**Porphyromonas gingivalis and its CRISPR-Cas system**

Tsute Chen and Ingar Olsen

*Department of Microbiology, The Forsyth Institute, Cambridge, MA, USA; Department of Oral Medicine, Infection, and Immunity, Harvard School of Dental Medicine, Boston, MA, USA; Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway*

**ABSTRACT**

The clustered regularly interspaced short palindromic repeats (CRISPRs) and their associated proteins (Cas) are immune systems in prokaryotes present in most Bacteria and Archaea. They provide adaptive immunity against foreign elements such as bacteriophages/viruses, plasmids and transposons. During immunization a small sequence of foreign DNA, a so-called spacer, is integrated into the CRISPR locus in the host cell. Spacers are then transcribed into small RNA guides that direct cleavage of foreign DNA by Cas nucleases. Immunization through spacer acquisition is transferred vertically to the progeny. It is possible that this genetic immune system of bacteria participates in modulating the microbiome of ‘chronic’ periodontitis, in which *Porphyromonas gingivalis* has been identified as a keystone pathogen causing microbial dysbiosis. An in-depth review of our current knowledge on the CRISPR-Cas systems in *P. gingivalis* is given in this paper with the attempt to understand how this anaerobic bacterium may protect itself in the periodontal pocket where bacteriophages are abundant and even out-number bacteria.

Clustered regularly interspaced short palindromic repeats CRISPRs belong to a family of DNA sequences characterized by short direct repeats (DRs) that are separated by spacers [1]. Almost half (45%) of the species in Bacteria and the majority (80%) in Archaea contain CRISPR-Cas systems [2]. Through an adaptation process, the system is able to store memories of previous exposure to foreign DNA in the spacer sequences across generations of progenies [3–5]. Upon invasion of the same foreign DNA to the ‘immunized’ cells, the spacer sequences are transcribed into small CRISPR RNAs (crRNAs) which was used by the Cas proteins as a guide to cleave the foreign DNAs through reverse sequence complementarity [4,6]. Hence, the spacers serve as the ‘acquired memory’ of this adaptive immunization system and are at the center of CRISPR defense providing immunity against specific phages or plasmids. Cas proteins are encoded by the cas genes that are located proximal to the CRISPR array. The mechanism of the CRISPR-Cas system was first demonstrated in 2007 in *Streptococcus thermophilus* [7], a bacterium widely used in the dairy industry to make yogurt and cheese. The Cas protein that is being used for genome editing was engineered from the cas9 gene of *S. pyogenes*, which is a pathogen that causes group A streptococcal (GAS) infection in humans [8].

CRISPR-Cas is a defense system commonly used by bacteria to fight phages/viruses, plasmids, transposons, integrative conjugative elements and genomic islands [2,9,10]. It is the only adaptive immune system in prokaryotes recognized so far. The system is also promising for programmable targeting of undesirable bacteria in microbial consortia. Interestingly, a significant association has been found between absence of CRISPR-Cas and the presence of antibiotic resistance [11]. *Porphyromonas gingivalis* is a Gram-negative anaerobic rod widely held as a keystone pathogen in ‘chronic’ periodontitis where it causes microbial dysbiosis [12,13]. It has also been associated with systemic diseases, e.g. cardiovascular and respiratory diseases, rheumatoid arthritis, premature birth and Alzheimer’s disease [14,15]. Almost 95% of clinical strains of *P. gingivalis* has been found to harbor CRISPR arrays [2]. The oral cavity is a frequent site for bacteriophages [16]. The vast majority of viruses detected in dental plaque has been identified as bacteriophages with only a few eukaryotic viruses such as herpesviruses and circoviruses present [17]. Bacteriophages may be active pathogens [16], This was supported by Lum et al. [18] who found that many of the CRISPR loci in the mouth were transcribed and matched oral phages.

