J Periodont Res 2005; 40; 193–198
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JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2005.00783.x

Sequence variations in *rgpA* and *rgpB* of *Porphyromonas gingivalis* in periodontitis

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Beikler T, Peters U, Prior K, Ehmke B, Flemmig TF. Sequence variations in rgpA *and* rgpB *of* Porphyromonas gingivalis *in periodontitis. J Periodont Res 2005; 40: 193–198. © Blackwell Munksgaard 2005*

Objective: The aim of the present study was to determine sequence variations in the active centre of the Arg-X-specific protease encoding genes *rgpA* and *rgpB* of clinical *Porphyromonas gingivalis* isolates and to analyse their prevalence in periodontitis patients before and 3 months after mechanical periodontal therapy.

Background: Genetic diversity at nucleotides 281, 283, 286 and 331 has been shown to result in amino acid substitutions in the catalytic domain of RgpA and RgpB that affect the substrate specificity and thus may influence the efficacy of Arg-X-protease specific inhibitors.

Methods: Sequence analysis of *rgpA* and *rgpB* genes in clinical *P. gingivalis* strains isolated from subgingival plaque samples of 82 periodontitis patients before and 3 months after mechanical supra- and subgingival debridement was performed.

Results: No specific variation within the *rgpA* sequence was observed. However, the *rgpB* sequence in the region of the active centre showed five different *rgpB* genotypes, which were named NYPN, NSSN, NSSK, NYPK and DYPN according to the derived amino acid substitution. *Porphyromonas gingivalis* genotype NYPN was detected in 27 patients (32.9%) before and in 8 patients (9.8%) after therapy, NSSN in 26 (31.7%) and 10 (12.2%), NSSK in 22 (26.8%) and 2 (2.4%), NYPK in 5 (6.2%) and 1 (1.2%), and DYPN in 1 patient (1.2%) and 0 patients (0%), respectively. Only one patient (1.2%) harboured two *P. gingivalis rgpB* genotypes (NSSK/NYPN) before treatment; these were no longer detected after therapy.

Conclusion: The results indicate that five *rgpB* genotypes are maintained in natural populations of *P. gingivalis*. These data may be of importance with regard to the development of specific *rgpB* inhibitors.

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Key words: gingipain; *Porphyromonas gingivalis*; *rgpA*; *rgpB*; sequence

Accepted for publication September 14, 2004

Porphyromonas gingivalis is a Gram-negative, black-pigmented strictly anaerobic bacterium strongly associated with chronic and aggressive periodontitis (1). The pathogenicity of *P. gingivalis* has been attributed to a number of virulence factors including lipopolysaccharide, fimbriae, haemagglutinin, haemolysin, and extracellular hydrolytic enzymes, especially proteinases (2–7). Among these the Arg-X-specific (Arg-gingipains RgpA and RgpB) and Lys-X-specific (kgp) cysteine proteinases appear to play a major role in the pathogenesis of *P. gingivalis*-associated periodontitis (4, 8, 9). In this regard, null mutants of *P. gingivalis* for Arg-X-specific cysteine protease enzymes were found to show a marked decrease in virulence in an *in vivo* model (10). Immunization with peptides corresponding to the N-terminal sequence of the catalytic domain of Arg-X-specific cysteine proteases has been shown to protect against infection by bacteria in mouse models (11, 12), indicating the overall importance of the Arg-gingipains in the pathogenesis of the disease.

The *P. gingivalis* gingipains-R are encoded by two genes, *rgpA* and *rgpB* (13). RgpA consists of a 50 kDa catalytic domain (RgpAcat) and a 95 kDa

high molecular mass, non-covalent complex of catalytic and haemagglutinin/adhesin domains (HRgpA) (14), the former responsible for proteolytic activity and the latter for adhesion to extracellular matrix proteins and red blood cells. In contrast to *rgpA*, the *rgpB* gene is lacking in almost the entire section encoding the haemagglutinin/adhesin domains, and its product occurs predominantly as a soluble 50 kDa protein (RgpB) (15). Despite the fact that the catalytic domain of RgpB is almost identical to that of RgpAcat at the primary structure level, the different cleavage of substrates has been demonstrated to result only from four amino acid substitutions at the active site of the *rgpA* gene product vs. those of *rgpB* gene (16). As the data set out above have been obtained solely from *P. gingivalis* laboratory strains, no information is available on the prevalence of these substitutions in clinical *P. gingivalis* isolates. Such information is, however, of great importance for the development of drugs, e.g. inhibitors targeting Arg-X-specific proteases.

