Characterization of RagA and RagB in Porphyromonas gingivalis: study using gene-deletion mutants

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INTRODUCTION

Porphyromonas gingivalis, a Gram-negative, asaccharolytic anaerobe, is a major causative agent in the initiation and progression of periodontal disease (Lamont & Jenkinson, 1998). This organism possesses a variety of virulence factors, including fimbriae and haemagglutinins, as well as strong proteolytic enzymes such as Lys- and Arg-gingipains. The outer membrane of the Gram-negative bacterium directly interacts with other bacteria, host cells and their environment. These interactions must be closely related to growth, colonization, biofilm formation, accomplishment of infection and development of diseases. We have established an isolation method for the outer membrane and identified major outer-membrane proteins designated Pgm1–7 from P. gingivalis strain ATCC 33277T (hereafter referred to as 33277) (Murakami et al., 2002, 2004). Among the major outer-membrane proteins, Pgm1 and Pgm4 were identified as RagA and RagB, respectively, and their peptide fingerprinting patterns and N-terminal or internal amino acid sequences were compared with those of P. gingivalis strain W83, whose complete genome sequence has been published (Nelson et al., 2003). We have also identified major outer-membrane proteins from W83 (Imai et al., 2005). RagA is observed as a 110 kDa protein by SDS-PAGE in both strains, although the mobility of RagB is different, appearing as 47 and 55 kDa proteins in 33277 and W83, respectively.

RagA and RagB were originally identified as immuno-dominant surface antigens recognized by sera from periodontitis patients (Curtis et al., 1991). It has been reported that ragB, which is located adjacent to and downstream of ragA, is co-transcribed with ragA as a

Abbreviations: CAT, chloramphenicol acetyltransferase; CBB, Coomassie brilliant blue; DAB, 3,3′-diaminobenzidine.

The GenBank/EMBL/DDBJ accession number for the P. gingivalis ATCC 33277T ragA, ragB and flanking region sequence is AB205195.
polycistronic message, and that expression of ragAB is influenced by temperature (Hanley et al., 1999; Bonass et al., 2000). RagA has homology to TonB-linked outer-membrane receptors, which are involved in the recognition and active transport of specific external ligands by a wide range of Gram-negative species (Curtis et al., 1999). RagB is predicted to be a lipoprotein from the primary amino acid sequence and is more diverse than RagA among the species, with only short regions of sequence conservation (Hall et al., 2005; Imai et al., 2005). Based on circumstantial evidence, Hanley et al. (1999) claimed that RagA and RagB might form a functionally linked complex on the outer surface of P. gingivalis, involved in a TonB-dependent, active process.

Both 33277 and W83 are widely used for P. gingivalis studies. 33277 has both long and short fimbriae, encoded by fimA and mfa1, respectively (Yoshimura et al., 1984; Hamada et al., 1996), whereas W83 lacks both of these, although it possesses apparently intact fimA (PG2132, as annotated in the P. gingivalis W83 genome database) and mfa1 (PG0178) disrupted with an insertion element (ISpg4). As whole-genome sequencing of 33277 has not yet been carried out, we wanted to analyse the rag locus from 33277.

In this study, we determined the DNA sequence of the ragAB region from 33277 and compared it with that from W83. Then we constructed three mutants from W83 by deleting the ORFs of ragA and/or ragB, and examined the physiological and pathological functions of RagA and RagB. We also present experimental evidence that RagA and RagB, localized on the cell surface, are physically and functionally associated with each other.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are shown in Table 1 (Gardner et al., 1996; Nelson et al., 2003). All P. gingivalis strains were grown at 37°C under anaerobic conditions [10% CO₂, 10% H₂ and 80% N₂] on Brucella HK agar (Kyokuto Pharmaceutical) supplemented with 5% laked rabbit blood, 2.5 µg haemin ml⁻¹, 5 µg menadione ml⁻¹ and 0.1 mg dithiothreitol ml⁻¹ (BHK agar), and in trypticase soy broth (Becton, Dickinson) supplemented with 2.5 mg yeast extract ml⁻¹, 2.5 µg haemin ml⁻¹, 5 µg menadione ml⁻¹ and 0.1 mg dithiothreitol ml⁻¹ (sTSB). For growth experiments, we also used Dulbecco's

