molecular oral microbiology

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In-vivo-induced antigenic determinants of *Fusobacterium nucleatum* subsp. *nucleatum*

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SUMMARY

***Fusobacterium nucleatum* plays a pivotal role in dental plaque biofilm formation and is known to be involved in chronic inflammatory systemic disease. However, limited knowledge of *F. nucleatum* genes expressed *in vivo* interferes with our understanding of pathogenesis. In this study, we identified *F. nucleatum* genes induced *in vivo* using *in-vivo*-induced antigen technology (IVIAT). Among 30,000 recombinant clones screened, 87 reacted reproducibly with pooled sera from 10 patients with periodontitis. The clones encoded for 32 different proteins, of which 28 could be assigned to their functions, which were categorized in translation, transcription, transport, energy metabolism, cell envelope, cellular process, fatty acid and phospholipid metabolism, transposition, cofactor biosynthesis, amino acid biosynthesis, and DNA replication. Putative virulence factors detected were ABC transporter, butyrate-acetoacetate CoA-transferase, hemin receptor, hemolysin, hemolysin-related protein, LysR family transcriptional regulator, serine protease, and transposase. Analysis of immune responses to the *in-vivo*-induced (*ivi*) antigens in five patients demonstrated that most were reactive to these proteins, confirming results with pooled sera. IVIAT-identified *F. nucleatum* genes in this study may accelerate the elucidation of *F. nucleatum*-mediated molecular pathogenesis.**

INTRODUCTION

Fusobacterium nucleatum, a gram-negative anaerobic and non-motile bacterium, has been implicated in periodontal disease as well as several inflammatory systemic diseases (Li *et al.*, 2000; Kim & Amar, 2006; Elkaïm *et al.*, 2008). *Fusobacterium nucleatum* includes five subspecies that differ morphologically and phenotypically. Among these, the most frequently encountered species in the gingival crevice are *F. nucleatum* ssp. *nucleatum*, *F. nucleatum* ssp. *polymorphum* and *F. nucleatum* ssp. *vincentii* (Dabija-Wolter *et al.*, 2009). These three *F. nucleatum* subspecies have been frequently detected in periodontitis lesions compared with gingivitis and periodontally healthy sites (Bolstad *et al.*, 1996). *Fusobacterium nucleatum* is known for its invasive activity with epithelial cells, gingival keratinocytes and endothelial cells (Saito *et al.*, 2008), which eventually transports other non-invading bacteria into human epithelial cells (Edwards *et al.*, 2006). After invading host cells, the organism induces proinflammatory cytokine production (Han *et al.*, 2000; Ji *et al.*, 2009).

Fusobacterium nucleatum mediates multiple interactions with early colonizers and late colonizers in dental plaque biofilm. Because of its length and adhesive property, it is referred to as a 'bridge organism' between commensal and putative pathogens in periodontal disease. It has been shown to serve as an important bridge for increasing the pathogenic properties of other periodontal pathogens, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. To date, several *F. nucleatum* virulence factors, including FomA, RadD, FadA, class D β-lactamase, enolase, ABC transporter ATP-binding protein, toxic metabolites, serine protease, glycosyltransferase, and poly-γ-glutamate, have been studied (Bolstad *et al.*, 1996; Bachrach *et al.*, 2004; Al-Haroni *et al.*, 2008; Kim *et al.*, 2008; Candela *et al.*, 2009; Ikegami *et al.*, 2009; Kaplan *et al.*, 2009; Nakagaki *et al.*, 2010). However, these virulence factors were characterized using various *in vitro* methods.

