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Sequence Diversity and Antigenic Variation at the *rag* Locus of *Porphyromonas gingivalis*

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The *rag* locus of *Porphyromonas gingivalis* W50 encodes RagA, a predicted *tonB*-dependent receptor protein, and RagB, a lipoprotein that constitutes an immunodominant outer membrane antigen. The low G+C content of the locus, an association with mobility elements, and an apparent restricted distribution in the species suggested that the locus had arisen by horizontal gene transfer. In the present study, we have demonstrated that there are four divergent alleles of the *rag* locus. The original *rag* allele found in W50 was renamed *rag-1*, while three novel alleles, *rag-2* to *rag-4*, were found in isolates lacking *rag-1*. The three novel alleles encoded variants of RagA with 63 to 71% amino acid identity to RagA1 and each other and variants of RagB with 43 to 56% amino acid identity. The RagA/B proteins have homology to numerous *Bacteroides* proteins, including SusC/D, implicated in polysaccharide uptake. Monoclonal and polyclonal antibodies raised against RagB1 of *P. gingivalis* W50 did not cross-react with proteins from isolates carrying different alleles. In a laboratory collection of 168 isolates, 26% carried *rag-1*, 36% carried *rag-2*, 25% carried *rag-3*, and 14% carried *rag-4* (including the type strain, ATCC 33277). Restriction profiles of the locus in different isolates demonstrated polymorphism within each allele, some of which is accounted for by the presence or absence of insertion sequence elements. By reference to a previously published study on virulence in a mouse model (M. L. Laine and A. J. van Winkelhoff, Oral Microbiol. Immunol. 13:322–325, 1998), isolates that caused serious disease in mice were significantly more likely to carry *rag-1* than other *rag* alleles.

Porphyromonas gingivalis is a gram-negative, anaerobic coccobacillus that is strongly associated with destructive periodontal disease. *P. gingivalis* is frequently isolated in high numbers from severely inflamed periodontal pockets in patients with periodontitis; it is isolated less frequently and in lower numbers from periodontally healthy individuals. Molecular typing studies have suggested that strains of some genotypes may be more commonly associated with disease than others (2, 11, 21, 24). Experiments using animal models and in vitro systems have also indicated that certain strains are more pathogenic than others; the significance of fimbrial genotypes and capsular serotypes has been subject to particular attention (9, 17, 25, 31), but neither accounts fully for the differences in pathogenicity between strains. A greater understanding of the factors governing strain variation in pathogenic potential has implications for the development of improved diagnostic tools and therapeutic strategies.

Analysis of the immunoglobulin G serum antibody response of periodontitis patients has led to the identification of three immunodominant surface antigens (115, 55, and 47 kDa) expressed by *P. gingivalis* W50 (8). We have previously cloned the gene *ragB* (receptor antigen gene B), encoding the 55-kDa antigen, and found it to be located immediately downstream of a cotranscribed gene, *ragA*, coding for a putative *tonB*-dependent receptor (12). Several factors led to the conclusion that the *rag* locus has arisen by horizontal gene transfer and may represent a pathogenicity island in *P. gingivalis* W50: the locus has a G+C content of 42%, compared to a mean value of 48% for the complete genome; it is flanked by an insertion sequence (IS*1126*, now known as IS*Pg1*) and a sequence with similarity to mobile elements; PCR and Southern blots indicated that the locus has a restricted distribution within the species; and PCR of subgingival samples suggested an association of the locus with deep periodontal pockets (12). The aim of the present study was to investigate the chromosomal location and extent of polymorphism of the *rag* locus in a diverse collection of isolates of *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Isolates were grown on fastidious anaerobic agar plates with 5% defibrinated horse blood or brain heart infusion (BHI) broth with 5 μg ml^{-1} hemin at 37°C in an anaerobic cabinet (Don Whitley Scientific) with an atmosphere of 80% N_2, 10% H_2, and 10% CO_2.

A total of 168 isolates of *P. gingivalis* from our laboratory collection were investigated, including isolates generously supplied by numerous colleagues. All isolates had been recovered from human sources, mostly from patients with periodontal disease, and were from 15 countries. Isolates were confirmed to be *P. gingivalis* by PCR with species-specific primers to 16S rRNA genes as described previously (3, 12).

DNA manipulations. Total genomic DNA was isolated from stationary-phase bacteria using a Puregene DNA isolation kit reagent (Flowgen) according to the manufacturer's instructions. Briefly, 1 ml of brain heart infusion cultures grown overnight were lysed with 600 μl cell lysis solution at 80°C for 5 min. Cells were treated with 3 μl RNase A solution at 37°C for 1 h. Protein was removed by precipitation with guanidine thiocyanate, and the DNA was precipitated and

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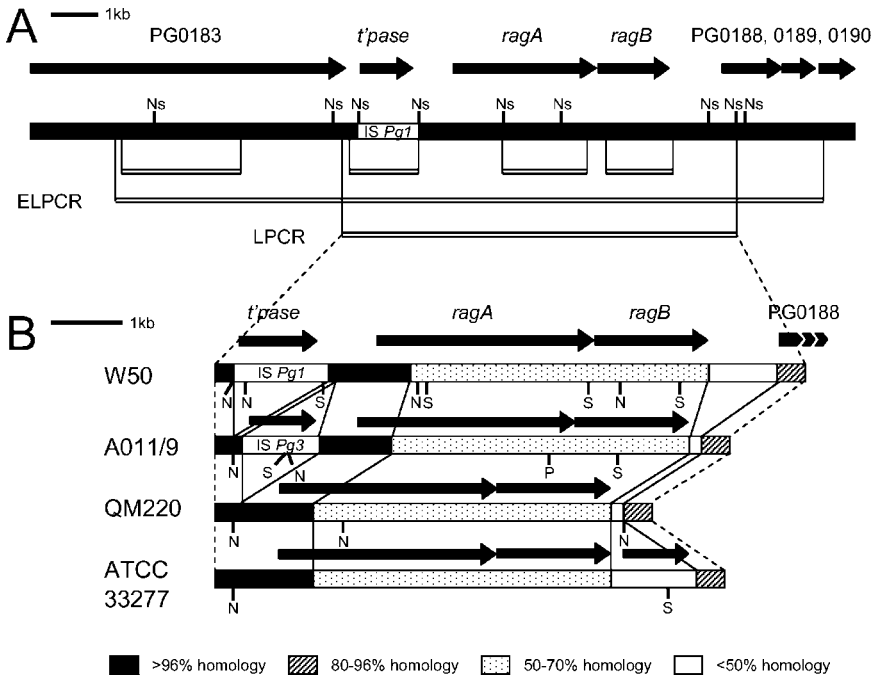

FIG. 1. (A) Map of the *rag* locus from the genome sequence of *P. gingivalis* W83 (26). Arrows represent open reading frames. Vertical bars joined by double lines represent the positions of PCR primers and the corresponding PCR products. NspI sites located within the ELPCR product are indicated by Ns. (B) Map of the LPCR product in four different isolates. Shading of the bars represents the degree of homology between different isolates, as shown in the key. Arrows represent open reading frames. NcoI (N), SacI (S), and SphI (P) sites are indicated.

cleaned with successive washes of 100% isopropanol and 70% ethanol. Purified DNA was resuspended in 50 μl TE (10 mM Tris, 1 mM EDTA, pH 8.0).