The aim of this review is to answer the question, to what extent *P. gingivalis*, recovered from periodontitis-affected sites and other locations, is equipped with CRISPR-Cas systems and how the CRISPR-Cas may function to protect *P. gingivalis*. An overview of most of the CRISPR-Cas systems studied so far in *P. gingivalis* will be given. A broader survey of the CRISPR-Cas systems in the genus *Porphyromonas* is also being conducted and the results will be presented elsewhere.
Diversity, classification and evolution of CRISPR-Cas systems in bacteria and archaea

There is considerable diversity in the CRISPR-Cas systems of bacteria and archaea with respect to protein composition, effector complex structure, genome locus architecture, mechanisms of adaptation, pre-CRISPR (cr)RNA processing and interference [19]. The CRISPR-Cas system includes two main classes and six different types [20–22]. It has multi-subunit effector complexes in Class 1 and single protein modules in Class 2 [19]. In Class 2, two new types and several subtypes were recently identified [19]. The type VI systems were the first among CRISPR-Cas variants to exclusively target RNA. Also, in some of the Class 2 systems, the effector protein was additionally responsible for the pre-crRNA processing. It was suggested that Class 2 systems had evolved from mobile genetic elements on multiple, independent occasions.

Classes and types of the CRISPR-Cas system in P. gingivalis

In P. gingivalis two different classes and four types of Cas systems were detected in 19 P. gingivalis genomes examined, based on the presence of different signature genes [23]. Watanabe et al. [24] detected six CRISPR/Cas types classified by sequence similarity of repeats in 13 P. gingivalis strains and 12 other types in 46 other Porphyromonas species. The CRISPR spacers with potential targets in the genus Porphyromonas were approximately 23 times more abundant than those with possible targets in other genera (1,720/6,896 spacers vs. 74/6,896 spacers) [25]. Porphyromonas CRISPR/Cas may therefore act by selective interference against intra- and interspecies nucleic acids as part of its genome plasticity.

CRISPR structure and operons in P. gingivalis

Detailed structure of all the CRISPRs detected in 19 P. gingivalis genomes [23] is given in Table 1. To more comprehensively reveal the Cas proteins rather than relying on the somewhat incomplete default protein annotation provided by NCBI, all the protein sequences from the 19 P. gingivalis genomes were searched against a set of known Cas proteins to identify potential homologues. A total of 217,660 Cas proteins were downloaded from NCBI and used to search for the Cas homologues in P. gingivalis. This gave a complete picture of the Cas proteins in all P. gingivalis genomes that had been examined at that time. In addition, a few more CRISPR-associated genes were detected in P. gingivalis compared to those reported previously [23]. Figure 1 depicts the cas gene operons of the four types of CRISPR systems detected in P. gingivalis. The operon structure of each type of CRISPR systems is quite conserved in this species.

High diversity of CRISPR spacer contents in P. gingivalis

Preliminary examination of CRISPRs in three P. gingivalis strains (TDC60:89, W83:44 and ATCC33277:137) showed that CRISPR spacers with high nucleotide similarity to regions of P. gingivalis genomes were present and that the number of spacers was diverse among the three genomes [24]. In another 60 isolates of P. gingivalis the high diversity in CRISPR spacer contents was confirmed [25]. When the analyzed clusters were summed into four clusters, each type had distinct characters. The contents of the spacers differed amongst almost all the isolates, even if they were from the same patient. In another study the total number of spacers and unique spacers in P. gingivalis were 267/244 [26]. The 44 spacers in the genome of W83 had no significant similarity with any known sequences, but four spacers were similar to sequences of bacteria found in the oral cavity and gastrointestinal tract [2].

P. gingivalis may selectively acquire DNA by CRISPRs for survival

Watanabe et al. [24] suggested that P. gingivalis might be a useful model to reveal the biological significance of CRISPR spacers with high nucleotide similarity to the genome of the same species; also, that P. gingivalis may selectively acquire useful DNA sequences for its own survival and evolution by the CRISPR function. They concluded that the CRISPR spacers in P. gingivalis possibly inhibit both genomic rearrangements and intercellular recombination among P. gingivalis strains. Furthermore, three P. gingivalis genomes harbored cas genes for DNA and RNA targeting. This supported the notion that CRISPRs in P. gingivalis may provide resistance to foreign RNA, as well as to DNA [20,27].