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. gingivalis</td>
<td></td>
<td></td>
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<tr>
<td>W83</td>
<td>Parental strain, genome sequenced</td>
<td>Nelson et al. (2003)</td>
</tr>
<tr>
<td>ATCC 33277ᵀ</td>
<td>Wild-type type strain</td>
<td>ATCC</td>
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<tr>
<td>ΔragA</td>
<td>ragA-deletion mutant from W83; Cmᴿ</td>
<td>This study</td>
</tr>
<tr>
<td>ΔragB</td>
<td>ragB-deletion mutant from W83; Cmᴿ</td>
<td>This study</td>
</tr>
<tr>
<td>ΔragAB</td>
<td>ragA and ragB-deletion mutant from W83; Cmᴿ</td>
<td>This study</td>
</tr>
<tr>
<td>ΔragB + ragB₃₃₂₇₇</td>
<td>ΔragB carrying pTCBex₂ :: ragB₃₃₂₇₇</td>
<td>This study</td>
</tr>
<tr>
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<td>ΔragB carrying pTCBex :: ragB₈₈₃</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>Used as chemically competent cells</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S17-1</td>
<td>Used for mobilizing pT-COW to Bacteroides and P. gingivalis via conjugation</td>
<td>Gardner et al. (1996)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
<tr>
<td>pCR-Blunt II-TOPO</td>
<td>A cloning vector, linearized with DNA topoisomerase I bound to the 3’ end of each DNA strand; Kmᴿ</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pKD260</td>
<td>A derivative of pACYC184 with deletion of a 1.1 kb HincII fragment, bearing the cat gene; Cmᴿ</td>
<td>A gift from K. Nakayama†</td>
</tr>
<tr>
<td>pT-COW</td>
<td>Apᴿ and Tcᴿ in E. coli, Tcᴿ in Bacteroides and P. gingivalis; Mobᴿ + Rep +</td>
<td>Gardner et al. (1996)</td>
</tr>
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<td>pTCB</td>
<td>A Tcᴿ gene-deleted pT-COW; Apᴿ in E. coli, Tcᴿ in P. gingivalis</td>
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<td>pTCBex</td>
<td>A pTCB derivative containing a fimR promoter region; Apᴿ in E. coli, Tcᴿ in P. gingivalis</td>
<td>This study; Nishikawa &amp; Yoshimura (2001)</td>
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<td>pTCBex₂</td>
<td>A pTCB derivative containing a rag promoter region; Apᴿ in E. coli, Tcᴿ in P. gingivalis</td>
<td>This study</td>
</tr>
<tr>
<td>pTCBex₂ :: ragB₃₃₂₇₇</td>
<td>A pTCBex derivative containing ragB ORF from 33277; Apᴿ in E. coli, Tcᴿ in P. gingivalis</td>
<td>This study</td>
</tr>
<tr>
<td>pTCBex₂ :: ragB₈₈₃</td>
<td>A pTCBex2 derivative containing ragB ORF from W83; Apᴿ in E. coli, Tcᴿ in P. gingivalis</td>
<td>This study</td>
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</table>

*Cmᴿ, chloramphenicol resistance; Tcᴿ, tetracycline resistance; Apᴿ, ampicillin resistance; Kmᴿ, kanamycin resistance.
†Division of Microbiology and Oral Infection, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences.
modified Eagle’s medium (DMEM) supplemented with 1% (w/v) trypotide (Becton, Dickinson), 1% (w/v) neopeptone (Becton, Dickinson), 1% (w/v) BSA (fraction V; Miles), or 1% (w/v) BSA digested with 0.25% trypsin (Invitrogen) for 10 h at 37°C. *Escherichia coli* strains were cultivated in Luria–Bertani medium supplemented with the necessary antibiotics. Bacterial growth was monitored by measuring the OD_{600}.

**DNA manipulation.** Restriction enzymes were purchased from Takara. Plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen). Genomic DNA was isolated with a Master Pure DNA Purification kit (Epicentre). Standard PCR experiments were performed using a high-fidelity Pyrobest DNA polymerase (Takara) with a PCR Thermal Cycler Dice (Takara). DNA and PCR products were purified with a Freeze ‘N Squeeze DNA Gel Extraction kit (Bio-Rad).

**DNA sequencing.** A purified PCR product and plasmid DNA were usually used as templates for the DNA cycle sequencing with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The products of the DNA cycle sequencing reaction were purified and analyzed using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

**Table 2. Primers used in this study**

<table>
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<tr>
<th>Name/FPF</th>
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<td>AGU01*</td>
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<tr>
<td>AGU02*</td>
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<td>AGU51</td>
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<td>AGU60</td>
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<td>fimRPSpeF</td>
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<td>fimRPBamF</td>
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</tr>
<tr>
<td>ragPBamF</td>
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<td>TTAAGAAACAGGATCATGAAAAATATTTATGTTGGTTG</td>
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<td>AAGATCTTACAAAGCCTTATATGCTCGGAGAGATAATTTATGAGGTG</td>
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<tr>
<td>33277ragBHindR</td>
<td>GAAACATCTAAAGCCTTTATTTTTGATCGCCAGTATTGATAAAC</td>
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</tbody>
</table>

