Involvement of extracytoplasmic function sigma factors in virulence regulation in *Porphyromonas gingivalis* W83

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Abstract

Extracytoplasmic function (ECF) sigma factors are known to play an important role in the bacterial response to various environmental stresses and can significantly modulate their pathogenic potential. In the genome of *Porphyromonas gingivalis* W83, six putative ECF sigma factors were identified. To further evaluate their role in this organism, a PCR-based linear transformation method was used to inactivate five ECF sigma factor genes (*PG0162*, *PG0214*, *PG0985*, *PG1660*, and *PG1827*) by allelic exchange mutagenesis. All five isogenic mutants formed black-pigmented colonies on blood agar. Mutants defective in *PG0985*, *PG1660* and *PG1827* genes were more sensitive to 0.25 mM of hydrogen peroxide compared to the wild-type strain. Isogenic mutants of *PG0162* and *PG1660* showed a 50 percent decrease in gingipain activity. RT-PCR analysis showed that there was no alteration in the expression of *rgpA*, *rgpB*, and *kgp* gingipain genes in these mutants. Hemolytic and hemagglutination activities were decreased by more than 50 percent in the *PG0162* mutant compared to the wild-type. Taken together, these findings suggest that ECF sigma factors can modulate important virulence factors in *P. gingivalis*. ECF sigma factors encoded by the *PG0162* and *PG1660* genes might also be involved in the post-transcriptional regulation of the gingipains.

Keywords

Porphyromonas gingivalis W83; ECF sigma factor; virulence

Introduction

The response and adaptation of bacteria to environmental stress is known to be mostly regulated at the level of transcription initiation. This regulation primarily involves alternative sigma factors which recruit RNA polymerase and facilitate specific promoter recognition and transcription initiation (Paget & Helmann, 2003). Extracytoplasmic function (ECF) sigma factor, the largest group of alternative sigma factors, plays a key role in adaption to environmental conditions (Staron *et al.*, 2009). Furthermore, because bacteria-host interaction via surface structures is important in bacterial pathogenesis, ECF sigma factors also regulate virulence factors (Staron *et al.*, 2009). This is well documented in *Mycobacterium tuberculosis* (Hahn *et al.*, 2005), *Staphylococcus aureus* (Shaw *et al.*, 2008), *Pseudomonas aeruginosa* (Wood & Ohman, 2009, Llamas *et al.*, 2009), and *Enterococcus faecalis* (Le *et al.*, 2010).

Porphyromonas gingivalis, an anaerobic gram negative bacterium, is an important etiological agent in adult chronic periodontitis. This organism possesses several cell surface

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associated virulence factors (e.g. hydrolytic enzymes, fimbriae, hemagglutinin, capsule, and lipopolysaccharide) that can directly or indirectly affect the periodontium (Yoshimura *et al.*, 2009). In addition, to survive in the microenvironment of an advanced periodontal pocket it is necessary that the bacteria has the capacity to respond to environmental changes including temperature, pH, concentration of some nutrients, and oxygen tension. To date, little is known about the relationship between the regulation of adaptive mechanisms, virulence, and sigma factors in *P. gingivalis*.

The *P. gingivalis* W83 genome encodes 8 sigma factors, 6 of which belong to the extracytoplasmic function (ECF) sigma factor subfamily (PG0162, PG0214, PG0985, PG1318, PG1660, and PG1827) (Nelson *et al.*, 2003). The PG1318 ECF sigma factor was recently shown to be involved in the regulation of mutation frequency in *P. gingivalis* (Kikuchi *et al.*, 2009). In this study, we used a PCR-based linear transformation strategy to inactivate the remaining five putative ECF sigma factors, and analyzed the virulence related characteristics of these proteins in *P. gingivalis* W83. We now report that several of the ECF sigma factors may play a role in virulence regulation and adaptation to oxidative stress. ECF sigma factors encoded by the *PG0162* and *PG1660* genes are likely involved in the post-transcriptional regulation of the gingipains.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 1. *P. gingivalis* strains were grown in Brain Heart Infusion (BHI) broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), hemin (5μg/ml), vitamin K (0.5μg/ml), and cysteine (0.1%) (Sigma-Aldrich, St. Louis, Mo). *P. gingivalis* strains were maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 10% H_2, 10% CO_2, and 80% N_2 at 37°C. Growth rates for *P. gingivalis* strains were determined spectrophotometrically by measuring optical density at 600 nm [OD_{600}]. Antibiotics were used at the following concentrations: erythromycin, 10μg/ml; tetracycline, 3μg/ml.