CRISPRs may limit IS transposition and DNA uptake from other P. gingivalis cells

Watanabe et al. [24] also showed that P. gingivalis has high intra-species diversity caused by frequent insertion sequence (IS) transposition. Seven spacers (7/19) revealed high nucleotide similarity to regions related to insertion segments in the genome of P. gingivalis [24]. CRISPRs may limit both IS transposition and the introduction of foreign DNA, mainly from other P. gingivalis cells.
CRISPRs provide immunity against transposons

Transposase is an enzyme that binds to the two ends of a transposon and bring them together to form a loop. It then catalyzes movement of the transposon to another part of the genome by a cut and paste mechanism or replicative transposition. In the study by Chen et al. [27] who did comparative genomics of 19 *P. gingivalis* strains, a high prevalence of transposase proteins was found encoded in *P. gingivalis* (Table 2); actually as much as 149 copies in strain A7436. In another study transposases were found in all of the 35 genomes of *P. gingivalis* examined, varying in number from 8 (strains Ando, F0185, SDJ5) to 103 (A7436) [28]. The lower number of transposases detected in the original genomes that were not completely sequenced in this study was most likely due to the in-between-contig sequence gaps that may contain highly repeated sequences such as transposases and IS elements. The completed genome with the lowest number of mobility-related genes was that of strain A7A1-28, where 68 transposases were detected.

### Table 1. Detailed structure of all the CRISPRs detected in 19 *P. gingivalis* genomes (from ref [23]).

| Genome          | Number of CRISPRs detected | DR size (bps)*number of DRs |
|-----------------|-----------------------------|----------------------------|
| ATCC33277       | 3                           | 36bps*13; 36bps*5; 30bps*120 |
| HG66            | 3                           | 36bps*97; 36bps*11; 37bps*6 |
| 381             | 3                           | 36bps*11; 36bps*5; 30bps*120 |
| W83             | 4                           | 36bps*8; 36bps*8; 37bps*8; 30bps*24 |
| W50             | 5                           | 36bps*6; 36bps*8; 30bps*24; 37bps*4; 37bps*8 |
| A7436           | 5                           | 35bps*6; 36bps*8; 37bps*7; 37bps*5; 30bps*17 |
| AJW4            | 2                           | 46bps*4; 36bps*12 |
| F0570           | 3                           | 37bps*4; 30bps*6; 36bps*9 |
| JCVI SC001      | 3                           | 31bps*4; 36bps*6; 46bps*5 |
| SJ2             | 3                           | 36bps*9; 36bps*5; 46bps*22 |
| F0568           | 7                           | 30bps*4; 30bps*35; 30bps*10; 30bps*16; 36bps*6; 32bps*5; 32bps*5 |
| F0569           | 22                          | 30bps*8; 30bps*11; 30bps*5; 30bps*6; 30bps*5; 30bps*4; 30bps*4; 30bps*4; 30bps*5; 30bps*8; 30bps*4; 30bps*13; 30bps*10; 30bps*4; 30bps*8; 30bps*4; 30bps*5; 33bps*4; 36bps*4; 36bps*5; 46bps*5 |
| Ando            | 3                           | 47bps*15; 36bps*4; 36bps*16 |
| F0185           | 15                          | 36bps*9; 27bps*6; 27bps*5; 31bps*4; 31bps*5; 31bps*4; 31bps*4; 31bps*13; 31bps*4; 31bps*4; 31bps*5; 31bps*5; 31bps*6; 31bps*4; 31bps*4; 31bps*5 |
| W4087           | 4                           | 30bps*19; 30bps*7; 36bps*4; 36bps*5 |
| MP4-504         | 3                           | 36bps*6; 36bps*13; 30bps*37 |
| A7A1-28         | 4                           | 36bps*7; 30bps*26; 30bps*64; 37bps*9 |
| F0566           | 7                           | 32bps*5; 32bps*4; 30bps*11; 30bps*19; 46bps*4; 46bps*4; 46bps*10 |
| TDC60           | 5                           | 37bps*5; 30bps*17; 30bps*66; 30bps*16; 36bps*4 |
| PaDSM20707      | 2                           | 33bps*30; 23bps*4 |