Therefore, the aim of the present study was to analyse the prevalence of sequence variations in the Arg-gingipain genes that encode for these substitutions and their persistence following mechanical periodontal therapy.

Material and methods

Study subjects

Eighty-two systemically healthy Caucasian patients with moderate to severe chronic or aggressive periodontitis were recruited from the Department of Periodontology, University of Münster, Germany. Patients were excluded from the study if any of the following conditions applied: professional tooth cleaning, periodontal therapy or antibiotic medication during the 6 months preceding the study, antibiotic premedication requirement, and any disorder associated with immunodepression. All patients enrolled into the study signed the informed consent form approved by the Ethics Committee of the Medical Chamber, Westphalia-Lippe, Münster, Germany.

Treatment

All patients received full mouth supra- and subgingival scaling and oral hygiene instruction. Supra- and subgingival scaling was performed under local anaesthesia and completeness of supra- and subgingival debridement, i.e. with all pathologically exposed subgingival root surfaces feeling hard and smooth, was determined by using a fine explorer.

Clinical examinations and sample collection

Pocket probing depths and bleeding on probing were assessed at baseline and 3 months after mechanical periodontal therapy in all patients at six sites per tooth with a periodontal probe (PCP 15, Stoma, Storz am Mark, Germany). Before and 3 months after mechanical periodontal therapy, subgingival plaque samples were obtained with sterile paper points (for *P. gingivalis* isolation) and a sterile curette (for DNA isolation) from the six deepest periodontal pockets, each representing the most severely affected site per sextant. For DNA isolation, the plaque samples were pooled in 200 µl of phosphate-buffered saline and placed in an ultrasonic cleaning bath (Bransonic 1510E, Branson, Dietzenbach, Germany) for 5 min at room temperature (~20°C) in order to disperse the plaque. For isolation of *P. gingivalis,* the sampled paper points were pooled in 200 µl of 0.25% Ringer solution and placed in an ultrasonic cleaning bath for 10 s at room temperature in order to disperse the plaque.

Isolation of *P. gingivalis*

The bacterial suspension was diluted in 10-fold steps. Then, 100 µl of both the undiluted and the diluted suspensions were streaked on non-selective blood agar (CDC agar) plates containing 5% defibrinated sheep blood supplemented with 5 mg/l hemin (Merck, Darmstadt, Germany) and 1 mg/l vitamin K1 (17). The plates were incubated in an atmosphere containing N_2 (85%), H_2 (10%), and CO_2 (5%) for 7 days. Colonies of *P. gingivalis* were selected from their colony morphology for identification. From each of these selected colonies a subculture was prepared, also utilizing non-selective blood agar base (CDC).

Identification of *P. gingivalis* by polymerase chain reaction

To confirm *P. gingivalis* in subgingival plaque and in clinical isolates a *P. gingivalis* specific polymerase chain reaction (PCR) was used (18), with a 548 bp fragment from the central portion of the collagenase gene *prtC* being amplified using primers Coll-1 (5′-ACAATCCACGACACCATC-3′) and Coll-2 (5′-TTCAGCCACCGAGAC-3′). Each sample was amplified by 30 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C. Eighteen microliters of the PCR product were subjected to agarose gel electrophoresis on 1.8% agarose gels. The gels were stained with ethidium bromide (1 µg/ml) and assessed under UV light. Precautions as described by Kwok and Higuchi were taken to prevent contamination (19).

Identification of *rgpA* and *rgpB* genotypes by direct sequencing of PCR products