*The same primers were used by Nagano et al. (2005).*
or finR with the ragB ORF from W83 or 33277. To generate restriction sites at the 5’ and 3’ ends of the promoters by PCR, the primers ragPNotF and ragPBamR were used to generate a 482 bp fragment of the rag promoter, and finRPspeF and finRPBamR to generate a 460 bp fragment of the finR promoter (Nishikawa & Yoshimura, 2001) (Table 2). The finR and rag promoters amplified and digested with appropriate restriction enzymes (SpeI/BamHI and NotI/BamHI, respectively) were ligated into pTCB digested with the same enzymes to create pTCBex and pTCBex2, respectively (Table 1).

The ragB ORF of W83 (ragBw83) was amplified with primers W83ragB BamF and W83ragB HindR from the W83 genome. The ragB ORF of 33277 (ragB33277) was amplified with primers 33277ragBBamF and 33277ragBHindR from the 33277 genome. Amplified ragBw83 or ragB33277 was digested with BamHI and HindIII and inserted into pTCBex or pTCBex2 digested with the same enzyme.

The recombinant plasmid vector was introduced into E. coli S17-1 by electroporation. We confirmed that the DNA sequence of the insert had no unintentional base changes after extraction of the plasmid from the transformants. The purified plasmid was then introduced into ΔragB via conjugation under aerobic mating conditions at 37 °C for 12 h and the mating bacteria were spread on BHK agar containing 100 µg gentamicin ml\(^{-1}\) and 1 µg tetracycline ml\(^{-1}\) (Nishikawa & Yoshimura, 2001). After the plates had been incubated anaerobically for about 1 week at 37 °C, the plasmid was extracted again from black-pigmented transformants and verified by PCR and restriction enzyme digestion analysis.

Cell fractionation, SDS-PAGE and Western blotting. Preparation of the bacterial whole-cell lysate and envelope fraction, SDS-PAGE and Western blotting analyses were performed essentially as described previously (Murakami et al., 2002; Nagano et al., 2005). For co-immunoprecipitation, whole-cell lysate was prepared using BugBuster HT protein extraction reagent according to the manufacturer’s instructions (Novagen). The gels were stained with Coomassie brilliant blue R-250 (CBB). As primary antibodies in Western blotting, we used antigen-specific antisera against RagA and RagB obtained from a rabbit immunized with purified RagA protein from 33277 (Murakami et al., 2004) and recombinant RagB constructed on the basis of the W83 sequence (Imai et al., 2005), respectively.

Animal experiments. P. gingivalis strains were grown in sTSB for 24 h until the early stationary phase, harvested and washed twice with 10 mM PBS (pH 7.4). The bacterial concentration (c.f.u. ml\(^{-1}\)) was adjusted with PBS by reference to a standard curve of concentration vs. OD\(_{660}\). The numbers of viable cells prepared were counted against OD\(_{660}\). The reaction was stopped by the addition of 0.1 M glycine. Coupling was carried out at 20 °C for 1 h and terminated by the addition of 1 M ethanolamine/HCl (pH 7.4). After incubation, envelope fractions were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Biotinylation was detected using peroxidase-conjugated streptavidin using 3,3’-diaminobenzidine (DAB). Dextran-labelled proteins were detected by Western blotting using anti-RagA and anti-RagB antibodies developed with a Pro-Q Western blot stain kit (Invitrogen).

Chemical cross-linking. Washed P. gingivalis cells were suspended in 0.1 M triethanolamine/HCl buffer (pH 8.5). The cross-linker disuccinimidyl suberate (11 Å length; Sigma-Aldrich) dissolved in DMSO was added to a final concentration of 50 mM. After incubation of the reaction mixture at 4 °C for 2 h, the cross-linking reaction was stopped by the addition of 0.1 M Tris/HCl buffer (pH 8.0). The envelope fraction was prepared from the cells and analysed by SDS-PAGE and Western blotting using anti-RagA and anti-RagB antibodies developed with DAB.

