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Antibody-based Screening of *Porphyromonas gingivalis* Proteins Specifically Produced in Patients with Chronic Periodontitis

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***Porphyromonas gingivalis* is among the major etiological pathogens of chronic periodontitis. The virulence mechanisms of *P. gingivalis* is yet to be identified as its activity is largely unknown in actual disease process. The purpose of this study is to identify antigens of *P. gingivalis* expressed only in patients with chronic periodontitis using a unique immunoscreening technique. Change Mediated Antigen Technology (CMAT), an antibody-based screening technique, was used to identify virulence-associated proteins of *P. gingivalis* that are expressed only during infection stage in patients having chronic periodontitis. Out of 13,000 recombinant clones screened, 22 tested positive for reproducible reactivity with rabbit hyperimmune anti-sera prepared against dental plaque samples acquired from periodontitis patients. The DNA sequences of these 18 genes were determined. CMAT-identified protein antigens of *P. gingivalis* included proteins involved in energy metabolism and biosynthesis, heme and iron binding, drug resistance, specific enzyme activities, and unknown functions. Further analysis of these genes could result in a novel insight into the virulence mechanisms of *P. gingivalis*.**

Key words: In vivo, Virulence factors, Antigens, Chronic periodontitis, *Porphyromonas gingivalis*

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Introduction

Dental plaque is a biofilm containing more than 500 different bacterial species [1-3], and behaves as a dynamic community affecting the host, which can lead to periodontal disease resulting from dysbiosis state [4]. It has been found that a population change toward increased composition of gram-negative bacteria within the dental plaque biofilm can lead to the development of periodontal diseases [2,5]. It is therefore crucial to understand the complicated interactions between the host and anaerobic gram-negative bacteria to better understand the microbial pathogenesis of periodontal diseases.

Although numerous data have been accumulated for the molecular pathogenicity of *P. gingivalis* [6-9], the exact mechanisms by which *P. gingivalis* behave in actual infectious processes are not fully understood. In order to assess the in vivo activity of *P. gingivalis* in periodontal disease, we applied a novel antibody-screening technique termed change mediated antigen technology (CMAT). CMAT is an immune-screening

technique used to identify bacterial antigens produced only in the host when cells undergo change such as in infection [10]. This technology utilizes hyperimmune antisera raised against bacterial cells obtained from disease sites, which are later adsorbed with bacterial cells grown in vitro. This procedure produce an antibody probe that is reactive only with in vivo-expressed proteins. Using this approach, we were able to identify in vivo-induced antigens of *Enterococcus faecalis*, a major endodontic pathogen in our previous study [11]. The purpose of the present study was to detect in vivo-expressed proteins of *P. gingivalis* using CMAT.

Materials and Methods

Bacterial strains and growth conditions

P. gingivalis ATCC 33277 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultivated on tryptic soy agar (Becton Dickson, Sparks, MD, USA) supplemented with 0.5% (w/v) yeast extract (Becton Dickson), 5% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), and 0.001% N-acetylmuramic acid (Sigma, St. Louis, MO, USA) at 37ºC under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for 5-7 days using a Forma 1025 Anaerobic Chamber (Thermo, Marietta, OH, USA). *P. gingivalis* cells were collected from the agar plates and washed, and kept at −80ºC until used. *E. coli* BL21(DE3)/pLysS Competent Cells (Stratagene, La Jolla, CA, USA) were grown on LB agar plates or broth containing kanamycin (30 μg/ml) at 37 ºC overnight.