Sensitivity to hydrogen peroxide

Sensitivity of *P. gingivalis* strains to hydrogen peroxide was tested as previously described (Henry *et al.*, 2008). Briefly, *P. gingivalis* strains were grown to early log phase (OD_{600} ~0.2) in BHI broth. Hydrogen peroxide at a final concentration of 0.25 mM was then added to the cultures and further incubated at 37°C for 24 hours. The OD_{600} was measured at 3-hour intervals over a 24 hour period. Cell cultures without hydrogen peroxide were used as controls.

Construction of ECF sigma factor defective mutants

Long PCR-based fusion of several fragments was done as previously described (Shevchuk *et al.*, 2004). Primers used in this study are listed in Table 2. One kilobase flanking fragments both upstream and downstream of the target genes were PCR amplified from chromosomal DNA of *P. gingivalis* W83. The *ermF* cassette was amplified from the pVA2198 (Fletcher *et al.*, 1995) plasmid with oligonucleotide primers that contained overlapping nucleotides for the upstream and downstream fragments. These three fragments were fused together by using the forward primer of the upstream fragment and the reverse primer of the downstream fragment. The fusion PCR program consisted of 1 cycle of 5min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 55°C, and 4 min at 68°C, with a final extension of 5 min at 68 °C. This PCR-fused fragment was used to transform *P. gingivalis* W83 by electroporation as previously described (Abaibou *et al.*, 2001). The cells were plated on BHI agar containing 10 μg/ml of erythromycin and incubated at 37 °C for 7 days.

The correct gene replacement in the erythromycin resistant mutants was confirmed by colony PCR and DNA sequencing.

Complementation of the *PG0162-defective* mutant (FLL350)

A DNA fragment containing the *PG0162* ORF with an upstream regulatory region was amplified from chromosomal DNA of *P. gingivalis* W83 using primer sets PG0162_Com_F and PG_0162_Com_R (Table 2). A *BamH*I restriction site was designed at the 5' end of both primers to facilitate the subcloning of the PCR fragment. Both pT-COW (Gardner *et al.*, 1996) and the *BamH*I-digested PCR fragment were ligated together and used to transform *E. coli* DH5α. The purified recombined plasmid designated pFLL350a was used to transform *P. gingivalis* FLL350 (PG0162::*ermF*) by electroporation. The transformants were selected on BHI agar plates with erythromycin and tetracycline.

Hemolytic activity assay

Hemolytic activity was determined as previously reported (Vanterpool *et al.*, 2004). Briefly, bacterial cells from 24 hour cultures were harvested by centrifugation (10,000 g for 10 min), washed three times with phosphate buffered saline (PBS, 0.147 M NaCl, 0.01 M sodium phosphate, pH 7.4), and then resuspended to a final OD_{600} of 1.5. Sheep erythrocytes (Hemostat Laboratories, Dixon, CA) were harvested by centrifugation (4,400 g for 20 min) and washed with PBS until the supernatant was visibly free of hemoglobin pigment. The washed erythrocytes were suspended to a final concentration of 1% in PBS. Hemolytic activity was determined by mixing an equal volume of bacterial cells with 1% erythrocytes in PBS. This mixture was then incubated at 37°C for 4 hours. Samples (500 μl) were withdrawn and further spun (1,300 g for 5 min) in an Eppendorf 5403 centrifuge at room temperature. The OD_{405} of supernatant was determined by spectrophotometry. As a negative control, erythrocytes were used alone.

Hemagglutination studies

Hemagglutinin activity was determined as previously reported (Vanterpool *et al.*, 2005a). Twenty-four hour cultures of *P. gingivalis* W83 and mutants were harvested by centrifugation (10,000 g for 10 min). Cells were washed twice in PBS buffer and resuspended to a final OD_{600} of 1.5. Sheep erythrocytes were washed twice with 1×PBS and resuspended in 1×PBS to a final concentration of 1%. An aliquot (100-μl volume) of the bacterial suspension was serially diluted two-fold with PBS in wells of a round-bottom 96-well microtiter plate. An equal volume (100 μl) of 1% sheep erythrocytes was mixed with each dilution and incubated at 4°C for 3 hours. Hemagglutination was visually assessed and the hemagglutination titer was determined as the last dilution that showed complete hemagglutination.