DNA purification to remove primers, enzymes, and other reagents was undertaken using a QIAgen gel extraction kit. Briefly, DNA was bound to a silicon-based ion-exchange matrix under high-salt conditions in Qiagen buffer PB. The DNA was cleaned with two washes of Qiagen buffer PE and eluted in 50 μl TE.

Restriction digestion of genomic DNA and PCR products was performed using Amersham Pharmacia or New England Biolabs enzymes and buffers. Reaction mixtures were incubated at 37°C for at least 2 h. DNA electrophoresis was performed in 0.8% agarose with Tris-borate-EDTA (0.09 M Tris-borate, 0.002 M EDTA). Ethidium bromide-stained gels were viewed under UV light, and the image was captured with a Syngene Imager. Southern hybridizations were performed by standard methods, with DNA immobilized on HyBond N+ (Amersham Pharmacia). PCR products used as probes were labeled with digoxigenin and detected by color precipitate using a DIG labeling and hybridization kit (Roche). Hybridization was at 65°C, and final washes were in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 15 min at 65°C.

PCR. All amplification reactions were performed by using an Omnigene Life Sciences International thermal cycler. Standard PCRs used 50-μl volumes with 0.5 μg each primer and 0.5 μl chromosomal DNA template in ABgene PCR Reddy Load Master Mix (ABgene) with 1.5 mM $MgCl_2$. Amplification of longer products was performed with ABgene Extensor Long Master Mix 2 (ABgene).

The following primers (with positions of primers and products indicated in Fig. 1) were used: PG0183F (forward) (CAA CTA CCT TCA CTT CCC), PG0183R (reverse) (AAC CTT CAA TCT CCT CCA C), ISPg1F (GGC TCC CCC TTG ATT TTT CTC), ISPg1R (AAG GAG GTG GAG GGA AGA GGA) W83 ragAF (TGT CTG GTG CGA TAG CGA ATA G), W83 ragAR (TAC ATA GGT GAG TTT GAG ATT C), W83 ragBF (AAT ACT GAA AAT CCA CGA), W83 ragBR (TAG GGG CTG CGA CAA AAA), ELPCRF (TAT CGT TTC GCC CAG CCA AGA), ELPCRR (TCC TTC GCA TCG CTC TTT CCC), LPCRF (CAA AGT CCT GCC ACG AGT AGC), and LPCRR (CGT TTT CTC GCC ACT TTC GTC). Primers designed to be specific to *ragB* from each of the four *rag* alleles were rag1F (CGC GAC CCC GAA GGA AAA GAT T) and rag1R (CAC GGC TCA CAT AAA GAA CGC T) (positions 70 to 697 of *ragB1*), rag2F (GCT TTG CCG CTT GTG ACT TGG) and rag2R (CCA CCG TCA CCG TTC ACC TTG) (positions 56 to 1034 of *ragB2*), rag3F (CCG GAA GAT AAG GCC AAG AAA GA) and rag3R (ACG CCA ATT CGC CAA AGC T) (positions 76 to 498 of *ragB3*), and rag4F (CCG GAT GGA AGT GAT GAA CAG A) and rag4R (CGC GGT AAA CCT CAG CAA ATT) (positions 82 to 820 of *ragB4*).

Standard programs for PCR comprised 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50°C, and extension for 1 to 3 min at 72°C. The annealing temperature was changed to 55°C for *ragA* primers and 60°C for IS*Pg1* primers. For extra-long PCR (ELPCR), the annealing temperature was 60°C, the extension time was 20 min, and 25 cycles were performed. For long PCR (LPCR), 10 cycles with annealing at 52°C and an extension time of 6 min 40 s at 68°C were followed by 15 cycles with annealing at 60°C and a 7-min extension step at 68°C.

Pulsed-field gel electrophoresis (PFGE). Bacterial growth from 4-day-old cultures grown on fastidious anaerobic agar was washed and resuspended in 0.5 ml of PIV buffer (10 mM Tris [pH 7.6], 1 M NaCl). The cell suspension was added to 0.5 ml molten 2% CHEF agarose (Bio-Rad) at 50°C, mixed, and gently pipetted into a CHEF plug mold (Bio-Rad). Plugs were incubated at 37°C in 2 ml lysis buffer (6 mM Tris [pH 6.7], 1 M NaCl, 100 mM EDTA, 0.5% Brij 58 solution, 0.2% deoxycholate, 0.5% *N*-lauroylsarcocine, 20 μg/ml RNase A, 100 μg/ml lysozyme) for 2 h. Plugs were washed in 2 ml TE (CHEF) (10 mM Tris [pH 7.5], 0.1 mM EDTA) at 50°C for 5 min and then incubated in 2 ml ESP buffer (0.5 M EDTA [pH 9 to 9.5], 1% *N*-lauroylsarcosine, 50 μg/ml proteinase K) at 50°C for 2 h and then washed twice in 2 ml TE (CHEF) at 37°C for 30 min. Plugs were stored in TE at 4°C in the dark. For restriction, plug slices approximately 1 mm thick were taken from three separate blocks and placed into 1.5-ml microcentrifuge tubes and equilibrated in restriction buffer, and then DNA was digested overnight at 37°C in 200 μl buffer plus XbaI. Electrophoresis was performed with a CHEF DRII system (Bio-Rad) for 19 h at 6 V cm^{-1}, with a switch time of 1 to 30 s and with a 1% agarose gel in 0.5× Tris-borate-EDTA buffer at 15°C.