**P. gingivalis** may limit genetic exchange in populations predisposed to exchange

Phillips et al. [29] found highly expressed transcripts of CRISPR regulatory small non-coding regulatory RNA (sRNA) in the intergenic sequences (IGS) upstream of CRISPR-associated (cas) gene arrays in *P. gingivalis* W83. Identification of these highly expressed sRNA transcripts indicated another mechanism that *P. gingivalis* uses to limit genetic exchange in a population with a genotype predisposed to a high degree of such exchange.

![Figure 1. Four types of CRISPR-Cas systems detected by us in *P. gingivalis.*](image-url)
Cas proteins do not necessarily follow CRISPRs in *P. gingivalis*

In the study by Chen et al. [23] CRISPR arrays were detected in all the 19 genomes examined, including the outgroup *Porphyromonas asaccharolytica* PaDSM20707 (Table 3). In contrast, Cas proteins were found only in select strains. Of 35 strains of *P. gingivalis* examined strain 13_1 encoded as many as 19 Cas homologous proteins and five different CRISPR systems were observed [28]. On the other hand, JSVI_SC001, which is an environmental strain, contained no detectable proteins even though three CRISPR-like DNA sequence structures were detected.

Previously, the structure of the *cas* gene in *P. gingivalis* was reported to be conserved at two CRISPR loci, type 1-C and III-B [24]. By using the online software CRISPR finger (http://crispr.i2bc.paris-saclay.fr/Server) strain F0569 was found to have the highest number of CRISPR arrays (Table 3), but this strain did not have the highest number of Cas proteins [23]. The length of the direct repeat (DR) elements ranged from 23 to 47 bps among all the CRISPR arrays detected while the number of DRs in the arrays varied from five to 121 copies. The closely related strains ATCC33277 and 381 had three copies of almost identical CRISPR arrays with 121 DR sequences and 120 spacer sequences.

The number of CRISPR systems in the genome may indicate the difficulty in incorporating foreign DNA, whereas the number of DRs in the CRISPR elements may indicate CRISPR activity in the past for the genome. Zhou et al. [30] reported slightly higher Shannon-Wiener diversities of DRs in periodontal disease than in periodontal health, but the spacer composition exhibited significantly higher diversities in healthy periodontium. This may imply that healthy people have a robust and functional bacterial community able to resist invasion from phages. Strain JSVI_SC001 of *P. gingivalis*, isolated from single cells in the biofilm of a hospital bathroom sink, had three copies of CRISPR arrays with DRs of 31, 26 and 45 bps and repeat numbers of five, seven and six, respectively [23]. It might be that this strain had a type Cas protein clearly different from those in the oral strains. However, if this strain lacked any functional Cas protein, it could be susceptible to bacteriophage infection or activation by prophages possibly present, as indicated by the detection of 25 copies of phage-related proteins in this strain [23].

**Proteins related to reproduction of phage may be related to the presence of CRISPR/Cas systems in *P. gingivalis***

Many proteins related to reproduction of phage were detected in all the 19 strains of *P. gingivalis* examined [23]. This may help explain the abundant prevalence of CRISPR/Cas systems in this species.
Functional activity of *P. gingivalis* CRISPRs detected in vivo

All four CRISPR regions in *P. gingivalis* W83 were found to be transcribed [2]. For one of the four arrays, activity was demonstrated in vivo against double-stranded DNA constructs that contained protospacer sequences accompanied by the 3’ end of an NGG protospacer-adjacent motif (PAM) (N is any nucleobase followed by two guanine (G) nucleobases). This was concluded from introduction of the flanked protospacer to foreign DNA, which caused specific degradation.

