Distribution of *Porphyromonas gingivalis* Biotypes Defined by Alleles of the *kgp* (Lys-Gingipain) Gene

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Paired subgingival plaque samples representing the most-diseased and least-diseased sites were collected from 34 adult patients with diagnosed chronic periodontitis. The percentage of *Porphyromonas gingivalis* relative to the total anaerobic and gram-negative bacterial load at each site was determined by real-time PCR. Based on variations in the noncatalytic C terminus of the Lys-gingipain (Kgp), it was reasoned that DNA sequence variation in the 3'-coding region of the *kgp* gene might determine functional biotypes. Perusal of the available sequence information in GenBank indicated three such forms of the *kgp* gene corresponding to *P. gingivalis* strains HG66, 381, and W83. Analysis of patient samples revealed the presence of a fourth genotype (W83v) that showed duplication of a sequence recognized by the W83 reverse primer. The four biotypes, HG66, 381, W83, and W83v, were present in the study group in the ratio 8:11:6:5, respectively. Each subject was colonized by one predominant biotype, and only three patients were colonized by a trace amount of a second biotype.

Although a variety of technologies have been employed to demonstrate the genetic diversity of *Porphyromonas gingivalis*, it remains unclear if there are virulent biotypes of this bacterium. While analysis by restriction fragment length polymorphism (1) indicated that the same ribotypes of *P. gingivalis* are found in subgingival plaque samples of periodontitis patients in different countries, data obtained by random amplified primer detection and multilocus enzyme electrophoresis methods (19, 21) suggested that multiple strains are associated with disease, leading to the conclusion that infection by this bacterium is largely opportunistic. There is no consensus from studies employing *fimA* genotyping, heteroduplex formation, and capsular antigen serotyping (2, 3, 8, 16).

Diversity in *P. gingivalis* appears to have arisen by genetic recombination rather than mutation (7, 15, 24). Accordingly, the structure and function of major virulence factors, such as the gingipain cysteine proteinases, provide key insights into the pathogenicity of different strains (17). The use of inactivation mutants (18, 26) and specific inhibitors (5) has demonstrated the important role of the Lys-gingipain, Kgp, in the acquisition of essential heme for growth in vitro and the virulence of *P. gingivalis* in animal models. The form and partitioning of Kgp between the cell and the extracellular milieu have been reported to show variations (28) that correlate with distinct changes in the functional properties of *P. gingivalis* strains (12, 22). Differences have also been observed in the pathogenic potential of laboratory strains, depending on the animal model (4, 13) or the in vitro assay employed (10, 11). The latter differences in virulence may be a function of the heterogeneity within virulence genes such as *kgp*.

The *kgp* gene codes for a protein composed of five domains:

a propeptide, a catalytic domain, and three adhesin domains, Kgp39, HA2, and Kgp44. A variable region exists in the Kgp44 domain of different strains of *P. gingivalis* (Fig. 1). The DNA sequence of the 3'-hemagglutinin region of the *kgp* gene is also found in multiple copies in the hemagglutinin gene, *hagA* (9). On the basis of evidence currently available, the distribution of biotypes based on variation of Kgp structure in patients with periodontal disease appears to be important. The present study was therefore undertaken to explore the feasibility of detecting different biotypes of *P. gingivalis* based on sequence variation of the *kgp* gene as a basis for defining the distribution of these forms in clinical samples.

Adult patients attending the periodontal clinics of the United Dental Hospital and Westmead Centre for Oral Health were selected on the basis of no history of systemic disease known to affect the periodontium, no periodontal therapy for the preceding 3 years and a diagnosis of generalized or localized periodontitis. After removal of supragingival plaque, subgingival plaque was obtained by curette sampling from the most diseased and least diseased sites of 34 patients (mean age, 51 ± 10 years; 18 males and 16 females). DNA was extracted from reference strains and from subgingival plaque samples by using the ATL buffer reagent (Qiagen, Victoria, Australia), which efficiently releases DNA from gram-negative bacteria as well as those cultured under anaerobic conditions (20, 23). Total bacterial and *P. gingivalis* loads were quantified in the subgingival plaque by real-time PCR on an ABI-PRISM 7700 sequence detection system (Applied Biosystems, Foster City, Calif.) using a universal primer and probe set and a *P. gingivalis*-specific primer and probe set, respectively, to detect 16S rRNA genes (20, 23). The amount of DNA in each sample was measured on a standard graph of *P. gingivalis* DNA (DNA range of 3.6 fg to 3.6 ng) and was converted to theoretical cell numbers based on the assumption that each cell contained 2.37 fg of DNA (20). The relative abundance of *P. gingivalis* was therefore expressed as a percentage of the total anaerobic plus