In order to identify sequence variations within the *rgp* genes, the *rgp* genes of 23 *P. gingivalis* isolates were sequenced. Bacterial DNA was isolated by Spin Mini Kit (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. The PCR products were obtained using primers designed according to the *P. gingivalis rgpA* and *rgpB* gene sequence (*rgpA*: Accession number U15282; *rgpB*: Accession number AF007124). The sequences were mapped on the *P. gingivalis* genome according to TIGR (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database = gpg) and the PCR-primers were designed not to bind to repeat regions at other loci within the *P. gingivalis* genome (primer sequences can be obtained from the corresponding author). For PCR reaction, 100 ng DNA, 200 µM of each of the four dNTPs, 2.0 mM $MgCl_2$, and 2.0 U Bio-X-Act Long DNA Poly-

merase (Bioline, London, UK) were added for a final volume of 50 µl. Each sample was amplified by 30 cycles of 30 s at 95°C, 30 s at 52°C, and 120 s at 72°C. The resulting PCR products were cleaned with exonuclease I and shrimp alkaline phosphatase and subsequently sequenced using the ABI BigDye Terminator version 1.1 cycle sequence kit (Applied Biosystems, Weiterstadt, Germany) with nested primers. The products were further purified by ethanol/sodium acetate precipitation, resuspended in deionized formamide, and loaded in the ABI PRISM 310 genetic analyser (Applied Biosystems) for sequence analysis. The alignment of the resulting sequences was analysed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST) (20).

Statistical analysis

Double data entry by two individuals was performed before statistical analysis. This permitted flaws in the data input to be detected and corrected by comparison with the original clinical and experimental data. Differences in the prevalence of the various *rgpB P. gingivalis* genotypes before and after therapy were assessed by chi-squared analysis. The association of *P. gingivalis* genotypes to clinical parameters at baseline as well as bleeding on probing and the percentage of sites showing improved pocket probing depths (greater than or equal to 2 mm compared to baseline), stability (1 mm better or poorer compared to baseline) or deterioration (greater than or equal to −2 mm compared to baseline) after therapy were analysed by Fisher's exact test. To analyse differences in the percentage of sites with improvement, stability or deterioration of pocket probing depths, the raw data were subjected to an arcus–sinus transformation before Fisher's exact test was performed. The data of the two patients colonized by NSSK/NYPN and DYPN were excluded from statistical analysis. The significance level of all statistical tests was set at $p < 0.05$.

Results

Demographics and periodontal conditions

Eighty-two patients (53 females, 29 males) subgingivally harbouring *P. gingivalis* were enrolled in the study. Their demographics and periodontal conditions are summarized in Table 1.

Sequencing of the *rgpA* and *rgpB* gene

The sequence analysis of the *rgpA* and *rgpB* gene in the 23 clinical isolates revealed a heterogeneous nucleotide sequence leading to numerous substitutions in the deduced amino acid sequences (RgpA: 44, and RgpB: 34). Compared to the reference sequence of *rgpA* (Accession number U15282), 26 conservative and 18 non-conservative amino acid substitutions were observed in the deduced amino acid sequence. In the *rgpB* gene (reference sequence: Accession number AF007124) the nucleotide variations accounted for 18 conservative and 16 non-conservative substitutions in the derived amino acid sequence. Moreover, 11 homologous amino acid substitutions derived from the *rgpA* and *rgpB* nucleotide sequence were observed.

The four amino acid substitutions that accounted for the different substrate specificity of RgpA and RgpB were at positions 281, 283, 286 and 331 (16). With regard to the encoding nucleotide sequences (between nucleotide 1524 and 1682, see Fig. 1) no heterogeneity could be detected in the *rgpA* gene. In contrast, the direct sequencing of this critical *rgpB* gene region in the 82 clinical isolates resulted in the identification of five different genotypes, which were named according to the resulting amino acid sequences, i.e. NYPN, NSSN, NSSK, NYPK and DYPN (Fig. 1).

Prevalence of *rgpB* genotypes in *P. gingivalis*-positive patients before and after therapy

At baseline all but one of the 82 *P. gingivalis*-positive patients were found to be subgingivally colonized by only one *rgpB* genotype: *P. gingivalis* genotype NYPN was detected in 27 patients (32.9% of all patients), NSSN in 26 (31.7%), NSSK in 22 (26.8%), NYPK in five (6.2%) and DYPN in one patient (1.2%). Only one patient (1.2%) was found to harbour two *P. gingivalis rgpB* genotypes (NSSK/NYPN) subgingivally (Table 1).