**CRISPR RNAs in the *P. gingivalis* CRISPR-Cas 1-C system**

CRISPR RNAs (crRNAs) are important elements in the CRISPR-Cas system being responsible for the system’s selectivity and effectiveness [6]. crRNAs contain a spacer sequence flanked with 5’ and 3’ handles that originate from repeat sequences important for recognition of small RNAs by Cas. Burmistrz et al. [6] found that the 5’ handle of crRNA in *P. gingivalis* was essential for the activity while the repeat element localized to the 3’ handle was less important. In addition, modification of the structure of the repeat element inhibited its activity, possibly by interacting with the primary processing nuclease.

**Concluding remarks**

The CRISPR/Cas system is known to prevent acquisition of conjugative plasmids, integrative conjugative elements and environmental DNA via natural transformation. To what extent this occurs in *P. gingivalis* is not yet clear. However, the widespread distribution of CRISPRs in *P. gingivalis* strains indicates that CRISPRs have an important role in this species. Interestingly, due to the fact that the spacer sequences derived more from its own genome, rather than foreign DNA, CRISPRs in this species may have developed as a selective system helping to regulate gene transfer. In contrast to CRISPRs, Cas proteins are only present in select *P. gingivalis* strains. The reason for this is also unclear. Previously, the structure of the *cas* gene in *P. gingivalis* was reported to be conserved at CRISPR loci. Most importantly, the CRISPR-Cas system of *P. gingivalis* has been found active in vivo providing protection against foreign genetic elements. This was supported by the observation that in most *P. gingivalis* genomes CRISPR-associated genes were found together as an operon and not scattered around the genome. More studies of the CRISPR systems in *P. gingivalis* both in wet laboratory and in-silico are needed to understand the true role of the CRISPR system in this periodontal pathogen and to what extent it is shaping the periodontal microbiome and the biological “arms race” occurring there. The CRISPR-Cas system
may be involved in more functions than just the ‘immune’ system against the invader. A deeper understanding of this particular system may even shed more light on why *P. gingivalis* deserves the term keystone pathogen in periodontitis.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

TC is funded by Grant R37-DE016937 “A Foundation for the Oral Microbiome and Metagenome” from The National Institute of Dental and Craniofacial Research. IO is grateful for funding through the European Commission (FP7-HEALTH-306029 “TRIGGER”).

**References**

[1] Jansen R, van Embden JDA, Gaastra W, et al. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43:1565–1575.

[2] Burmistrz M, Dudek B, Staniec D, et al. Functional analysis of *Porphyromonas gingivalis* W83 CRISPR-Cas systems. J Bacteriol. 2015;197:2631–2641.

[3] Sampson TR, Weiss DS. CRISPR-Cas systems: new players in gene regulation and bacterial physiology. Front Cell Infect Microbiol. 2014;4:37.

[4] Marraffini LA. CRISPR-Cas immunity in prokaryotes. Nature. 2015;526:55–61.

[5] Sternberg SH, Richter H, Charpentier E, et al. Adaptation in CRISPR-Cas systems. Mol Cell. 2016;61:797–808.

[6] Burmistrz M, Rodriguez Martinez JL, Krochmal D, et al. Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) RNAs in *Porphyromonas gingivalis* CRISPR-Cas I-C system. J Bacteriol. 2017;199 pii: e00275–17.

[7] Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315:1709–1712.

[8] Cunningham MW. Pathogenesis of group A streptococcal infections and their sequelae. Adv Exp Med Biol. 2008;609:69–72.

[9] Zhang Q, Rho M, Tang H, et al. CRISPR-Cas systems target a diverse collection of invasive mobile genetic elements in human microbiomes. Genome Biol. 2013;14:R40.

