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The *rag* locus of *Porphyromonas gingivalis* might arise from *Bacteroides* via horizontal gene transfer

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Abstract *Porphyromonas gingivalis* is regarded as one of the risk factors of periodontitis. *P. gingivalis* exhibits a wide variety of genotypes. Many insertion sequences (ISs), located in their chromosomes, made *P. gingivalis* differentiate into virulent and avirulent strains. In this research, we investigated the prevalence of *P. gingivalis* in the gingival crevicular fluid (GCF) among periodontitis patients from Zhenjiang, China, detected the *P. gingivalis rag* locus distributions by multiplex polymerase chain reaction (PCR), and analyzed the origin of the *P. gingivalis rag* locus based on evolution. There were three *rag* locus variants co-existing in Zhenjiang. The results showed that the *rag* locus may be associated with severe periodontitis. This work also firstly ascertained that the *rag* locus might arise, in theory, from *Bacteroides* sp. via horizontal gene transfer.

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Introduction

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium and is considered as a major etiological agent in destructive periodontal diseases [1, 2]. It is frequently detected in a high ratio from severely inflamed subgingival lesions, whereas it may also be isolated from the healthy population at a very lower percentage, and these *P. gingivalis* constituted a significant proportion of the oral microflora. Previous studies have also shown phenotypic differences in the pathogenicity of strains of *P. gingivalis* in animal models [3] and in humans [4]. Therefore, *P. gingivalis* might be an important potential pathogen [5]. Recently, DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis have confirmed that the highly virulent *P. gingivalis* isolates always carry an *rag* locus, whereas the avirulent strain does not contain the *rag* locus [6–8].

The *rag* locus of virulent *P. gingivalis* encodes *RagA* (receptor antigen gene A), a 115-kDa outer membrane protein with features of a TonB-dependent receptor, and *RagB*, a 55-kDa antigen which makes periodontal patients induce an elevated immunoglobulin G response. The *RagA* and *RagB* form a putative co-distribution and co-transcription complexes on the surface of *P. gingivalis*. The complexes constitute a membrane transporter system, which involves the uptake or recognition of a specific carbohydrate or glycoprotein [9–11]. Four *rag* locus variants have been detected from periodontal patients [2]. The *rag* locus has three characteristics: (1) the

locus has a lower G+C content compared with the average values (48%) of the complete genome; (2) there is an insertion sequence (IS) 1126 with 1,338 bp (IS1126, now known as ISPg1), located upstream of the *rag* locus and flanked by 12-bp inverted repeats, which is a mobile element that generates a 5-bp target site duplication [12] and contains a single open reading frame (ORF) encoding the transposase which belongs to the IS5 family [13]; (3) polymerase chain reaction (PCR) and Southern blots results indicate that the *rag* locus has a restricted distribution within the species (only existing in virulent genotypes); the *rag* locus is also amplified from the subgingival samples of undetected *P. gingivalis*. The characteristics mentioned above make the experts speculate that the *rag* locus has arisen from exogenous genes and is obtained via horizontal gene transfer, and that the *rag* locus may represent a pathogenicity island in *P. gingivalis*. However, so far, no report has indicated from where the *rag* locus arises.

Since from where the *rag* locus arises is still unknown, the present work was to investigate the prevalence of *P. gingivalis* in the gingival crevicular fluid (GCF) of periodontitis patients from Zhenjiang, China, to detect the *rag* locus distributions among periodontitis patients infected by *P. gingivalis* using multiplex PCR, and to analyze the origin of the *P. gingivalis rag* locus based on evolution.