Three months after mechanical sub- and supragingival debridement, the prevalences of *P. gingivalis* decreased for all genotypes. Interestingly, only two of the 22 patients (2.4% of all patients) initially tested positive for the NSSK *P. gingivalis* genotype were found to harbour this pathogen

Table 1. Demographics and severity of periodontal disease in study patients, categorized according to the subgingival identification of *rgpB Porphyromonas gingivalis* genotypes NYPN, NSSN, NSSK, NYPK, DYPN and NSSK/NYPN (mean ± SD)

					PPD		
Patients (*n*)	Smokers (*n*) (%)	Age (years)	Teeth (*n*)	BOP (%)	1–3 mm (%)	4–6 mm (%)	≥ 7 mm (%)
NYPN (27)	7 (25.9)	54.1	22.3	46.6	40.6	46.4	13.1
		13.3	4.6	24.7	18.7	12.1	14.3
NSSN (26)	9 (34.6)	48.2	24.0	47.9	41.2	46.0	12.8
		11.0	5.1	29.5	21.9	16.8	10.8
NSSK (22)	4 (18.2)	51.7	23.3	58.0	43.4	38.9	17.8
		10.2	4.7	22.8	22.1	12.7	16.0
NYPK (5)	1 (20)	46.8	26.8	49.7	51.8	37.4	10.7
		6.9	2.7	33.2	27.8	18.7	14.4
DYPN (1)	0 (0)	54	22	74.2	45.5	34.1	20.5
		–	–	–	–	–	–
NSSK/NYPN (1)	0 (0)	27	27	68.5	31.5	61.7	6.8
		–	–	–	–	–	–
All (82)	21 (25.6)	50.8	23.4	50.8	42.2	43.7	14.1
		11.7	4.7	26.2	20.8	14.5	13.6

PPD, pocket probing depth; BOP, bleeding on probing. Values represent means ± (SD).