Gingipain activity assays

The presence of Arg-X- and Lys-X-specific cysteine protease (Rgp and Kgp) activity was determined with a microplate reader (Bio-Rad, Hercules, CA) as previously reported (Vanterpool *et al.*, 2005b). In brief, activity of arginine gingipains was measured with 1mM BAPNA (Nα-benzoyl-DL-arginine-p-nitroanilide) in activated protease buffer (0.2 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 10 mM L-cysteine, pH 7.6). Lysine gingipain activity was measured with ALNA (Ac-Lys-p-nitroanilide HCl). After incubating the substrate and culture, the reaction was stopped by the addition of 50 μl of glacial acetic acid. The O.D. at 405 nm was then measured against a blank sample containing no bacteria.

Reverse transcription (RT)-PCR analysis

Total RNA from *P. gingivalis* strains was extracted by using the SV Total RNA Isolation System (Promega Corp. Madison, WI) according to the manufacturer's instructions. Complementary DNA was synthesized by using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Corp., Indianapolis, IN). The primers used as listed in Table 2. The PCR program consisted of 1 cycle of 5 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 54°C, and 1 min at 68°C, with a final extension of 5 min at 68 °C.

Results and discussion

Construction of ECF sigma factor mutants in *P. gingivalis* W83

To construct ECF sigma factor isogenic mutants, PCR was used to fuse the upstream and downstream fragments of the target gene to *ermF*, generating a 3 kb-length fragment, which was then electrotransformed into *P. gingivalis* W83. To the promoter region upstream of the ATG start codon of *ermF*, we added a 20 base oligonucleotide 5'-TGACTAACTAGGAGGAATAA-3' containing three stop codons separated by one nucleotide. An 18 base oligonucleotide 5-TACCTGGAGGGAATAATC-3' containing a ribosome binding site was added downstream of the TAG stop codon of *ermF*, to eliminate the influence on expression of the other genes downstream of the target gene. Though W83 lacks a TraP, which was previously shown to be required for plasmid transfer in *P. gingivalis* (Tribble *et al.*, 2007), PCR-based transformation worked with high efficiency in W83. We were able to construct 5 ECF sigma factor deletion mutants (*PG0162, PG0214, PG0985, PG1660*, and *PG1827*). These mutants were confirmed by colony PCR (Fig. 1a) and sequencing (data not shown). To rule out polar mutations arising from the inactivation of these genes, RT-PCR was used to amplify the sigma factor encoding genes and the downstream genes (Fig. 1b). As shown in Figure 1c, inactivation of the ECF genes had no effects on the expression of the downstream genes. FLL355 (*PG1827::ermF*) showed a slower growth rate compared to the other ECF mutants which were similar to the wild-type strain (Figure 2a). However, similar to the wild-type strain, all five ECF isogenic deletion mutants were black-pigmented on blood agar plates (data not shown).

Growth in the presence of hydrogen peroxide

The sensitivity to several environmental stresses including oxidative stress and involvement in pathogenesis of ECF sigma factor mutants have been described for several bacteria (Kallifidas *et al.*, 2010, Staron *et al.*, 2009, White *et al.*, 2010). In the human oral cavity, *P. gingivalis* encounters oxidative stress from exposure to air and reactive oxidative species (ROS) generated by neutrophils or from other oral bacteria. ROS can cause damage to cell membranes, nucleic acids, and proteins (Imlay, 2003). While several organisms have evolved various mechanisms to protect themselves against oxidative stress, little is known about ROS sensing and adaptation/protection in anaerobic bacteria.

In order to evaluate the relationship between the sensitivity of *P. gingivalis* to hydrogen peroxide and ECF sigma factors, isogenic mutants defective in these factors were exposed to hydrogen peroxide. As shown in Figure 2, the growth of *P. gingivalis* isogenic mutants defective in *PG0985* (FLL352), *PG1660* (FLL354), and *PG1827* (FLL355) was more retarded in the presence of hydrogen peroxide compared to the wild-type. *PG0162* (FLL350) and *PG0214* (FLL351) isogenic mutants and the wild-type showed a similar sensitivity to hydrogen peroxide (data not shown). This suggests that ECFs PG0985, PG1660, and PG1827 may play a role in H_2O_2-induced oxidative stress resistance in *P. gingivalis*.