Antiserum production and Western blotting. Preparation of extracts, electrophoresis on 10% polyacrylamide gels, and detection of the 55-kDa outer membrane antigen RagB with monoclonal antibodies B15 and E38 were performed as previously described (20). Polyclonal antiserum to recombinant W50 RagB, synthesized as previously described (29), was produced in rabbits. Pooled dialyzed fractions of recombinant protein in phosphate-buffered saline (0.7 mg/ml) were incubated with a fresh precipitate of alum. The precipitate was washed and resuspended in saline for injection. Primary immunization was with 175 μg protein subcutaneously over four sites. Two more immunizations of 100 μg were

TABLE 1. PFGE types and detection of genes for 38 isolates of *P. gingivalis*

PFGE type	Detection of *rag* genes[a]	Detection of PG0183[b]	Isolate(s)
A	+	+	W50, W83, PGF112, OMZ308, HG66, 73.3.3, 73.3.2, 34.2.3, 32.2.4, 13JC, 501
B	+	+	15.41, PGF140
C	+	+	10.72
D	+	+	LB13D3
E	+	+	SUDAN93
F	−	+	A007/5, A009/1, A009/4, A009/6, A009/8, A009/9, A009/10, A010/4, A010/9, A010/10, A011/9
G	−	+	N107/9
H	+	+	AHN9292
I	+	+	A7436
J	−	−	381, G102, ATCC 33277^T, NCTC11834^T
K	+	+	ROMANIA93
L	+	+	ATCC 49417
M	−	+	YH522[c]
ND[d]	−	−	23A4

[a] Presence of W50-type *ragA* and *ragB* was determined by PCR and Southern hybridization.
[b] Presence of PG0183 was determined by PCR.
[c] A faint signal of hybridization to W50 *ragA* was detected in YH522.
[d] Not determined, as DNA consistently degraded.

given 3 weeks apart. Blood was withdrawn and separated for antiserum after an additional 3 weeks.

DNA sequence determination. Long PCR products from A011/9, QM220, and ATCC 33277^T (strain obtained as NCTC11834^T) were purified and sent to MWG-Biotech for sequence determination using their publication-grade sequencing service. Open reading frames were identified with FramePlot 2.3.2 online software (13).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this study have been assigned GenBank accession numbers AY842852 (NCTC11834^T), AY842853 (QM220), and AY842854 (A011/9).

RESULTS AND DISCUSSION

Detection of *rag* genes and genomic location in diverse isolates. We previously proposed that *P. gingivalis* W50 had acquired the *rag* locus by horizontal gene transfer (12), raising the possibility that the site of insertion could differ between isolates. We had also demonstrated that the *rag* locus of W50 was not present in all isolates. In order to identify isolates that were genetically diverse for further investigation of the locus, we first typed 42 isolates originating from several countries. PFGE identified 13 different types (A to M) among 37 isolates (Table 1). Four types were shared by two or more isolates, while nine were unique. Five isolates consistently failed to yield readable band patterns; 23A4 was retained for further investigation, as this is a well-characterized isolate, but the other four untyped isolates were not studied further.

The presence of genes corresponding to *ragA* and *ragB* of W83/W50 was investigated by PCR using primers located as illustrated in Fig. 1. In 20 isolates of nine PFGE types, both genes were detected, while in 18 isolates (including the type strain) of four PFGE types or that were untyped, *ragA* and *ragB* PCR failed to yield a product (Table 1). Southern hybridization of chromosomal DNA with probes comprising PCR products from W50 *ragA* and *ragB* (Fig. 1) confirmed the PCR results in all cases. PCR with primers in the gene upstream of the *rag* locus in W83, PG0183 (separated from *ragA* by IS*Pg1* [Fig. 1]), was also negative in 5 of the 18 *rag*-negative isolates. In all cases, identical PCR results were obtained for isolates having the same PFGE type (Table 1).

Western blots were performed on representative isolates of *rag*-positive PFGE types A, B, C, D, and I and *rag*-negative PFGE types F, G, and J plus untyped isolate 23A4 by using monoclonal antibodies (MAbs) specific to RagB (MAb B15 and MAb E38). The 55-kDa RagB was detected in all *rag*-positive isolates and was absent in all *rag*-negative isolates.

Publication of the complete genome sequence of *P. gingivalis* W83 allowed examination of the region surrounding the locus (26). PCR was performed on representatives of all PFGE types with primers from sequences in PG0183, upstream of the *rag* locus in the sequenced isolate W83, and PG0190 (*uppS*), downstream of the locus (ELPCR in Fig. 1). A PCR product of similar length (13.5 to 15 kb, established by adding the sizes of fragments after digestion with BglII and NspI) was obtained with isolates of the nine *rag*-positive PFGE types and a *ragB* probe hybridized to a 2.5-kb BclI fragment from the PCR product in all cases. This indicated that the *rag* locus was in the same genomic location (i.e., flanked by the same genes) in diverse isolates. Variations in the length of the long PCR product and in the sizes of restriction fragments suggested that there was nevertheless some polymorphism between isolates in this region.

Polymorphism among *rag*-positive isolates. Polymorphism in the *rag* locus of isolates representing the nine *rag*-positive PFGE types was investigated further. Long PCR products spanning the *rag* locus (ELPCR, PG0183 to PG0190), as described above, were digested with NspI and BglII. Fragments were identified by size comparison to those of W83 and by hybridization with probes from *ragA*, *ragB*, and a fragment overlapping IS*Pg1*. All isolates with PFGE types other than type A had a deletion of 1.3 kb between PG0183 and *ragA*, relative to W50 and W83. The restriction fragments obtained were compatible with the absence of IS*Pg1* in these isolates. Isolate LB13D3 had an insertion of approximately 1 kb in the intergenic region between *ragB* and PG0188, and a number of restriction sites were absent in individual isolates, suggesting a degree of sequence polymorphism in the locus.

The locus in *rag*-negative isolates. The same long PCR primers (ELPCR) were used to amplify DNA from isolates in which *ragA* and *ragB* gave negative results but PG0183 PCR was positive. In the absence of *ragA* and *ragB*, a PCR product of no more than 10 kb would be predicted; unexpectedly, products of

TABLE 2. Restriction profiles of *rag* locus long PCR digested with NcoI, SacI, and SphI

Restriction profile[a]	*rag* allele[b]	No. of isolates	Isolate from which *rag* locus sequence was determined	Country of origin[c]
1a	*rag-1*	25	W50[d], W83[d]	France, Germany, The Netherlands, UK
1b	*rag-1*	14		Kenya, Germany, The Netherlands, Romania, Sweden, UK, US
1c	*rag-1*	2		The Netherlands, UK
1d	*rag-1*	1		Sudan
1e	*rag-1*	1		Finland
2a	*rag-2*	14	A011/9[e]	Thailand
2b	*rag-2*	43		Finland, The Netherlands, Sweden, UK
2c	*rag-2*	1		Belgium
2d	*rag-2*	1		France
2e	*rag-2*	1		UK
3a	*rag-3*	40	QM220[e]	Finland, Germany, Japan, The Netherlands, Sweden, Thailand, UK, US
3b	*rag-3*	2		The Netherlands
4a	*rag-4*	14	ATCC 33277^{T}[e]	Canada, Sweden, UK, US
4b	*rag-4*	4		UK
4c	*rag-4*	3		The Netherlands, UK
ND	*rag-3*	2		Switzerland
Total		168		

[a] Isolates with the same number and different letter differ by 1 to 3 bands, consistent with a difference due to a single genetic event.
[b] *rag* allele determined by PCR.
[c] Country from which isolates were sent. UK, United Kingdom; US, United States.
[d] Sequence determined previously (12, 26).
[e] Sequence determined in this study.

