学位論文

Role of ECF sigma factors in biofilm formation of *Porphyromonas gingivalis*

(歯周病原細菌 *Porphyromomas gingivalis* の バイオフィルム形成における ECF シグマ因子の役割)

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松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

Role of ECF sigma factors in biofilm formation of *Porphyromonas gingivalis*

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要　旨

【背景と目的】

歯周病原細菌 *Porphyromonas gingivalis*（以下 *P. gingivalis* と略す）は、偏性嫌気性グラム陰性桿菌であり、血液寒天培地上での黒色集落形成、赤血球凝集性、ヘモグロビン吸着性、糖非発酵性、強力なプロテアーゼの産生など興味深い性状を示す細菌である。

P. gingivalis は、歯周ポケット底部の嫌気度の高い部位にバイオフィルム（デンタルプラーク）を形成し慢性感染する結果、病原性を発現する。口腔内は、人体の中でも外界に開かれた環境下にあり、温度、酸素、pH、栄養状態、他の細菌や宿主細胞など周囲環境からの影響をうけやすい。よって、その環境下で慢性感染することが可能な *P. gingivalis* は、周囲環境ストレスを回避する何かしらの防御機構を備えていることが予測される。

原核生物において周囲環境ストレスを回避するうえで重要な役割を果たす ECF(extra cytoplasmic function)シグマ因子は大腸菌を代表とするあらゆる細菌において存在が確認され性状解析されているが、歯周病原細菌の ECF シグマ因子に関する報告は少数である。

そこで本研究は、歯周病原細菌 *P. gingivalis* の ECF シグマ因子と、周囲環境ストレスを回避するうえで重要な役割を担っているバイオフィルムの形成との関連性について検討した。

【材料と方法】

用いた細菌株は *P. gingivalis* ATCC33277 と、それを親株とした ECF シグマ因子変異株及び相補株である。最初に ECF シグマ因子遺伝子(PGN_0274, PGN_0319, PGN_0450, PGN_0970, PGN_1740)のクローニングを行い、その後エリスロマイシンカセットをそれぞれの遺伝子の中間に挿入したターゲッティングベクターを構築した。そのベクターをエレクトロポレーターGene Pulser Xcell（バイオ・ラッド　ラボラトリーズ株式会社）にて *P. gingivalis* ATCC33277 に導入し、形質転換を起こさせることによって ECF シグマ因子変異株を得た。

また PGN_0274 と PGN_1740 については、両遺伝子とその周辺部を pT-COW ベクターに組み込み、エレクトロポレーション法にてこれらのプラスミドをそれぞれの変異株に導入して、プラスミド性の PGN_0274 相補株と PGN_1740 相補株を得た。

次に野生株と ECF シグマ因子変異株を 3 日間嫌気培養し、デジタル比色計 miniphoto 518R（タイテック株式会社）を用いて増殖速度を測定した。

バイオフィルムの形成については、前培養した野生株と ECF シグマ因子変異株および相補株の濁度を OD660nm=0.1 に揃え、コラーゲンⅠコート 12 ウェルプレートに 1.5ml ずつ植菌し、2 日間嫌気培養を行った。その後浮遊細菌を除去し、クリスタルバイオレット染色を 15 分間行い、PBS にて洗浄した。乾燥後、1% SDS 0.5ml を用いて溶出させ、その吸光度（OD600nm）をマイクロプレートリーダー（モレキュラーデバイスジャパン株式会社）にて測定した。

【結果】

(1)親株と比較し、増殖速度の遅滞と静止期における濁度の低下を示したECFシグマ因子変異株が認められた。その程度は様々であるが、その中でもPGN_1740変異株が顕著な値を示した。

(2)野生株と比較し、PGN_0274変異株, PGN_0319変異株, PGN_1740変異株はバイオフィルム形成能の有意な増加を認めた。その中でも、PGN_0274変異株とPGN_1740変異株にて顕著な増加を認めた。

