Effect of extracytoplasmic function sigma factors on autoaggregation, hemagglutination, and cell surface properties of *Porphyromonas gingivalis*

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**Abstract**

*Porphyromonas gingivalis* is a bacterium frequently isolated from chronic periodontal lesions and is involved in the development of chronic periodontitis. To colonize the gingival crevice, *P. gingivalis* has to adapt to environmental stresses. Microbial gene expression is regulated by transcription factors such as those in two-component systems and extracytoplasmic function (ECF) sigma factors. ECF sigma factors are involved in the regulation of environmental stress response genes; however, the roles of individual ECF sigma factors are largely unknown. The purpose of this study was to investigate the functions, including autoaggregation, hemagglutination, gingipain activity, susceptibility to antimicrobial agents, and surface structure formation, of *P. gingivalis* ECF sigma factors encoded by SigP (PGN_0274), SigCH (PGN_0319), PGN_0450, PGN_0970, and SigH (PGN_1740). Various physiological aspects of the **sigP** mutant were affected; autoaggregation was significantly decreased at 60 min (**p** < 0.001), hemagglutination activity was markedly reduced, and enzymatic activities of Kgp and Rgps were significantly decreased (**p** < 0.001). The other mutants also showed approximately 50% reduction in Rgps activity. Kgp activity was significantly reduced in the **sigH** mutant (**p** < 0.001). No significant differences in susceptibilities to tetracycline and ofloxacin were observed in the mutants compared to those of the wild-type strain. However, the **sigP** mutant displayed an increased susceptibility to ampicillin, whereas the **PGN_0450** and **sigH** mutants showed reduced susceptibility. Transmission electron microscopy images revealed increased levels of outer membrane vesicles formed at the cell surfaces of the **sigP** mutant. These results indicate that SigP is important for bacterial surface-associated activities, including gingipain activity, autoaggregation, hemagglutination, vesicle formation, and antimicrobial susceptibility.
Introduction

Bacteria are exposed to physiological stresses such as temperature, oxygen tension, and pH in natural environments that they inhabit. They adapt to these environmental stresses using multiple regulatory pathways [1, 2]. Extracytoplasmic function (ECF) affects bacterial signaling [3] and is associated with signals relaying extracytoplasmic conditions to the cytoplasm. ECF sigma factors, which are the largest group of alternative sigma factors, are activated by environmental stresses and recruit RNA polymerases to directly transcribe genes that encode proteins with environment-specific functions [4, 5].

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium frequently isolated, together with Treponema denticola and Tannerella forsythia, from human chronic periodontal lesions [6]. This microorganism produces cell surface-associated virulence factors, such as cysteine proteases (gingipains), lipopolysaccharides, and fimbriae [7–9], and can invade epithelial cells [10]. It is considered to be a key microorganism in the development of periodontal disease [11]. To colonize the gingival crevice, P. gingivalis has to adapt to the environment. Six putative ECF sigma factors, namely, PGN_0274, PGN_0319, PGN_0450, PGN_0970, PGN_1108, and PGN_1740, were identified in the genome of P. gingivalis ATCC 33277 (GenBank: AP009380) [12].

The functions of ECF sigma factor have been reported in previous studies. For example, PG1318 in strain W83 [P. gingivalis ATCC 33277 open reading frame (ORF) number: PGN_108] is involved in the regulation of mutation frequency in P. gingivalis [13]. P. gingivalis W83 mutants PG0162 (P. gingivalis ATCC 33277 ORF number: PGN_0274) and PG1660 (ATCC 33277 ORF number: PGN_0450) showed decreased gingipain activity [14]. PG1827 (SigH) in strain W83 (ATCC 33277 ORF number: PGN_1740) plays a key role in regulation and adaptation to oxidative stress [15]. PGN_0274 and SigH play key roles in biofilm formation [16]. The expression of approximately 24% of P. gingivalis W83 genes was shown to be altered with over-expression of PG0162 [17]. SigP (PGN_0274) regulates the type IX secretion system (T9SS) via the two-component system PorXY [18]. SigCH (PGN_0319) acts as a direct regulator of cdhR and hmuYR, and involved in hemin utilization [19]. However, the involvement of ECF sigma factors in shaping P. gingivalis cell surface characteristics is largely unknown. In the present study, we used sigP, sigCH, PGN_0450, PGN_0970, and sigH mutants to investigate the roles of ECF sigma factors, focusing on P. gingivalis cell surface characteristics.

Materials and methods

Bacterial culture conditions

Bacterial strains used in this study are listed in Table 1. P. gingivalis ATCC 33277 (wild-type) and sigP, sigCH, PGN_0450, PGN_0970, and sigH mutants were used. P. gingivalis strains were maintained anaerobically (10% CO₂, 10% H₂, and 80% N₂) at 37˚C on enriched tryptic soy (TS) agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.5% brain heart infusion (BHI) (Becton Dickinson), 0.1% cysteine (Wako Pure Chemical Industries, Osaka, Japan), 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μg/mL menadione (Nacalai Tesque, Kyoto, Japan), and 5% defibrinated horse blood (Nippon Bio-Test Laboratories, Tokyo, Japan). When required, erythromycin (15 μg/mL) was added to the medium. For liquid cultures, P. gingivalis cells were grown in enriched BHI medium supplemented with 0.5% yeast extract (Becton Dickinson), 0.1% cysteine, 5 μg/mL hemin, and 0.5 μg/mL menadione [20].

Mice

The animal protocols were approved by the Tokyo Dental College Animal Ethics Committee (Approval Number: 290601). All experiments were performed in accordance with the...
Guidelines for the Treatment of Experimental Animals at Tokyo Dental College. Female BALB/c mice (8–10 weeks of age) obtained from Sankyo Labo Service (Tokyo, Japan) were housed five per cage and allowed to acclimate for 1 week. All surgery was performed under pentobarbital (30 mg/kg of body weight) and sevoflurane anesthesia. All mice were euthanized with pentobarbital (200 mg/kg of body weight) at the end of the study. All efforts were made to minimize animal suffering and to reduce the number of mice used.

Construction of ECF sigma factor mutants and complemented strains

ECF sigma factor mutants in ATCC 33277 were generated by double recombination of the targeted genes and the introduction of erythromycin resistance genes as previously described [16]. Flanking fragments, both upstream and downstream of each ECF sigma factor, were amplified by PCR from the chromosomal DNA of P. gingivalis ATCC 33277. The 2.1-kb ermF ermAM cassette was inserted into the BamHI or BglII site within the ECF sigma factor gene to yield plasmids for mutagenesis. The plasmids were digested with NotI and introduced into P. gingivalis ATCC 33277 by electroporation. For complementation of SigP, the whole sigP region with its upstream and downstream flanking regions (0.5 kb) was PCR amplified from the genomic DNA, and the complete fragment was cloned into the shuttle vector pT-COW [22].

Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Reference or source |
|------------------|-------------|---------------------|
| **Escherichia coli strain** | | |
| DH5α | General-purpose host strain for cloning | Thermo Fisher Scientific |
| **Porphyromonas gingivalis** | | |
| ATCC 33277 | wild type | American Type Culture Collection |
| KDP314 | sigP (PGN_0274)::ermF ermAM, Em' | [16] |
| KDP315 | sigCH (PGN_0319)::ermF ermAM, Em' | [16] |
| KDP316 | PGN_0450::ermF ermAM, Em' | [16] |
| KDP317 | PGN_0970::ermF ermAM, Em' | [16] |
| KDP319 | sigH (PGN_1740)::ermF ermAM, Em' | [16] |
| KDP314C | sigP::ermF ermAM, Em' pT-COW-sigP, Tc' | [16] |
| W83 | wild type | [13] |
| KDP307 | sigP (PG0162)::ermF ermAM, Em' | this study |
| KDP307C | sigP (PG0162)::ermF ermAM, fimA::tetQ sigP' sigP' | this study |
| **Escherichia coli plasmid** | | |
| pGEM-T Easy | Ap', plasmid vector for TA cloning | Promega |
| pKD355 | Ap', contains the ermF ermAM DNA cassette between EcoRI and BamHI of pUC18 | [13] |
| pKD806 | Ap', contains the 2.0-kb PCR-amplified fragment (PG0162 region) in pGEM-T Easy | this study |
| pKD811 | Ap' Em', contains the ermF ermAM DNA cassette at BamHI site within PG0162 of pKD806 | this study |
| pKD703 | Ap', contains 0.77 kb fimA-upstream and 0.78 kb fimA downstream DNA fragments | [21] |
| pKD713 | Ap', Tc' contains the tetQ DNA cassette at BamHI site of pKD703 | [21] |
| pKD831 | Ap', Tc' contains the 1.28 kb sigP (PG0162) region at DNA cassette at EcoRI site of pKD713 | this study |

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The resulting plasmid was introduced into sigP mutant by electroporation. Transformants were selected on enriched TS agar containing 15 μg/mL erythromycin and 1 μg/mL tetracycline.

To generate a sigP mutant in W83, the coding region of sigP (PG0162) was amplified by PCR from the chromosomal DNA of P. gingivalis W83 using primers PG0162-U-F and PG0162-D-R. The amplified DNA fragment (2.0 kb) was cloned into a pGEM-T Easy vector, resulting in pKD806. The ermF-ermAM cassette of pKD355 was inserted into the BamHI site within sigP of pKD806 to yield pKD811. pKD811 was digested with NotI and introduced into P. gingivalis W83 by electroporation, resulting in strain KDP307. To construct the sigP+-complemented mutant, the whole sigP gene region with its upstream and downstream flanking regions (1.28 kb) was PCR-amplified from W83 chromosomal DNA using the primer pair PG0162-COMP-U-F and PG0162-COMP-D-R, digested with BamHI and PstI, and inserted into the BamHI and PstI site of pKD713, resulting in pKD831. pKD831 was linearized by NotI digestion and introduced into KDP307, resulting in strain KDP307C.

Autoaggregation assay

The autoaggregation assay was performed as described previously [23]. Briefly, P. gingivalis strains were cultured anaerobically at 37˚C for 2 days in enriched BHI broth and then harvested by centrifugation at 8,000 × g for 10 min at 4˚C. The pellets were gently washed with 20 mM phosphate-buffered saline (PBS) twice and resuspended in the same buffer. Suspension turbidities were adjusted to OD660 = 1.0 using a spectrophotometer (Mini Photo 518R; Taitec, Saitama, Japan). Aliquots (5 mL) of each sample were placed in test tubes (18-mm diameter) and shaken at 37˚C at a speed of 120 strokes/min. Autoaggregation was monitored at 25˚C, by measuring the decrease in turbidity (660 nm) for 60 min.

Hydrophobicity assay

A surface hydrophobicity assay was performed according to the method of Naito et al. [24]. Briefly, P. gingivalis strains were cultured anaerobically at 37˚C for 2 days in enriched BHI broth and then harvested by centrifugation at 8,000 × g for 10 min at 4˚C. The pellets were gently washed with the phosphate urea magnesium sulfate (PUM) buffer (pH 7.2) three times and then resuspended in the same buffer. Suspension turbidities were adjusted to OD550 = 0.6 using a spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan). A sample (3 mL) of each bacterial suspension was placed in a test tube (13-mm diameter) and mixed with 400 μL of hexadecane (Wako Pure Chemical Industries). Following vigorous shaking, the tubes were allowed to stand for 15 min. The percent hydrophobicity was calculated as follows: [(A550 without hexadecane − A550 with hexadecane) / A550 without hexadecane] × 100. Each isolate was assayed twice, and the values obtained were averaged. Experiments were performed with triplicate samples and were repeated three times to verify the results.

Hemagglutination activity (HA) assay

Hemagglutination assay was performed as described previously [13]. Briefly, P. gingivalis cells were cultured in enriched BHI medium to the stationary phase, and then the samples were centrifuged, washed with 10 mM PBS (pH 7.4), and suspended in PBS to adjust the turbidity to 1.8 (OD600) as determined by a spectrophotometer (Shimadzu UV-2550). Bacterial suspensions were diluted twofold in PBS using a round-bottom microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and equal volumes of 1% horse erythrocytes suspended in PBS were added. HA was evaluated visually after 3 h of incubation at room temperature.
Enzymatic assays

Lys-X cysteine proteinase (Kgp) and Arg-X cysteine proteinase (RgpA and RgpB) activities were determined using synthetic substrates N-(p-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (Sigma-Aldrich Japan, Tokyo, Japan) and benzoyl-L-arginine p-nitroanilide monohydrochloride, respectively (Peptide Institute, Inc., Osaka, Japan). Samples were prepared as previously described, with some modifications [13]. Briefly, the absorbances (OD_{600}) of *P. gingivalis* cultures were measured after 48 h of anaerobic incubation at 37˚C. Culture aliquots (3.0 mL) were centrifuged at 8,000 × g for 10 min at 4˚C, and the resulting supernatant was used as “culture supernatant” in the assays. Pelleted cells were washed twice with 10 mM HEPES/NaOH (pH 7.4) and then suspended in the same buffer (OD_{600} = 1.0) for use as “whole cells.” Gingipain activities were determined in 200 μL of reaction buffer containing 0.2 mM substrate, 50 mM Tris-HCl (pH 8.0), and 10 mM DTT. Reactions were initiated by the addition of 4.0 μL of culture supernatant or whole cells. The reaction mixtures were incubated at 37˚C for 30 min. Acetic acid (50%, 40 μL) was added, and the release of the cleaved product, p-nitroanilide, was determined by absorbance measurements at 405 nm using a Spectra Max M5 (Molecular Device, Sunnyvale, CA, USA). Proteinase activity values were divided by OD_{600} cell density values to normalize the data to OD_{600} units.