[10] Briner AE, Barrangou R. Deciphering and shaping bacterial diversity through CRISPR. Curr Opin Microbiol. 2016;31:101–108.

[11] Burley KM, Sedgley CM. CRISPR-Cas, a prokaryotic adaptive immune system, in endodontic, oral, and multidrug-resistant hospital-acquired *Enterococcus faecalis*. J Endod. 2012;38:1511–1515.

[12] Hajishengallis G, Darveau RP, Curtis MA. The keystone pathogen hypothesis. Nat Rev Microbiol. 2012;10:717–725.

[13] Hajishengallis G, Lamont RJ. Breaking bad: manipulation of the host response by *Porphyromonas gingivalis*. Eur J Immunol. 2014;44:328–338.

[14] Olsen I. From the Acta Prize Lecture 2014: the periodontal-systemic connection seen from a microbiological standpoint. Acta Odontol Scand. 2015;73:563–568.

[15] Olsen I, Singhrao SK. Poor oral health and its neurological consequences: mechanisms of *Porphyromonas gingivalis* involvement in cognitive dysfunction. Curr Oral Health Rep. 2019;6(2):120–129.

[16] Edlund A, Santiago-Rodriguez TM, Boehm TK, et al. Bacteriophage and their potential roles in the human oral cavity. J Oral Microbiol. 2015;7:27423.

[17] Naidu M, Robles-Sikisaka R, Abeles SR, et al. Characterization of bacteriophage communities and CRISPR profiles from dental plaque. BMC Microbiol. 2014;4:175.

[18] Lum AG, Ly M, Santiago-Rodriguez TM, et al. Global transcription of CRISPR loci in the human oral cavity. BMC Genomics. 2015;16:401.

[19] Koozin EV, Makarova KS, Zhang F. Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol. 2017;37:67–78.

[20] Makarova KS, Haft DH, Barrangou R, et al. Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol. 2011;9:467–477.

[21] Makarova KS, Wolf YI, Alkhnbashi OS, et al. An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol. 2015;13:722–736.

[22] Shmakov S, Smargon A, Scott D, et al. Diversity and evolution of class 2 CRISPR-Cas systems. Nat Rev Microbiol. 2017;15:169–182.

[23] Chen T, Siddiqui H, Olsen I. In silico comparison of 19 *Porphyromonas gingivalis* strains in genomics, phylogenetics, phylogenomics and functional genomics. Front Cell Infect Microbiol. 2017;7:28.

[24] Watanabe T, Nozawa T, Aikawa C, et al. CRISPR regulation of intraspecies diversification by limiting IS transposition and intercellular recombination. Genome Biol Evol. 2013;5:1099–1114.

[25] Watanabe T, Shibasaki M, Maruyama F, et al. Investigation of potential targets of *Porphyromonas* CRISPRs among the genomes of *Porphyromonas* species. PLoS One. 2017;12:e0183752.

[26] Endo A, Watanabe T, Ogata N, et al. Comparative genome analysis and identification of competitive and cooperative interactions in a polymicrobial disease. Isme J. 2015;9:629–642.

[27] Sorek R, Kunin V, Hugenholtz P. CRISPR—a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol. 2008;6:181–186.

[28] Olsen I, Chen T, Tribble GD. Genetic exchange and reassignment in *Porphyromonas gingivalis*. J Oral Microbiol. 2018;10(1):1457373.

[29] Phillips PL, Progulske-Fox A, Grieshaber NA. Expression of *Porphyromonas gingivalis* small RNA in response to hemin availability identified using microarray and RNA-seq analysis. FEMS Microbiol Lett. 2014;351:202–208.

[30] Zhou H, Zhao H, Zhen J, et al. CRISPRs provide broad and robust protection to oral microbial flora of gingival health against bacteriophage challenge. Protein Cell. 2015;6:541–545.

[31] Kanchisw M, Sato Y, Morishima K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. J Mol Biol. 2016;428:726–731.