Materials and methods

Subjects, selection of sites, and sampling and DNA extraction

A total of 110 GCF samples were obtained from 110 patients (58 female and 52 male, aged between 15 and 68 years) going to hospital for periodontal diseases at the First Affiliated Hospital of Jiangsu University, China. Patients were screened for periodontal diseases by radiographic evidence of bone loss, or with visible plaque, marginal bleeding, bleeding on probing (BOP), and clinical attachment loss (CAL) and periodontal probing depth (PD) ≥4 mm at two or more sites [14, 15]. Exclusion criteria were pregnancy, smoking, and systemic antimicrobial/anti-inflammatory drug therapy within 6 months before the baseline visit. The 110 patients were classified by the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions [16, 17]: 57 patients were localized chronic periodontitis, 33 were generalized periodontitis, and 20 were necrotizing periodontal diseases (three patients were accompanied with diabetes and five patients had cardiovascular disease). The present study was conducted in accordance with the Declaration of Helsinki. All patients gave informed consent and the research protocol was approved by the Committee for Ethical Affairs of Jiangsu University.

GCF samples were obtained from the two deepest periodontal pockets in the maxilla according to Rüdin et al's technique [18]. Before sampling, the respective site was cleaned by cotton rolls; a gentle air stream of 5-s duration with a 90° angle to the tooth axis was used and supragingival plaque was eliminated. Standardized paper strips were inserted 1 mm into the sulcus and held for 30 s. Papers with blood contamination were discarded. To avoid evaporation, paper strips with GCF were immediately put into a sterile Eppendorf tube and stored at −20°C until laboratory analysis.

The isolates in the DNA were extracted by the boiling lysis method [19]. The templates were prepared by suspending in 200 mL of sterile water, followed by boiling for 10 min and centrifuging for 3 min.

PCR-detected *P. gingivalis* and multiplex PCR amplification of *rag* locus genes

The 16S rRNA specific primers were used to detect *P. gingivalis* and four different *rag* locus variants primers were used to amplify the *rag* locus among GCF samples containing *P. gingivalis* by multiplex PCR; the primers are listed in Table 1 [2, 20]. PCR assays were performed in volumes of 15 µl under the following conditions: 1.5 µl 10×PCR buffer ($Mg2^+$), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.1 µM of primers, and 1.5 units of

Table 1 Primers used in this study

No.	Primers	Sequences	Sizes
1	*16S rRNAF*	TGT AGA TGA CTG ATGGTG AAA ACC	197 bp
2	*16S rRNAR*	ACG TCA TCC CCA CCT TCC TC	
3	*rag1F*	CGC GAC CCC GAA GGA AAA GAT T	628 bp
4	*rag1R*	CAC GGC TCA CAT AAA GAA CGC T	
5	*rag2F*	GCT TTG CCG CTT GTG ACT TGG	979 bp
6	*rag2R*	CCA CCGTCA CCG TTC ACC TTG	
7	*rag3F*	CCG GAAGAT AAG GCC AAG AAA GA	422 bp
8	*rag3R*	ACG CCA ATT CGC CAA AGCT	
9	*rag4F*	CCG GAT GGA AGT GAT GAACAG A	738 bp
10	*rag4R*	CGC GGT AAA CCT CAG CAA ATT	

Taq DNA polymerase (Takara Biotechnology [Dalian] Co., Ltd.) for 10 min at 94°C for 35 cycles, with each cycle consisting of 30 s at 94°C, 45 s at 50°C (for 16S rRNA) and 54°C (for the *rag* locus), and 1 min at 72°C, with a final step of 10 min at 72°C for standard PCR assays.

Databases and search strategies

A comprehensive search by PSI-BLAST was performed in the National Center for Biotechnology Information (NCBI) protein database (Reference proteins, RefSeq proteins) using the published *ragA1* and *ragB1* proteins of *P. gingivalis* as query sequences. The cutoff values were *e-100* for *ragA1* and *e-10* for *ragB1*, respectively. The redundant sequences were removed by the DAMBE program, and the nucleotide sequences were also retrieved from GenBank.

Additionally, the function-similar proteins of the *rag* locus were also retrieved from GenBank, and the similar proteins included hemin/hemoglobin receptor protein (*HmuR*), TonB-linked adhesin (*Tla*) of *P. gingivalis*, TonB-dependent colicin I receptor (*CirA*) of *Escherichia coli* and *Salmonella enterica* [9, 21], and the lactoferrin and transferrin binding systems in *Neisseria* and *Haemophilus* species (*TBP2* and *LBP2*), *P. gingivalis orf3*, *Vibrio cholerae*-IS1358, and *E. coli orfH*. These similar proteins were used to analyze the gene characteristics.