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FIG. 1. Location of *kgp* biotype-specific primers in the *kgp* gene of laboratory strains of *P. gingivalis*. Processed domains of expressed Kgp are labeled above the figure. The hemagglutinin region is shaded gray, and the variable region in the Kgp44 domain is shaded black. Primers used to amplify biotype-specific *kgp* amplicons are denoted by arrowheads, and the predicted sizes of the PCR amplicons are in parentheses. Biotype-specific sequences of *kgp* were obtained from GenBank, and their accession numbers are as follows: HG66, accession no. U54691; W83, accession no. AF017059; and 381, accession no. D83258. W83v was a variant observed in this study (accession no. AY559244).

gram-negative load determined by using a universal bacterial amplicon (20, 23). Thirty-one patients were positive for *P. gingivalis*, with the relative abundance of this organism varying from 0.003 to 38% of the total anaerobic and gram-negative load (see Fig. 3). The samples that were positive for *P. gingivalis* were further tested for different biotypes based on variations in the *kgp* gene.

In order to determine the feasibility of discriminating biotypes of *P. gingivalis* based on the *kgp* gene, DNA was first extracted from individual batch cultures (20) of the *P. gingivalis* strains HG66, 381, and W83, which express three different alleles of the *kgp* gene (27). To discriminate strains 381 and W83, a biotype-specific region of the *kgp* gene was amplified by PCR using the *kgp*-forward primer, *kgp*F, complementary to a region of identity in all strains, and either the strain-specific reverse primer 381R or W83R, which were complementary to unique DNA sequences within the 3'-variable region of the gene (Table 1 and Fig. 1). To further discriminate these two alleles from a third expressed by strain HG66, the HG66F primer, complementary to a DNA sequence within the region coding for the Kgp39 domain, and the HG66R primer, complementary to a DNA sequence coding for a region within the Kgp44 domain, were used (Table 1 and Fig. 1).

PCR was carried out with HotStarTaq Master Mix (Qiagen, Australia) with an initial enzyme activation at 95°C for 15 min followed by 40 cycles of 15 s of denaturation at 95°C and 5 min of annealing and extension at 60°C. The capacity of the primer sets to differentiate the three structural homologs of *kgp* was established (Fig. 2). The primer set HG66F and HG66R amplified an HG66-biotype-specific amplicon of 1.9 kb, the primer set *kgp*F and 381R amplified a 381-biotype-specific amplicon of 3.4 kb, and the primer set *kgp*F and W83R amplified a W83-biotype-specific amplicon of 3.6 kb. All three primer sets were strain specific (Fig. 2). Analysis of patient samples, however, revealed a further *kgp* allele, identified as a variant of the W83 type (designated W83v; GenBank accession no. AY559244). This allele possessed a duplication of a sequence complementary to that of the W83R primer in the Kgp39 domain (Fig. 1) that resulted in the amplification of a 2.2-kb amplicon by PCR (Fig. 2I, gel C, samples 6D and 10D; Fig. 2II, gel C, samples 25D, 26D, and 27D).

Biotypes of *P. gingivalis* based on *kgp* heterogeneity were identified in the most-diseased sites of 27 patients (Fig. 2 and 3), in which the relative abundance of *P. gingivalis* exceeded 0.01% of total gram-negative and anaerobic bacterial load. This result indicated either that the detection of a specific biotype was less sensitive than bacterial detection based on the 16S rRNA gene or that samples with low levels of *P. gingivalis* contained an unknown biotype(s). *P. gingivalis* was detected in the least-diseased sites in 12 patients. In all patients in which *P. gingivalis* could be quantified at both sites, the same biotype(s) was detected (Fig. 2). Only three individuals carried more than one biotype: two harbored both the HG66 and W83v biotypes (Fig. 2I, 6D, and 2II, 25D), while one possessed a combination of *P. gingivalis* HG66 and 381 biotypes (Fig. 2I, 8D). The frequency of occurrence of biotype HG66 versus biotype 381 versus biotype W83 versus biotype W83v was 8:11:6:5.