Cell-surface labelling. P. gingivalis cells suspended in 10 mM PBS (pH 8.0) were labelled with sulfo-NHS-LC-biotin (Pierce) at 4 °C for 2 h. The reaction was stopped by the addition of 0.1 M glycine. Dextran-labelling was carried out by the method of Kamio & Nikaido (1977). Briefly, Dextran T-10 (Pharmacia) activated with CNBr was added to P. gingivalis cells suspended in 0.1 mM sodium carbonate buffer (pH 8.5). Coupling was carried out at 20 °C for 1 h and terminated by the addition of 1 M ethanolamine/HCl (pH 7.4). After labelling, envelope fractions were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Biotinylation was detected using peroxidase-conjugated streptavidin using 3,3’-diaminobenzidine (DAB). Dextran-labelled proteins were detected by Western blotting using anti-RagA and anti-RagB antibodies developed with a Pro-Q Western blot stain kit (Invitrogen).

RESULTS AND DISCUSSION

Comparison of RagA and RagB in W83 and 33277

ragA, ragB and their flanking regions in 33277 were sequenced. The amino acid sequences deduced from the nucleotide sequences of W83 and 33277 were aligned using CLUSTAL W (Thompson et al., 1994) as shown in Fig. 1. For RagA, a sequence identity of 70 % and a sequence similarity of 81 % were found between the two strains, and the N- and C-terminal domains were almost identical. The regions boxed in RagA in Fig. 1 are conserved in TonB-linked proteins (Hanley et al., 1999). These regions were completely or highly conserved between the strains. In contrast, RagB in W83 and 33277 showed only 48 % identity and 63 % similarity over the whole region. When

minocycline and norfloxacin (Sigma-Aldrich). MICs were evaluated by an agar double-dilution assay, as recommended by the NCCLS (1997). Briefly, serial dilutions of the corresponding antibiotics were added to BHK agar. After the bacteria had been grown to the early stationary phase in sTSB, 2 µl bacterial culture was spotted on the antibiotic-containing agar. After 2 days of anaerobic incubation, the susceptibility breakpoints were determined.

Electron microscopy. P. gingivalis cells were grown to the early stationary phase in sTSB and directly spotted onto carbon-coated grids. After negative staining with 2 % (w/v) uranyl acetate, the cells were observed using a JEM-1210 electron microscope (JEOL).

Characterization of RagA and RagB in P. gingivalis

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the nucleotide sequences of W83 and 32727 were also compared, ragA showed a higher sequence identity (72%) than ragB (59%). The ragB locus including ragA was found only in 17 of the 132 P. gingivalis strains and was present mostly in virulent

Fig. 1. Alignment of the RagA and RagB sequences of P. gingivalis W83 (W83) and ATCC 33277T (327) by CLUSTAL W. In RagA, TonB Box I is identified as the signal peptidase cleavage site. In RagB, the predicted signal peptidase II cleavage site is shown by a box. The conserved hexapeptide motif and the conserved TonB C-terminus are shown by asterisks and two dots, respectively. Identical, strongly conserved and weakly conserved residues are indicated by two dots, one dot and a dash, respectively. The arrow in RagA indicates the signal peptidase cleavage site at the threonine residue as the N-terminus, identified by the N-terminal sequence (Murakami et al., 2002). The arrowhead in RagB indicates the predicted signal peptidase II cleavage site at the cysteine residue as the N-terminus. Asterisks, two dots and one dot indicate identical, strongly conserved and weakly conserved residues, respectively, between the two sequences of the strains.
Construction of deletion mutants

Three deletion mutants from W83 were constructed. In these ORFs, ragA and/or ragB were deleted and replaced by cat using the PCR-based overlap-extension method. The overall design for construction of the mutants is shown in Fig. 2(a). To confirm the replacement of target ORFs by cat, the cloning sites were amplified from flanking regions by PCR using each mutant chromosome as a template with primers of AGU59 and AGU60 designed to anneal outside the cloning sites as shown in Fig. 2(b). Amplified PCR products were then digested with selected restriction enzymes to verify the genotypes of the putative mutants. As shown in Fig. 2(c), gel electrophoresis of digested DNA yielded the predicted fragments. Thus three mutants of W83 with deletion of ragA (ΔragA), ragB (ΔragB) or both (ΔragAB) were obtained.

Complementation of RagB

Complementation analysis was performed with shuttle plasmid pT-COW derivatives incorporating the ragA and fimR promoters, presumed to be strong and weak promoters, respectively (Gardner et al., 1996; Nishikawa & Yoshimura, 2001). Although we tried to construct a series of ΔragB complemented with ragB, namely ragB from W83 (ragB\textsubscript{W83}) or ragB from 33277 (ragB\textsubscript{33277}) under the rag promoter and ragB\textsubscript{W83} or ragB\textsubscript{33277} under the fimR promoter, we obtained only two strains: ragB\textsubscript{W83} downstream of the rag promoter (ΔragA + ΔragB\textsubscript{W83}) and ragB\textsubscript{33277} downstream of the fimR promoter (ΔragB + ΔragB\textsubscript{33277}) (Table 1). Although further research is required to determine the reason for this, various explanations include the possibility that: (i) RagB\textsubscript{33277} may not be compatible with RagB\textsubscript{W83} because of their low homology (Hall et al., 2005); and (ii) full expression of RagB\textsubscript{33277} via the rag (strong and authentic) promoter in ΔragB in the presence of RagA\textsubscript{W83} may cause cell viability to deteriorate, resulting in failure to establish the complementation strain carrying pTCEBex2::ragB\textsubscript{33277}. Also, insufficient expression of RagB\textsubscript{W83} via the fimR