Sequence comparison and phylogenetic analysis

Multiple sequence alignments were performed using the ClustalX package, and then the results were manually corrected. All alignment gap sites were eliminated before phylogenetic analyses. The phylogenetic trees were reconstructed with the neighbor-joining (NJ) and minimum evolution (ME) methods implemented in MEGA4.0. The bootstrap values were estimated with 1,000 replications. Multiple sequence alignments were also carried out between the *rag* locus and highly homologous sequences by ClustalX in order to further identify conserved protein motifs. The alignments were shaded by the BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html).

Tests for selection

To test whether the *rag* locus was evolving neutrally, we aligned the nucleotide sequences and then performed two likelihood ratio tests. Firstly, we compared a model in which all branches had the same dN/dS ratio of 1, in which each branch had its own ratio, to determine whether it was appropriate to use branch-specific tests for selection. As the free-ratio model was a significantly better fit to the data ($P>0.0001$), we then performed a second set of tests, comparing a model in which the dN/dS ratio was fixed to 1 with a model in which the dN/dS values of all branches were freely estimated. Model likelihoods were estimated using PAML [22].

Results

The prevalence of *P. gingivalis* and four *rag* locus variant gene distributions

We detected 29 *P. gingivalis* from 110 GCF samples, one from 57 cases of localized chronic periodontitis, nine from 33 generalized chronic periodontitis patients, and 19 from 20 necrotizing periodontal patients. Among the 29 GCF samples containing *P. gingivalis*, only 19 cases were amplified from the *rag* locus genes (ten to *rag1*, six to *rag2*, and three to *rag3*), while *rag4* was undetected. Among the 19 cases containing the *rag* locus, 18 cases were amplified from necrotizing periodontal patients and one from a generalized chronic periodontitis patient. In particular, three patients were accompanied with diabetes and five patients who suffered from cardiovascular diseases also amplified the *rag* locus, and all of the 19 cases were from at least three sites with a PD of 8 to 10 mm, accompanying clearly visible plaque or BOP and CAL, whereas none of the localized chronic periodontitis patients amplified the *rag* locus and the PD of the localized chronic periodontitis patients was normally between 4–6 mm (Fig. 1).

Phylogenetic analysis

A total of 153 homologous amino acid sequences were retrieved from the RefSeq proteins. The abundant sequences were removed by the DAMBE program and a total 50 protein sequences were included in the final dataset. In order to determine the evolutionary relationships between the *P. gingivalis rag* locus and homologous sequences, the phylogenetic trees were reconstructed by two different methods (ME and NJ methods) with high bootstrap values. There were three major clusters (I, II, and III), which were statistically supported (Fig. 2). Cluster I mainly included *Bacteroides* sp. TonB-dependent receptor (*SusC/CirA*), *P. gingivalis ragA*, *Porphyromonas* sp *ragA*, and *Capnocytophaga* sp. TonB-dependent receptor; cluster II was constituted of TonB-dependent receptor of *Parabacteroides* sp., *Dyadobacter fermentans*, *Robiginitalea biformata*, *Capnocytophaga ochracea*, *Flavobacterium johnsoniae*, *Croceibacter atlanticus*, *Bacteroides* sp., *Dyadobacter fermentans*, and *Parabacteroides* sp.; and *P. gingivalis ragB*, *Porphyromonas* sp. *ragB* and *SusD* of *Prevotella* sp., *Bacteroides* sp., and *Capnocytophaga* sp. were involved in the cluster III.