FLL350 and FLL354 showed decreased gingipain protease activity

Several reports have documented the multiple effects of gingipains, a major virulence factor of *P. gingivalis* (Sheets *et al.*, 2006, Sheets *et al.*, 2008). These gingipains, which are both extracellular and cell membrane-associated, are essential for growth and can also play a role in oxidative stress resistance (Sheets *et al.*, 2008). In order to identify whether the sigma factors were involved in gingipain regulation, gingipain activity was measured in ECF sigma factor mutants. In comparison to the wild-type, Rgp gingipain activity was decreased by 50% and 60% in FLL350 (*PG0162::ermF*) and FLL354 (*PG1660::ermF*) respectively (Fig. 3a). Kgp activity was decreased by 20% in FLL350 and 50% in FLL354 compared to the wild-type strain (Figure 3a). Because gingipain activity can be regulated at the transcriptional and post-transcriptional levels (Tokuda *et al.*, 1998), oligonucleotide primers as previously described (Vanterpool *et al.*, 2005a) were used in RT-PCR analysis to determine whether these two sigma factors were involved in the transcriptional regulation of gingipain encoding genes. As shown in Figure 3b, the inactivation of *PG0162* and *PG1660* had no effect on the expression of *rgpA*, *rgpB*, or *kgp* at the transcriptional level. In FLL355 (*PG1827::ermF*), the Kgp activity showed a 25% increase over the wild-type. No change was observed in the transcription of the *kgp* gene in FLL355 (data not shown). Taken together, these results suggest that ECF sigma factors may be involved in the post-transcriptional regulation of gingipains. Post-transcriptional regulation of the gingipains in *P. gingivalis* is associated with its maturation pathway that is linked to the biosynthesis of surface carbohydrates (Paramonov *et al.*, 2005, Shoji *et al.*, 2002) and several other proteins including the PorR (Shoji *et al.*, 2002), PorT (Nguyen *et al.*, 2009, Sato *et al.*, 2005), Sov (Saiki & Konishi, 2010) and VimA (Vanterpool *et al.*, 2006). It is unclear how these factors are modulated by the ECF sigma factors and is an active area of further exploration in the laboratory.

Hemagglutination activity is decreased in the ECF sigma factor-defective mutants

The correlation between gingipain activity and hemagglutination in *P. gingivalis* (Lewis *et al.*, 1999, Shi *et al.*, 1999, Vanterpool *et al.*, 2005a) is related to the similar adhesion domains encoded by the *hagA*, *rgpA*, and *kgp* genes (Chen & Duncan, 2004). The hemagglutination potential of ECF sigma factor defective mutants was assessed. In comparison to the wild-type strain, there was a decrease in the hemagglutination activity in all the mutants. In FLL350, the level of hemagglutination activity was comparable to the negative control. This is in contrast to FLL354, which had the greatest reduction in gingipain activity but a higher hemagglutination activity. RT-PCR using *hagA* specific primers indicate no change in the expression of that gene in FLL350 (Fig. 4c).

PG0162 can regulate hemolytic activity

While gingipains have been observed to have hemolytic activity (Lewis *et al.*, 1999, Shah & Gharbia, 1989), hemolysin can be independent of their catalytic association (Deshpande & Khan, 1999). Several putative hemolysin genes have been identified in the *P. gingivalis* genome (Nelson *et al.*, 2003) and cloned in *E. coli* (Karunakaran & Holt, 1993). The hemolysins produced by *P. gingivalis* provide the bacterium with heme-containing molecules that are required for their in vivo survival. Hemolytic activities of all the ECF-defective mutants in this study were similar to those of the wild-type except FLL350 (Figure 4d). FLL350 mutant showed a 50% reduction in those activities compared to the parent strain. Complementation of FLL350 with a wild-type copy of *PG0162* restored some of the hemolytic activity of this mutant [FLL350c, (Figure 4)]. Gingipain and hemagglutination activities were also restored in FLL350c (Figure 3 and 4), thus confirming a role for *PG0162* in virulence regulation in *P. gingivalis* W83. It is noteworthy that although FLL354 had the lowest gingipain activity, its hemolysin profile was similar to the wild-type strain.

Taken together, this is consistent with previous observations suggesting that hemolysin and gingipain activities can be distinct from each other (Deshpande & Khan, 1999).