Construction of genomic expression libraries of *P. gingivalis ATCC 33277*

P. gingivalis genomic DNA was extracted using a G-spinTM Genomic DNA Extraction Kit for Bacteria (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) according to the manufacturer's instruction. The genomic DNA was then cut by sonication using a SONOPULS Ultrasonic homogenizer (Bandelin, Berlin, Germany) for 6 seconds at the 100% power setting in order to produce DNA fragments sized 1−5 kb. After they were fractionated by agarose gel electrophoresis to eliminate very small and too large fragments, the DNA fragments were treated with the End-It^{TM} DNA End-Repair Kit (Epicentre, Madison, WI, USA) and subjected to phenol-chloroform extraction procedure to remove T4 polynucleokinase activity. A recombinant plasmid vector, pET-30c(+) (Novagen, Madison, WI, USA), was treated with *EcoR*V, and subsequently purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). pET-30c(+)/*EcoR*V and blunt-ended *P. gingivalis* DNA fragments (1-5 kb) were ligated using T4 DNA ligase (New England Biolab, Ipswich, MA, USA). Then, the mixture was used to transform *E.coli* DH5α (Takara, Daejon, Korea), and transformants were chosen on LB plates containing kanamycin antibiotics. Ten randomly selected colonies were screened using colony PCR with T7 promoter primers and checked to ensure self-ligation frequency was lower than 10%. Then, purified recombinant DNA mixture was used to transform *E. coli* BL21(DE3)/pLysS Competent Cells (Stratagene, La Jolla, CA, USA). Transformed cells were grown on LB agar plates containing kanamycin (30μg/ml) at 37 ºC overnight.

Collecting dental plaque samples containing *P. gingivalis* and preparing hyperimmune antisera

The protocol for collecting the dental plaque samples was approved by the Institutional Review Board (Approval number: 1-2008-05-22, Chonnam National University Hospital, Gwangju, Korea). Dental plaque samples were obtained from 10 patients who had moderate or severe chronic periodontitis, collected from the subgingival pockets of maxillary first molars. After supragingival plaque and calculus were removed, sterile #14 Gracey curette was inserted into the subgingival pockets, and plaque samples were obtained with a single stroke. The plaque samples were transferred aseptically to 1.5 ml microcentrifuge tubes containing 1 ml TE buffer, vortexed for 10 s, and centrifuged at 100 rpm for 10s to collect samples. Then, the presence of *P. gingivalis* in the samples was confirmed by PCR analysis as described before [12]. *P. gingivalis*-containing samples were immediately formalin fixed to preserve the integrity of protein antigens present at the time of harvest. Finally, tissue samples were pooled together and immediately stored at -70ºC until use. The samples were then sent to a commercial vendor (Young In Frontier, Seoul, Korea) for preparing rabbit polyclonal antibodies. The presence of high-titer rabbit polyclonal antibodies produced against *P. gingivalis* was confirmed by ELISA and western blot analysis.

Serum adsorption

In order to obtain an antibody probe that reacted only with in vivo-expressed *P. gingivalis* antigens, hyper-immune rabbit anti-sera were adsorbed with *P. gingivalis* whole cells cultivated in vitro. First, the rabbit polyclonal antiserum was mixed with

1 x 10^9 *P. gingivalis* cells with slow agitation (30 rpm) at 4˚C for 1 h; the cells were removed by centrifugation at 5,000 × g for 10 min, and the antisera were obtained. This step was repeated 4 times. Cell lysates were bound to NC membrane and added to the serum after whole cells were removed and then were agitated overnight at 4˚C; the next day, the serum was collected. To remove antibodies reacted with *E. coli* proteins, the serum was agitated again by adding nitrocellulose membrane-bound *E. coli* BL21 harboring only pET-30c(+).

To confirm adsorption efficiency, ELISA was performed as described before [13]. Briefly, a 96-well plate was coated with *P. gingivalis* protein extracts (0.1mg/100μl) in a coating buffer (0.1M sodium carbonate, pH9.6) overnight at 4℃. After washing, 2% skim milk in PBS was added and incubated for 1 hr at 37℃, followed by re-washing with PBS. Then, adsorbed rabbit antisera were added to each well using serial dilution starting at the ratio of 1:1000 and incubated at 37℃ for 2 hr. After the incubation, the plated was washed 3 times with 1× TBS-Tween. Then, 50 μl of 1:10000 diluted goat anti-rabbit IgG conjugated with horse radish peroxidase (Abcam, Hanam, Korea) was added to each well and incubated for 1 hr at 37℃. After washing with TBS-Tween (3X), TMB solution (GenDEPOT, Barker, TX, USA) was mixed and incubated for 2 min. The reaction was interrupted by adding 1N H_2SO_4 (100 μl). The reactivity was measured with absorbance at 450 nm in a spectrometer.