Fig. 1a–c The prevalence of *Porphyromonas gingivalis* and four *rag* locus variant gene distributions. **a** Agarose gel electrophoresis image of *P. gingivalis* detection. *Lane 1* marker, *lane 2* positive control, *lane 11* negative control, *lanes 3–10* samples. **b** Image of four *rag* locus variants gene distributions. *Lane 1* marker, *lane 2* positive control, *lane 11* negative control, *lanes 3–10* were samples. **c** Case numbers of varying degrees of periodontitis, the number of *P. gingivalis* detected in different periodontitis patients, and the four *rag* locus variant gene distributions
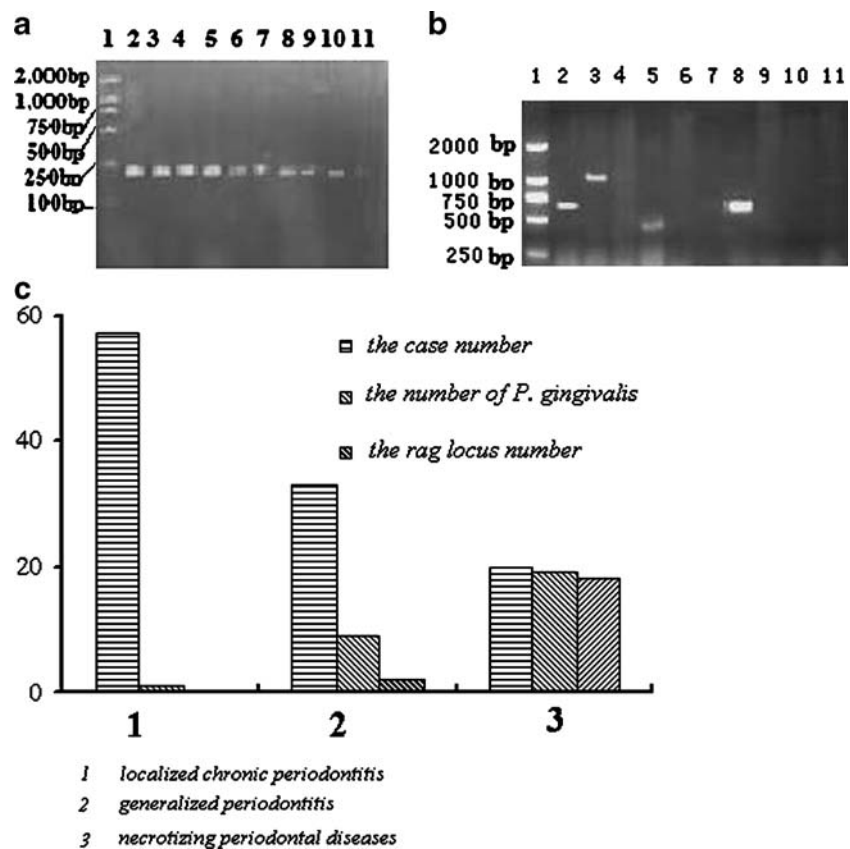
This tree showed that branches 1 and 2 in cluster I were the paralogy, and in the cluster III, the two branches were also paralogy. The *ragA* was highly similar to the TonB-dependent receptor of *Bacteroides* sp., and *ragB* was analogous to *ragB/SusD* of *Prevotella* sp., *Bacteroides* sp., and *Capnocytophaga* sp., respectively.

Analysis of G+C content

The G+C contents of the *rag* locus, homologous genes, and function-similar genes were analyzed by MEGA4.0 (Table 2). From this table, we could divide these gene sequences into three clusters: lower G+C contents (<40%), moderate (between 40% and 50%), and higher G+C contents (>50%). The *P. gingivalis rag* locus gene G+C contents were close to *Bacteroides* sp. *Sus*.

Multiple alignments of the *rag* locus and *Sus*

The *P. gingivalis rag* locus and *Bacteroides* sp. *Sus* were aligned for further evolutionary analysis (Fig. 3). The *ragA* and *SusC* proteins had three domains: the carboxy-peptidase regulatory region, TonB-dependent receptor plug Domain, TonB-dependent receptor (also named TonB box I, box II, and box III), and the *ragB* and *SusD* were also constituted of two domains, lipoprotein and *RagB/SusD*. All of these domains are shown in Fig 3. As shown

in Fig. 3a, the *RagA* motifs generally showed a good match to the three domains of *Bacteroides* sp. *SusC*, for example, P××GA×××V×G×TT×G××TD×DG×F×LS at the TonB box I; LKDA××T×IYG×RA×NGV at the TonB box II. However, the amino acids were often substituted in other domains. There are also some conserved positions in lipoprotein and *RagB/SusD* domains indicated in Fig. 3b. Nevertheless, the major positions in other domains showed far less conservation.