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from *P. gingivalis* cells grown to an OD_{660} of 0.6–0.8 using TRIzol reagent (Thermo Fisher Scientific, MA, USA) and following the protocol described by the manufacturer. The RNA samples were treated using the TURBO DNA-free Kit (Thermo Fisher Scientific, MA, USA) at 37˚C for 15 min to eliminate contaminating genomic DNA, and then PCR was performed to confirm the lack of detectable genomic DNA in the RNA samples. RNA was reverse transcribed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) following the manufacturer’s instructions. Each reaction mixture contained 1 μL of cDNA mixed with 10 μL of Taqman Fast Universal PCR MasterMix (Thermo Fisher Scientific), 1 μL gene-specific primers and probes mixture (Table 2) and 8 μL of RNase-free water. PCR was performed using a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). Experiments were performed in triplicate with three biologically independent replicates. Data were normalized to the mRNA level of the housekeeping gene *glk* [25]. All primers used in this study are listed in Table 2.

Antimicrobial susceptibility testing

Susceptibility of *P. gingivalis* strains to three antimicrobial agents, ampicillin, tetracycline, and ofloxacin, was investigated as described previously [26]. Briefly, TS blood agar plates with doubling concentrations of 0.125–2.0 μg/mL of ampicillin, 0.0625–0.5 μg/mL of tetracycline, and 0.0625–0.5 μg/mL of ofloxacin were made. The *P. gingivalis* strains were cultured overnight in enriched BHI broth. The turbidity of late log phase cultures (OD_{660}) was adjusted to 0.2 with fresh medium, and the diluted cultures were grown overnight. The new late log phase cultures were diluted in the same manner to OD_{660} = 0.2. Culture aliquots (2 μL) were spotted on TS blood agar plates with or without antimicrobials and incubated anaerobically at 37˚C for 7 days. Minimum inhibitory concentration (MIC) values were considered the lowest antimicrobial agent concentrations that resulted in no growth of the bacteria in the inoculum. Each antibiotic sensitivity assay was performed at least three times.

Transmission electron microscopy (TEM)

*P. gingivalis* strains were cultured in enriched BHI broth at 37˚C for 2 days. Late exponential phase cultures (OD_{660} = 0.8) were harvested and then fixed with 2.5% glutaraldehyde for 2 h at
4˚C. Bacterial cells were washed with PBS, fixed with 1% aqueous OsO₄ for 2 h, and washed again with PBS. The cells were dehydrated by incubation in an ethanol series (70–100% for 15 min) and suspended in propylene oxide solution for 30 min. Samples were embedded in an Epon 812/resin mixture and allowed to polymerize at 60˚C for 48 h. Thin sections (70 nm) were cut by cryo-ultramicrotome (Leica Biosystems, Wetzlar, Germany), stained for less than 1 min with 4% uranyl acetate and 0.2% lead citrate solutions, and examined under TEM (H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan). The outer membrane vesicles (OMVs) were semi-quantified by manually counting the number of OMVs per 1.4 μm² in 15 specimens (one cell per field) [27, 28]. In addition, inter-membrane distances (between the outer and cytoplasmic membranes) of at least 20 specimens were determined from TEM images using ImageJ software (version 1.44p; http://imagej.nih.gov/ij/).

**Mouse virulence assay**

The virulence of the *P. gingivalis* W83 *sigP* mutant and the complemented *sigP* mutant strain were determined by mouse subcutaneous infection experiments [29, 30]. Each *P. gingivalis* strain was cultured 2 days in enriched BHI broth until an OD₆₀₀ = 1.0 was reached. The concentration of this sample was approximately 1×10⁹ CFU/mL. The samples were centrifuged and suspended in fresh BHI to adjust the concentration of approximately 1 × 10¹¹ CFU/mL. For each *P. gingivalis* strain, five mice were inoculated subcutaneously with 0.1 mL of bacterial suspension at two sites on the depilated dorsal surface (0.2 mL per mouse). Survival was observed daily. Three sets of experiments were carried out, using 15 mice in total. For data analysis, Kaplan-Meier plots were constructed, and the log rank test was used to evaluate the differences in mean survival rates between mice infected with the W83 parent strain, *sigP*

| Primer/probe          | Nucleotide Sequence (5'-3')                     |
|-----------------------|-------------------------------------------------|
| For construction of the *sigP* (PG0162) mutant and complemented strain |                                                 |
| PG0162-U-F            | TCGACAGTTGATTTGCGAT                              |
| PG0162-D-R            | TGGATCGTTCTCCAGGATT                              |
| PG0162-COMP-U-F       | CCTGACGGCTGCTACGTTCGCCAGGTT                     |
| PG0162-COMP-D-R       | CCGCTAGCGAAACAAACACTCAGGAGTT                    |
| For qRT-PCR            |                                                 |
| PGN_0381 (glk) probe  | 56-FAM/TTCCAGACT/CEN/ACGCGGAGCCATCTTC/3IABkFQ   |
| PGN_0381 (glk)-F       | GATGGCGCTTGGCTAGAGAT                             |
| PGN_0381 (glk)-R       | GGTCAACCTGCTTCTGTTGATA                           |
| PGN_1970 (rgpA) probe | 56-FAM/ATGCAACCA/CEN/CTAAATACGCTGCGA/3IABkFQ    |
| PGN_1970 (rgpA)-F      | AGTAACGCTCAAGTGGGATG                             |
| PGN_1970 (rgpA)-R      | CATCGCTGACGACGAGAA                               |
| PGN_1466 (rgpB) probe | 56-FAM/AACGTAGA/CEN/AAGTCCTGCTGCGGG/3IABkFQ     |
| PGN_1466 (rgpB)-F      | GCCGACGTAGCCAAATGAA                              |
| PGN_1466 (rgpB)-R      | CGGCCGTCAATACGTGAGAT                             |
| PGN_1728 (kpg) probe  | 56-FAM/TGCGCTGGTTATGCCTTA/3IABkFQ               |
| PGN_1728 (kpg)-F       | CGCGCTAATAGCTGGAAATA                             |
| PGN_1728 (kpg)-R       | CAGAAGACGAGGAGGATATA                             |
| PGN_1733 (hagA) probe | 56-FAM/AAGGACCTCTCcen/CCTCTGAGCGAC/3IABkFQ      |
| PGN_1728 (hagA)-F      | CGCGCTATATCTTCTTATG                              |
| PGN_1728 (hagA)-R      | CGGAGATCTTACCTTGGGAT                            |

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mutant, and complemented strain in three experiments using GraphPad Prism (version 7.0 for Windows, GraphPad Software, La Jolla, CA, USA).