Screening for recombinant proteins uniquely expressed in vivo

Recombinant *E. coli* clones were detected using the adsorbed rabbit antisera as described previously [10]. Briefly, *E. coli* clones showing kanamycin resistance, about 200 per plate, were transferred to a nitrocellulose membrane. *P. gingivalis* and *E. coli* containing pET-30(+) were spotted on the membrane as positive and negative controls for screening, respectively. The membrane was then transferred to a LB agar plate containing 1 mM IPTG to induce protein expression. After the incubation at 37˚C for 3 hrs, the adhered cells were lysed by chloroform vapor. Then, the membrane was treated with PBS (5% skim milk) for 1 h, washed with PBS-Tween 20, and subjected to overnight incubation with the adsorbed rabbit antisera (1:500) at 4˚C. After the incubation, the membrane was screened with goat anti-rabbit IgG conjugated with horse radish peroxidase (1:1,000) for 1 hr at room temperature. 1-Step Chloronaphthol (4CN) (Pierce Biotechnology, Rockford, IL, USA) was used to identify reactive clones. The reactive clones were removed from the master plate and subsequently cultivated on a LB-kanamycin plate, and their relativities were confirmed.

Identifying genes isolated by CMAT

Plasmid DNA from positive clones was isolated with a QIAprep Spin Miniprep Kit (Qiagen), and DNA sequences of inserted fragments were determined using a T7 promoter primer with an ABI Prism 377 automatic DNA sequencer at GenoTech (Daejeon, Korea). Identified gene sequences were compared with the Oral Pathogen Sequence Databases (http://www.oralgen.org), where the entire genome sequence of *P. gingivalis* ATCC 33277 is available. Additionally, these gene sequences were compared with the DNA and protein databases using BLAST (http://www.ncbi.nlm.nih.gov) and analyzed with Invitrogen Vector NTI software (Thermo, CA).

Predicting functions of antigens identified by CMAT

Functional classification of identified antigens was based, when available, on published studies of identified proteins of *P. gingivalis*. When functional studies for *P. gingivalis* were not available in the literature, protein functional classification was predicted on the description posted on the Oral Pathogen Sequence Databases.

Results

Adsorption of rabbit hyper-immune antisera

Hyper-immune rabbit antisera produced against dental plaque samples containing *P. gingivalis* were successively adsorbed against *P. gingivalis* cells grown in vitro. Adsorption efficiency was determined by analyzing the reactivity of antisera using ELISA after each adsorption step with in vitro-grown *P. gingivalis*. Fully adsorbed serum exhibited significantly lower reactivity with in vitro-grown *P. gingivalis* (as shown in Figure 1). This result suggested that the adsorption process efficiently removed antibodies against most *P. gingivalis* proteins produced during in vitro cultivation.

Genomic library construction and screening

Ten colonies were randomly selected, and their plasmids were subjected to DNA sequencing to ensure the presence of the DNA inserts present in the recombinant plasmids. It was found that 8 out of 10 colonies had DNA inserts in the