Selection

We investigated whether the *rag* locus was evolving neutrally and examined the ratios of nonsynonymous to synonymous changes (dN/dS) along each branch of the tree. The *ragA* locus had a dN/dS of 0.23, and the *ragB* locus had a dN/dS of 0.19. Both these values were significantly lower than a neutral dN/dS of 1 ($P>0.0001$, likelihood ratio test, Bonferroni correction for multiple tests), which indicated that these genes were probably evolving under long-term purifying selection.

Discussion

Though the exact roles that *P. gingivalis* played in the initiation and progression of periodontitis remain unclear,

Fig. 2 Phylogenetic relationship between the *P. gingivalis ragA/B* and highly homologous protein sequences
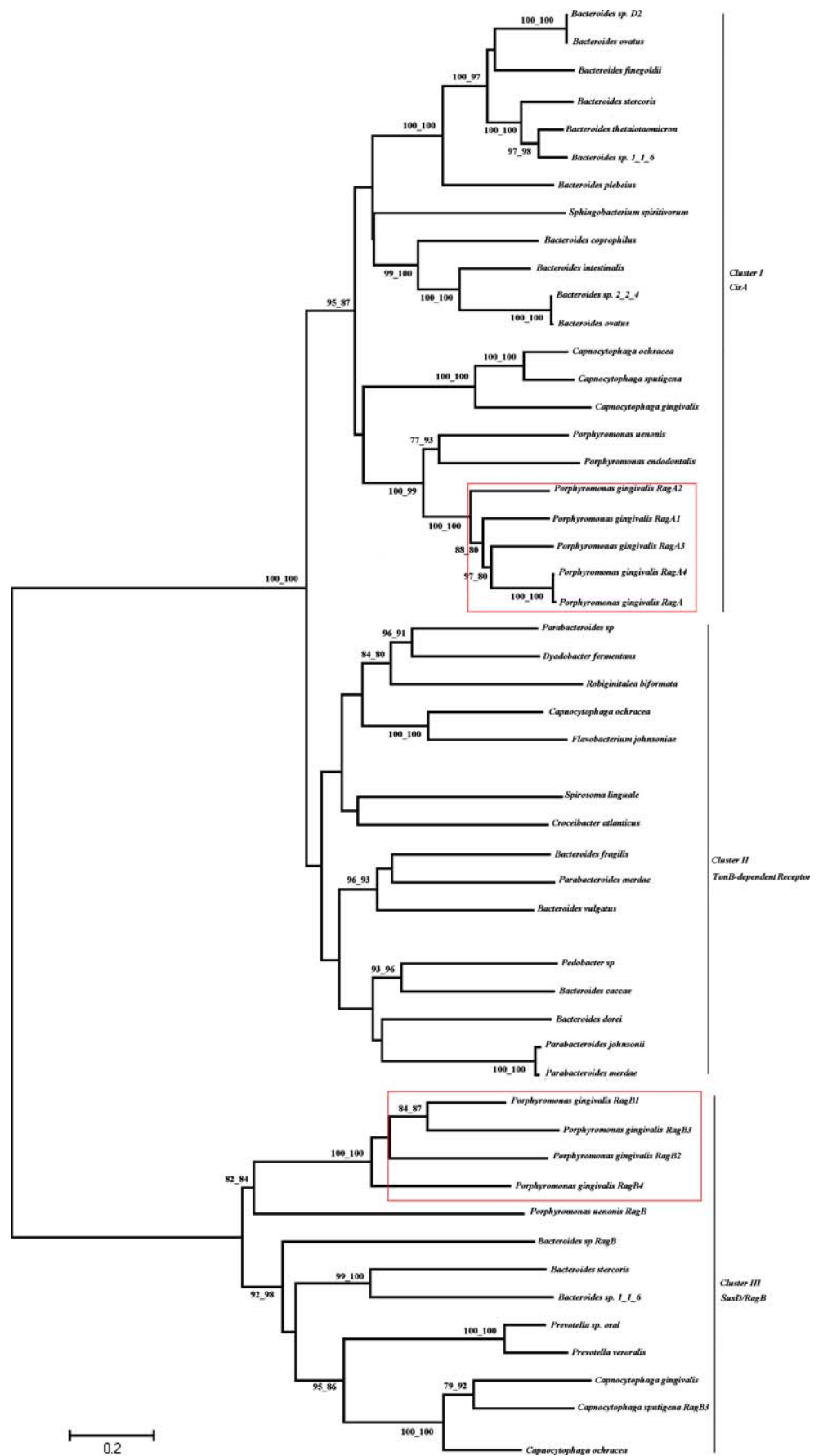
Table 2 G+C contents of the *rag* locus, homologous genes, and function-similar sequences