Fig. 2. Construction of ragA and ragB deletion mutants from P. gingivalis W83. (a) Gene arrangement in the chromosome, procedure for construction of allele-exchange gene-deletion and the location of primers. (b) Restriction sites and predicted lengths of the DNA regions in each genotype. The sizes (bp) of the DNA regions are indicated. (c) Verification of the mutants by gel electrophoresis of PCR products. After amplification by PCR with primers AGU59 and AGU60 shown at the top of (b) as 59 and 60, respectively, the PCR products were digested with restriction enzymes described below and subjected to electrophoresis. ragA, dotted arrows; ragB, diagonal-line arrows; cat, shaded arrows; overlap regions on set, wavy lines. Underlined numbers indicate simplified primer designation (see Table 2 for details). U, Undigested; E, digested with EcoRI; A, digested with AciI; P, digested with PshBl. DNA size marker bands are indicated (kb).

strains, but not in 381 and 33277 (Hanley et al., 1999; Frandsen et al., 2001). However, we have reported that 33277 expresses both RagA and RagB in the outer membrane, based on peptide mapping and internal sequence analysis (Murakami et al., 2002, 2004) and Western immunoblotting (Imai et al., 2005). In this study, we verified by sequencing that the genes ragA and ragB were present in the 33277 chromosome. More recently, Hall et al. (2005) reported sequence diversity and antigenic variation at the rag locus, which was classified into four alleles. The rag locus was in the same genomic location; however, there was some polymorphism. W83 and 33277 represented rag-1 and rag-4, respectively, in the report. The ragAB nucleotide sequence from 33277 in our study (GenBank accession no. AB205195) was identical to that derived by Hall et al. (2005) (GenBank accession no. AY842852). The degree of similarity between RagB variants (43–56 % identity at the protein level and 53–62 % identity at the nucleotide level) would not have been detected by Southern blotting under high-stringency conditions (Frandsen et al., 2001).
(weak) promoter may make cells unstable in the presence of sufficient RagA_W83 in ΔragB as shown in the following section. We did not attempt to complement ragA into ΔragA because of the lower viability during experiments with the mutant (see section on physical and morphological characteristics later).

**SDS-PAGE and Western blot analyses of envelope fractions**

Envelope fractions denatured in SDS with 2-mercaptoethanol were subjected to SDS-PAGE and visualized with CBB (Fig. 3a). The 110 kDa band of RagA was clearly observed at high intensity in 33277 and W83, and at low intensity in ΔragB, whereas such bands were not observed in ΔragA or ΔragAB. Complemented ΔragB +ragB_w83 was able to recover the 110 kDa band (RagA) with higher intensity than ΔragB, whereas RagA was recovered in ΔragB +ragB_33277 at a low level similar to ΔragB. RagB bands of high intensity were clearly observed at 47 and 55 kDa in 33277 and W83, respectively, and with low intensity in ΔragB +ragB_w83; however, corresponding bands were not observed in the other strains, ΔragA, ΔragB, ΔragAB and ΔragB +ragB_33277. RagA and RagB could be detected on SDS-PAGE gels even in whole-cell lysates, although their contents were higher in the envelope fraction, indicating that the proteins in the mutants localized on the membrane as in the parent.

To verify these assignments, envelope fractions separated by SDS-PAGE were subjected to Western blot analyses with antigen-specific antisera against RagA (Fig. 3b) and RagB (Fig. 3c). The anti-RagA antibody detected a strong band of 110 kDa in 33277 and W83 and several weaker bands of lower molecular masses, which appeared to be degradation products. In ΔragB, a new strong 60 kDa band was detected, although a weak signal was seen at the 110 kDa position. Such bands were not observed in ΔragA or ΔragAB. ΔragB +ragB_w83 was able to recover a strong 110 kDa band together with several lower-molecular-mass bands, including a faint 60 kDa band, although overall signals appeared to be slightly weaker than those of W83. In contrast, in ΔragB +ragB_33277, the signal intensity of RagA did not increase; the 110 kDa band and several other bands were not recovered to the level of ΔragB +ragB_w83. The anti-RagB antibody detected weak and strong signals in 33277 (47 kDa) and W83 (55 kDa), respectively. As antiserum against the RagB antigen from W83 was used, the signal for RagB from 33277 was weak, indicating an antigenic difference between the two strains (note the two equivalent CBB-stained RagB bands of lanes 1 and 2 in Fig. 3a compared with the two clearly different immunostained bands in the same lanes in Fig. 3c). The migration position of RagB following SDS-PAGE was also clearly different between the two strains. The precise reason is unclear; however, we speculate that the degree of lipid modification might be different in lipoprotein RagB, as the

