Oral Microbiology Immunology 2008: 23: 84–87
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Short communication

Identification of the genes associated with a virulent strain of *Porphyromonas gingivalis* using the subtractive hybridization technique

M. Tachibana-Ono[1], A. Yoshida[1], S. Kataoka[2], T. Ansai[1], Y. Shintani[1], Y. Takahashi[1], K. Toyoshima[2], T. Takehara[1]

Divisions of [1]Community Oral Health Science, [2]Oral Histology and Neurobiology, Kyushu Dental College, Kokurakita-ku, Kitakyushu, Japan

Tachibana-Ono M, Yoshida A, Kataoka S, Ansai T, Shintani Y, Takahashi Y, Toyoshima K, Takehara T. Identification of the genes associated with a virulent strain of Porphyromonas gingivalis *using the subtractive hybridization technique. Oral Microbiol Immunol 2008: 23: 84–87.* © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Background/aims: *Porphyromonas gingivalis*, a major etiological organism implicated in periodontal disease, can be classified into virulent and avirulent strains. Our aim was to identify a gene for the virulence of *P. gingivalis*.

Methods: The subtractive hybridization technique was employed to identify the genes specific to *P. gingivalis* W83, a virulent strain. In this study, *P. gingivalis* W83 was used as the tester strain, and *P. gingivalis* ATCC 33277 was the driver strain. The prevalence of W83-specific genes was determined by Southern blot analysis of several *P. gingivalis* strains.

Results: We obtained 575 colonies using the subtractive hybridization technique. From among these, 26 DNA fragments were subjected to a homology search using the BLAST program. Compared with strain ATCC 33277, strain W83 contained 12 unique clones. The specificities of the isolated DNA fragments were analyzed among four *P. gingivalis* strains by Southern blot analysis. Five genes showed specificity for strain W83 compared with strain ATCC 33277. All five genes were also identified in strain W50.

Conclusions: The subtractive hybridization technique was effective in screening the two strains for specific DNA sequences, some of which might be responsible for determining virulence. The results suggested that several genes specific to strain W83 were associated with its virulence. Further analysis of these DNA fragments will provide important information on the pathogenesis of virulent *P. gingivalis* strains.

Key words: periodontitis; *Porphyromonas gingivalis;* subtractive hybridization technique; virulence

Akihiro Yoshida, Division of Community Oral Health Science, Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan
Tel.: +81 93 582 1131 (Ext. 2103);
fax: +81 93 591 7736;
e-mail: akihiro@kyu-dent.ac.jp
Accepted for publication May 7, 2007

Periodontitis is one of the most common infectious diseases worldwide (1, 3, 5, 7, 8, 12). The infection appears to result from the inflammatory response of the host to mixed anaerobic bacterial infections of supportive tissues of teeth (3, 7). *Porphyromonas gingivalis*, a gram-negative, black-pigmented anaerobe, is recognized as one of the primary pathogens in severe manifestations of adult periodontitis (1, 3, 5, 7, 8, 10, 12, 13). Furthermore, virulent strains of *P. gingivalis* have been isolated frequently from severe periodontal lesions, whereas avirulent strains were detected more commonly in healthy subjects (6). In recent years, the search for virulence factors of this organism has been greatly facilitated by molecular genetics (8). The analysis of genetic elements is crucial to understanding the properties and roles of virulence factors, and the subtractive

hybridization (SH) technique has great potential in comparing genomic sequences between related bacterial strains that differ in virulence (2).

In this study, our aim was to identify a gene associated with the virulence factors of *P. gingivalis*. Using the SH technique, we detected a unique gene in the virulent strain *P. gingivalis* W83 by comparing its chromosomal DNA with that of the avirulent strain *P. gingivalis* ATCC 33277.