12.6 to 14 kb were obtained. A simple insertion/deletion event was therefore unlikely to account for the difference between these *rag*-negative isolates and *rag*-positive isolates. Southern hybridization of digested ELPCR products with W50 *ragA* and *ragB* probes again confirmed the absence of sequences with homology to these genes, except for isolate YH522, in which a weak but distinct hybridization signal to a 6.6-kb BglII fragment was detected with the W50 *ragA* probe.

An alternative primer pair was designed to amplify the *rag* locus with less additional flanking sequence by using primers closer to the 3′ end of PG0183 and, in PG0188, the gene immediately downstream of *ragB* (LPCR in Fig. 1). These should result in an 8.35-kb product in W83. Amplification with the alternative primer pair yielded fragments of 6 to 8.5 kb in isolates representative of all PFGE types, including those that had been negative for PG0183 by PCR. Again, this suggested that the locus had been replaced rather than deleted in isolates that both PCR and Southern blotting had indicated did not contain W50 *ragA* and *ragB*. It also suggested that the failure to detect PG0183 by PCR in some isolates might be due to local sequence polymorphism rather than absence of the gene.

Novel variants of the *rag* locus. Digestion of LPCR products spanning the region between PG0183 and PG0188 in *rag*-negative isolates yielded restriction fragments that were of very different sizes from *rag*-positive isolates. To determine the extent of polymorphism in this region, a much larger collection of isolates was screened by digestion of the LPCR product with NcoI, SacI, and SphI. From a total of 168 isolates, 15 different restriction profiles were obtained; for two isolates, no specific amplification product could be obtained. The profiles fell into four main groups on the basis of common restriction fragments, designated 1 to 4; within each group, profiles differed by up to three bands, consistent with a single polymorphism, and were designated with a lowercase letter (1a, 1b, etc.) (Table 2). The profiles most frequently observed were 2b (43 isolates), 3a (40 isolates), 1a (25 isolates), and 1b, 2a, and 4a (14 isolates each) (Fig. 2). W83 and W50 both had profile 1a; profile 1b was highly related and was observed in isolates for which the *rag* locus had already been shown to differ from W50 only in the absence of IS*Pg1*.

The LPCR product extending from the 3′ end of PG0183 to the 5′ end of PG0188 was sequenced in isolates A011/9 (profile 2a), QM220 (profile 3a), and ATCC 33277^{T} (profile 4a) in order to find out what genes had replaced *ragA* and *ragB* of W50/W83. Analysis of open reading frames revealed that each of the three isolates carried different novel genes with homology to *ragA* and *ragB* of W50/W83. Sites of PCR primers used to detect *ragA* and *ragB* were absent or divergent in each iso-

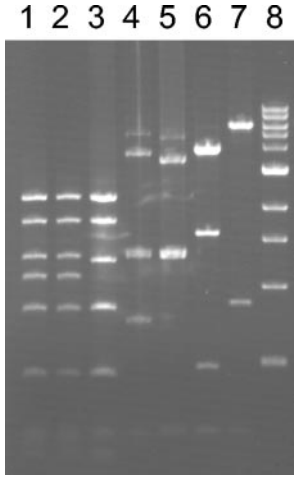

FIG. 2. LPCR products from different isolates digested with NcoI, SacI, and SphI. Lanes: 1, W50 (restriction profile 1a); 2, 61.3.3 (1a); 3, 15-041 (1b); 4, A011/9 (2a); 5, 01-013 (2b); 6, QM220 (3a); 7, NCTC 11834^{T} (4a); 8, 1-kb ladder.

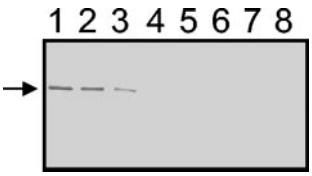

FIG. 3. Western blots of *P. gingivalis* whole-cell proteins from different isolates probed with polyclonal anti-RagB1 antiserum. Lanes: 1, W83 (RagB1); 2, W50 (RagB1); 3, A7436 (RagB1); 4, A011/9 (RagB2); 5, 15.17 (RagB4); 6, 12.08 (RagB2); 7, N107/9 (RagB3); 8, 381 (RagB4). Arrows mark the position of RagB at 55 kDa.

late, and the regions corresponding to probes were less than 60% identical to the corresponding genes in W50/W83, accounting for the failure to detect *rag* genes in these isolates.

Between PG0183 and *ragA* in A011/9 was a gene with 98% nucleotide homology to the transposase of IS*Pg3* (formerly IS*195*), an insertion sequence found previously in *P. gingivalis* (18). Examination of the flanking sequences identified a 10-bp direct repeat element (TTACATCACA), possibly a target for insertion. On the basis of restriction analysis described above, it could be deduced that isolates with LPCR restriction profile 2b carried the same *rag* locus as A011/9 but lacked IS*Pg3*.

In ATCC 33277^T, an additional open reading frame was found downstream of *ragB*. The protein encoded had no significant homology to known proteins, except for a C-terminal 80-amino-acid sequence with 50% similarity to the C-terminal domain shared by gingipain precursor proteins and hemagglutinins of *P. gingivalis* (7).

To distinguish the different *rag* loci, the following designations were assigned: *rag-1*, representing the locus found in W50 and W83; *rag-2*, representing the locus of A011/9; *rag-3*, representing the locus of QM220; and *rag-4*, representing the locus of ATCC 33277^T. PCR primers were designed to specifically amplify *ragB* of each of the four *rag* loci and distinguish between them (further details of this PCR assay and its use in clinical material will be described in a separate publication). When applied to the full collection of isolates, PCR confirmed the groups assigned by LPCR restriction profile (Table 2); *rag-3* was detected in the two isolates for which LPCR was unsuccessful.

Antigenic variation and expression. Western blots of isolates with different PFGE types had already demonstrated that two monoclonal antibodies recognizing RagB1 from W50 did not cross-react with proteins from isolates lacking the *rag-1* locus. To further investigate antigenic cross-reactivity between the different variants of RagB, polyclonal antiserum was raised against recombinant RagB1 from W50. Western blots with representative isolates carrying the four alleles of the *rag* locus demonstrated that neither the monoclonal antibodies nor the polyclonal antiserum to RagB1 were able to recognize RagB2, RagB3, or RagB4 (Fig. 3).

