pigmented variants showed very weak Kgp activity in the cell extract and the culture supernatant. These values were comparative to those of KDP129 (*kgp*) and KDP136 (*rgpA rgpB kgp*) (27), suggesting that the non-pigmented variants show little or no Kgp activity. The pigmented variants showed higher Kgp activity both in the cell extract and the culture supernatant than that of the non-pigmented KDP313. Rgp activity in the non-pigmented variants in all samples was similar to the activities of the Rgp-defective mutants KDP133 (*rgpA rgpB*) and KDP136 (*rgpA rgpB kgp*) (Table 2). Taken together, the results indicate that activity of Kgp and Rgp in the non-pigmented variants of KDP313 was very weak.

Table 1. Appearance of colonial variants during passages

Passage number	Pigmentation on blood agar after 14 days	Numbers of colonies (percentage of colonies) W83	KDP313
1	Black → black	66 (100.0)	5 (6.4)
	Black → weak pigmentation or white	0 (0.0)	73 (93.6)
	White → white	–	99 (96.1)
	White → weak pigmentation or black	–	4 (3.9)
2	Black → black	35 (100.0)	18 (50.0)
	Black → weak pigmentation or white	0 (0.0)	18 (50.0)
	White → white	–	48 (100.0)
	White → weak pigmentation or black	–	0 (0.0)
3	Black → black	166 (100.0)	6 (5.9)
	Black → weak pigmentation or white	0 (0.0)	96 (94.1)
	White → white	–	186 (100.0)
	White → weak pigmentation or black	–	0 (0.0)

Each colony was inoculated into the enriched brain–heart infusion (BHI) broth. The cultures were incubated anaerobically at 37°C. After 2 days, they were diluted $1 : 10^7$ with fresh BHI broth and 100 μl of the diluted culture were plated onto enriched tryptic soy agar plate and grown for 14 days. Three serial experiments were carried out and colonial variants were counted.

Table 2. Rgp and Kgp activities of various *Porphyromonas gingivalis* mutants

Strain	Cell extract (relative activity) Rgp	Kgp	Culture supernatant (relative activity) Rgp	Kgp
W83	100.0 ± 0.5	100.5 ± 0.8	100.0 ± 1.7	98.0 ± 4.5
KDP313 (PG1318)-B1	27.3 ± 0.7	29.6 ± 0.6	42.4 ± 1.2	47.0 ± 2.4
KDP313 (PG1318)-B2	30.0 ± 0.9	30.4 ± 0.7	95.9 ± 1.5	130.5 ± 1.7
KDP313 (PG1318)-B3	91.3 ± 0.7	104.2 ± 0.7	96.9 ± 4.1	146.9 ± 3.9
KDP313 (PG1318)-B4	95.1 ± 1.7	119.2 ± 0.7	55.2 ± 2.2	101.0 ± 2.9
KDP313 (PG1318)-W1	19.2 ± 0.3	8.6 ± 0.1	15.8 ± 0.6	4.7 ± 0.4
KDP313 (PG1318)-W2	16.3 ± 0.3	15.8 ± 0.2	13.4 ± 0.5	14.4 ± 0.3
KDP313 (PG1318)-W3	7.4 ± 0.3	8.1 ± 0.2	2.8 ± 0.4	4.2 ± 0.4
KDP313 (PG1318)-W4	8.7 ± 0.1	10.1 ± 0.2	21.4 ± 0.7	22.9 ± 0.9
33277	103.0 ± 1.1	116.8 ± 3.4	92.0 ± 0.7	89.5 ± 0.9
KDP129 (*kgp*)	89.5 ± 4.3	13.7 ± 5.0	464.5 ± 13.3	10.6 ± 1.5
KDP133 (*rgpA rgpB*)	12.5 ± 2.7	101.2 ± 3.4	5.4 ± 0.9	111.4 ± 2.1
KDP136 (*rgpA rgpB kgp*)	14.8 ± 0.2	15.9 ± 0.1	5.4 ± 0.9	8.2 ± 1.6

Forty-eight-hour cultures of *P. gingivalis* strains in enriched brain–heart infusion broth were centrifuged. The cell extracts and culture supernatants were used for the determination of Rgp and Kgp activities. Data are the means ± SE of three independent experiments.