(3)PGN_0274変異株とPGN_1740変異株におけるバイオフィルム形成量の増加は、それぞれの相補株にて野生株と同程度に回復した。

【考察と結論】

PGN_0274変異株とPGN_1740変異株に認められるバイオフィルム形成能の増加は、それぞれの相補株にて野生株と同程度にまで回復した。このことより、PGN_0274とPGN_1740は、*P. gingivalis*のバイオフィルム形成に関わるタンパク質の遺伝子発現を調節している可能性が示唆された。PGN_0274は*P. gingivalis*の病原因子ジンジパインの膜輸送に、PGN_1740は*P. gingivalis*の酸化ストレス回避機構に関わると過去に報告されているので、これらのECFシグマ因子を標的とした抗菌薬を創薬することが実現すれば、選択毒性の高い優れた薬剤となることが示唆される。

Introduction

The anaerobic Gram-negative bacterium *Porphyromonas gingivalis* is considered to be one of the important etiological agents of periodontal disease (1). To colonize and survive in 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. This bacterium and other bacteria can survive in the oral cavity by forming biofilm to prevent the environmental stress. Dental plaque, a multispecies biofilm, is organized on the tooth surface and periodontal tissues of the human oral cavity (2). Oral bacteria in the biofilms on teeth prevent the environmental stress and survive in the gingival crevice for a long time, and then gingivitis can progress into periodontitis. Understanding how bacteria escape the environmental stress is very important for the prevention of periodontal disease.

Extracytoplasmic function (ECF) sigma factors serve as bacterial transcriptional regulators in the response to various stresses. The *P. gingivalis* 33277 genome encodes six ECF sigma factors (PGN_0274, PGN_0319, PGN_0450, PGN_0970, PGN_1108 and PGN_1740) (3). PGN_1108 (W83 ORF number: PG1318) plays a role in the regulation of mutation frequency in the bacterium (4). PGN_0274 (W83 ORF number: PG0162) and PGN_0450 (W83 ORF number: PG1660) may be involved in the post-transcriptional regulation of gingipain (5), and PGN_1740 (W83 ORF number: PG1827) is required for survival of the bacterium in the presence of oxygen and oxidative stress, hemin uptake and virulence (5, 6). In this study, to analyze the

functions of these proteins in the organism, we constructed *P. gingivalis* mutants that were defective in five ECF sigma factors. Because the PGN_1108-defective mutant may have a mutator phenotype, we ruled it out in this study (4). The PGN_0274 and PGN_1740-defective mutants exhibited enhanced biofilm formation, but the complemented strains did not. These results suggest that the PGN_0274 and PGN_1740 ECF sigma factors are involved in the regulation of biofilm formation in the bacterium.

Materials and Methods

Bacterial strains and cell culture conditions

All bacterial strains and plasmids used in the present study are listed in Table 1. P. gingivalis cells were grown anaerobically (10% CO2, 10% H2 and 80% N2) in enriched brain heart infusion (BHI) broth and on enriched tryptic soy (TS) agar (7). For blood agar plates, defibrinated laked sheep blood was added to enriched TS agar at 5%. For selection and maintenance of antibiotic-resistant *P. gingivalis* strains, the following antibiotics were added to the medium: 15 μg/ml erythromycin (Em), and 0.7 μg/ml tetracycline (Tc).

Construction of ECF sigma factor mutants and complemented mutants

To disrupt the ECF sigma factor genes, PGN_0274-, PGN_0319-, PGN_0450-, PGN_0970- and PGN_1740-encoding genes were PCR-amplified from the chromosomal DNA of *P. gingivalis* 33277 using Takara Ex Taq (Takara Bio, Otsu, Japan) and the gene-specific primers listed in Table 2. Namely, a DNA fragment