In a previous study, we had investigated outer membrane proteins in strains W50, W83 (both *rag-1*), and NCTC11834^T (*rag-4*) (8). Retrospective examination of the published gel images demonstrates that while W50 and W83 both produced a prominent band at 55 kDa, which later proved to represent RagB, at least one protein of similar molecular weight but which was apparently less abundant was also expressed by NCTC11834^T. In the absence of a specific antibody to Rag4, it was not possible to confirm the identity of this protein. However, Murakami et al. recently determined by N-terminal sequencing that both RagA and a variant of RagB that can now be confirmed to be RagB4 are expressed in ATCC 33277^T (*rag-4*), where they represent two of the seven major outer membrane proteins (22, 23).

Sequence comparisons of the four *rag* alleles. Multiple alignment of the four RagA proteins using ClustalW (32) demonstrated a high level of sequence homology at the N terminus of the proteins, with only one amino acid polymorphism in the first 150 residues (Fig. 4). Through the rest of the protein, there were local regions of high homology interspersed with regions with little sequence conservation. The RagB proteins were more diverse, with only short regions of sequence conservation (Fig. 5). Alignment of the DNA sequences revealed that the region between PG0183 and *ragA* was highly conserved between isolates, apart from the presence of insertion sequences in W50/W83 and A011/9. Only 29 (3.1%) of 939 nucleotides in this intergenic region were found to be polymorphic. Conversely, there was no significant sequence homology detected between any of the isolates in the intergenic region between *ragB* and PG0188, apart from the last 46 nucleotides upstream of PG0188 (Fig. 1).

All four variants of RagA and RagB were submitted to the NCBI Conserved Domain Search and BLAST analysis (1, 19). Homology to the N-terminal region of conserved domains "ligand_gated_channel, TonB dependent (cd01347)," "TonB_dep_rec, TonB dependent receptor (pfam00593)," "FepA, outer membrane receptor for ferrienterochelin and colicins (COG 4771)," "CirA, outer membrane receptor proteins, mostly Fe transport (COG1629)," and "BtuB, outer membrane cobalamin receptor protein" was detected from approximately amino acids 100 to 250 of all RagA variants. Further homology to "ligand_gated_channel, TonB_dep_rec" and CirA was also detected over the less conserved part of the protein, especially amino acids 500 to 800 of all variants. No conserved domains were detected in the RagB proteins.

All RagA variants demonstrated extensive homology to members of the large families of putative outer membrane proteins (over 50 in each) from the complete genome sequences of *Bacteroides thetaiotaomicron* (35) and *Bacteroides fragilis* (15). Many of these outer membrane protein genes have a putative lipoprotein gene immediately downstream, in an arrangement analogous to the *rag* locus, and RagB in turn had significant homology to some of these lipoproteins. For all Rag variants, the homology to other *P. gingivalis* Rag protein variants was always much greater than the homology to the most closely related *Bacteroides* protein (Tables 3 and 4); the same *B. thetaiotaomicron* locus ranked as the highest score for each of the RagA and RagB variants.

The most similar protein to RagA with an experimentally proven function was SusC (27), involved in starch uptake in *B. thetaiotaomicron*, as noted previously (12) (Tables 3 and 4). SusD, a lipoprotein functionally associated with SusC and encoded immediately downstream (28) had significant similarity to RagB2 over a short region, but similarity to the other RagB variants was scored as nonsignificant by standard BLASTP analysis. By analogy to SusC/D, the families of related outer membrane proteins and lipoproteins in both *Bacteroides* species have been interpreted as systems for the binding and up-