Strains	Encode protein	T (%)	C (%)	A (%)	G (%)	G+C (%)
Flavobacterium sp.	TonB-dependent receptor	31.7	16.4	34.9	16.9	33.3
Croceibacter sp.	TonB	35.1	20.4	29.2	15.3	35.7
H. influenza	TBP2	36.3	18.5	27.4	17.8	36.3
Haemophilus	TonB-dependent receptor	34.4	20	27.4	18.2	38.2
Capnocytophaga sp.	TonB-dependent receptor	31.3	20.2	28.8	20.7	40.9
E. coli	orfH	25.8	17.6	32.5	24.2	41.8
V. cholerae	IS1358	27.9	17.9	29.9	24.3	42.2
P. gingivalis	orf3	31	20.8	25.8	22.3	43.1
P. gingivalis	RagB	29.4	19.5	27	23.9	43.4
Bacteroides sp.	SusC	28.0	23.5	26.5	22.0	43.5
P. gingivalis	RagA	29	20.5	26.7	23.9	44.4
Parabacteroides	TonB-dependent receptor	27	19.8	26.9	26.3	46.1
N. meningitidis	LBP2	31.5	25.4	22.1	20.9	46.3
P. gingivalis	hmuR	26.2	24.8	27.2	21.9	46.7
N. meningitidis	TBP2	20.2	23	32.4	24.4	47.4
P. gingivalis	hemR	26.8	25.2	25.8	22.2	47.4
P. gingivalis	tla	24.2	24.6	26.7	24.4	49
E. coli	CirA	21.5	24.9	26.2	27.3	52.2
Robiginitalea sp.	TonB	26.7	29.6	20.1	23.5	53.1
Salmonella enterica	CirA	20.8	25.8	24.6	28.8	54.6

it is definite that *P. gingivalis* has been known to be a risk factor for periodontitis. Furthermore, accumulated evidence shows that an infection with *P. gingivalis* might predispose an individual to suffer from cardiovascular disease, diabetes, and the delivery of preterm infants [23–26]. All of these factors highlighted the importance of preventing infection from this bacterium. In this respect, information on the virulent factors and genotype of *P. gingivalis* among individuals becomes important for developing treatment strategies and preventing the transmission of the pathogen to uninfected individuals [12]. Previous researches stated that *P. gingivalis* produced a number of well-characterized virulence factors, including proteases, fimbriae, and capsule [27]. Recently, a significant association was observed between a carrier of the *rag* locus and a highly virulent phenotype in a murine model of soft tissue destruction [3, 4, 10, 28].

In order to further determine whether the *P. gingivalis* *rag* locus was associated with periodontitis, we investigated the prevalence of *P. gingivalis* and *rag* locus distributions in periodontitis patients in this study. We detected 29 *P. gingivalis* out of 110 patient GCF samples. However, the prevalence of *P. gingivalis* was lower in localized chronic periodontitis patients (1.75%) and generalized chronic periodontitis patients (27%) [29]. We also suspected that the method of samples collection was insensitive, but others reported that the prevalence of *P. gingivalis* was high by this method [30, 31]. At the same time, the ratios of