containing part of the 5' end of each ECF sigma factor gene and the upstream region of the ATG initiation codon, and a DNA fragment containing the 3' end of each sigma factor gene and the downstream region of its stop codon, were amplified. Both fragments were then ligated into the multiple cloning site of T-vector (pGEM-T Easy Vector, Promega, Tokyo, Japan). A BamHI-SacI fragment (BglII-SacI fragment for PGN_0970) containing the 3' end of each sigma factor gene was extracted from the resulting plasmid and ligated into the BamHI-SacI site (BglII-SacI fragment for PGN_0970) of the plasmid containing the 5' end of the corresponding ECF gene. The *ermF-ermAM* cassette of pKD355 (8) was inserted into the BamHI site within PGN_0274 of pKD817, PGN_0319 of pKD818, PGN_0450 of pKD814 and PGN_1740 of pKD821, or the BglII site within PGN_0970 of pKD819 to yield pKD822, pKD823, pKD824, pKD827 and pKD825, respectively. These plasmids were linearized by NotI digestion and introduced into *P. gingivalis* 33277 cells by electroporation as described previously (9), resulting in KDP314 (PGN_0274::ermF ermAM), KDP315 (PGN_0319::*ermF ermAM*), KDP316 (PGN_0450::ermF ermAM), KDP317 (PGN_0970::*ermF ermAM*) and KDP319 (PGN_1740::*ermF ermAM*), respectively. Correct gene replacement of these strains, which had been generated by double crossover recombination events, was verified by PCR and Southern blot analysis (data not shown).

For complementation of PGN_0274 and PGN_1740, the whole ECF sigma factor gene region with its upstream and downstream flanking regions (0.5 kb) was PCR-amplified from the chromosomal DNA using Takara Ex Taq with the upper and

lower primers (Table 2). The amplified DNA fragments were ligated into the multiple cloning site of pGEM T-Easy vector. The SphI-BamHI fragment of PGN_0274 or the BamHI fragment of PGN_1740 were extracted from the resulting plasmid and ligated into the SphI-BamHI or BamHI site of pT-COW. The shuttle vector pT-COW was kindly provided by Professor N.B. Shoemaker (10). The resulting plasmids, pKD828 and pKD829, were introduced into KDP314 or KDP319 by electroporation, resulting in KDP314C and KDP319C, respectively, after 7 d incubation on enriched TS agar containing 0.7 μg/ml tetracycline. The proper replacement of these strains was verified by PCR and Southern blot analysis (data not shown).

Evaluation of biofilm formation ability

Biofilm development was examined by the modified protocol of Saito (11). Briefly, *P. gingivalis* cells were inoculated into BHI broth, and precultured anaerobically at 37°C for 2 d. Full growth cultures of the *P. gingivalis* strains were adjusted for the turbidity to $OD_{660} = 0.1$ in a fresh medium, then aliquots (1.5 ml) were inoculated into collagen type-I-coated 12-well flat-bottom microplates (IWAKI Glass Co., Funahashi, Japan) and cultured anaerobically at 37°C for 2 d. The culture medium was then removed from each well and 0.5 ml of 0.1% crystal violet solution was added. After 15 min, the wells were rinsed three times with PBS and air-dried. The crystal violet remaining in the biofilm was solubilized and extracted with 0.5 ml of 1% SDS. Biofilm mass was evaluated at OD_{600} using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

The Kruskal-Wallis Nonparametric ANOVA Test/Dunn's Multiple Comparison Test was used to compare the differences between 33277 and ECF mutants using GraphPad Prism version 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Data were considered significant if $p < 0.05$.

Results

Construction of *P. gingivalis* ECF sigma factor mutants

To analyze the biological significance of ECF sigma factors in *P. gingivalis* cells, we constructed ECF sigma factor mutants with the ermF-ermAM cassette inserted into each of the ECF sigma factor genes. The ECF sigma factor genes disrupted by insertion of the *ermF-ermAM* cassette were introduced into cells of the *P. gingivalis* wild-type strain (33277) by electroporation. Erythromycin-resistant colonies on blood agar plates were obtained. To confirm the disruption of the ECF sigma factor gene in the mutants, Southern blot hybridization and RT-PCR analyses were performed, revealing proper construction of all of the ECF sigma factor mutants (data not shown).

Growth and biofilm formation ability of the *P. gingivalis* ECF sigma factor mutants

To colonize and survive in 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. Most of these activities involve ECF sigma factors, thus we

analyzed biofilm formation of the ECF sigma factor mutants. All five ECF mutants' growth yields were slightly lower than that of the wild-type strain following a 48-h incubation under anaerobic conditions (Fig. 1). Next, we investigated the microtiter plate biofilms stained with crystal violet after 48 h. The biomass of the PGN_0274 and PGN_1740 mutants was enhanced in comparison with the wild-type strain (Fig. 2).