Collectively, our study showed that ECF sigma factors PG0162 and PG1660 play an important role in the regulation of gingipain, hemolytic, and hemagglutination activities, and could likely modulate the virulence potential of *P. gingivalis*. Because the exterior surface structures and factors of the infectious bacteria are the first to come in contact with the host and must respond and adapt to the host environment, our observations are consistent with the role of ECF sigma factors in the regulation of virulence associated genes [reviewed in (Brooks & Buchanan, 2008)]. The activity of ECF sigma factors is most often negatively regulated by direct interaction with cognate anti-sigma factors, which prevent their association with the core RNA polymerase or facilitate holoenzyme dissociation [reviewed in (Staron *et al.*, 2009)]. While PG1660 appear to have a putative cognate anti-sigmafactor PG1659 (http://www.oralgen.lanl.gov/, http://www.cbs.dtu.dk/services/TMHMM/), a similar putative component is missing for PG0162. It is noteworthy that regulation of PG0162 on virulence observed in this study is occurring at the post-transcriptional level. It is likely that PG0162 may be involved in a unique and complex regulatory mechanism and that requires further evaluation.

Acknowledgments

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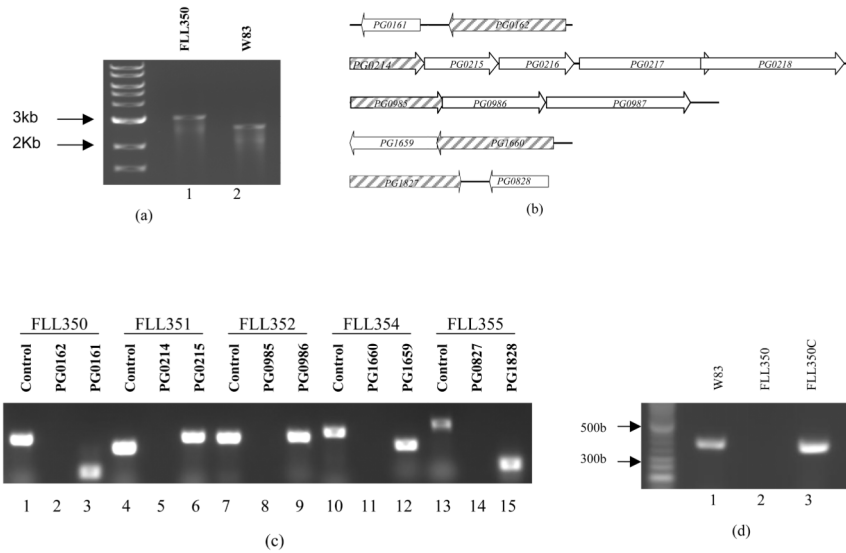
Fig. 1.
(a) Colony PCR confirmation of the mutant FLL350. The primers used were PG_0162_F1 and PG_0162_ R3. Lane 1, FLL350 PCR product (3 kb). Lane 2, W83 PCR product (2.5 kb). (b) Diagram of chromosomal structure of *PG0162*, *PG0214*, *PG0985*, *PG1660* and *PG1827*. Hatched arrows indicate the ECF sigma factor encoding genes and white arrows indicate genes located downstream of ECF sigma factor genes. Arrow indicates the transcription orientation. (c) RT-PCR confirmation showing the non-polar effects of the inactivated ECF sigma factor genes is isogenic defective mutants. Lane 1, *PG0162* product using W83 cDNA as template (379 bp). Lane 2, *PG0162* product using cDNA of FLL350 as template. Lane 3, *PG0161* product using cDNA of FLL350 as template (150 bp). Lane 4, *PG0214* product using W83 cDNA as template (308 bp). Lane 5, *PG0214* product using cDNA of FLL351 as template. Lane 6, *PG0215* product using cDNA of FLL351 as template (382 bp). Lane 7, *PG0985* product using W83 cDNA as template (367 bp). Lane 8, *PG0985* product using cDNA of FLL352 as template. Lane 9, *PG0986* product using cDNA of FLL352 as template (376 bp). Lane 10, *PG1660* product using W83 cDNA as template (414 bp). Lane 11, *PG1660* product using cDNA of FLL354 as template. Lane 12, *PG1659* product using cDNA of FLL354 as template (310 bp). Lane 13, *PG1827* product using W83 cDNA as template (491 bp). Lane 14, *PG1827* product using cDNA of FLL355 as template. Lane 15, *PG1828* product using cDNA of FLL355 as template (165 bp). (d) Transcriptional analyses of FLL350C by RT-PCR. Primers used were PG0162_RT_F and PG0162_RT_R as listed in Table 2. Lane 1, W83 cDNA. Lane 2, FLL350 cDNA. Lane 3, FLL350C cDNA.