The *P. gingivalis* strains were grown at 37°C under anaerobic conditions (85% N_2, 10% H_2, and 5% O_2) in GAM broth (Nissui Medical, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). The SH technique was performed as previously described (Fig. 1) (2, 14). Briefly, chromosomal DNA from *P. gingivalis* W83 (target) was completely digested with *Sau*3AI and purified using a polymerase chain reaction (PCR) purification kit (Qiagen GmbH, Hilden, Germany). The *Sau*3AI-digested DNA fragments of *P. gingivalis* W83 were ligated with the oligonucleotide adapters RBam12 and RBam24 (Table 1) for 18 h at 11°C and purified with a PCR purification kit. To prepare the driver DNA, chromosomal DNA from *P. gingivalis* ATCC 33277 was digested with *Hin*dIII and *Eco*RI. After digestion, the DNA was precipitated with ethanol-sodium acetate and dissolved in distilled water. The first subtractive hybridization was performed in 10 μl of a reaction mixture containing 2 μg of the driver DNA from *P. gingivalis* ATCC 33277, 20 ng of the R-adaptor-linked *Sau*3AI fragments from *P. gingivalis* W83, 10 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS), and 1 mM ethylenediaminetetraacetic acid (EDTA). The DNA mixture was denatured at 100°C for 2 min and then placed at 55°C. After adding 2 μl 5 M NaCl, the mixture was left to hybridize at 55°C for 24 h. A 2-μl aliquot of the reaction mixture was diluted to 40 μl with a PCR mixture containing 100 pmol RBam24, 0.25 mM of each deoxynucleoside triphosphate, 5 U *ExTaq*, and 1× Ex*Taq* buffer (Takara Bio, Shiga, Japan) to fill in the ends corresponding to the RBam24 adaptor. After denaturation for 5 min at 94°C, the mixtures were amplified by PCR for 30 cycles of 1 min at 70°C, 3 min at 72°C, and 1 min at 94°C, followed by 1 min at 94°C and 10 min at 72°C. The PCR products were purified using a PCR purification kit. The RBam24 adaptor was removed from the PCR products by digestion with *Sau*3AI, and the DNA fragments were purified using the PCR purification kit. In a 40-μl volume, 2 nmol of the second adaptors, JBam12 and JBam24 (Table 1), were ligated for 18 h at 11°C and purified using a PCR purification kit. The second-round subtractive hybridization was performed with 2 ng DNA from the first-round PCR products and 2 μg of the driver DNA from *P. gingivalis* ATCC 33277, prepared as described above. The second-round PCR products were digested with *Sau*3AI, cloned into *Bam*HI-digested pBluescript II SK^+ (Stratagene, La Jolla, CA), and then used to transform *Escherichia coli* DH5α (Invitrogen, San Diego, CA). We then determined the nucleotide sequences of the inserts.

Following SH, we obtained 575 colonies. After the DNA fragments had been isolated and sequenced we had 26 different sequences; these were subjected to a homology search using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and were subsequently identified. Oligonucleotide primers specific to the 26 DNA fragments were designed, and their specificities were analyzed by conventional PCR (data not shown). Twelve clones were identified as containing DNA fragments unique to strain W83 as compared with strain ATCC 33277 (Table 2). Digoxigenin-labeled PCR probes were generated using the oligonucleotide primers listed in Table 1 and a PCR DIG probe