Complementation of the PGN_0274- and PGN_1740-defective mutants

To determine if the enhancement in biofilm mass was caused by the deletion of PGN_0274 and PGN_1740, we constructed strains where the PGN_0274 and PGN_1740 were restored. The PGN_0274 and PGN_1740 complemented strains were constructed by introduction of the pT-COW containing wild-type PGN_0274 and PGN_1740 DNA into each of the mutants. This complementation restored the biofilm formation ability to wild-type levels (Fig. 3). These results support the concept that PGN_0274 and PGN_1740 play an important role in controlling *P. gingivalis* biofilm formation.

Discussion

Bacteria sometimes encounter an environment unfavorable for their survival. The human oral microbiota is also often influenced by various stresses, hence it must possess the ability to defend itself. 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. Therefore, the various stress response systems have to interact with cytoplasmic and extracytoplasmic regions. Two principal regulatory mechanisms interact with cytoplasmic and extracytoplasmic regions via alternative sigma factors (ECF sigma factors) and phosphorylation-dependent response regulators (two-component systems: TCS). 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 (12). Bacterial core RNA polymerase (composed of two α subunits, ß subunit and ß' subunit) binds sigma factors. Multiple sigma factors are the bacterial transcription initiation factors that enable specific binding of RNA polymerase to gene promoters (6). TCS typically consist of a membrane-bound histidine kinase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response, mostly through differential expression of target genes (13). Interestingly, the transcriptional regulator PhyR, which combines domains of both systems, has been identified (14). ECF sigma factors and TCS are essential factors that protect the bacteria from the environmental stress.

The *P. gingivalis* W83 genome encodes six ECF sigma factors (PG0162, PG0214, PG0985, PG1660 and PG1827). Recent studies have revealed that these factors have a role in regulating the response to DNA damage (PG1318), oxidative stress (PG0985, PG1660 and PG1827), and gingipain activity (PG0162 and PG1660) in *P. gingivalis* (4,

5, 6). Nevertheless, there is relatively little information on the ECF sigma factors that may operate in this bacterium in response to entrance into biofilm formation. In *Bacillus subtillis* and *Pseudomonas aeruginosa*, ECF sigma factors are involved in regulating biofilm development (15, 16). In this study, we investigated whether biofilm formation of *P. gingivalis* is regulated by ECF sigma factors. Because *P. gingivalis* W83 strain appears to be afimbriate (17), W83 cannot form mature biofilms on collagen type-I-coated microplates (data not shown). We therefore investigated whether ECF sigma factors in *P. gingivalis* ATCC 33277 are associated with biofilm formation. This study demonstrated that PGN_0274 and PGN_1740 mutants yielded higher biofilm formation than that obtained with the wild type or the other ECF sigma factor mutants. Fimbriae and minor fimbriae influence monospecies biofilms (18). Also, the inactivation of PGN_1740 increased the expression of *fimS* at the transcriptional level (6). However, further work is needed to clarify this point.

The crystal violet remaining in the wild type, PGN_0319, PGN_0450, PGN_0970 and PGN_1740 mutant biofilm was solubilized and extracted with ethanol, but in the PGN_0274 mutant it was not. Ethanol had no significant effect on the attachment of PGN_0274 mutant biofilm. Thus, the biofilm mass was dissolved with SDS and measured. These results show that the matrix is composed predominantly of a protein component. The biofilm extracellular polymeric substances (EPS), composed of exopolysaccharides, proteins, nucleic acids and lipids, play a well-known role as a defense structure, protecting bacteria from the host immune system and

antimicrobial therapy (19). Protein is a major component among the EPS (20). As the metabolism pathway of the PGN_0274 mutant is changed by the loss of the PGN_0274 ECF sigma factor, the protein yields in the PGN_0274 mutant are more abundant than those in the wild type and the other mutants. The *sinR* ortholog PGN_0088 acts as a negative regulator of exopolysaccharide accumulation in *P. gingivalis* ATCC 33277 (21). PGN_0274 has the same role as PGN_0088, but in protein regulation.