(a)   (b)
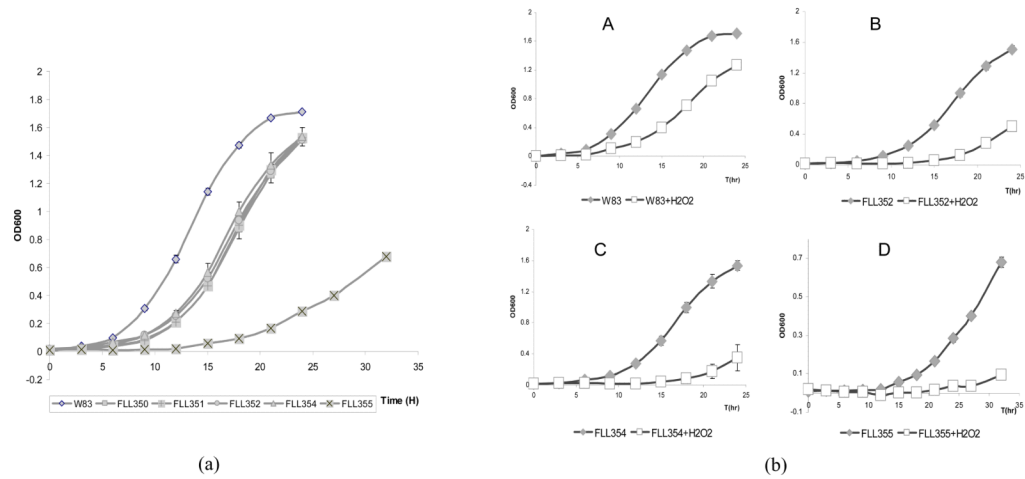
Fig. 2.
Growth and H_2O_2 sensitivity of ECF sigma factor isogenic mutants. Strains grown to early log phase were treated with 0.25 mM H_2O_2 and further incubated for 24 hr. The cultures without H_2O_2 were used as controls. The results shown were representative of at least three independent replicates. Panel (a), Growth of *P. gingivalis* W83 and ECF sigma factor isogenic mutants in the absence of H_2O_2. Panel (b), Growth of *P. gingivalis* W83 and ECF sigma factor isogenic mutants in the presence of H_2O_2. A, W83. B, FLL352. C, FLL354. D, FLL355. Error bars represent the standard deviation.
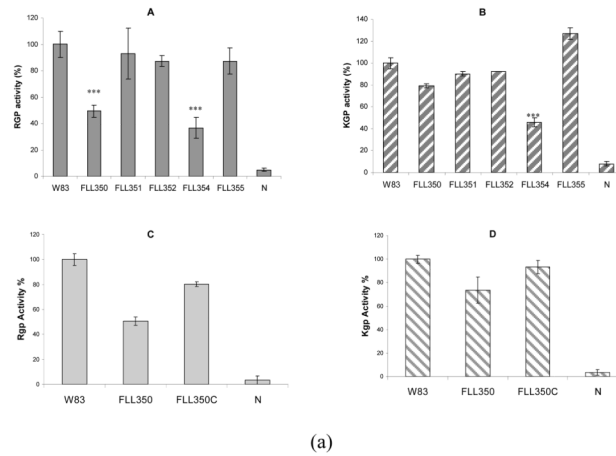
(a)
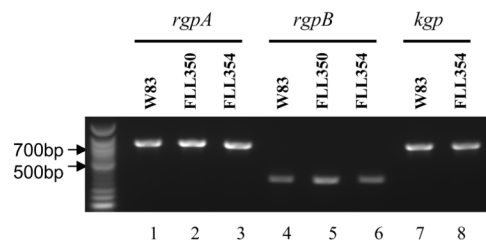
(b)