**Statistical analysis**

One-way ANOVA test and Dunnett’s or Kruskal-Wallis multiple comparison test were used to compare the data for *P. gingivalis* wild type and the ECF sigma factor mutants using the GraphPad Prism, except for data from the mouse virulence assay. Differences were considered significant if *p* < 0.05.

**Results**

**Autoaggregation is most affected in *P. gingivalis* sigP mutant**

Several studies have indicated that *P. gingivalis* autoaggregation is associated with cell wall components and characteristics such as fimbriae, Kgp, and hydrophobicity [23, 31, 32]. We compared the autoaggregation of wild-type cells and ECF sigma factor mutants to determine whether ECF sigma factors modulate cell wall architecture. As shown in Fig 1A and 1B, autoaggregation of the *sigP* mutant was significantly reduced at all time points (10–60 min) compared to that of the wild type and *sigCH* and *PGN_0970* mutants (*p* < 0.05). The *sigP* mutant also showed a significant reduction in autoaggregation when compared with *PGN_0450* and *sigH* mutants at all time points except at 20 and 30 min (*p* < 0.05). To determine whether the reduction in autoaggregation was caused by the deletion of SigP, we constructed the SigP-complemented strain. Although a significant difference compared to the *sigP* mutant, the SigP-complemented strain failed to restore the wild-type phenotype (Fig 1C). In addition, the hydrophobicity assay revealed that the *sigP* mutant was less hydrophobic than the wild-type and complemented strains (Fig 1D).

**P. gingivalis sigP and sigH mutants display reduced HA**

To further confirm the relation between ECF sigma factors and colonial pigmentation, we examined black pigmentation in colonies. As shown in Fig 2A, the *sigP* mutant and *sigH* mutants exhibited non-pigmented or less-pigmented colonies, whereas the wild type, *sigCH*, *PGN_0450*, and *PGN_0970* mutants showed black pigmentation. Next, we compared the HAs of ECF sigma factor mutants, because membrane-associated protein Hgp44 is important for hemagglutination in *P. gingivalis* [33]. The results are shown in Fig 2B and 2C. The *sigP* mutant exhibited hemagglutination titers more than five dilutions higher than those of the wild type. The *sigH* mutant exhibited hemagglutination titers one dilution higher than those of the wild type. The *sigP*-complemented strain restored the HA levels to the same as those of the wild type (Fig 2C).

**Rgp and Kgp activities are differentially affected in *P. gingivalis* ECF sigma factor mutants**

A previous study suggested an association between HA and gingipain activity [34]. We therefore examined Rgp and Kgp activities in whole cells and culture supernatants of ECF sigma factor mutants. Rgp and Kgp activities were almost completely abolished in both whole cell and supernatant samples of the *sigP* mutant (Fig 3A). The Rgp activities of other ECF sigma factor mutants were approximately half (whole cells) or 40% (supernatants) of those of the wild type. In addition, Kgp activity was significantly reduced in both whole cells and supernatant of the *sigH* mutant. Complementation of the *sigP* mutant with wild-type *sigP* restored Kgp and partially Rgp activity (Fig 3B).
Fig 1. Autoaggregation of *Porphyromonas gingivalis* wild type and extracytoplasmic function (ECF) sigma factor mutants. (A) Representative images of strains at 60 min. The OD₆₆₀ of ECF sigma factor mutants (B) or the *sigP* mutant and *sigP*-complemented strain (C) were measured at 0, 10, 20, 30, and 60 min. Relative turbidities were calculated as follows: relative turbidity (%) = (turbidity at each time point / turbidity at 0 min) × 100. (D) Hydrophobicity. All assays were performed three times, and means ± SD (standard deviations) are shown.

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Fig 2. Morphology and hemagglutinin activity of *Porphyromonas gingivalis* wild type and ECF sigma factor mutant strains. (A) Colony pigmentaton. The strains were grown anaerobically on enriched tryptic soy agar plates supplemented with 5% defibrinated horse blood at 37°C for 7 days. (B and C) Hemagglutination. The OD$_{600}$ of *P. gingivalis* ECF sigma factor mutant (B) or the sigP mutant and sigP-complemented strain (C) cultures were adjusted to 1.8, and the cultures were diluted 1 to 32 times. After diluting, horse erythrocyte suspensions (1% in PBS) were added to each of the wells.

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Fig 3. Gingipain activities of the indicated Porphyromonas gingivalis strains. Following 48 h of growth, P. gingivalis cultures were centrifuged. Gingipain activities in "culture supernatants" and "whole cells" of ECF sigma factor mutants (A) or the sigP mutant and its
Two separate genes (rgpA and rgpB) and a single gene (kgp) have been shown to be involved in Rgp and Kgp activity [35]. In addition, the Hgp44 domain of RgpA, Kgp, and HagA mediate hemagglutination [33]. Therefore, rgpA, rgpB, kgp, and hagA were analyzed by qRT-PCR. The results showed that kgp was downregulated in the sigP and sigH mutants, whereas rgpA and rgpB exhibited almost the same levels of transcription as that of the wild type (Fig 4A). hagA expression was downregulated in the sigH mutant (Fig 4A). Complementation of the sigP mutant with wild-type sigP restored kgp expression (Fig 4B).

**ECF sigma factor deletions affect *P. gingivalis* MICs of ampicillin but not tetracycline or ofloxacin**

Table 3 shows MIC values of the wild-type and ECF sigma factor mutant strains. Three antimicrobial agents were tested. All mutants showed the same levels of susceptibility to tetracycline and ofloxacin as those of the wild-type strain. Compared with the wild type, susceptibility to ampicillin was increased in the sigP mutant and decreased in the PGN_0450 and sigH mutants. Susceptibility to ampicillin of the sigP-complemented strain was not different from that of the wild type.

**OMV formation and intermembrane distances are affected in some ECF sigma factor mutants**

TEM images of *P. gingivalis* wild type and mutant strains are shown in Fig 5. OMV formation at the cell surface was more pronounced in the sigP mutant than in the wild-type strain. The number of OMVs formed by the sigP mutant was significantly higher than that in the wild type (Fig 6A), and inter-membrane distances were significantly reduced in the sigP mutant (Fig 6B).

**Influence of SigP ECF sigma factor on the virulence of *P. gingivalis* W83**

BALB/c mice were inoculated subcutaneously with 0.1 mL of bacterial suspension (2 × 10⁷ CFU per animal), and their survival was monitored for 10 days. The mouse mortality resulting from subcutaneous injection of *P. gingivalis* W83 and the sigP mutant and sigP-complemented strain is summarized in Fig 7. The log rank test showed no relationship between *P. gingivalis* virulence and the SigP ECF sigma factor.