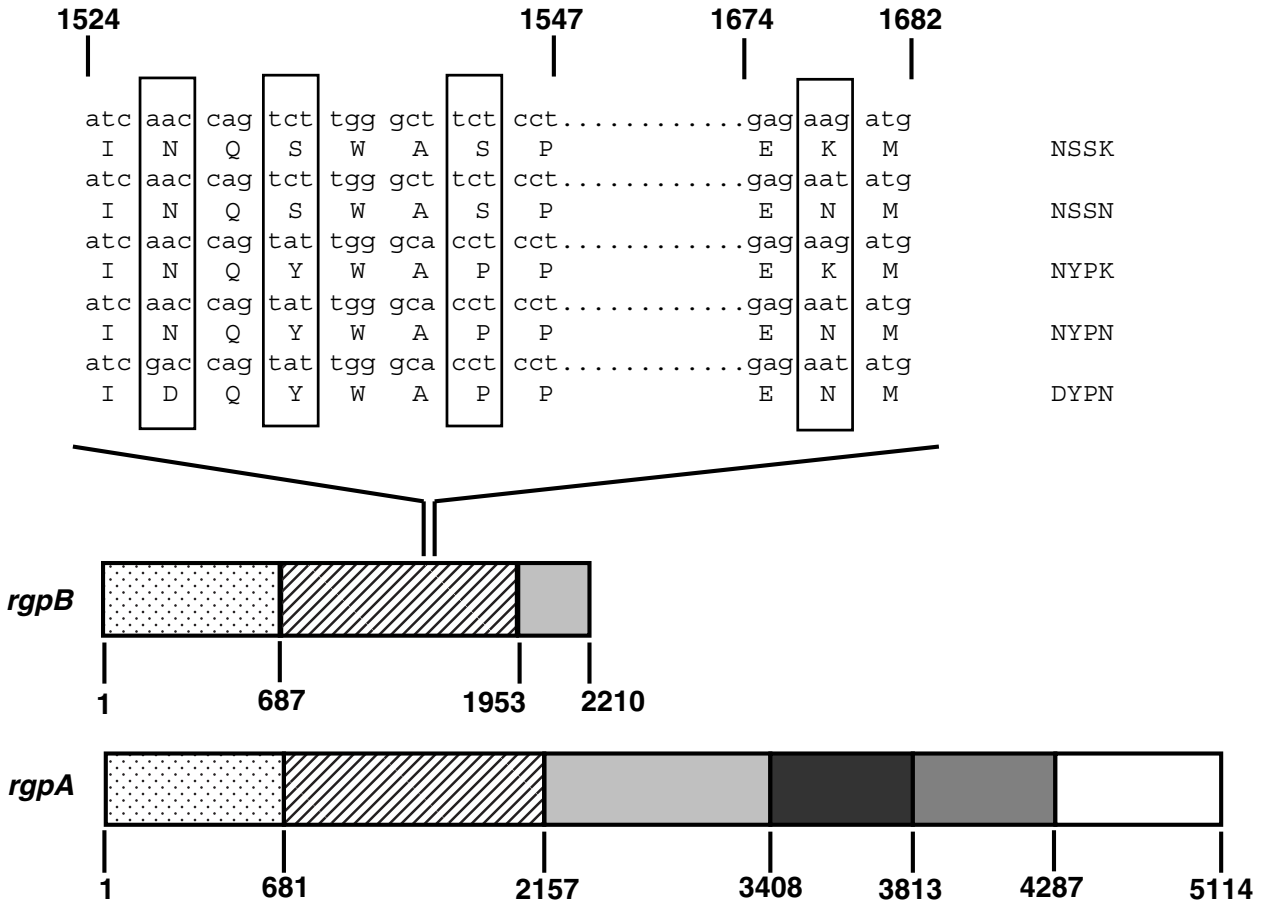

Fig. 1. Nucleotide sequence of *rgpB* and depiction of the different *rgpB* genotypes and the corresponding deduced amino acid sequences in the active site of RgpB.

subgingivally 3 months after therapy. Compared to the prevalences of *P. gingivalis* genotypes NYPN (9.7% of all patients) and NSSN (12.1% of all patients), this reduction was statistically significant ($p = 0.029$). The other *P. gingivalis* genotypes were reduced (NYPK, 1.2%) or could not be detected three months after therapy (DYPN, NSSK/NYPN) (Table 2).

Association of subgingival *rgpB* genotype colonization with pocket probing depths, bleeding on probing, number of teeth, and age

At baseline there was no statistically significant difference between patients carrying the different *rgpB* genotypes with respect to the frequency of sites with bleeding on probing, sites with a pocket probing depth of 1–3 mm, 4–6 mm, and sites with a pocket probing depth equal to or greater than 7 mm. Furthermore, no intergroup differences were observed regarding the number of teeth present and the age of the patients (Table 1).

Table 2. Number and percentage of patients that tested subgingivally positive for *rgpB Porphyromonas gingivalis* genotype NYPN, NSSN, NSSK, NYPK, DYPN or NSSK/NYPN before (before Tx) and after supra- and subgingival mechanical debridement (after Tx)

	P. gingivalis positive	
	Before Tx *n*	After Tx *n* (%)
NYPN	27	8 (29.6)
NSSN	26	10 (38.5)
NSSK	22	2 (9)
NYPK	5	1 (20)
DYPN	1	0
NSSK/NYPN	1	0
All	82	21 (25.6)

Association of subgingival *rgpB* genotype colonization with outcome 3 months after therapy

In patients subgingivally harbouring the *rgpB* genotypes NYPN, NSSN, NSSK or NYPK the percentage of sites showing an improvement of 2 mm or more in pocket probing depth varied between 18.5% ± 11.6% and 24.3% ± 18.4%. The percentage of stable sites (change of pocket probing depth from −1 mm to +1 mm compared to baseline) was between 75.0% ± 20.1% and 79.8% ± 11.4%, and the percentage of sites showing a further increase of 2 mm or more in pocket probing depth between 1.3% ± 1.4% and 1.7% ± 2.6% (Table 3). Bleeding on probing was reduced in all patients (Table 4). However, no significant difference with regard to changes in pocket probing depth and bleeding on probing could be

Table 3. Percentage of sites (± SD) showing improvement (≥ 2 mm compared to baseline), stability (−1 mm to +1 mm compared to baseline) or deterioration (≤ 2 mm compared to baseline) in pocket probing depth following supra- and subgingival mechanical debridement in each patient category (defined as subgingivally harbouring rgpB genotype NYPN, NSSN, NSSK, NYPK, DYPN or NSSK/NYPN)

	NYPN		NSSN		NSSK		NYPK		DYPN		NSSK/NYPN	
	%	± SD	%	± SD	%	± SD	%	± SD	%	± SD	%	+ − SD
Improvement	18.5	11.6	20.2	16.4	24.3	18.4	23.3	20.8	27.1	–	10.4	–
No change	79.8	11.4	78.3	15.8	74.4	17.9	75.0	20.1	72.9	–	86.5	–
Deterioration	1.7	1.8	1.5	2.0	1.3	1.4	1.7	2.6	–	–	3.1	–