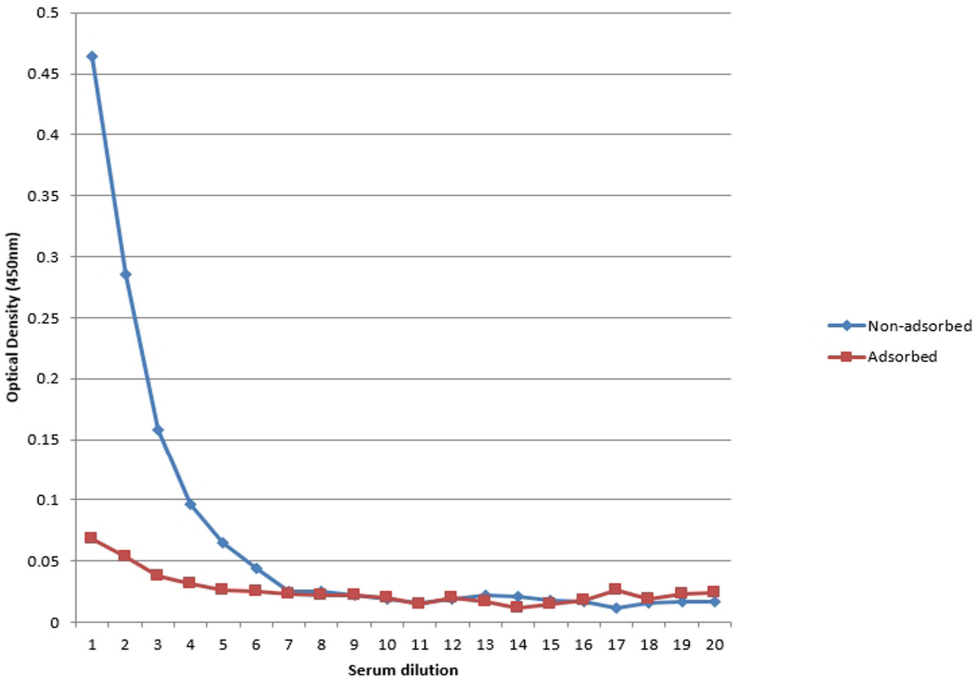

Figure 1. Effectiveness of antibody adsorption. Rabbit antisera were adsorbed with *P. gingivalis* whole cells cultivated in vitro, and the results were confirmed with ELISA.

Table 1. *P. gingivalis* protein antigens identified by CMAT

GeneID	Definition	Function
chlI	Magnesium chelatase subunit ChlI	Energy metabolism
PGN_0492	Cation-transporting ATPase	Energy metabolism
PGN_1761	V-type ATPase subunit B	Energy metabolism
PGN_0556	Putative cobalamin biosynthesis-related protein	Biosynthesis
PG_0085	Alpha-galactosidase	Energy metabolism
PGN_1207	Putative transport multidrug efflux protein	Drug resistance
hmuR	TonB-dependent hemoglobin receptor HmuR	Heme binding
mutS	DNA mismatch repair protein MutS	Biosynthesis
porU	Por secretion system protein porU	Cysteine-type peptidase activity
lysS	Lysine--tRNA ligase	Energy metabolism
ftn	Ferritin	Iron binding
aspS	Aspartate-tRNA ligase	Energy metabolism
dpp7	Dipeptidyl peptidase 7	Aminopeptidase activity
engB	Probable GTP-binding protein EngB	Energy metabolism
PGN_1670	Conserved hypothetical protein	Unknown
PGN_0684	Conserved hypothetical protein	Unknown
PG_0534	Conserved hypothetical protein	Unknown
PG_0326	Conserved hypothetical protein	Unknown

pET-30c(+) vector. A total of approximately 13,000 recombinant clones were screened with hyper-immune rabbit antisera. Initially, 28 immunoreactive clones were detected. Twenty-two of these reproducibly exhibited reactivity with the adsorbed serum, and these were chosen for further study in gene sequencing.

Identification and functional classification of *P. gingivalis* genes identified by CMAT

Sequencing of the DNA inserts from the 22 positive clones revealed that 18 ORFs had been identified; the gene sequences from four clones could not be obtained. These gene sequences were analyzed using a BLAST program against the total genome sequence of *P. gingivalis* in the database at NCBI (http://www.ncbi.nlm.nih.gov/). CMAT-identified IVI genes and their predicted functions are listed in Table 1.

It was determined from sequence analysis that many of these genes were enzymes implicated in housekeeping functions with intermediary energy metabolism and biosynthesis. Also, a small number of enzymes that possessed peptidase activity, and heme and iron binding proteins were also identified. In addition, proteins involved in drug resistance and proteins with unknown function were identified.