RT-PCR and southern blot analyses of the PG1318-defective colonial variants

To determine what causes the decrease in Kgp activity of non-pigmented variants, we performed RT-PCR and southern blot analyses of the *kgp* gene of the PG1318-defective colonial variants. To determine the presence of *kgp* messenger RNA, total RNA was isolated from the wild-type W83 and the colonial variants of KDP313 grown to mid-log phase. Specific primers for *kgp* and the PG1318-encoding gene were used in RT-PCR to amplify a 0.5-kb region of the transcripts. In the *kgp* transcript, no amplified products were observed for the non-pigmented variants W1 and W3 (Fig. 2A). All of the other variants showed *kgp* transcript levels the same as or higher than that of the wild-type (Fig. 2A). Next, southern blot analysis was performed to determine whether the *kgp* gene of the PG1318-defective

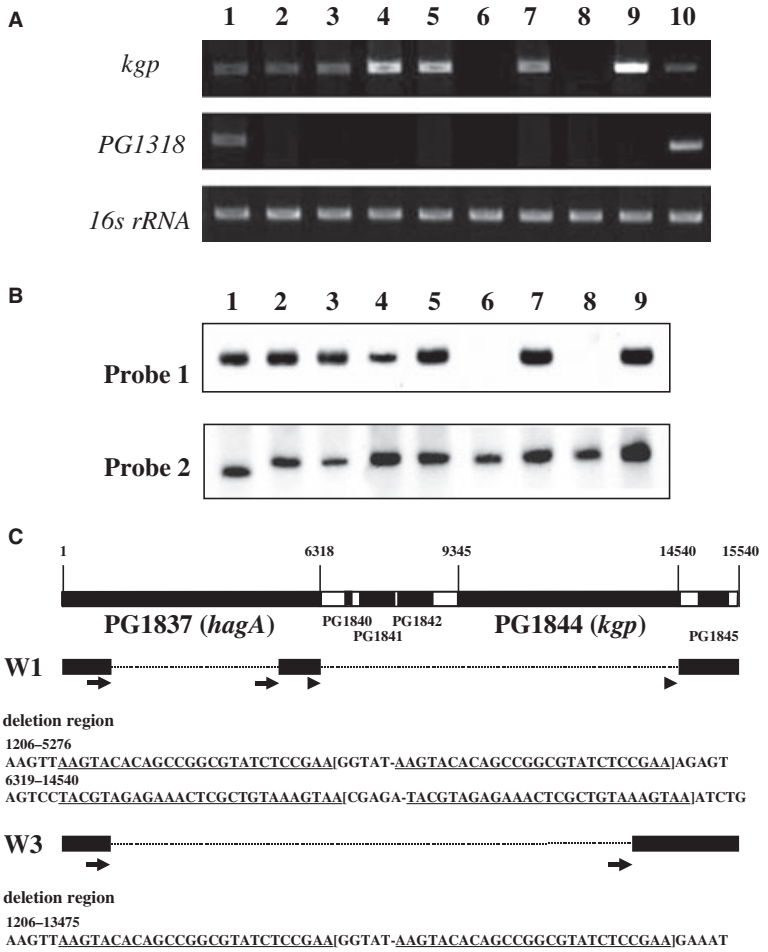

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis, Southern hybridization, and DNA sequence of the *kgp* gene. (A) Intragenic primers of *kgp* were used for RT-PCR. Lane 1, W83; lanes 2–5, black pigmented variants of KDP313 (*PG1318*::*ermF*) (B1–B4); lanes 6–9, non-pigmented variants of KDP313 (W1–W4); lane 10, positive control (W83 genome). All lanes contain 2 μl of the amplified mixture. (B) Southern hybridization of the *PG1318* insertion mutant. Chromosomal DNA from KDP313 and W83 (wild-type) were digested with *Bam*HI (for *kgp*-specific probe; Probe 1) or *Eco*RV (for *PG1318*-specific probe; Probe 2) and subjected to Southern hybridization using the *kgp*-specific probe (as the same of Southern hybridization probe). Lane 1, W83; lanes 2–5, black-pigmented variants of KDP313 (B1–B4); lanes 6–9, non-pigmented variants of KDP313 (W1–W4). (C) Structure of the *PG1837* (*hagA*) to *PG1844* (*kgp*) region on the *P. gingivalis* chromosome W1 and W3. Arrows and arrow heads below the map indicate repeated sequences. The precise positions of the breakpoint in brackets in W1 and W3 could lie within the repeated sequences (underlined).