It has been reported that periodontal pathogens have different gene expression patterns between *in vivo* and *in vitro* conditions (Cao *et al.*, 2004; Yoo *et al.*, 2007). Therefore, identification of genes that are expressed specifically during *in vivo* infection is important for understanding the virulence of *F. nucleatum* in the host condition. *In-vivo*-induced antigen technology (IVIAT) is an immunoscreening assay used to identify immunogenic bacterial genes expressed specifically during host infection (Handfield *et al.*, 2000; Rollins *et al.*, 2008). It does not depend on animal models of infection and uses patient serum, which is adsorbed with *in-vitro*-cultured bacteria to produce a probe that is reactive only with protein expressed *in vivo*. So far, IVIAT has been successfully applied to identify *in-vivo*-induced genes in several pathogenic bacteria, including *Vibrio cholerae*, *Vibrio vulnificus*, *Escherichia coli* O157:H7, *Bacillus anthracis*, *Salmonella enterica*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, group A *Streptococcus*, *Streptococcus suis* serotype 2 and periodontal pathogens including *A. actinomycetemcomitans*, *P. gingivalis* and *Tannerella forsythia* (Deb *et al.*, 2002; Song *et al.*, 2002; Hang *et al.*, 2003; Kim *et al.*, 2003; Cao *et al.*, 2004; John *et al.*, 2005; Rollins *et al.*, 2005, 2008; Salim *et al.*, 2005; Harris *et al.*, 2006; Yoo *et al.*, 2007; Gu *et al.*, 2009), where several putative virulence factors involved in the pathogenicity of these pathogenic bacteria have been identified and characterized.

The purpose of this study was to identify *F. nucleatum* genes expressed specifically during *in vivo* infection. We applied IVIAT to identify upregulated genes *in vivo* and used real-time reverse transcription–polymerase chain reaction (RT-PCR) to confirm expression after human oral epithelial cell infection with *F. nucleatum*.

METHODS

Bacterial strains and growth conditions

The *F. nucleatum* ssp. *nucleatum* (ATCC 25586) was grown in brain–heart infusion broth supplemented with hemin (5 μg ml^{-1}) and menadione (0.2 μg ml^{-1}) at 37°C under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2) for 3 days. Bacterial cells were harvested and washed, and the cell pellets were kept at −80°C until used. *E. coli* DH5α and BL21 (DE3) were grown in Luria–Bertani (LB) broth at 37°C.

Mammalian cell culture

Human epithelial cell line HOK-16B cells were cultured in KBM medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco BRL), 100 μg streptomycin and 100 U penicillin per ml (Gibco BRL). Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Construction of an *F. nucleatum* genomic DNA expression library

Fusobacterium nucleatum genomic DNA was purified by a phenol extraction, partially digested with *Sau*3AI, and separated by electrophoresis on a 0.8% low-melting-point agarose gel. DNA fragments of 0.5–3.0 kilobases were collected from the agarose gel and extracted using a Dyne Power Gel Extraction kit (DYNE BIO, Seoul, Korea). The purified DNA fragments were ligated into the pET30 a, b, c expression vector (Novagen, Darmstadt, Germany), which had been restricted with *Bam*HI and treated with antarctic phosphatase (New England Biolabs, Beverly, MA). Electrocompetent *E. coli* DH5α cells were used for electroporation of ligation products, and the transformants were spread on LB agar plates containing 30 μg ml^{-1} kanamycin. After overnight incubation at 37°C, colonies were scraped and frozen at −70°C

until use. The frozen aliquot of the library was grown in LB broth, the plasmids were isolated, and were used to transform the *E. coli* BL21 (DE3) expression host by electroporation.

Adsorption of periodontitis patient sera with *in-vitro*-expressed *F. nucleatum* antigens