![Fig. 3. Protein patterns following SDS-PAGE and Western blotting of envelope fractions. SDS-PAGE gels were stained with CBB (a) and subjected to Western blot analysis with anti-RagA (b) and anti-RagB (c) sera using the chromogenic substrate 4-chloro-1-naphthol. Lanes: 1, 33277; 2, W83; 3, ΔragA; 4, ΔragB; 5, ΔragAB; 6, ΔragB supplemented with ragB from W83; 7, ΔragB supplemented with ragB from 33277. Asterisks and double asterisks indicate RagB from 33277 and W83, respectively.](image-url)
calculated molecular mass and isoelectric point were almost the same in the two strains. When the protein motif was analysed by the Motif Scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan), nine possible N-myristoylation sites were detected in 33277, whereas there were four sites in W83. RagB was not detected in ΔragB and ΔragAB, whereas a faint band was detected in ΔragA. The marked decrease in RagB may not be a polar effect in ΔragA as the inserted cat gene was supposed to be a non-polar cassette. Instead, the decrease in RagB may have been caused by a deficiency in RagA due to instability of RagB alone in the outer membrane as discussed later.

As production of RagA was decreased by deletion of RagB as described above, both RagA and RagB may exist as mutually associated structures, and their interaction is probably important in their stabilization in cells. Expression of RagA and RagB in ΔragB + ragB\textsubscript{w83} was almost fully recovered and the degradation product of RagA (60 kDa protein) disappeared. In contrast, recovery of RagA and RagB in ΔragB + ragB\textsubscript{33277} was marginal, although the 60 kDa protein derived from RagA decreased and RagB\textsubscript{33277} was not detected, partially due to the use of an antisera with less specificity for RagB\textsubscript{33277}. This also suggested that RagB\textsubscript{33277} under the fimR promoter was not expressed sufficiently and might not interact with RagA\textsubscript{W83} adequately because of their incompatibility.

Physical and morphological characteristics

ΔragA and ΔragAB tended to lose their viability during experiments. We therefore investigated whether they became more oxygen sensitive than the parental strain W83. However, we did not observe any difference in oxygen sensitivity between them (data not shown). These mutants probably had decreased resistance to physical stresses such as centrifugal force, mixing and pipetting. It is possible that the complete disappearance of RagA existing on the outer membrane in large amounts might destabilize the membrane and make it fragile. Such fragility was not observed in ΔragB, presumably due to the presence of RagA to some degree. We did not observe any morphological changes in the three mutants when they were compared with W83 by negative staining in electron microscopy (data not shown).

Growth in nutrient-rich and synthetic media

An early stationary-phase culture in sTSB, a nutrient-rich medium, was inoculated at a 1 : 20 ratio into several media. In sTSB (Fig. 4a), the three mutants ΔragA, ΔragB and ΔragAB showed growth rates similar to the parental strain W83 and reached the stationary phase within 24 h. The three mutants also showed growth curves similar to the parental strain in DMEM supplemented with 1 % tryptone (Fig. 4b) and 1 % neopeptone (data not shown). In DMEM supplemented with 1 % BSA, the parental strain reached the stationary phase within 36 h, slightly later than in sTSB, and ΔragB reached it even later, whereas ΔragA and ΔragAB grew considerably slower and reached the same level as the parental strain at about 72 h (Fig. 4c). However, in DMEM supplemented with BSA pre-digested with trypsin, ΔragA and ΔragAB showed growth curves similar to the parental strain and ΔragB (Fig. 4d). It has been reported that P. gingivalis utilizes proteins such as serum albumin as nutrition by digestion with gingipains (Grenier et al., 2001). The amounts and activities of gingipains were almost the same among the strains used here (data not shown). Therefore, the mutants, especially

![Fig. 4. Growth curves in various media. An early-stationary-phase culture in sTSB was inoculated at a 1 : 20 ratio into sTSB (a) or into DMEM supplemented with 1 % tryptone (b), 1 % BSA (c) or 1 % pre-digested BSA (d). Growth experiments were repeated at least twice and typical results are shown. ](http://jmm.sgmjournals.org)
Animal experiments

It has been reported that injection of *P. gingivalis* cells results in necrotizing ulcer formation in the abdomen (Kumagai *et al.*, 2000). Although *P. gingivalis* inhabits periodontal tissue, this experimental model is often used to evaluate pathogenicity.