Discussion

It is not known how *P. gingivalis* actually behaves within the host and ultimately mediates periodontal infection that can lead to tissue destruction. Logically, it can be assumed that genes (proteins) expressed in vivo are essential in survival in the host and serve as potential virulence factors. It was our major objective to identify proteins from *P. gingivalis* that were exclusively produced in vivo during the actual disease process. For this purpose, we used an immunoscreening technique called CMAT in the present study; CMAT was designed to detect immunoreactive proteins produced only in vivo. The technique is a modification of the in vivo-induced antigen technology (IVIAT) that has allowed many researches to identify IVI proteins directly from human hosts rather than from potentially misleading animal models [10,14]. In many pathogenesis studies, IVIAT has been used to identify potential virulence-associated antigens from *Candida albicans* [15], *Vibrio cholera* [16], *Mycobacterium tuberculosis* [17], *Vibrio vulnificus* [18], *Escherichia coli* [19], *Salmonella enterica* [20], Group A *Streptococcus* [21], and periodontal pathogens including *Aggregatibacter actinomycetemcomitans* [22], *P. gingivalis* [23], and *Tannerella forsythia* [13].

Many IVI genes identified by CMAT in this study were enzymes associated with housekeeping functions including energy metabolism, nucleotide biosynthesis, and other cellular processes, as shown in Table 1. These genes were similar to those identified in previous IVIAT studies [13,24], and they are thought to play roles that are not required during in vitro growth [25]. It has been observed that housekeeping genes are not constitutively expressed at the transcriptional level in bacteria as was previously assumed [26-28], indicating that housekeeping metabolism is a dynamic process that is highly capable of adapting to different growth conditions. Therefore, it is plausible that these genes are up-regulated, and thus expressed only *in vivo*

Two important proteins related to the physiology and virulence of *P. gingivalis* were identified: HmuR and ferritin. HmuR, a TonB-dependent hemoglobin receptor, is a component of the *hmu* operon system that mediates heme and iron acquisition by *P. ginigvalis* [29,30]. Heme binding activity can directly affect virulence of *P. gingivalis*, as heme was demonstrated to have a virulence-enhancing effect [31]. It was also shown that HmuR is required for *P. gingivalis* growth [32]. Separately, ferritin is one of the intracellular iron-storage proteins, and this protein may be important for *P. gingivalis* survival under iron-depleted conditions [33]. Because heme and iron are required by most bacterial pathogens for infection development and progression [30,34], in vivo expression of HmuR and ferritin may be responsible for enhancing the virulence potential for *P. gingivalis* inside the host. We found it of interest that we identified a putative transport multidrug efflux protein that could be recognized as the major player in antimicrobial resistance. It is plausible for *P. gingivalis* to express a novel antimicrobial resistance using this mechanism inside the host, in addition to other conventional antimicrobial resistance mechanisms [35-37].

We also identified dipeptidyl peptidase 7 (DPP7) that possessed aminopeptidase activity; this enzyme is involved in detaching dipeptides from the N-terminal end of oligopeptides and is therefore crucial for the growth of *P. gingivalis* utilizing amino acids as energy sources. It was suggested that this protein is involved in facilitating dental plaque development and exerts cytotoxicity, leading to increased pathogenicity of *P. gingivalis* [38]. We also identified another enzyme, PorU, with the cysteine-type peptidase activity that is involved in the hydrolysis of peptide bonds. It is very likely that both DPP7 and PorU utilize peptides in vivo given that *P. gingivalis* is asaccharolytic, and therefore can only metabolize amino acids.

In summary, we identified 18 IVI antigenic bacterial proteins of *P. gingivalis* that are expressed uniquely in vivo and are reactive with rabbit hyperimmune sera produced against *P.*

gingivalis contained in the dental plaque samples. The results of this pilot study suggest that CMAT is a useful approach to identifying and characterizing potential virulence factors of *P. gingivalis*. The antigens identified in this study can be further analyzed by conventional techniques in order to refine the exact functions. Subsequent studies can test the functional roles of these identified genes by producing isogenic mutants for the purpose of studies using animal or in vitro models. These efforts will ultimately allow us to achieve better insights into the virulence mechanisms of *P. gingivalis*. This effort can lead to the advancement of new diagnostic, therapeutic, and preventive measures for periodontal and other infections associated with *P. gingivalis*.

Conflict of interest

There are no potential conflicts of interest relevant to this article.

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