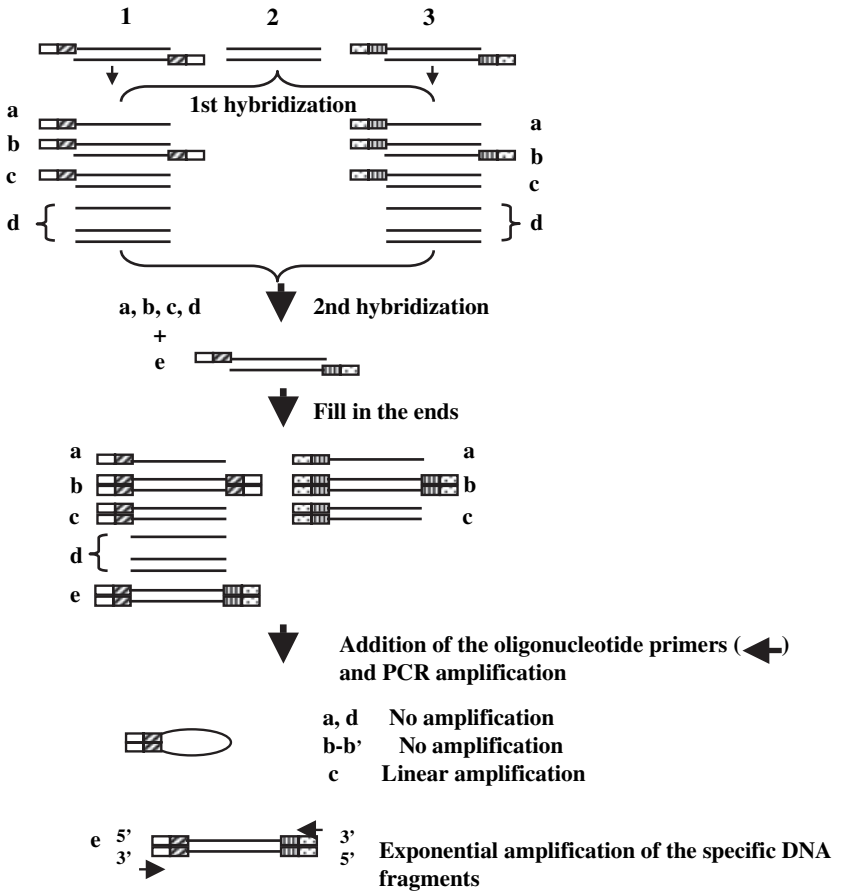

Fig. 1. The principle of subtractive hybridization. *Porphyromonas gingivalis* W83 was used as the tester strain, and ATCC 33277 was the driver strain. Genomic DNA from *P. gingivalis* W83 was subtracted using DNA from the avirulent strain ATCC 33277. 1: Adapter1-binding tester (*P. gingivalis* W83); 2: Driver DNA (*P. gingivalis* ATCC 33277); 3: Adapter2R-binding tester DNA (*P. gingivalis* W83). The first hybridization was performed as follows. An excess of driver DNA was added to each adaptor-ligated tester sample. The samples were denatured and allowed to anneal, generating the type a, b, c, and d molecules in each sample. During the second hybridization, the two primary hybridization samples were mixed together without denaturing and formed new type e hybrids. These new hybrids were tester molecules with different ends, which corresponded to the sequences of Adaptors 1 and 2R. After the ends were filled in by DNA polymerase, the type e molecules had different primer annealing sites on their 5′ and 3′ ends. The entire population of molecules was subjected to PCR to amplify the desired tester-specific sequences. During this PCR, only type e molecules, the equalized differentially expressed tester-specific molecules with two different adaptors, could be amplified exponentially.

Table 1. Oligonucleotide primers used in this study

Oligonucleotide primer	Sequence (5′ to 3′)	Amplicon size (bp)	Gene
RBam12	GATCCTCGGTGA		SH[1]
RBam24	AGCACTCTCCAGCCTCTCACCGAG		SH
JBam12	GATCCGTTCATG		SH
JBam24	ACCGACGTCGACTATCCATGAACG		SH
W83-1 F[2]	GACTGCCTATGCAGTGAT	231	Capsular polysaccharide biosynthesis gene
W83-1 R[3]	GCCAGTCTAGTTCTGTCT		
W83-9 F	GAACGAGAAGGCTTACTC	290	Hypothetical protein
W83-9 R	CCTAACATTGCCTCTCCT		
W83-11, 101, 165 F	ACGCAAGGCAAGTACACT	223	Transposase (ISPg4)
W83-11, 101, 165 R	TTTGCACCTCTACGGCAT		
W83-16 F	TTGGATGGTGAAGGTCTC	236	Hypothetical protein
W83-16 R	TCTAACAACGTCTCCCTG		
W83-43 F	CATATAGCGGACAAGGCA	227	Membrane-bound lytic murein transglycosylase D
W83-43 R	AGGATTTGGAGAGACTCC		
W83-97 F	CGCCAAACAGATGCAAGA	239	Sensor histidine kinase
W83-97 R	GGAGTCTGTTTCTGGTGT		
W83-149, 363 F	CTAACGGGTTACCTACCT	199	Helicase
W83-149, 363 R	GTACCCTTTTGTTGCGAC		
W83-230 F	TCGGCCGCTTCTATCATA	245	Fibronectin type III domain protein
W83-230 R	TGGAGCCAATGTATGGCA		
W83-282 F	GTACGTTCGTTGCGAGAA	231	Hypothetical protein
W83-282 R	CAATAGTGGCATTGCTGC		
W83-362 F	GCTGAATTTGCACCGGAT	212	Hypothetical protein
W83-362 R	GAGTAGATGCGAAGGTCT		
W83-367 F	TCTTCAGCCAGTATGGAG	262	Helicase (*cas3*)
W83-367 R	CGAGTGGAGTTAGCTTGT		
W83-558 F	TCGTGGTATTTACCGCCT	238	Sensor histidine kinase
W83-558 R	ACGTACACCATCTCATCG		

[1]SH, adaptors for subtractive hybridization; [2]F, forward primer; [3]R, reverse primer.