Fig. 3.
(a) Proteolytic activity of ECF sigma factor mutants. *P. gingivalis* strains were grown to stationary phase in BHI media supplemented with hemin and vitamin K. Activities against Rgp or Kgp were tested in whole cell culture. The gingipain activities were normalized to W83 being 100% and the mutants reported as a percentage thereof. Asterisks indicate results significantly different from those of W83 (P<0.005). Error bars represent the standard deviation. A, Rgp activity. B, Kgp activity. C, Rgp activity of FLL350 complemented with *PG0162*. D, Kgp activity of FLL350 complemented with *PG0162*. (b) RT-PCR analysis of gingipain encoding genes in W83, FLL350 and FLL354.
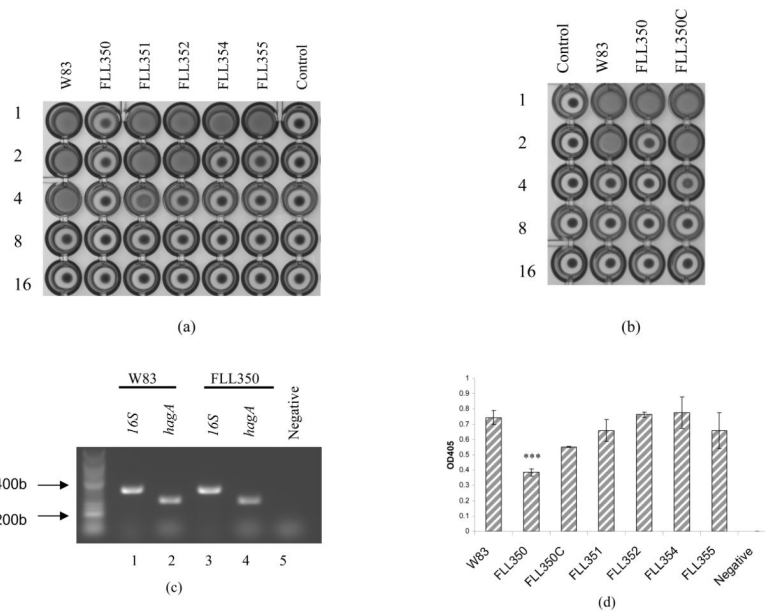
Fig. 4.
(a) Hemagglutination activity of ECF sigma factor mutants. Hemagglutinin activities of *P. gingivalis* were assessed in cells serially diluted and incubated with sheep erythrocytes at 4°C for 3 h in a round-bottom microtiter plate. The titer was defined as the last dilution that showed full agglutination. Dilution folds were listed on the left. (b) Hemagglutination activity of ECF sigma factor mutants. Dilution folds were listed on the left. (c) RT-PCR analysis of *hagA* in FLL350. Lane 1, 16S rDNA, W83 cDNA as template (404b). Lane 2, *hagA*, W83 cDNA as template (312b). Lane 3, 16SrDNA, FLL350 cDNA as template. Lane 4, *hagA*, FLL350 cDNA as template. Lane 5, *hagA*, FLL350 RNA as template. (d) Hemolytic activity ECF sigma factor mutants. Erythrocytes were used as negative control. The hemolytic activity of W83 was normalized as 100% and the mutants reported here as percentage. Error bars represent the standard deviation. The data shown are an average of three or more replicates. Asterisks indicate results significantly different from that of W83 (P<0.005).

Table 1

Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	References
Strains		
P. gingivalis		
W83	Wild-type	Henry *et al.* (2008)
FLL350	*PG0162*-defective	This study
FLL351	*PG0214*-defective	This study
FLL352	*PG0985*-defective	This study
FLL354	*PG1660*-defective	This study
FLL355	*PG1827*-defective	This study
FLL350 C	FLL350 with pFLL350a	This study
Plasmids		
pVA2198	Sp^r, *ermF-ermAM*	Fletcher *et al.* (1995)
pT-COW	Ap^r, *tetQ*	Gardner *et al.* (1996)
pFLL350a	Ap^r, *tetQ*, *Tc::PG0162*	This study