colonial variants is changed at the DNA level. The non-pigmented variants W1 and W3 showed no band hybridizing to the *kgp* catalytic domain-specific probe DNA, whereas all of the other variants showed the band (Fig. 2B). These results suggested that there was a deletion at the *kgp* locus in the non-pigmented variants W1 and W3. To identify the deletion in the *kgp* locus of W1 and W3 genomes, we performed direct sequencing of the *PG1837*(*hagA*) to *PG1844*(*kgp*) region in W1 and W3 genomes. Figure 2C shows the deletion sites identified in W1 and W3. Two deletions in W1 and one deletion in W3 were flanked by a direct repeat. Two deletions (nucleotide 1206–5276 in W1, nucleotide 1206–13475 in W3) had the same 26-bp direct repeat. We also analysed the *kgp* region sequence in W2 and W4. Although there was no deletion site within *kgp*, CCC bases were inserted between A (13804) and T (13805). The same insertion was found in W3. These insertions might have no effect on *kgp* transcription because that occurred downstream of the Kgp protease domain.

Frequencies of spontaneous mutations

To examine whether the PG1318-defective mutant has the mutator phenotype, we determined the frequency of mutation causing resistance to TFM in 10 clones of *P. gingivalis* strains W83 and KDP313 (Table 3). The mutation frequency of W83 varied between 1.7×10^{-6} and 2.2×10^{-7}, while that of KDP313 varied between 7.6×10^{-6} and 1.6×10^{-6}. The average mutation frequency of KDP313 was statistically ($P < 0.001$) greater than that of the wild-type parent strain W83.

Discussion

Bacteria sometimes encounter an environment that is unfavorable for their survival. Through the course of evolution and continuous adaptation, bacteria have developed several methods to cope with a variety of environmental stresses. Therefore, response to stresses is a major determinant for bacterial survival in various environments. There are two types of stress, cytoplasmic stress and extracytoplasmic stress. Cytoplasmic stress results in the generation of misfolded proteins in the cytoplasm, whereas extracytoplasmic stress results in the generation of misfolded proteins in the membrane or periplasm. Many of these responses are controlled by ECF sigma factors that respond to external signals. ECF sigma factors have been shown to regulate cell envelope-related processes (involving maintenance of the membrane/periplasmic architecture), such as secretion, synthesis of exopolysaccharides, iron export, and efflux synthesis of extracellular proteases (3).

P. gingivalis is implicated as a major pathogen in the development and progression of chronic periodontitis (14). Although this bacterium has numerous potential virulence factors, the proteolytic enzymes are most important because gingipains destroy periodontal tissue directly or indirectly (16). *P. gingivalis* produces large amounts of Kgp and Rgp proteinase in both cell-free and cell-associated forms. Three genes encode the gingipains and these have been designated *rgpA*, *rgpB*, and *kgp* (5). A previous study demonstrated that Hgp15, a 19-kDa polypeptide derived from the adhesin domains of the *rgpA*, *kgp*, and *hagA* gene products, could bind hemoglobin (21). It was revealed that Kgp activity was essential to cleave Hgp15 from these domains, because a *kgp* mutant showed a white colony phenotype on blood plates (22).

In this study, a PG1318-defective mutant showed a high frequency of non-pigmented colonies on blood agar plates. We also disrupted genes for five other ECF sigma factors (PG0162, PG0214, PG0985,