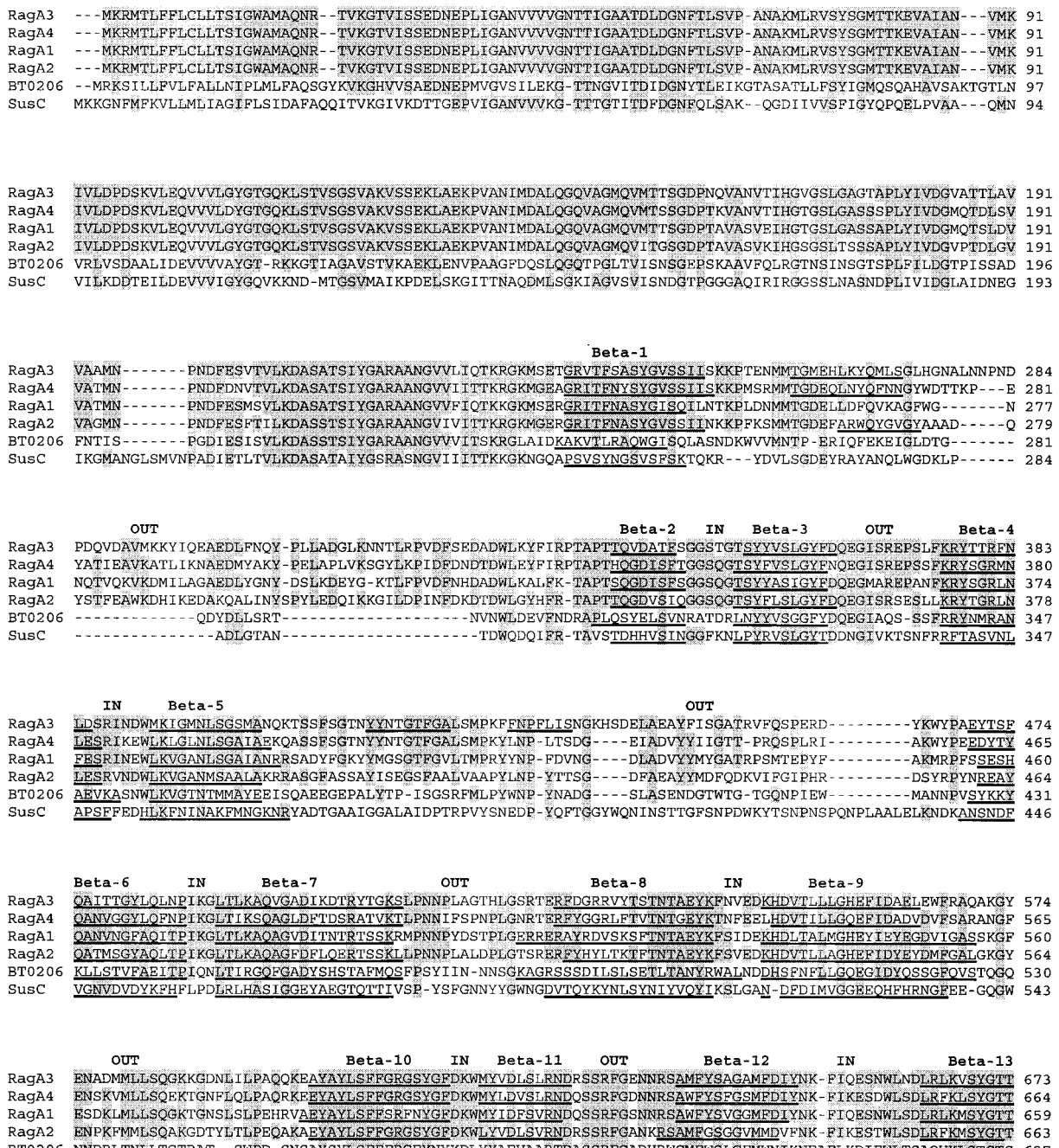

FIG. 4. Sequences of RagA1-4 aligned with BT0206 and SusC from *B. thetaiotaomicron* using ClustalW (32). Residues identical in at least three proteins are highlighted. Regions of at least 7 amino acids predicted by 3D-PSSM (14) to form β-strands are underlined. Conserved β-strands likely to form the 22-strand β-barrel are numbered, and the orientation of the linking loops relative to the outer membrane is designated IN (i.e., on the periplasmic surface) or OUT (i.e., on the outer surface) by analogy with the structure of FepA (5).

take of a wide range of polysaccharides from their environment in the large intestine (15, 35). Circumstantial support for this role comes from the finding of *susCD*/*ragAB*-related genes on a plasmid required for agar degradation by a marine bacterium identified as a *Microscilla* species (36). The occurrence of such a function in *P. gingivalis* is surprising given that this species is asaccharolytic, requiring protein as a carbon source.

The RagA sequences were submitted to the web-based program for protein fold recognition, 3D-PSSM (14). In accordance with the results of the Conserved Domain Search, all four RagA variants had the greatest structural homology (>95% confidence level) to the TonB-dependent outer membrane transporter proteins FepA, FhuA, and BtuB from *Escherichia coli*. FepA, FhuA, and BtuB all form 22-stranded antiparallel β-barrels, with an N-terminal domain that forms a plug within the barrel and interacts with TonB (5, 6, 10). Alignment

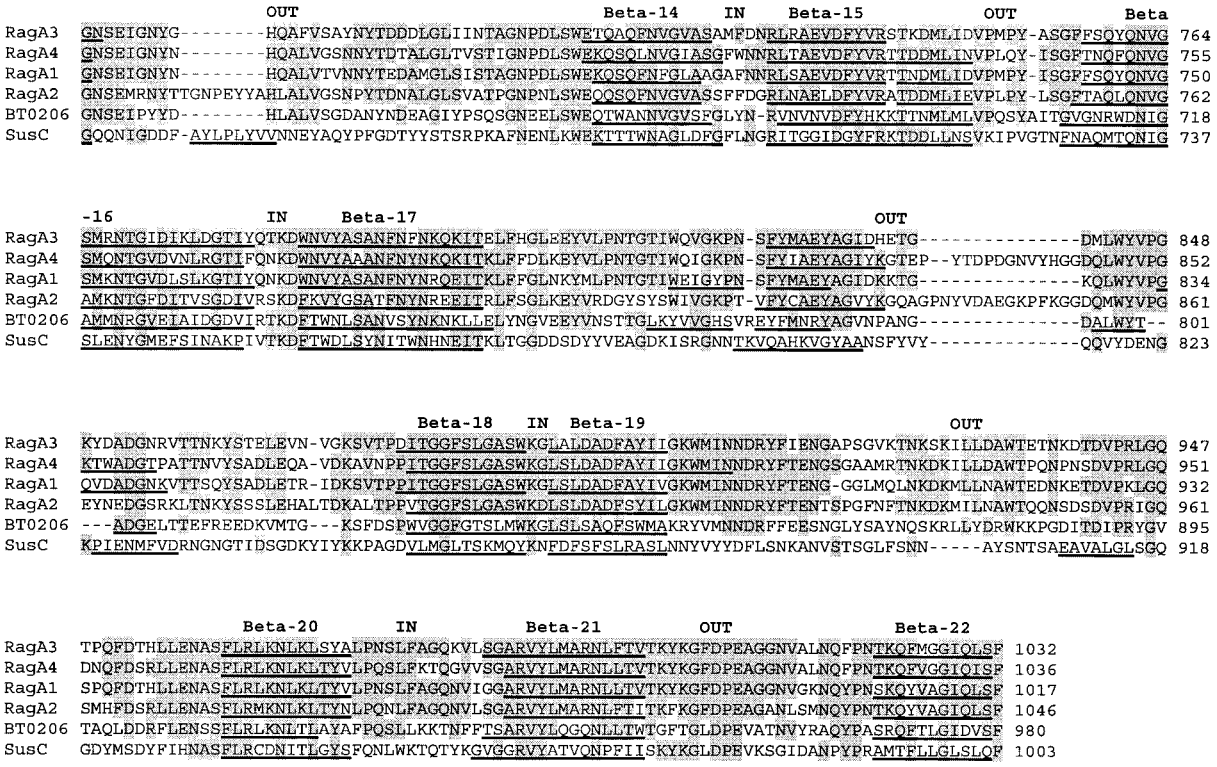

FIG. 4—*Continued.*

of the sequences of the four RagA variants, the *B. thetaiotaomicron* homologue BT0206, and SusC demonstrated strong conservation in the position of predicted barrel region β-strands despite extensive sequence divergence (Fig. 4). (The structural similarity of RagA1 to FepA had previously been noted by Wexler et al. [34], who have characterized the locus encoding the *omp200* porin complex of *Bacteroides fragilis* and demonstrated a gene organization similar to that of the *rag* locus.)

Analysis of the RagB sequences by 3D-PSSM revealed a predicted similarity to the alpha/alpha-6 barrel structure of 1AYX, a glucoamylase of yeast belonging to a sequence family 15 of glycosyl hydrolases (30), although the significance score was over 95% only for RagB4. This prediction was intriguing given the role of the Sus operon in polysaccharide degradation and uptake (27, 28). Sequence alignment of the RagBs, BT0207 (the putative lipoprotein encoded downstream of BT0206), and SusD demonstrated close correspondence in the positions of 11 predicted alpha helices (Fig. 5), but the location and spacing of alpha helices did not match well to those of 1AYX (not shown).

Distribution of the *rag* alleles. The collection of *P. gingivalis* investigated here comprises isolates from many sources, including well-characterized laboratory strains as well as more recent clinical isolates. A majority of isolates are from western European countries, with few from North America. The collection may not be fully representative of the *P. gingivalis* population currently circulating, nor is it possible to rule out that some isolates may be duplicates either from the same patient or from laboratory strains that have been renamed by different laboratories. Nevertheless, some general comments may be made about the prevalence and geographic distribution of the four *rag* alleles.