In conclusion, we have identified that PGN_0274 and PGN_1740 play a key role in the formation of biofilm by *P. gingivalis*. PGN_0274 is involved in the post-transcriptional regulation of gingipains (5), which is consistent with our findings (data not shown). Gingipain is a very important virulence factor in *P. gingivalis*, because gingipains destroy periodontal tissue, immunoglobulins and complement factors (22, 23). As PGN_1740 plays a significant role in oxidative stress responses in the bacterium (5, 6), the survival of the PGN_1740 mutant was reduced in the presence of host cells (6). We also observed this in Ca9-22 cells (data not shown). Taken together, these results show that ECF sigma factors PGN_0274 and PGN_1740 are involved in the virulence of *P. gingivalis*. Further studies on the roles of the *P. gingivalis* ECF sigma factors, PGN_0274 and PGN_1740, will help us understand the strategy of *P. gingivalis* for colonizing and surviving in the gingival crevice, and becoming a human pathogen.

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strain		
DH5α	General-purpose host strain for cloning	Invitrogen
P. gingivalis strain		
ATCC 33277	wild type	ATCC
KDP314	PGN_0274::*ermF ermAM*, Em^r	This study
KDP315	PGN_0319::*ermF ermAM*, Em^r	This study
KDP316	PGN_0450::*ermF ermAM*, Em^r	This study
KDP317	PGN_0970::*ermF ermAM*, Em^r	This study
KDP319	PGN_1740::*ermF ermAM*, Em^r	This study
KDP314C	KDP314/pKD828, Em^r Tc^r	This study
KDP319C	KDP319/pKD829, Em^r Tc^r	This study
E. coli plasmid		
pGEM-T Easy	Ap^r, plasmid vector for TA cloning	Promega
pKD355	Ap^r, contains the *ermF ermAM* DNA cassette between EcoRI and BamHI of pUC18	8
pKD814	Ap^r, contains the 1.0-kb PCR-amplified fragment (PGN_0450 region) in pGEM-T Easy	This study
pKD817	Ap^r, contains the 1.5-kb PCR-amplified fragment (PGN_0274 region) in pGEM-T Easy	This study
pKD818	Ap^r, contains the 2.0-kb PCR-amplified fragment (PGN_0319 region) in pGEM-T Easy	This study
pKD819	Ap^r, contains the 2.0-kb PCR-amplified fragment (PGN_0970 region) in pGEM-T Easy Ap^r	This study
pKD821	Ap^r, contains the 2.0-kb PCR-amplified fragment (PGN_1740 region) in pGEM-T Easy Ap^r	This study
pKD822	Ap^r Em^r, contains the *ermF ermAM* DNA cassette at BamHI site within PGN_0274 of pKD817	This study
pKD823	Ap^r Em^r, contains the *ermF ermAM* DNA cassette at BamHI site within PGN_0319 of pKD818	This study

pKD824	Ap^r Em^r, contains the *ermF ermAM* DNA cassette at BamHI site within PGN_0450 of pKD814	This study
pKD825	Ap^r Em^r, contains the *ermF ermAM* DNA cassette at BglII site within PGN_0970 of pKD819	This study
pKD827	Ap^r Em^r, contains the *ermF ermAM* DNA cassette at BamHI site within PGN_1740 of pKD821	This study
P. gingivalis plasmid		
pT-COW	Ap^r Tc^r, *E. coli-P. gingivalis* shuttle plasmid	10
pKD828	Ap^r Tc^r, pT-COW- PGN_0274^+	This study
pKD829	Ap^r Tc^r, pTCB- PGN_1740^+	This study