Table 3. Mutation frequency for L-trifluoromethionine (TFM) resistance

P. gingivalis strain	TFM-resistant cells (CFU/ml)	Total viable cells (CFU/ml)	Mutation frequency
W83-1	5.2×10^3	4.2×10^9	1.2×10^{-6}
W83-2	4.0×10^3	5.4×10^9	7.4×10^{-7}
W83-3	3.2×10^3	5.5×10^9	5.9×10^{-7}
W83-4	1.4×10^3	5.0×10^9	2.8×10^{-7}
W83-5	4.4×10^3	4.3×10^9	1.0×10^{-6}
W83-6	6.2×10^3	3.6×10^9	1.7×10^{-6}
W83-7	2.6×10^3	5.3×10^9	4.9×10^{-7}
W83-8	7.2×10^3	4.2×10^9	1.7×10^{-6}
W83-9	2.4×10^3	4.6×10^9	5.2×10^{-7}
W83-10	1.2×10^3	5.4×10^9	2.2×10^{-7}
KDP313-1	3.7×10^4	5.3×10^9	6.9×10^{-6}
KDP313-2	3.7×10^4	4.9×10^9	7.6×10^{-6}
KDP313-3	4.0×10^4	5.7×10^9	7.0×10^{-6}
KDP313-4	2.8×10^4	4.7×10^9	6.0×10^{-6}
KDP313-5	1.1×10^4	6.9×10^9	1.6×10^{-6}
KDP313-6	2.6×10^4	7.2×10^9	3.5×10^{-6}
KDP313-7	1.8×10^4	4.4×10^9	4.1×10^{-6}
KDP313-8	3.9×10^4	2.4×10^9	1.6×10^{-6}
KDP313-9	3.9×10^4	5.4×10^9	7.2×10^{-6}
KDP313-10	3.8×10^4	4.9×10^9	7.7×10^{-6}

Ten parallel cultures per *Porphyromonas gingivalis* strain were first inoculated with a small number (OD_{600} = 0.1) of bacterial cells. Cultures were anaerobically grown to steady state for 2 days. The number of resistant mutants that emerged in each culture was determined by plating 5.0 μl of these cultures on tryptic soy (TS) agar plates containing 1.0 mM L-trifluoromethionine (TFM). The total number of cells was determined by plating 10^{-6} dilution of these cultures on non-selective TS agar plates. Colonies on both selective and non-selective plates were counted after anaerobic incubation for 7 days. The frequency of resistant mutants was expressed at the number of resistant cells divided by the total number of viable cells per culture.

PG1660, and PG1827) individually, but these mutants had black pigmentation (data not shown). All of the non-pigmented variants of the PG1318-defective mutant showed severe defects in gingipain activities. The results of RT-PCR, southern blot analyses, and DNA sequence revealed that the non-pigmented variants W1 and W3 had a DNA deletion in the *kgp*. Figure 2C shows a slipped mispairing model that has been proposed to account for these deletion mutations. This model describes what occurs when a replication fork slips down a repeated region of the DNA strand during DNA replication (17). Taken together, these results showed that the PG1318 might control the expression of proteins involved in DNA replication, such as exonucleases or helicases. However, the defect in the *kgp* gene of the non-pigmented variants W1 and W3 cannot account for the decreased Rgp activities of the variants because a *kgp* mutant has been reported to show normal Rgp activity (22). Several studies have indicated that a number of *P. gingivalis* genes (*porR*, *porT*, *vimA*, *vimE*, *vimF*, *gppX*, PG1140-encoding gene, and *sov*) contribute to colonial pigmentation (1, 2, 4, 9, 24, 26, 28, 30, 31). The non-pigmented variants of the PG1318-defective mutant including W1 and W3 might have some changes in such genes and/or the gingipain-encoding genes. Spontaneous mutation frequency in TFM resistance of the PG1318-defective mutant was higher than that of the wild-type parent. Taken together, the results suggest that the PG1318-defective mutant has a mutator phenotype. A mutator phenotype generally results from a defect in a gene whose normal product is part of some error-avoidance or DNA repair pathway (8). In *Bacillus subtilis*, functional roles of ECF σ^M factor-regulated genes may include DNA monitoring and repair (6). However, there have been no other reports that an ECF sigma factor is involved in mutagenesis and DNA repair. Further study is needed to clarify the relationship between the PG1318 ECF sigma factor and mutation frequency.

In *E. coli*, genetic and biochemical data indicate that at least two genes (*rseA* and *rseB*) are involved in the transduction of the envelope stress signal. Both *rseA* and *rseB* are encoded directly downstream of *rpoE* in an operon (23). RseA (transmembrane anti-sigma factor) and RseB are negative regulators of the pathway that is required for transduction of the envelope stress signal. Interestingly, no RseA or RseB homologue is encoded in the genome of *P. gingivalis*, suggesting that the signaling mechanisms and/or functions of the ECF sigma factors in *P. gingivalis* are different from those in other bacteria such as *E. coli*. Further studies on the roles of the *P. gingivalis* ECF sigma factors in various stress conditions might help us to understand the strategy of *P. gingivalis* for coping with environmental stresses.

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