**Discussion**

In the present study, we determined that autoaggregation, hemagglutination, gingipain activities, and OMV formation, which all contribute to *P. gingivalis* virulence, were altered in the sigP mutant. Compared with the wild-type strain, Kgp activity was reduced in the sigH mutant, and Rgp activity was decreased in all mutants. In addition, the sigP, PGN_0450, and sigH mutants displayed altered ampicillin MICs. These results suggested that adaption to various environmental changes governed by ECF sigma factors plays a key role in the virulence of *P. gingivalis*.

In the present study, the activities of Kgp and Rgps were reduced most in the sigP strain compared to the other strains. In a previous report, inactivation of PG0162 and PG1660 (PGN_0274 and PGN_0450 in strain ATCC 33277) resulted in a 50% decrease in gingipain
Fig 4. Expression levels of *rgpA*, *rgpB*, *kgp*, and *hagA* in the indicated *Porphyromonas gingivalis* strains. qRT-PCR analysis of the *rgpA*, *rgpB*, *kgp*, and *hagA* genes in ECF sigma factor mutants (A) or the *sigP* mutant and its complemented strain (B). Total RNA was extracted from *P. gingivalis* cells grown to an OD\(_{660}\) of 0.6–0.8. All assays were performed nine times, and means ± SD (standard deviations) are shown. *, \( p < 0.05; \) **, \( p < 0.01; \) ***, \( p < 0.001. \)

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activity, and an RT-PCR analysis showed no changes in the expression of \textit{rgpA}, \textit{rgpB}, and \textit{kgp} gingipain genes in these mutants [14]. Gingipains are exported by a type IX secretion system [36]. SigP and the two-component system PorXY regulates type IX secretion systems [18].

Table 3. Antimicrobial susceptibilities of the \textit{Porphyromonas gingivalis} ECF sigma factor mutants.

| Strain                     | Ampicillin (μg/mL) | Tetracycline (μg/mL) | Ofloxacin (μg/mL) |
|---------------------------|--------------------|----------------------|-------------------|
| \textit{P. gingivalis} ATCC 33277 | 1.0                | 0.25                 | 0.25              |
| \textit{P. gingivalis} \textit{sigP} mutant | 0.5                | 0.25                 | 0.25              |
| \textit{P. gingivalis} \textit{sigCH} mutant | 1.0                | 0.25                 | 0.25              |
| \textit{P. gingivalis} PGN_0450 mutant | 2.0                | 0.25                 | 0.25              |
| \textit{P. gingivalis} PGN_0970 mutant | 1.0                | 0.25                 | 0.25              |
| \textit{P. gingivalis} \textit{sigH} mutant | > 2.0              | 0.25                 | 0.25              |

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Fig 5. Transmission electron microscopy images of \textit{Porphyromonas gingivalis} wild-type and ECF sigma factor mutant strains. The cells were stained for less than 1 min in 4% uranyl acetate and 0.2% lead citrate solutions. Scale bars are shown. The arrows indicate outer membrane vesicles.

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This suggests that the precursor forms of Kgp and Rgps may accumulate in the periplasmic space of the sigP mutant. In addition, we found that the expression of rgpA and kgp were decreased in the sigP mutant (Fig 4B). Kadowaki et al. showed, based on a transcriptional analysis by microarray or qRT-PCR, that rgpA, rgpB, and kgp did not change significantly in the

Fig 6. Vesicle numbers and intermembrane distances in Porphyromonas gingivalis wild-type and ECF sigma factor mutant strains. The number of vesicles per 1.4 μm² (15 specimen counted) (A) and inter-membrane distance (distance between the outer and cytoplasmic membranes) (at least 20 specimen counted) (B) were established from TEM images. Data are expressed as means ± SD. *, p < 0.05; ***, p < 0.001.

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This suggests that the precursor forms of Kgp and Rgps may accumulate in the periplasmic space of the sigP mutant. In addition, we found that the expression of rgpA and kgp were decreased in the sigP mutant (Fig 4B). Kadowaki et al. showed, based on a transcriptional analysis by microarray or qRT-PCR, that rgpA, rgpB, and kgp did not change significantly in the

Fig 7. Virulence of Porphyromonas gingivalis W83 and the sigP mutant strain in a murine model. Five BALB/c mice were inoculated subcutaneously with 0.1 mL of bacterial suspension at two sites on the depilated dorsal surface (0.2 mL per mouse), and the survival of the mice was monitored daily for up to 10 days. Three sets of experiments were carried out (15 mice in total). For the data analysis, Kaplan-Meier plots were constructed, and the log rank test was used to evaluate the differences in mean survival rates between mice infected with the W83 parent strain, sigP mutant, and complemented strain in three experiments.

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sigP mutant [18]. Nevertheless, we found that the expression of rgpA and kgp decreased by approximately 60% in the sigP mutant and was restored in the complemented strain (Fig 4B). The exact reasons for this discrepancy in gene expression results are not known. However, we propose three possible reasons. First, microarray and qRT-PCR data often disagree [37, 38]. Second, the methods of sigP mutagenesis used were not the same. We used an erythromycin resistance cassette containing both ermF and ermAM, whereas Kadowaki et al. used only a ermF cassette. Third, Kadowaki et al. analyzed expression by qRT-PCR using SYBR Green dye, whereas we used a Taqman probe assay. However, we believe that our qRT-PCR data is reliable, because the complementing the sigP mutant with wild-type sigP restored rgpA and kgp expression. Further analysis is required to clarify whether SigP is involved in the regulation of rgpA and kgp. RgpA, Kgp, and HagA possess adhesion and hemagglutinin domains (Hgp44), and inactivation of rgpA significantly reduced HA [33, 39]. However, hagA expression was not downregulated in the sigP mutant. Thus, the reduction in HA may also be attributable to the decrease in gingipain activity of the sigP mutant.

Although the gingipain activities of sigP and PGN_0450 mutants in the present study were different, those of sigP and PG1660 mutants in the P. gingivalis W83 background were similar to what has been reported previously [14]. The difference between the present and previous studies may be attributable to the experimental conditions used. A difference was also observed in HA. Insertion Sequences (ISs) and miniature inverted-repeat transposable elements were detected in the P. gingivalis genome, and genome rearrangements were detected [12, 40]. Variations in fimbriae and capsules were reported [41, 42], as well as differences in the virulence of strains [43]. Further study is needed to clarify the relationship between ECF sigma factors and gingipain activity.