Bacterial cells of W83 (2 × 10⁹ or 1 × 10⁹ c.f.u.) or ΔragB (4 × 10⁹ or 2 × 10⁹ c.f.u.) were injected into five mice each. ΔragA and ΔragAB were not used in animal experiments because they tended to lose their viability during the experimental procedure, as described above. One day after injection, ruffled hair was observed in every mouse. Although body weight fell from 2 to 4 days after injection, it then recovered and began to increase again. All of the animals injected survived for the experimental period of 2 weeks because the cell numbers used were much lower than those used by others (Kumagai *et al.*, 2000). Abdominal ulcers were observed within 2 days in all mice injected with 2 × 10⁹ c.f.u. W83 and in two mice injected with 1 × 10⁹ c.f.u. W83. Ulcer formation was also observed in all mice within 2 days when 4 × 10⁹ c.f.u. ΔragB was injected; however, ulcer size was clearly smaller than that induced by 2 × 10⁹ c.f.u. W83. Only small ulcers were formed in three mice after injection of 2 × 10⁹ c.f.u. ΔragB. These results suggested that ΔragB might be less virulent than the parental W83.

To examine the difference in virulence between the strains quantitatively, bacterial cell numbers in the spleen were also evaluated. As a pilot study, 1 × 10⁹ c.f.u. W83 was injected and bacterial cells in the spleen were counted in three mice every day from days 1 to 7. *P. gingivalis* was detected in all three mice at day 1 and in two of the three mice at day 2. At days 3 to 5, a small number of *P. gingivalis* cells were detected in a mouse with a large lesion. However, at days 6 and 7, *P. gingivalis* was not detected in any mouse. *P. gingivalis* was not detected in mice injected only with PBS as a control. Therefore, we decided to inject 1 × 10⁹ c.f.u. W83, ΔragB or ΔragB + ragBₘ₈₃ to count bacterial cell numbers in the spleen at days 1 and 2. At days 1 and 2, 10⁴–10⁷ c.f.u. W83 per spleen was detected in all four mice. In contrast, much lower cell numbers were detected in two mice and one mouse out of four at days 1 and 2, respectively, and almost no bacteria were detected in five other mice in the case of ΔragB (Fig. 5). Unexpectedly, bacteria were not detected when the complementation strain ΔragB + ragBₘ₈₃ was used. We do not know the exact reasons for this, but there are at least two possible explanations. First, although ΔragB almost recovered to the normal level, RagA was not fully stabilized, as shown in Fig. 3. Secondly, the strain, bearing a recombinant plasmid vector with a large size, might become weak in vivo.

It had been considered that ΔragB or the rag locus is related to virulence, as they are frequently detected in *P. gingivalis* strains isolated from deep periodontal pockets (Curtis *et al.*, 1999; Hanley *et al.*, 1999). However, we have recently reported that both RagA and RagB are widely expressed in various strains, including 33277, which has been considered a non-virulent strain, although RagB has several variations (Imai *et al.*, 2005). The relationship between RagB and periodontal disease is unclear, as a proper animal model for periodontal disease has not yet been developed. However, we showed that ΔragB deletion reduced virulence in mice, suggesting that RagA and RagB are functionally related to survival, growth and expression of virulence of *P. gingivalis in vivo* directly or indirectly. Very recently, Shi *et al.* (2007) reported that both RagA and RagB mutants are less virulent than the wild-type in a similar murine model.

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**Fig. 5.** Viable cell numbers of *P. gingivalis* in spleens of mice inoculated with 1×10⁹ c.f.u. W83 (lanes 1 and 3, open columns) or ΔragB (lanes 2 and 4, closed columns). Spleens were excised at 1 (lanes 1 and 2) and 2 (lanes 3 and 4) days after inoculation and spread on BHK agar after homogenization in sTBSB broth. After anaerobic culture, black colonies that appeared were counted as *P. gingivalis*. Circles and columns indicate values of individuals and means, respectively. Significant differences between the two groups (*P<0.05*) as determined by Student’s *t*-test are indicated by asterisks.
Antimicrobial susceptibility and other characteristics