Table 2. Primers used in this study

PGN0274-U-F: TCGACAGTTGATTGCCGAT
PGN0274-U-R-BamHI: GGGATCCCCATCGAAAGACTGCAATCTGG
PGN0274-D-F-BamHI: GGGATCCCATGACGACGCCGCTCCTGTCGAAA
PGN0274-D-R: TGTGCAAAAAAGGAAACAGC
PGN0319-U-F: GCTGCCGCTCCTTCTTCAT
PGN0319-U-R-BamHI: GGGATCCCAAAGGCAGATCGTCCGGTA
PGN0319-D-F-BamHI: GGGATCCCCTCCGATCATGCCCCTA
PGN0319-D-R: TCAGGCTCTTGTACAGATGGA
PGN0450-U-F: GGGATGTGGAGAAAAAGGAA
PGN0450-D-R: ATGACCACGGACAGGAAGAT
PGN0970-U-F: ACCGGGAAATAATTCTCAAGC
PGN0970-U-R-BglII: AAGATCTTCCAAAGAGGTCGGATAAGGA
PGN0970-D-F- BglII: AAGATCTTAGGCTGCCGAGGTACAGGA
PGN0970-D-R: ACACAAGCTACAGCCCCGTA
PGN1740-U-F: GAGGATCTCCCTGCCAATAAT
PGN1740-U-R-BamHI: GGGATCCCACCCAGCCTTTGAAGTTGACA
PGN1740-D-F-BamHI: GGGATCCCGCTCACTGTCATGCGAAAT
PGN1740-D-R: CCAACGGCTATTTAGCATCC
PGN0274-COMP-U-F-PstI: CCTGCAGGCTGCTACTGTCTCGGACGTG
PGN0274-COMP-D-R-BamHI: GGGATCCCGTTTGTGTTTGAGGCTGCAT
PGN1740-COMP-U-F-BamHI: CGGGATCCCGAGTGCGATATCGGGAATCAG
PGN1740-COMP-D-R-BamHI: CGGGATCCCGAGTTGATACGGCTGCTATGC

Restriction sites incorporated into oligonucleotides for subcloning are underlined.