Autoaggregation of the sigP mutant was significantly reduced compared to that of the wild-type strain. Our results suggested that the sigP mutant was less hydrophobic than the wild type and the complemented strain (Fig 1D). Several studies have indicated that the hydrophobicity of bacterial cells is related to autoaggregation. However, autoaggregation of the sigP-complemented strain was not as same as that of the wild type (Fig 1C). Autoaggregation in P. gingivalis might be correlated with the bacterial surface hydrophobicity, fimbriae, capsule, VimA, Kgp activity [32, 44–47]. First we speculated that another factor, P. gingivalis FimA fimbriae, contributed little to autoaggregation. To investigate the relationship between autoaggregation and FimA, we performed FimA polymerization using SDS-polyacrylamide gel electrophoresis under nonreducing conditions. However, there was no obvious alteration of ladder-like bands in all of strains, suggesting that autoaggregation of the sigP mutant cannot be explained by its FimA polymerization (S1 Fig). Further analysis will be required to clarify the autoaggregation mechanism.

In a previous study, susceptibility to antimicrobial agents changed because of efflux pump inactivation [26]. We used the same antimicrobial agents to investigate the role of the ECF sigma factors in susceptibility to antimicrobials. The MIC of ampicillin was lower with the sigP mutant than with wild type. In contrast, the MICs of ampicillin with the PGN_0450 and sigH mutants were higher. Ampicillin inhibits a transpeptidase involved in peptidoglycan synthesis. Intermembrane distances were also altered in sigP and sigH mutants. These results suggested that SigP and SigH affect cell wall structure in different ways. The expression of approximately 24% of P. gingivalis genes was affected by PG0162 overexpression [17]. It is possible that the three ECF sigma factors are involved in cell wall assembly. In Staphylococcus aureus, ECF sigma factor SigS was shown to be involved in the cellular response to cell wall-targeting antimicrobial agents [48]. Further research is needed to determine whether this mechanism plays a role in P. gingivalis.

The MICs of tetracycline and ofloxacin were the same for the wild type and all mutants tested. This indicated that the five ECF sigma factors were not involved in the response to stress caused by these antimicrobial agents. SigX, an ECF sigma factor in Pseudomonas.
*Pseudomonas aeruginosa*, was reported to affect the efflux pump MexXY/OprM, a key element of bacterial adaptation to ribosome-targeting antibiotics [49, 50]. Inactivation of the *P. gingivalis* efflux pump was reported to increase susceptibility to ampicillin, tetracycline, and norfloxacin [51]. Our results, therefore, suggest that the ECF sigma factors examined are not involved in regulation of an efflux pump specific for removal of these antibacterial agents.

The cell wall of the *sigP* mutant was obviously different from that of the wild-type strain and was characterized by increased OMV formation. There are two possible explanations for the increase in OMVs. One reason is that disruption of $\sigma^E$-regulated genes caused increased vesiculation in *Escherichia coli* [52]. Another reason is that overproduction of outer membranes relative to peptidoglycans caused blebbing of outer membranes [53]. Gui et al. [54] reviewed that the biogenesis of OMVs of *P. gingivalis* and another bacterium, *E. coli*. Overproduction of OMVs in *E. coli* was found to be related to ECF sigma factor ($\sigma^E$) stress-response pathways. $\sigma^E$ controls the expression of degP, which encodes a periplasmic serine protease. The DegP mutant accumulates misfolded proteins in the periplasm, increasing envelope stress, and, as a result, induces OMV's production. Therefore, we speculated that misfolded proteins or precursor forms of Kgp and Rgps may accumulate in the periplasmic space of the *sigP* mutant, causing increased budding of vesicles. In the present study, the ampicillin MIC of the *sigP* mutant was decreased relative to that of the wild type, suggesting that cell wall synthesis was affected by SigP inactivation. It is possible that the mutation affected the equilibrium between peptidoglycans and outer membrane components. The mechanism by which OMV formation increased in the *sigP* mutant should be investigated further.

As revealed by the mouse virulence assay, disruption of the *sigP* did not affect *P. gingivalis* virulence in mice. Previous studies have shown that Rgp and Kgp activity significantly reduced mouse virulence [30, 55]. This result may be attributed to an increase in a virulence factor other than Rgp and Kgp in the absence of SigP. However, we cannot yet explain why the virulence of the wild type and *sigP* mutant were similar in the mouse virulence assay. Further research is needed to clarify this finding.

Taken together, results of our study indicate that ECF sigma factor SigP is involved in regulating *P. gingivalis* virulence factors, including gingipain activity, autoaggregation, hemagglutination, and OMV formation.

**Supporting information**

**S1 Fig.** Polymerization patterns of FimA in the *sigP* mutant and the *sigP*-complemented strain. Whole-cell lysates were obtained from *P. gingivalis* ATCC 33277, *sigP* mutant and its complemented strain. Solubilized samples in the presence of $\beta$-mercaptoethanol were denatured at 100°C for 10 min, or 80°C for 10 min and then separated by SDS-PAGE and detected by anti-FimA antibody. (TIF)

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References

1. Eisenstark A, Calcutt MJ, Becker-Hapak M, Ivanova A. Role of Escherichia coli rpoS and associated genes in defense against oxidative damage. Free Radic Biol Med 1996; 21: 975–993. PMID: 8937883

2. Hengge-Aronis R. Signal transduction and regulatory mechanisms involved in control of the σs (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev 2002; 66: 373–395. https://doi.org/10.1128/MMBR.66.3.373-395.2002 PMID: 12208955

3. Mascher T. Signaling diversity and evolution of extracytoplasmic function (ECF) sigma factors. Curr Opin Microbiol 2013; 16: 148–155. https://doi.org/10.1016/j.mib.2013.02.001 PMID: 23466210

4. Staron A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. Mol Microbiol 2009; 74: 557–581. https://doi.org/10.1111/j.1365-2958.2009.06870.x PMID: 19737356

5. Brooks BE, Buchanan SK. Signaling mechanisms for activation of extracytoplasmic function (ECF) sigma factors. Biochim Biophys Acta 2008; 1778: 1930–1945. https://doi.org/10.1016/j.bbamem.2007.06.005 PMID: 17673165

6. Holt SC, Ebersole JL. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the ”red complex”, a prototype polybacterial pathogenic consortium in periodontitis. Periodontology 2000 2005; 38: 72–122. https://doi.org/10.1111/j.1600-0757.2005.00113.x PMID: 15853938

7. Guo Y, Nguyen KA, Potempa J. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon’s knife to a meat chopper-like brutal degradation of proteins. Periodontol 2000 2010; 54: 15–44. https://doi.org/10.1111/j.1600-0757.2010.00377.x PMID: 20712633

8. Jain S, Darveau RP. Contribution of Porphyromonas gingivalis lipopolysaccharide to periodontitis. Periodontol 2000 2010; 54: 53–70. https://doi.org/10.1111/j.1600-0757.2009.00333.x PMID: 20712633