Different alleles of *kgp* may be relevant to the virulence of *P. gingivalis* strains. First, the importance of Kgp in heme acquisition is evident from studies of *kgp* deletion mutants in *P. gingivalis* ATCC 33277 (*kgp* biotype 381) where the mutants are poorly pigmented in comparison with the wild type (29). This change in heme acquisition is reflected in the different biotypes that formed the basis of the current study, since colonies of *P. gingivalis* HG66 grown on blood agar are poorly pigmented. This phenomenon may be related to the observed release of gingipain moieties by strain HG66 compared with the primary retention of these complexes at the cell surface by strain 381 (28). Second, all biotypes (including W83v) possess a putative C-terminal membrane-anchoring peptide in the

TABLE 1. Strain-specific primers used in this study^a

Primer	Sequence (5'→3')	<i>T_m</i> (°C) ^b
HG66F	TGCACGTTATGACGATTTACAT	59.0
HG66R	ACTTCCGAATGTATTGTGATCGG	58.9
<i>kgp</i> F	ATGTATACTTTCCGTATGTCTGCTTCTTC	59.5
W83R	AAGCGTCGTTACCCGTAGAAGA	58.9
381R	ATCAGAAAGTTGTCGCGCTG	58.8

^a Primers were synthesized by Applied Biosystems (Foster City, Calif.).

^b *T_m*, melting temperature.

FIG. 2. Migration pattern of *kgp* biotype-specific PCR amplicons on ethidium bromide-stained 1% agarose gels. Amplicons were observed by using DNA from control batch cultures of *P. gingivalis* laboratory strains (HG66, gels Ia, Id, and IIa; 381, gels Ib, Ie, and IIb; and W83, gels Ic, If, and IIc), a no-template control (NTC), or DNA extracted from subgingival plaque samples of patients attending the United Dental Hospital, Sydney (I), and the Westmead Centre for Oral Health, Sydney (II). D, most-diseased site; H, least-diseased site. Molecular size standards in kilobases (MW) are listed to the left of gels a, b, and c in panels I and II.

adhesin domain and one in the variable region of the Kgp44 domain (25), while only one copy is to be found in the Kgp39 adhesin domain of other *P. gingivalis* strains. It is presently unknown if clinical isolates corresponding to the reference

Kgp44 domain (30). A region of identity extends from this anchoring peptide for a further 30 residues towards the N terminus of the protein to a site where processing of the protein is considered to occur based on the analysis of the X-ray crystallographic structure of the processed protein, RgpB, which belongs to a related family of gingipains (6). The region N terminal to this processing site is identical in the Kgp of *P. gingivalis* strains HG66 and 381 but differs in strains W83 and W83v. Accordingly, Kgp in W83 and W83v may be processed differently. Third, a segment within the variable region of W83 and W83v shows 65% identity to a synthetic 20-amino-acid peptide that can inhibit hemagglutination (14). The same segment is not observed in other strains. Fourth, variations in immunological reactivity have been noted. The Kgp from *P. gingivalis* HG66 has two copies of an epitope: one in the Kgp39

FIG. 3. Relationship between *P. gingivalis* and *kgp* biotype. Plaque from the least-diseased (open circles) and most-diseased (solid circles) sites from each patient was assayed for the percentage of the *P. gingivalis* population that belonged to a particular *kgp* biotype. The mean value for each group is represented by a solid bar, and statistical comparison between the groups was analyzed by the nonparametric, unpaired, two-tailed Mann-Whitney test with a 95% confidence interval. An asterisk denotes significance of $P < 0.05$.

P. gingivalis Kgp-based biotypes described here have similar properties with respect to Kgp partitioning and functionality. Future expanded analysis of the relationship of the biotypes to the structure and stage of the periodontal lesion will provide a basis for evaluating the association of *kgp* alleles with the virulence of this bacterium.

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