We also examined whether deletion of RagA and/or RagB influenced MICs to various antibiotics. The MIC of chloramphenicol for the wild-type was 4 μg ml⁻¹, whilst for the mutants it was 64 μg ml⁻¹, presumably due to the strong CAT expression. Strong CAT activity was detected only in mutants where cat had been introduced (data not shown). However, no significant difference in the MICs of other antibiotics was observed between the wild-type and mutants. Moreover, the various antibiotics used were effective against the *P. gingivalis* strains, showing considerably low MICs. It has been reported that *P. gingivalis* is susceptible to most antibiotics, including β-lactams, partially due to the absence of β-lactamase (Andrés et al., 1998; Kleinfelder et al., 1999; Ikeda & Yoshimura, 2002). Thus we concluded that RagA and RagB might not be involved in antimicrobial susceptibility.

In addition, we tested the effects of deletion of RagA and/or RagB on haemagglutinating activity as one of the representative characteristics of *P. gingivalis*; however, no difference from the parental strain was observed, implying that they were unrelated to this characteristic (data not shown).

Cell-surface labelling

To examine localization of RagA and RagB, we used different labelling molecules, namely biotin and dextran. Both RagA and RagB were strongly detected by a streptavidin-linked reagent after biotinylation (Fig. 6b), based on comparison with patterns after amido black staining (Fig. 6a). Sulfo-NHS-LC-biotin has been demonstrated predominantly to label cell-surface proteins of Gram-negative bacterial cells (Bradburne et al., 1993). However, as this reagent is relatively small (*M*ᵣ, 557) and hydrophilic, it may penetrate the outer membrane through diffusion pores such as porins, and outer-membrane proteins might be labelled from the periplasmic space. Therefore, we used macromolecular dextran as another labelling reagent that could not pass through the outer-membrane pores. After CNBr-activated dextran labelling, the intensities of RagA and RagB bands decreased, whereas thick smears appeared at the top of the separation gel (Fig. 7a), indicating that RagA and RagB were shifted up by conjugation with dextran. Western blotting using anti-RagA and anti-RagB antibodies confirmed that large quantities of both RagA and RagB existed in the high-molecular-mass smears after labelling (Fig. 7b, c). Consequently, these results suggested that both RagA and RagB were exposed on the cell surface.

Chemical cross-linking

After chemical cross-linking, both RagA and RagB bands at the appropriate positions almost disappeared; instead, heavily stained, diffuse bands appeared as high molecular masses of more than 200 kDa in CBB-stained gels (Fig. 8a). Western blotting using specific antibodies confirmed that most RagB migrated to much higher positions as high-molecular-mass bands, although RagA behaved in a slightly different manner (Fig. 8b, c). These results suggested that RagA and RagB were in close proximity and physically interacted with each other.

Co-immunoprecipitation

The anti-RagA antibody precipitated RagA and RagB proteins, and the anti-RagB antibody also precipitated both proteins (Fig. 9). Essentially, no other bands were stained. This strongly suggested that RagA and RagB have a physical molecular interaction that permits them to be chemically cross-linked as described above.

Conclusions

This is the first report on RagA and RagB characterized using deletion mutants. We have demonstrated that both *ragA* and *ragB*, tandemly arranged in this order, are required for stable expression of RagA and RagB. When *ragA* was deleted, RagB was expressed only at a negligible level. Deletion of *ragB* led to RagA degradation. We also showed that deletion mutants had retarded bacterial growth in a nutrient-poor synthetic medium and had decreased infective activity. Chemical cross-linking and
co-immunoprecipitation experiments revealed a physical, molecular association of RagA and RagB. Both RagA and RagB were identified as proteins exposed on the cell surface after labelling experiments. In conclusion, RagA and RagB, which stabilize each other, may form functionally associated complexes in the outer membrane of *P. gingivalis*.
Characterization of RagA and RagB in *P. gingivalis*

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Fig. 9. Co-immunoprecipitation of *P. gingivalis* W83 whole-cell lysate with anti-RagA and RagB antibodies. Co-immunoprecipitation was performed using a ProFound Co-Immunoprecipitation kit. Whole-cell lysate prepared with BugBuster HT protein extraction reagent was added to AminoLink Plus Gel coupled with an anti-RagA or anti-RagB antibody. After gentle rocking overnight at 4 °C, the beads were washed three times with Dulbecco’s PBS. Bound proteins were eluted with ImmunoPure IgG Elution Buffer and analysed by SDS-PAGE. The gel was stained with CBB. Lanes: 1, W83 whole-cell lysate as a control; 2, co-immunoprecipitates with anti-RagA; 3, co-immunoprecipitates with anti-RagB.

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