9. Enersen M, Nakano K, Armano A. Porphyromonas gingivalis fimbriae. J Oral Microbiol 2013; 5 https://doi.org/10.3402/jom.v5i0.20265 PMID: 23667717

10. Saito A, Inagaki S, Ishihara K. Differential ability of periodontopathic bacteria to modulate invasion of human gingival epithelial cells by Porphyromonas gingivalis. Microb Pathog 2009; 47: 329–333. https://doi.org/10.1016/j.micpath.2009.09.012 PMID: 19818393

11. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. Nat Rev Microbiol 2012; 10: 717–725. https://doi.org/10.1038/nrmicro2873 PMID: 22941505

12. Naito M, Hirakawa H, Yamasita A, Ohara N, Shoji M, Yukitake H, et al. Determination of the genome sequence of Porphyromonas gingivalis strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in P. gingivalis. DNA Res 2008; 15: 215–225. https://doi.org/10.1093/dnares/dsn013 PMID: 18524787

13. Kikuchi Y, Ohara N, Ueda O, Hirai K, Shibata Y, Nakayama K, et al. Porphyromonas gingivalis mutant defective in a putative extracytoplasmic function sigma factor shows a mutator phenotype. Oral Microbiol Immunol 2009; 24: 377–383. https://doi.org/10.1111/j.1399-302X.2009.00526.x PMID: 19702950

14. Dou Y, Osbourne D, McKenzie R, Fletcher HM. Involvement of extracytoplasmic function sigma factors in virulence regulation in Porphyromonas gingivalis W83. FEMS Microbiol Lett 2010; 312: 24–32. https://doi.org/10.1111/j.1574-6968.2010.02093.x PMID: 20807237

15. Yanamandra SS, Sarrafie SS, Anaya-Bergman C, Jones K, Lewis JP. Role of the Porphyromonas gingivalis extracytoplasmic function sigma factor, SigH. Mol Oral Microbiol 2012; 27: 202–219. https://doi.org/10.1111/j.2041-1014.2012.00643.x PMID: 22520899
16. Onozawa S, Kikuchi Y, Shibayama K, Kokubu E, Nakayama M, Inoue T, et al. Role of extracytoplasmic function sigma factors in biofilm formation of Porphyromonas gingivalis. BMC Oral Health 2015; 15: 4. https://doi.org/10.1186/1472-6831-15-4 PMID: 25596817

17. Dou Y, Aruni W, Muthiah A, Roy F, Wang C, Fletcher HM. Studies of the extracytoplasmic function sigma factor PG0162 in Porphyromonas gingivalis. Mol Oral Microbiol 2016; 31: 270–283. https://doi.org/10.1111/mom.12122 PMID: 26216199

18. Kadowaki T, Yukitake H, Naito M, Sato K, Kikuchi Y, Kondo Y, et al. A two-component system regulates gene expression of the type IX secretion component proteins via an ECF sigma factor. Sci Rep 2016; 6: 23288. https://doi.org/10.1038/srep23288 PMID: 26996145

19. Ota K, Kikuchi Y, Imamura K, Kita D, Yoshikawa K, Saito A, et al. SigCH, an extracytoplasmic function sigma factor of Porphyromonas gingivalis regulates the expression of cdhR and hmuYR. Anaerobe 2017; 43: 82–90. https://doi.org/10.1016/j.anaerobe.2016.12.006 PMID: 27940243

20. Ueshima J, Shoji M, Ratnayake DB, Abe K, Yoshida S, Yamamoto K, et al. Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe Porphyromonas gingivalis. Infect Immun 2003; 71: 1170–1178. https://doi.org/10.1128/IAI.71.3.1170-1178.2003 PMID: 12595429

21. Kikuchi Y, Ohara N, Sato K, Yoshimura M, Yukitake H, Sakai E, et al. Novel stationary-phase-upregulated protein of Porphyromonas gingivalis influences production of superoxide dismutase, thiol peroxidase and thioredoxin. Microbiol 2005; 151: 841–853. https://doi.org/10.1099/mic.0.27589-0 PMID: 15758230

22. Gardner RG RJ, Wilson DB, Wang GR, Shoemaker NB. Use of a modified Bacteroides-Prevotella shuttle vector to transfer a reconstructed β-1,4-β-endoglucanase gene into Bacteroides uniformis and Prevotella ruminicola B-4. Appl Environ Microbiol 1996; 62: 196–202. PMID: 8572695

23. Nishiyama S, Murakami Y, Nagata H, Shizukuishi S, Kawagishi I, Yoshimura F. Involvement of minor components associated with the FimA fimbriae of Porphyromonas gingivalis in adhesive functions. Microbiol 2007; 153: 1916–1925. https://doi.org/10.1128/mic.0.00556-0.2007 PMID: 17526848

24. Naito Y, Tohda H, Okuda K, Takazoe I. Adherence and hydrophobicity of invasive and noninvasive strains of Porphyromonas gingivalis. Oral Microbiol Immunol 1993; 8: 195–202. PMID: 7902556

25. Xie H, Zheng C. OxyR activation in Porphyromonas gingivalis response to a hemin-limited environment. Infect Immun 2012; 80: 3471–3480. https://doi.org/10.1128/IAI.00680-12 PMID: 22825453

26. Ikeda T, Yoshimura F. A resistance-nodulation-cell division family xenobiotic efflux pump in an obligate anaerobe, Porphyromonas gingivalis. Antimicrob Agents Chemother 2002; 46: 3257–3260. https://doi.org/10.1128/AAC.46.10.3257-3260.2002 PMID: 12234854

27. Huett A, Ng A, Cao Z, Kuballa P, Komatsu M, Daly MJ, et al. A novel hybrid yeast-human network analysis reveals an essential role for FNBPI1 in antibacterial autophagy. J Immunol 2009; 182: 4917–4930. https://doi.org/10.4049/jimmunol.1934276

28. Saie J. A single-cell imaging screen reveals multiple effects of secreted small molecules on bacteria. Microbiologyopen 2014; 3: 426–436. https://doi.org/10.1002/mbo3.176 PMID: 24910069

29. Kondo Y, Ohara N, Sato K, Yoshimura M, Yukitake H, Naito M, et al. Tetraexperimental peptide-associated proteins contribute to the virulence of Porphyromonas gingivalis. Infect Immun 2010; 78: 2846–2856. https://doi.org/10.1128/IAI.01148-09 PMID: 20351137

30. Taguchi Y, Sato K, Yukitake H, Inoue T, Nakayama M, Naito M, et al. Involvement of an Skp-like protein, PGN_0300, in the Type IX secretion system of Porphyromonas gingivalis. Infect Immun 2015; 83: 230–240. https://doi.org/10.1128/IAI.01308-15 PMID: 26502912