Table 2

Primers used in this study

Primers	Sequences (5'--3')
Primers for PG0162 mutant construction	
PG0162_F1	CGAAGAGGTGGAGGCCAACAATTACT
PG0162_R1	TACCTTATTCCTCCTAGTTAGTCATGTGGAAACTGCTCATAGGAGAATC
PG0162_F3	TTCGTAGTACCTGGAGGGAATAATCCATGTCGGCTTAGTCGATATTCCATA
PG0162_R3	GTGCATCCTCTGCCAGCGTACATAT
Primers for PG0214 mutant construction	
PG0214_F1	GCTGCTGCCACTATGCGCTTTTCGA
PG0214_R1	TACCTTATTCCTCCTAGTTAGTCACTATGGGCGAATGCTTCTACTTCCAT
PG0214_F3	TTCGTAGTACCTGGAGGGAATAATCGGCTCTCCAACTCAAACGATGATGA
PG0214_R3	CAGGAGCGATACCAGATCCTCGGAA
Primers for PG0985 mutant construction	
PG0985_F1	CTGACAGAAGAAGCTCGTAAGGAGT
PG0985_R1	TACCTTATTCCTCCTAGTTAGTCAGAAAGCGATAGTATTCACGTCGTACT
PG0985_F3	TTCGTAGTACCTGGAGGGAATAATCGAGGTGATGAAAGATGATGCGTG A
PG0985_R3	CGTGAATGTCCCTTCCATGACAAGT
Primers for PG1660 mutant construction	
PG1660_F1	CCCATGATAGAGACCATCTCCCCTT
PG1660_R1	TACCTTATTCCTCCTAGTTAGTCATAGCTGTCGGATTGCTTCTCCATCT
PG1660_F3	TTCGTAGTACCTGGAGGGAATAATCTGATGAGAAAATGAAACAGAATATA
PG1660_R3	CTCGCGTAAGGAGATACAGCTTGAGA
Primers for PG1827 mutant construction	
PG1827_F1	GACATAGGTGTTCGAACCGCCGATAT
PG1827_R1	TACCTTATTCCTCCTAGTTAGTCAATAATAGACGGCATCAATGACATGAA
PG1827_F3	TTCGTAGTACCTGGAGGGAATAATCAGGACATGAGATAACAGCCTGCTCA
PG1827_R3	GATGCGTACAGGTGATCTTGGCACCA
Erm_F_r(3)	GATTATTCCCTCCAGGTACTACGAAGGATGAAATTTTTCA
Erm_F_P_5'	TGACTAACTAGGAGGAATAAGGTATTTGCAACATCATAGAAA
Primers for FLL350 complementation	
PG0162_Com_F	ATGGATCCGCATTAGTCGGGTTGATAAGCAGT
PG0162_Com_R	TTGGATCCCTAAGCCGACATGCCCATCATTTT
RT-PCR primers	
PG0161_RT_F	CCTTTTGGCGAATATGCTTATGACAGA
PG0161_RT_R	GAGCAACGTGAACGGGCCATAAGTTT
PG0162-RT_F	TGATCCATACGCTTCGTCGCGGA
PG0162-RT_R	GCAGATTGATCAGCGCATATCGCATT
PG0214-RT_F	GACCACTGCTCGGCGAATAGCCT
PG0214-RT_R	GGAGAGCCAGTGCTGTACGCAGT
PG0215-RT_F	GAAGAGGATCGCCTGCGTCGCTAT
PG0215-RT_R	CGAAAAGCTCCTGTGCCAACTCCT

Primers	Sequences (5'--3')
PG0985-RT_F	CGACGACGAGGAGGCTGAGGAT
PG0985-RT_R	CACATTGCCCTCCGTCAGTCCCAA
PG0986-RT_F	CAAGAGGAGTTGGAGCTGTATTGCTT
PG0986-RT_R	CGAGACAATACTCCAATAGCTCCAT
PG1659-RT_F	GACTCAAACAACGCTTCGACGTGAT
PG1659-RT_R	CGATGCTTCGTGTTGCGATATAGTT
PG1660-RT_F	GTGGAGCGATACAGCGACATGCT
PG1660-RT_R	GTGGAGCTTGACCTTCACGTTGGA
PG1827-RT_F	CGCTGTTTATTAGGGTTTCTCACCTA
PG1827-RT_R	GTACATGGAGAATGGCTGCTTGAGA
PG1828-RT_F	GCTGTATCATTCGCTTCTTGCAACT
PG1828-RT_R	CTTGTTCAGTAGCTTCTTCAGCCTT
16S_rRNA_F	AGGCAGCTTGCCATACTGCG
16S_rRNA_R	ACTGTTAGCAACTACCGATGT
HagA_RT_F	GTGGTAGAGGCCGGTAAGACTTATCA
HagA_RT_R	CGTCACCTGTATGAGTAGCAGTGTT