Table 2. DNA fragments obtained by subtractive hybridization

Clone	Homology with BLASTX	G+C content (%)	Insert size (bp)	Accession number
Pg W83-1	Capsular polysaccharide biosynthesis gene	38.3	1224	AE017172
Pg W83-9, 538	Hypothetical protein	36.7	1500	AE017178
Pg W83-11, 101, 165, 339	Transposase (ISPg4)	45.8	1158	AE017177
Pg W83-16	Hypothetical protein	38.5	4032	AE017174
Pg W83-43	Membrane-bound lytic murein transglycosylase D	54.4	1356	AE017172
Pg W83-97	Sensor histidine kinase	39.1	1866	AE017177
Pg W83-149, 363	Helicase	48.0	3483	AE017175
Pg W83-230	Fibronectin type III domain protein	47.9	1623	AE017174
Pg W83-282	Hypothetical protein	39.7	1296	AE017175
Pg W83-362	Hypothetical protein	51.3	1506	AE017172
Pg W83-367	Helicase (*cas3*)	38.4	2337	AE017178
Pg W83-558	Sensor histidine kinase	46.3	1284	AE017174

Table 3. Southern blot analysis

Clone	Homology with BLAST	W83	33277	W50	381
Pg W83-1	Capsular polysaccharide biosynthesis gene	+[1]	–[2]	+	–
Pg W83-11, 101, 165, 339	Transposase (ISPg4)	+	–	+	–
Pg W83-43	Membrane-bound lytic murein transglycosylase D	+	–	+	+
Pg W83-230	Fibronectin type III domain protein	+	–	+	–
Pg W83-367	Helicase (*cas3*)	+	–	+	–

[1]+, positive for W83 probe; [2]–, negative for W83 probe.

synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany); the specificities were analyzed among four *P. gingivalis* strains by Southern blot analysis. The results are summarized in Table 3. Of the 12 clones, except for five hypothetical genes, five genes showed specificity to strain W83 compared with strain ATCC 33277, according to Southern blot analysis. In addition, these five genes were cross-reacted to strain W50 by Southern blotting (Table 3). The genes related to the histidine kinase sensor (*Pg* W83-97 and *Pg* W83-558) were not observed in strain ATCC 33277 by conventional PCR but were detected by Southern blot analysis. The existence of these genes in ATCC 33277 was therefore uncertain, and further research will be required on this issue. We additionally identified the gene associated with capsular polysaccharide synthesis. This gene is registered as PG0109 in the CMR database, TIGR. We found that the homologue of this gene was not conserved in strains ATCC 33277 and 381, based on Southern blot analysis (Table 3). As reported previously, this gene is involved in the

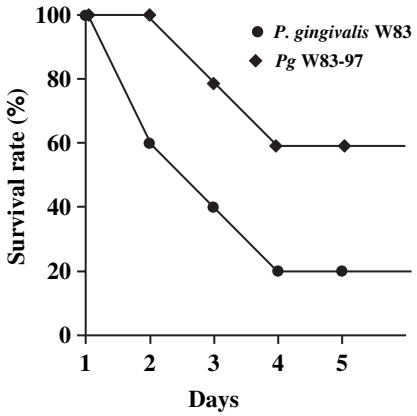

Fig. 2. Survival rates of mice challenged with *Porphyromonas gingivalis* W83 and the mutant for sensor histidine kinase (*Pg* W83-97). Female BALB/c mice were injected subcutaneously with 0.1 ml *P. gingivalis* suspension at two sites on the depilated dorsal surface (0.2 ml per mouse). The survival rate in each group was calculated as the number of survivors divided by the total number of mice ($n = 3$).

capsule locus of *P. gingivalis* W83 (4). We are now focusing on this gene in relation to the virulence of *P. gingivalis* strain W83.