31. Kuboniiwa M, Amano A, Hashino E, Yamamoto Y, Inaba H, Hamada N, et al. Distinct roles of long/short fimbriae and gingipains in homotypic biofilm development by Porphyromonas gingivalis. BMC Microbiol 2009; 9: 105. https://doi.org/10.1186/1471-2180-9-105 PMID: 19470157

32. Yamaguchi M, Sato K, Yukitake H, Noiri Y, Ebisu S, Nakayama K. A Porphyromonas gingivalis mutant defective in a putative glycosyltransferase exhibits defective biosynthesis of the polysaccharide portions of lipopolysaccharide, decreased gingipain activities, strong autoaggregation, and increased biofilm formation. Infect Immun 2010; 78: 3801–3812. https://doi.org/10.1128/IAI.00071-10 PMID: 20624909

33. Sakai E, Naito M, Sato K, Hotokezaka H, Kadowaki T, Kamaguchi A, et al. Construction of recombinant hemagglutinin derived from the gingipain-encoding gene of Porphyromonas gingivalis, identification of its target protein on erythrocytes, and inhibition of hemagglutination by an interdomain region of peptide. J Bacteriol 2007; 189: 3977–3986. https://doi.org/10.1128/JB.01691-06 PMID: 17384191

34. Sato K, Sakai E, Veith PD, Shoji M, Kikuchi Y, Yukitake H, et al. Identification of a new membrane-associated protein that influences transport/maturity of gingipains and adhesins of Porphyromonas gingivalis. J Biol Chem 2005; 280: 8668–8677. https://doi.org/10.1074/jbc.M413542400 PMID: 15634642

35. Nakayama K, Kadowaki T, Okamoto K, Yamamoto K. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of Porphyromonas gingivalis. J Biol Chem 1995; 270:23619–23626 PMID: 7559528
36. Sato K, Naito M, Yukitake H, Hirakawa H, Shoji M, McBride MJ, et al. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. Proc Natl Acad Sci U S A 2010; 107: 276–281. https://doi.org/10.1073/pnas.0912010107 PMID: 19966289

37. Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol Proced Online 2006; 8: 175–193. https://doi.org/10.1251/bpo126 PMID: 17242735

38. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, et al. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 2010; 16: 991–1006. https://doi.org/10.1261/rna.1947110 PMID: 20360395

39. Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of Porphyromonas gingivalis. Construction of mutants with a combination of rgpA, rgpB, kgp, and hagA. J Biol Chem 1999; 274: 17955–17960. PMID: 10364243

40. Nelson KE, Fleischmann RD, DeBoy RT, Paulsen IT, Fouts DE, Eisen JA, et al. Complete genome sequence of the oral pathogen Porphyromonas gingivalis strain W83. J Bacteriol 2003; 185: 5591–5601. https://doi.org/10.1128/JB.185.18.5591-5601.2003 PMID: 12994112

41. Laine ML, van Winkelhoff AJ. Virulence of six capsular serotypes of Porphyromonas gingivalis in a mouse model. Oral Microbiol Immunol 1998; 13: 322–325. PMID: 9807125

42. Amano A, Nakagawa I, Kataoka K, Morisaki I, Hamada S. Distribution of fimA genotypes in periodontitis patients. J Clin Microbiol 1999; 37: 1426–1430. PMID: 10203499

43. Ebersole JL, Kesavalu L, Schneider SL, Machen RL, Holt SC. Comparative virulence of periodontopathogens in a mouse abscess model. Oral Dis 1995; 1: 115–128. PMID: 8705817

44. Tokuda M, Duncan M, Chi MI, Kuramitsu HK. Role of Porphyromonas gingivalis protease activity in colonization of oral surfaces. Infect Immun 1996; 64: 4067–4073. PMID: 8926070

45. Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in Porphyromonas gingivalis. J Bacteriol 2006; 188: 5510–5523. https://doi.org/10.1128/JB.01685-05 PMID: 16855241

46. Osbourne DO, Aruni W, Roy F, Perry C, Sandberg L, Muthiah A, et al. Role of virnA in cell surface biofilm formation in Porphyromonas gingivalis. Microbiol 2010; 156: 2180–2193. https://doi.org/10.1099/mic.0.038331–0 PMID: 20378652

47. Gerits E, Verstraeten N, Michiels J. New approaches to combat Porphyromonas gingivalis biofilms. J Oral Microbiol 2017; 9: 1300366. https://doi.org/10.1080/20002297.2017.1300366 PMID: 28473880

48. Krute CN, Bell-Temin H, Miller HK, Rivera FE, Weiss A, Stevens SM, et al. The membrane protein Prs mimics σ^5 in protecting Staphylococcus aureus against cell wall-targeting antibiotics and DNA-damaging agents. Microbiol 2015; 161: 1136–1148. https://doi.org/10.1099/mic.0.000065 PMID: 25741016

49. Gicquel G, Boulfartiguès E, Bains M, Oxaran V, Rosay T, Lesouhaitier O, et al. The extra- cytoplasmic function sigma factor SigX modulates biofilm and virulence-related properties in Pseudomonas aeruginosa. PLoS One 2013; 8: e80407. https://doi.org/10.1371/journal.pone.0080407 PMID: 24260387

50. Muller C, Plesiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in Pseudomonas aeruginosa. Antimicrob Agents Chemother 2011; 55: 1211–1221. https://doi.org/10.1128/AAC.01252-10 PMID: 21149619

51. Inoue T, Nakayama M, Taguchi Y, Kano K, Ono M, Shimizu Y, et al. Characterization of the tripartite drug efflux pumps of Porphyromonas gingivalis ATCC 33277. New Microbiol 2015; 38: 101–108. PMID: 25742153

52. McBroom AJ, Johnson AP, Vermulapalli S, Kuehn MJ. Outer membrane vesicle production by Escherichia coli is independent of membrane instability. J Bacteriol 2006; 188: 5385–5392. https://doi.org/10.1128/JB.00498-06 PMID: 16855227

53. Wensink J, Witholt B. Outer-membrane vesicles released by normally growing Escherichia coli contain very little lipoprotein. Eur J Biochem 1981; 116: 331–335. PMID: 7018907

54. Gui MJ, Dashper SG, Slakeski N, Chen YY, Reynolds EC. Spheres of influence: Porphyromonas gingivalis outer membrane vesicles. Mol Oral Microbiol 2016; 31: 365–378. https://doi.org/10.1111/moi.12134 PMID: 26466922

55. Curtis MA, Aduse Opoku J, Rangarajan M, Gallagher A, Sterne JAC, Reid CR, et al. Attenuation of the virulence of Porphyromonas gingivalis by using a specific synthetic Kgp protease inhibitor. Infect Immun 2002; 70: 6968–6975. https://doi.org/10.1128/IAI.70.12.6968-6975.2002 PMID: 12438376