All four of the *rag* alleles were found among isolates from several countries; none was restricted to isolates from a single continent. Their relative prevalence in this collection was comparable, except that *rag-4* was found less frequently than the other alleles. *rag-1* was detected in isolates obtained from Germany, The Netherlands, Romania, Sweden, the United Kingdom, and Kenya. However, LPCR restriction profile 1a was frequently found in established laboratory strains such as W50 and W83, some of which may have a common origin, while profile 1b (lacking IS*Pg1*) was more frequent among isolates thought to have a recent clinical source. Profile 2a (*rag-2*) was found exclusively among a set of isolates from Thailand, but profile 2b, representing the *rag-2* locus without IS*Pg3*, was found in isolates from across Europe (Finland, The Netherlands, Sweden, and the United Kingdom). *rag-3* was found in numerous isolates from Europe and one isolate each from Japan and Thailand. Profile 4a (*rag-4*) was represented by strains ATCC 33277^T and 381, both of which are long-established laboratory strains, and in isolates from European countries.

Relationship of *rag* alleles to other markers of disease and virulence. The distribution of *rag* alleles was investigated relative to other polymorphic markers that have been used to type *P. gingivalis* and determine associations with disease (Table 5). A set of isolates kindly provided by M. L. Laine representing two to three isolates each of the six K-capsular serotypes (K1 to K6) plus unencapsulated types (K0) of *P. gingivalis* (16, 33) was included in the analysis. There was a limited degree of correlation between the *rag* allele and K serotype: K3 and K5

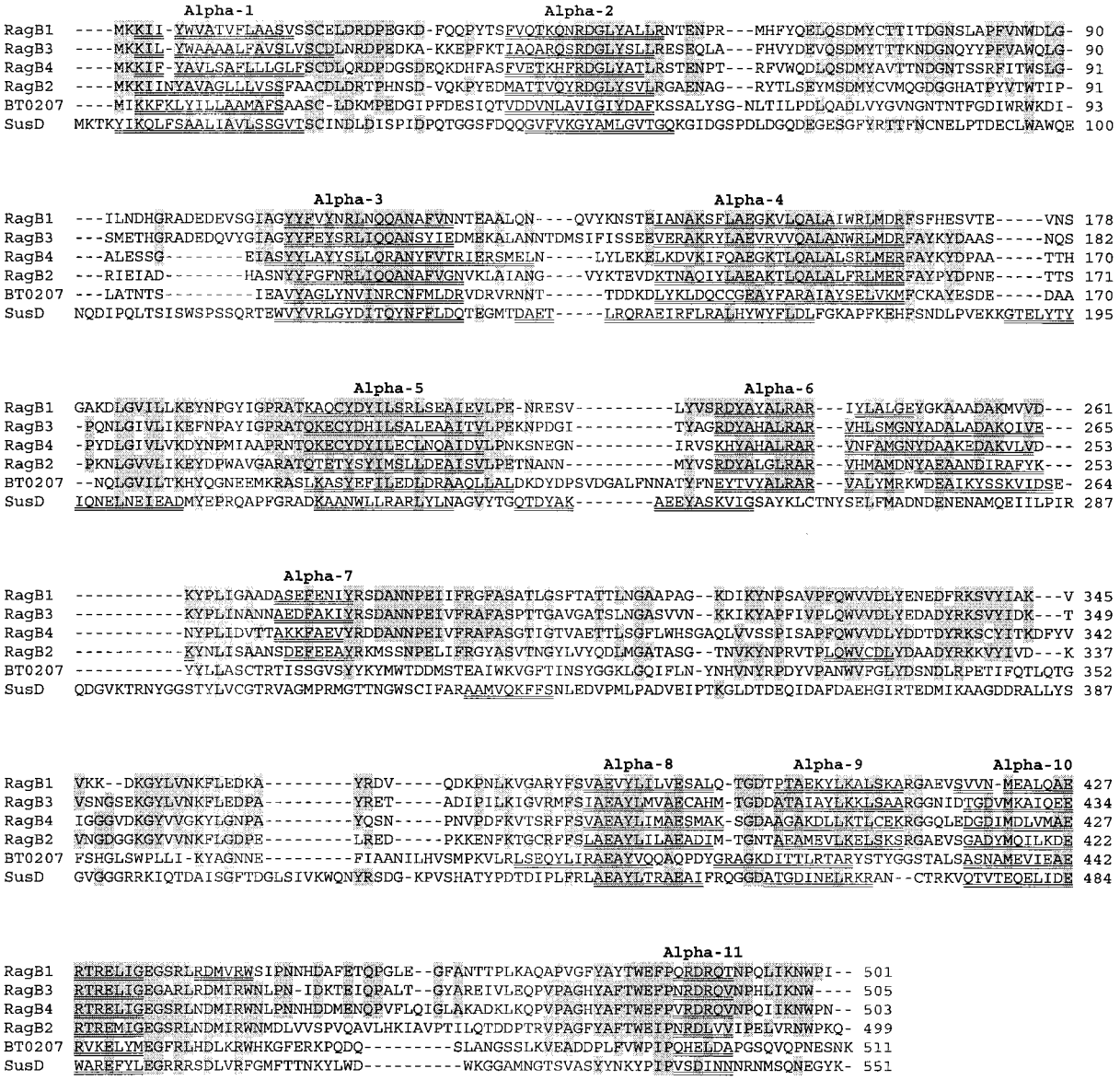

FIG. 5. Sequences of RagB1-4 aligned with BT0207 and SusD from *B. thetaiotaomicron* using ClustalW (32). Residues identical in at least three proteins are highlighted. Regions of at least 6 amino acids predicted by 3D-PSSM (14) to form alpha helices are underlined. Alpha helices conserved in all six proteins are numbered (the fifth, sixth, and seventh alpha helices of SusD are poorly aligned, but sequence elements such as LRAR in alpha-6 suggest an equivalence in these structures).

were found only in *rag-3* isolates, and K4 was found only in *rag-1* isolates, but K1, K2, and K0 were each associated with two different *rag* alleles; each *rag* allele was found in isolates of at least two different K serotypes (Table 5). The *fimA* genotype was known for eight isolates (25) and showed no clear association with the *rag* allele. Genotypes *fimA* II and Ib have been reported to be associated with periodontitis and recovery from deep pockets in epidemiological studies in Asia (2, 24), although the same association was not found in a study in Europe (4); these genotypes were found among isolates with three different *rag* alleles. The ribosomal intergenic spacer heteroduplex types were too diverse among the few isolates for which they were known to draw any conclusions, but again, types with an epidemiological association with periodontitis (11) were found in isolates of both *rag-1* and *rag-2*. We had

TABLE 3. Similarities between RagA proteins and their homologues

Rag protein (isolate)	BLAST score versus W50 RagA (% homology)	BLAST score versus *B. thetaiotaomicron* BT0206[a] (% homology)	BLAST score versus *B. thetaiotaomicron* SusC[b] (% homology)
RagA1 (W50)	1017/1017 (100)	378/1028 (35)	314/1081 (29)
RagA2 (A011/9)	679/1046 (64)	354/1059 (33)	321/1094 (29)
RagA3 (QM220)	716/1032 (69)	363/1041 (34)	107/281 (38)
RagA4 (ATCC 33277^T)	736/1040 (70)	369/1046 (35)	317/1105 (28)

[a] For all RagA proteins, the *B. thetaiotamicron* protein BT0206 (NP_809119.1) was scored by BLAST as having the most significant homology after *P. gingivalis* RagA proteins.