Biochemical approaches comparing the growth requirements and enzyme activities of *P. gingivalis* have failed to identify the differences between the virulent and avirulent strains (9). Recently, the SH technique has been developed as a tool for identifying genetic differences between closely related bacterial strains or strains associated with virulence or genomic mutation (2). In this study, we constructed the null mutant of the histidine kinase sensor gene (*Pg* W83-97) to analyze the pathogenicity of *P. gingivalis* strain W83. For this purpose, BALB/c mice were challenged with subcutaneous injections of the bacterial suspension at two sites on the depilated dorsal surface (15). Four days after the subcutaneous injections, about 20% of the mice challenged with W83 at a dose of 6×10^{10} colony-forming units were still alive. In contrast, 60% of the mice challenged with the histidine kinase sensor mutant at the same dose per mouse had survived (Fig. 2). A previous study reported that the polyphosphate kinase gene is associated with biofilm formation by *P. gingivalis* (3). Our result suggests that the histidine kinase sensor gene might also be involved in the virulence of strain W83. In this regard, the specificity of the histidine kinase gene to strain ATCC 33277 should be further analyzed.

A previous study using the SH technique revealed that the *P. gingivalis* insertion sequence ISPg4 transposase gene is specific for virulent strains of *P. gingivalis* (11). The study isolated only three genes unique to *P. gingivalis* strain W83. Therefore, we performed the SH analysis as an alternative procedure to identify genes unique to strain W83 compared with strain ATCC 33277.

In this study, SH analysis revealed five DNA fragments unique to *P. gingivalis* W83 compared with ATCC 33277. Of these, the capsular polysaccharide-associated genes were further isolated (11). Furthermore, the variance in W83-specific genes was compared using four representative *P. gingivalis* strains. Five W83 strain-specific DNA fragments were also identical to the W50 strain. Using the SH technique, we could analyze the genetic similarities among virulent and avirulent strains.

In conclusion, SH is an effective tool for comparing virulent and avirulent strains of oral pathogenic bacteria. Further experiments regarding virulence-associated genes are required to determine the virulence of *P. gingivalis* at periodontal sites.

Acknowledgments

This study was supported by Grants-in-Aid (B) 14370770 (to T. Takehara) (C) 13672164 (to T. Ansai), and Aid for the Encouragement of Young (C) 19592409 (to A. Yoshida) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Amano A. Molecular interaction of *Porphyromonas gingivalis* with host cells: implication for the microbial pathogenesis of periodontal disease. J Periodontol 2003: **74**: 90–96.
2. Bjourson AJ, Stone CE, Cooper JE. Combined subtraction hybridization and polymerase chain reaction amplification procedure for isolation of strain-specific *Rhizobium* DNA sequences. Appl Environ Microbiol 1992: **58**: 2296–2301.
3. Chen W, Palmer RJ, Kuramitsu HK. Role of polyphosphate kinase in biofilm formation by *Porphyromonas gingivalis*. Infect Immun 2002: **70**: 4708–4715.
4. Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. J Bacteriol 2006: **188**: 5510–5523.
5. Genco RJ. Host responses in periodontal diseases: current concepts. J Periodontol 1992: **63**: 338–355.
6. Grenier D, Mayrand D. Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. J Clin Microbiol 1987: **25**: 738–740.
7. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 1998: **62**: 1244–1263.
8. Nakayama K. Molecular genetics of *Porphyromonas gingivalis*: gingipains and other virulence factors. Curr Protein Pept Sci 2003: **4**: 389–395.
9. Neiders ME, Chen PB, Suido H et al. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J Periodontal Res 1989: **24**: 192–198.
10. Park Y, Simionato MR, Sekiya K et al. Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. Infect Immun 2005: **73**: 3983–3989.
11. Sawada K, Kokeguchi S, Hongyo H et al. Identification by subtractive hybridization of a novel insertion sequence specific for virulent strains of *Porphyromonas gingivalis*. Infect Immun 1999: **67**: 5621–5625.
12. Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984: **63**: 412–421.
13. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol 1992: **63**: 322–331.
14. Stauffer GV, Plamann MD, Stauffer LT. Construction and expression of hybrid plasmids containing the *Escherichia coli gly*A genes. Gene 1981: **14**: 63–72.
15. Yoshimura M, Nakano Y, Yamashita Y, Oho T, Saito T, Koga T. Formation of methyl mercaptan from L-methionine by *Porphyromonas gingivalis*. Infect Immun 2000: **68**: 6912–6916.