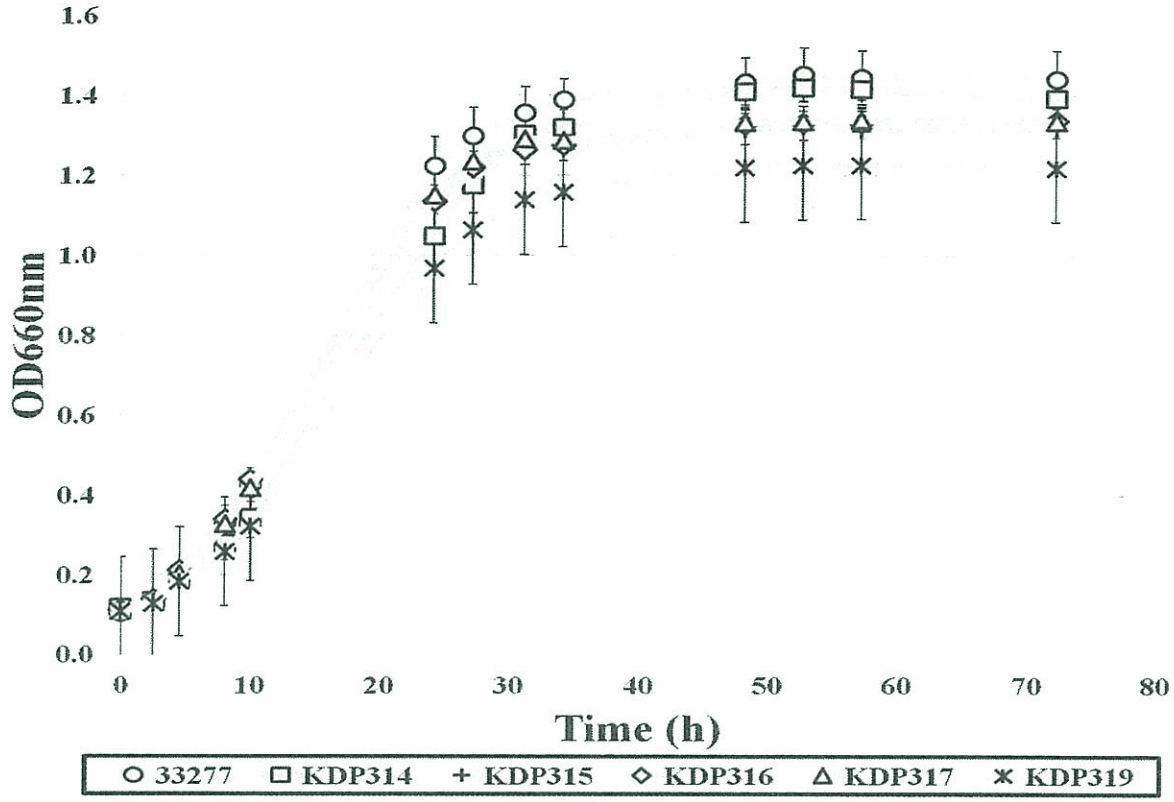

Fig.1 Growth curves of *P. gingivalis* 33277 (wild type) (circle), PGN_0274 mutant (KDP314) (rectangle), PGN_0319 mutant (KDP315) (plus), PGN_0450 mutant (KDP316) (diamond), PGN_0970 mutant (KDP317) (triangle), PGN_1740 mutant (KDP319) (cross) in enriched BHI broth. The data shown are mean ± SD of triplicate experiments.