[b] For all RagA proteins, *B. thetaiotamicron* SusC was scored by BLAST as the most significant homology for a protein with experimentally determined function.

TABLE 4. Similarities between RagB proteins and their homologues

Rag protein (isolate)	BLAST score versus W50 RagB (% homology)	BLAST score versus *B. thetaiotaomicron* BT0207[a] (% homology)	BLAST score versus *B. thetaiotaomicron* SusD (% homology)
RagB1 (W50)	474/501 (94)[b]	57/187 (30)	NS[c]
RagB2 (A011/9)	244/510 (47)	93/370 (25)	27/73 (36)
RagB3 (QM220)	284/507 (56)	114/515 (22)	NS
RagB4 (ATCC 33277^T)	237/512 (46)	121/469 (25)	NS

[a] For all RagB proteins, the *B. thetaiotamicron* protein BT0207 (NP_809120.1) was scored by BLAST as having the most significant homology after *P. gingivalis* RagB proteins.
[b] This value is less than 100% because the default low-complexity sequence filter setting in BLAST was applied.
[c] NS, no significant homology using default settings.

previously demonstrated the association of the *rag* locus, corresponding to *rag-1* in this study, with deep pockets in patients with periodontal disease (12). An epidemiological study comparing these various markers in isolates from patients with detailed clinical assessment is required to evaluate their relative strength of association with disease. Such a study would have the potential to indicate the significance of the three polymorphic surface antigens, RagAB, capsular polysaccharide, and fimbriae, to the outcome of *P. gingivalis* infection.

An alternative strategy to assess the significance of putative virulence factors is to use an animal model of infection, and Laine and van Winkelhoff (17) have reported the virulence of isolates with different serotypes in a mouse model. Encapsulated strains were significantly more virulent than K0 strains, but variability in virulence between strains with the same serotype suggested the importance of additional virulence factors that must differ between strains. A measure of virulence from the Laine and van Winkelhoff study has been included in Table 5, and it was apparent that most of the *rag-1* isolates had high scores for virulence. Chi-square analysis demonstrated that there was a significant correlation ($P < 0.05$) between carriage of *rag-1* (versus carriage of any other *rag* allele) and high virulence (2/3 or 3/3 mice seriously ill within 72 h of inoculation).

TABLE 5. Correlation of *rag* allele to published data on typing and virulence

Isolate	*rag* allele	K type[a]	*fimA* type[b]	Heteroduplex type[c]	Virulence[d]
W83	*rag-1*	K1	IV	hW83*	3
W50	*rag-1*	K1	IV	hW83*	–
22KN6-12	*rag-1*	K1	–	–	3
AJW5	*rag-1*	K2	–	–	3
ATCC 49417	*rag-1*	K4	II*	h49417*	3
HG1702	*rag-1*	K4	–	–	3
HG1703	*rag-1*	K4	–	–	0
HG1695	*rag-1*	K0	–	–	0
HG1706	*rag-2*	K2	–	–	0
HG1690	*rag-2*	K5	II*	–	3
HG1691	*rag-2*	K6	Ib*	hHG1691*	0
HG1708	*rag-2*	K6	–	–	0
HG1694	*rag-2*	K0	–	–	0
HG1700	*rag-3*	K1	–	–	0
A7A1-28	*rag-3*	K3	II*	hA7A1	2
CLN-17-6-1	*rag-3*	K3	–	–	0
HG1701	*rag-3*	K3	–	–	0
HG1704	*rag-3*	K5	–	–	3
HG1707	*rag-3*	K5	–	–	0
HG1705	*rag-4*	K6	–	–	0
381	*rag-4*	K0	I	h381	1
ATCC 33277^T	*rag-4*	–	I	h381	–

[a] Capsule type (17).
[b] Genotype of *fimA* (25); types with an asterisk have been found at a higher frequency in patients with periodontitis and associated with deep pockets (2).
[c] Ribosomal intergenic spacer region heteroduplex type (11); types with an asterisk have been found at a higher frequency in patients with periodontitis.
[d] Measure of virulence in a mouse model; the figure represents the number of mice (out of three) that demonstrated serious illness within 72 hours of inoculation, as determined previously by Laine and van Winkelhoff (17). –, not known.

Further discussion. In our initial investigation of the *rag* locus of *P. gingivalis*, it was concluded that the genes encoding this immunodominant surface antigen had a restricted distribution in the species (12). The present study has demonstrated that there are actually four distinct variants or alleles of the *rag* locus, each of which is common among the *P. gingivalis* population and none of which appear to be confined to specific geographic regions.

Although the function of RagA/B has not yet been experimentally determined, the similarity to SusC/D family proteins in *Bacteroides* species suggests a possible role in polysaccharide uptake. Expression of some of the SusC/D loci in *B. fragilis* has been found to be controlled by DNA inversion systems, including three clusters of loci where inversion of a promoter mediates alternative expression of different SusC/D cassettes (15). Other DNA inversion systems in *B. fragilis* affect the expression of other surface-expressed molecules such as capsular polysaccharides. These systems are likely to represent a mechanism for the high-frequency generation of antigenic variation and are thus implicated in evasion of the host response (15). While there is no evidence for high-frequency variation of RagA/B, the *rag* locus bears many of the hallmarks characteristic of genes involved in the production of variable surface antigens. These features include an association of insertion elements with the locus and a G+C content lower than what is typical of the species and are generally interpreted to suggest that the locus and its variant alleles have been acquired by horizontal gene transfer. The large number of genes in *Bacteroides* spp. with sequence homology to the *rag* locus would suggest a ready reservoir of genes available for recombination, although none of the genes available to date in sequence databases are sufficiently similar to suggest a close ancestry in common with the *P. gingivalis* locus.

The presence of variant alleles of the *rag* locus leads directly to the question of whether specific alleles are associated with different clinical conditions. We had previously demonstrated that *rag-1* was associated with deep periodontal pockets (12). The present study has revealed that strains shown by others to be more virulent in a mouse model (17) are significantly more likely to carry *rag-1* than other *rag* alleles. Further work is in progress to investigate the links between the *rag* locus and pathogenesis in more detail.

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