P. gingivalis in necrotizing periodontal patients (95%) were in accordance with other reports [32–34]. Among the 29 cases containing *P. gingivalis*, only 19 samples amplified the *rag* locus variants. And among 19 cases containing the *rag* locus, 18 (95%) were amplified from necrotizing periodontal patients and one (5%) from a generalized chronic periodontitis patient. All of the 19 cases containing the *rag* locus were from at least three sites with a PD of 8 to 10 mm, accompanying clearly visible plaque or BOP and CAL; at the same time, some *rag* locus carrier cases accompanied diabetes and cardiovascular disease, which indicated that the *rag* locus may be associated with severe periodontitis. Besides, we found that *rag1* locus variants may be the prevalent genotype in Zhenjiang; however, other *rag* locus variants were also common in other regions [29, 34–37], which showed that the *rag* locus distribution had the same obvious regional differences as the bacteria resistance to antibiotics [38].

The *rag* locus has a restricted distribution within the species and lower G+C contents. Furthermore, the *rag* locus is located downstream of IS1126. As was well known that the insertion sequence (IS) is a mobile element contributing to gene transposition which could lead to the inactivation of genes, the transcriptional activation of dormant genes, and genomic rearrangements, which are the main causes of the pathogenicity and genetic diversity of bacterial populations [39–41], we speculated that the *rag* locus might have arisen from exogenous genes and
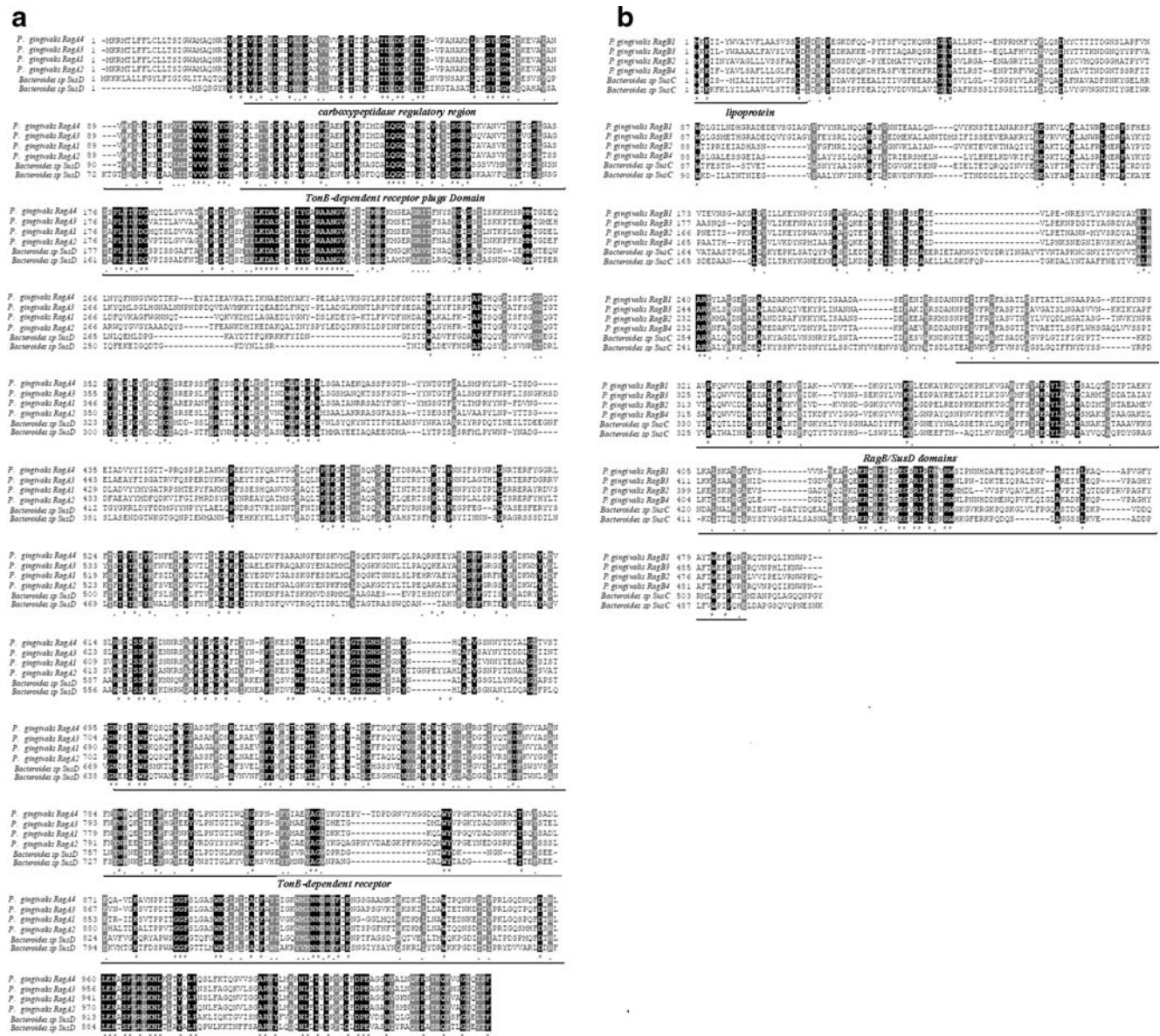
Fig. 3a, b Comparison of the *P. gingivalis rag* locus with *Bacteroides Sus*. **a**, **b** Multiple amino acid sequences alignment between the *P. gingivalis RagA* locus with *Bacteroides SusC*. The asterisks indicate positions within the proteins that were identical. The carboxypeptidase regulatory region, TonB-dependent receptor plug Domain, TonB-dependent receptor, lipoprotein, and *RagB/SusD* domains are shown