Table 4. Percentage of sites (± SD) with bleeding on probing before (before Tx) and after supra- and subgingival mechanical debridement (after Tx) in each patient category (defined as subgingivally harbouring rgpB genotype NYPN, NSSN, NSSK, NYPK, DYPN or NSSK/NYPN)

	NYPN		NSSN		NSSK		NYPK		DYPN		NSSK/NYPN	
	%	± SD	%	± SD	%	± SD	%	± SD	%	± SD	%	± SD
BOP before Tx	46.6	24.8	47.8	29.5	57.9	22.8	49.7	33.2	74.2	–	68.5	–
BOP after Tx	27.6	20.3	21.5	14.5	29.3	17.9	18.3	10.2	25.8	–	45.7	–
Δ BOP (before Tx – after Tx)	19.0	26.5	26.3.7	22.4	28.6	27.9	31.4	33.4	48.4	–	22.8	–

BOP, bleeding on probing; Δ, difference.

detected in subjects harbouring the different *rgpB* genotypes.

Discussion

Most *in vitro* or *in vivo* studies involving oral pathogens are conducted with laboratory strains that are not necessarily comparable to clinical strains either in virulence or in metabolic function. In this regard, a recent study indicates that clinical isolates of *P. gingivalis* bear little resemblance to *P. gingivalis* strain W83 (21). Therefore, to study the genetics or proteomics of *P. gingivalis* or to develop pharmacological therapies, the analysis of *P. gingivalis* laboratory strains, e.g. *P. gingivalis* W83, alone is not likely to be sufficient (21) and has to be extended to clinical strains.

In the *P. gingivalis* laboratory strain HG66 (also known as strain W83 (22)) four specific amino acid substitutions in RgpB compared to RgpA were found to account for the different substrate specificity of the two enzymes (16). However, the sequence analysis of *rgpA* in 23 clinical *P. gingivalis* isolates revealed only one *rgpA* genotype. Moreover, compared to *rgpB* the flanking up- and downstream regions (data not shown) of the analysed *rgpA* exhibited a more homogenous nucleotide sequence, thus indicating that *rgpA* is more conserved compared to *rgpB*. In line with this result, the present study demonstrated additional *rgpB* polymorphisms in clinical isolates, resulting in different amino acid substitutions at the identical locations within the *rgpB* gene.

As seen in the present and in previous studies (10, 23–28), some *P. gingivalis* genotypes are found in periodontitis patients more frequently than others. In this regard, the *rgpB* genotypes NYPN, NSSN, and NSSK were detected in a similar proportion of patients that was much higher compared to *rgpB* genotypes NYPK and DYPN, indicating that these genotypes might be better adapted to environmental challenges.

The finding of only one patient with simultaneous subgingival colonization by two *rgpB* genotypes is in accordance with previous studies reporting that the intra-individual heterogeneity of *P. gingivalis* is limited to one or two clones in most subjects (23, 24, 29–31).

The results of the present study may further be of relevance with regard to the development of alternative therapeutic strategies directed against the RgpB protein. In this regard, specific inhibitors of Arg-X-specific proteases have been found to effectively reduce the virulence of *P. gingivalis* (32, 33), and thus may be a promising alternative therapeutic option. In addition, immunization against RgpB was found to reduce alveolar bone loss in a primate model (34). Interestingly, in another study, immunization against RgpA but not against RgpB was found to result in protection from bone loss in mice (12). This might be due to the infection with *P. gingivalis* expressing RgpB that is not neutralized by the humoral immune response, stimulated by vaccination against a structurally different RgpB protein. The existence of various *rgpB* genotypes that presumably result in the production of different Rgp proteins might therefore influence the effectiveness of a vaccine or an RgpB inhibitor specifically targeted against only one RgpB protein.

However, further studies are needed to explore the structure of the different RgpB proteins, their putative different functions or activities, and their influence on disease progression. Only then can their role in the pathogenesis of *P. gingivalis*-associated periodontitis and as a target for vaccination or alternative drug therapy with specific *rgpB* inhibitors be fully appraised.

Acknowledgements

This work was supported by the Federal German Ministry of Education and Research (Fö. 01KS9604/0), and

the Interdisciplinary Center for Clinical Research, Münster (IZKF Project C18). The NCBI is gratefully acknowledged for providing access to the BLAST program.

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