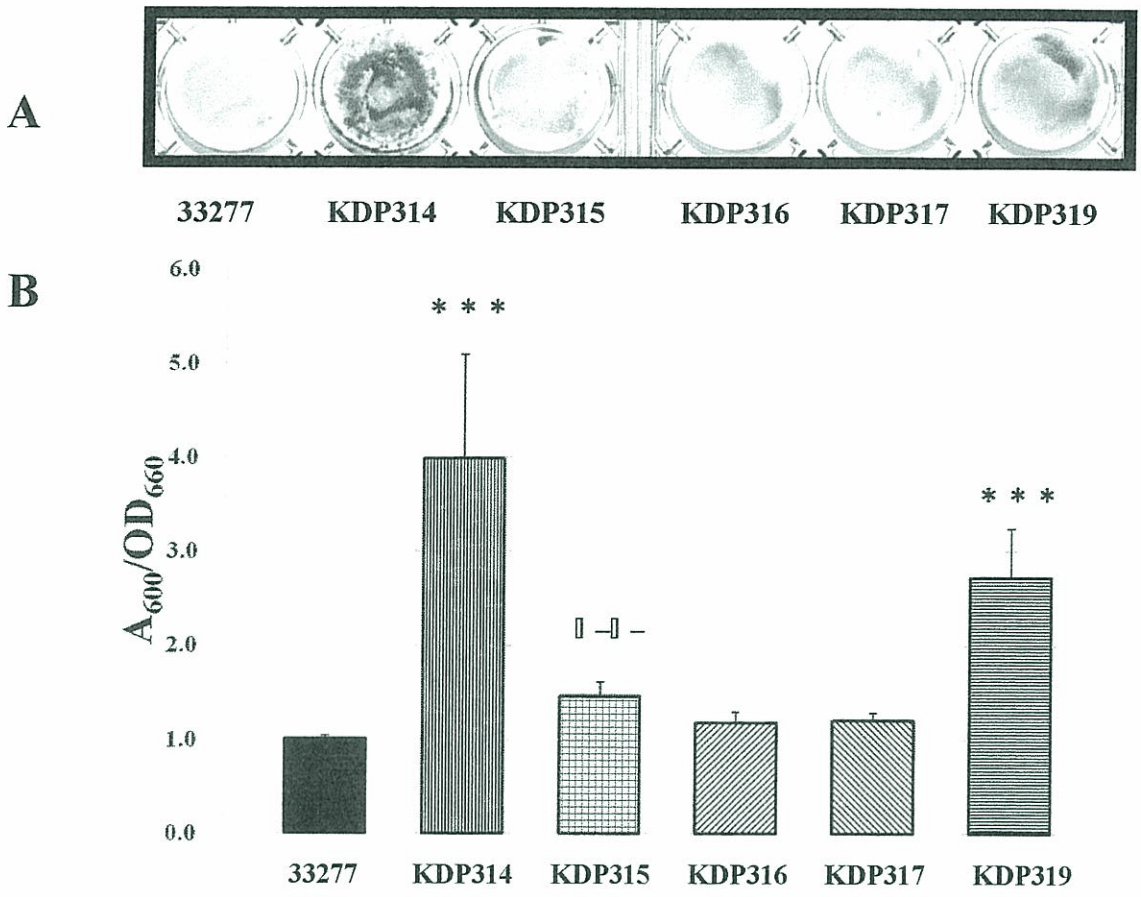

Fig.2 Biofilm formation by homotypic *P. gingivalis* 33277 or ECF sigma factor mutants. The strains were grown on enriched BHI broth anaerobically at 37°C in collagen type-I-coated 12-well flat-bottom microplates. After 48 h of cultivation, the organized biofilm mass was evaluated by staining with crystal violet. (A) The photographs are a representative sample of each experimental strain. (B) Biofilm formation determined by crystal violet staining and adjusted for growth (A_{600} units per OD_{660} unit). The data shown are mean ± SD of triplicate experiments. **, $p < 0.01$, and ***, $p < 0.001$, by a Kruskal-Wallis Nonparametric ANOVA Test/Dunn's Multiple Comparison Test.

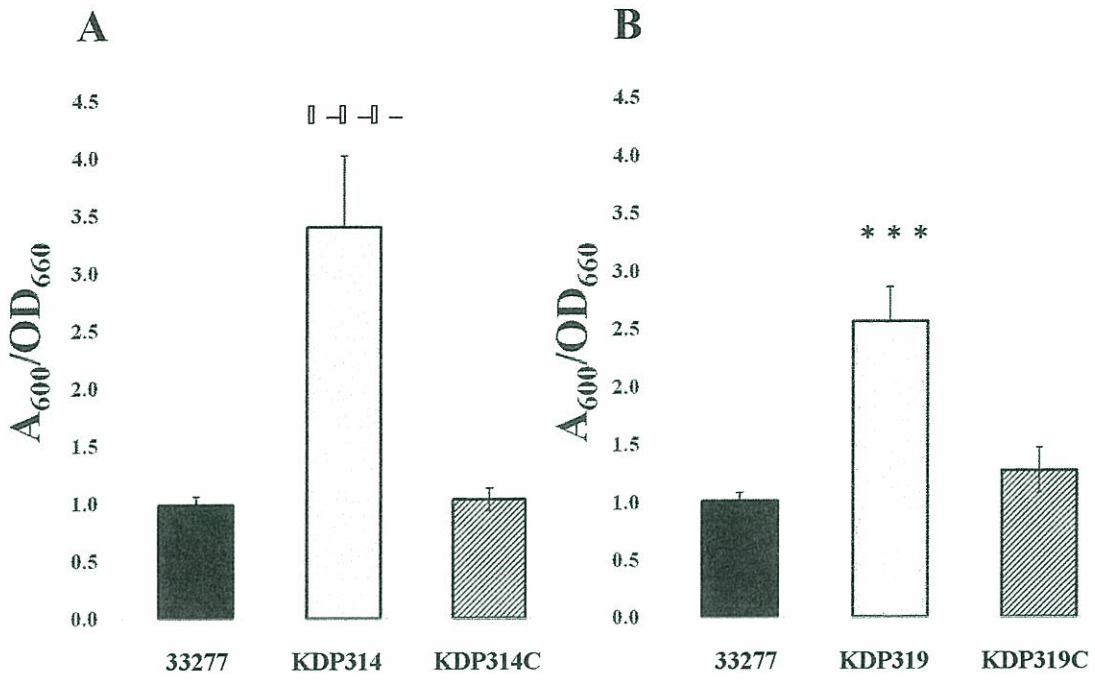

Fig. 3. Biofilm formation by homotypic *P. gingivalis* 33277 or ECF sigma factor mutans. The strains were grown on enriched BHI broth anaerobically at 37°C in collagen type-I-coated 12-well flat-bottom microplates. After 48 h of cultivation, the organized biofilm mass was evaluated by staining with crystal violet. (A) Biofilm formation of 33277, PGN_0274 mutant and the complemented mutant strain were compared. (B) Biofilm formation of 33277, PGN_1740 mutant and the complemented mutant strain were compared. Biofilm formation was determined by crystal violet staining and adjusted for growth (A_{600} units per OD_{660} unit). The data shown are mean ± SD of triplicate experiments. ***, $p < 0.001$, by a Kruskal-Wallis Nonparametric ANOVA Test/Dunn's Multiple Comparison Test.