obtained through transposition. In order to prove our speculations, a phylogenetic analysis was performed to determine the evolutionary relationships between the *P. gingivalis rag* locus and homologous sequences. Many proteins were analyzed, for example, *RagA/B*, *Sus*, and *CirA*. All of these proteins belonged to the family of TonB-linked outer membrane receptors which were involved in the recognition and active transport of specific external ligands [36]. In organisms, a TonB-linked outer membrane receptor was also co-transcribed with an outer membrane lipoprotein [42]. The co-distribution and co-transcription of *ragA* and *ragB* strongly suggested that they were function-

ally linked, and the products might form a complex on the outer surface of *P. gingivalis*, which was involved in a TonB-linked process. *RagA*, *Sus*, and *CirA* were involved in maltose uptake, and *RagB* was associated with the active transport of lactoferrin and transferrin iron sources. *RagB* was closely similar to *RagB/SusD* of *Prevotella* sp., *Bacteroides* sp., and *Capnocytophaga* sp. Furthermore, this tree also showed that branches 1 and 2 in cluster I were the paralogy, and in cluster III, the two branches were also paralogy, which indicated that *ragA* was highly similar to the TonB-dependent receptor of *Bacteroides* sp., and *ragB* was analogous to *ragB/SusD* of *Prevotella* sp., *Bacteroides*

sp., and *Capnocytophaga* sp., respectively. To further determine the origin of the *rag* locus, the G+C contents of highly homologous genes to *rag* were analyzed. The analysis of G+C contents revealed that the G+C contents of the *rag* locus were under the average level of the *P. gingivalis* complete genome, including *HmuR*, and that the G+C contents of the *rag* locus were close to *Bacteroides* sp. *Sus*. Moreover, multiple alignments between the *rag* locus and *Sus* showed that the *RagA* and *SusC* were both constituted of three domains (TonB I, II, and III); and *RagB* and *SusD* were constituted of two domains: Lipoprotein and *RagB/SusD*, respectively. The amino acids were highly conserved, and our tests for selection showed that the *rag* locus were probably evolving under long-term purifying selection, which also indirectly indicated that the rag locus might arise from horizontal gene transfer.

From all of the evidence mentioned above, we could conclude that the *rag* locus was not duplicated by self-*HmuR*, but instead arose from *Bacteroides* sp. *Sus* via horizontal gene transfer. The exogenous *rag* locus promoted the *P. gingivalis* expression of virulence gene or the activation of dormant genes. Thus, *P. gingivalis* was differentiated into virulent and avirulent genotype. The *rag* locus-carried genotype may be associated with severe periodontitis.

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