The subjects for this study included 15 patients with periodontitis who visited the Department of Periodontology at the Seoul National University Dental Hospital. Periodontal status was evaluated by periodontal probing depth and clinical attachment level. All subjects showed at least six sites with probing depth >6 mm and attachment loss >3 mm. Sera from 10 patients were used for screening the *F. nucleatum* expression library and sera from five patients were used for screening the *in-vivo*-induced (*ivi*) antigens with sera from individual periodontitis patients. The Institute Review Board at the Seoul National University approved the study, and written informed consent was obtained from all subjects. To eliminate variation in individual patient immune responses, we pooled equal volumes (200 μl) from the serum of 10 patients with periodontitis. The pooled patient sera were sequentially adsorbed against *in-vitro*-grown whole cells, cell lysates, heat-denatured whole cells, and heat-denatured cell lysates of *F. nucleatum* and *E. coli*. First, the pooled sera were mixed with 1×10^9 *F. nucleatum* cells immobilized on epoxy beads with agitation at 4°C for 2 h. The *F. nucleatum* immobilized epoxy beads were removed by centrifugation and the pooled sera were recovered. This adsorption step was repeated four times. Next, the sera were mixed with *F. nucleatum* cell lysates immobilized on a nitrocellulose membrane, and were agitated at 4°C for 2 h, and this was repeated four times. Adsorption of sera with whole cells and cell lysates of *E. coli* BL21 (DE3) was performed as it had been for *F. nucleatum*. After centrifugation, the adsorbed pooled serum was stored at −70°C until further use. To confirm adsorption efficiency, we performed Western blot analysis using *F. nucleatum* and *E. coli* whole cell protein extracts. The *F. nucleatum* and *E. coli* whole cell protein extracts (10 μg) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (on a 10% gel) and the gel was electrophoretically transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) for 1 h, washed with PBST, and incubated with the unadsorbed pooled serum and fully adsorbed pooled serum (diluted to 1 : 500) at 4°C for 1 h. After incubation, the membrane was washed three times with PBST and reacted with goat anti-human IgG conjugated with alkaline phosphatase (1 : 1000, Sigma, St Louis, MO) for 1 h at room temperature. After three washes in PBST, the membrane was developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP, 165 μg ml^{-1}; Sigma) and nitroblue tetrazolium (NBT, 330 μg ml^{-1}; Sigma).

Library screening for *in-vivo*-induced genes of *F. nucleatum*

Kanamycin-resistant *E. coli* recombinant clones were plated at a colony density of approximately 700 colonies per LB agar plate (100-mm diameter) and were lifted to nitrocellulose membranes. The membranes were transferred colony side up to an LB agar plate containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated at 37°C for 4 h to induce protein expression. After incubation, the colonies were lysed by chloroform vapor exposure for 15 min at room temperature. The membranes were then blocked with 5% skim milk in PBST for 1 h, washed with PBST, and incubated with the adsorbed pooled serum (diluted to 1 : 500) at 4°C for 1 h. After incubation, the membranes were washed three times with PBST and reacted with goat anti-human IgG conjugated with alkaline phosphatase (diluted to 1 : 1000) for 1 h at room temperature. After three washes in PBST, the membranes were developed with BCIP and NBT. After secondary screening of the colonies, plasmids from individual reactive clones were purified, and the insert DNA was sequenced in both directions using pET30-specific primers (Novagen, Madison, WI).

Gene identification

In-vivo-induced genes were identified by sequence comparison of the cloned insert DNA and their translated protein sequences with the genomic sequence of *F. nucleatum* (ATCC 25586) from the Oral Pathogen Sequence Database (http://www.oralgen.lanl.gov).

Screening of *ivi* antigens with individual periodontitis patient serum

As we used pooled sera from 10 patients with periodontitis to identify *ivi* antigens of *F. nucleatum*, we assessed the degree of reactivity of 32 antigens detected by IVIAT using five additional patient sera that were not included for the library screening. Each serum was adsorbed against *F. nucleatum* and *E. coli* grown *in vitro* as described above. The clones harboring 32 genes were transferred to nitrocellulose membranes and reacted with each serum after IPTG induction as described above. As negative controls, clones that harbored protein coding gene fragments and did not react with pooled sera were included.

Infection of oral epithelial cells with *F. nucleatum* and analysis of intracellular *F. nucleatum* gene expression

It has been reported that *F. nucleatum* is invasive to human gingival epithelial cells (Ji *et al.*, 2009). To obtain bacteria grown intracellularly, HOK-16B cells were seeded in 12-well plates at a density of 2.5×10^5 cells per well and cultured overnight. The cells were then infected with *F. nucleatum* at a 1000 multiplicity of infection in antibiotic-free medium for 12 h. After 12 h of internalization, cells were washed twice with PBS and incubated with amoxicillin (100 μg ml^{-1}) for 2 h to eliminate both extracellular bacteria and those attached to the cell membrane. Infected HOK-16B cells were washed three times with PBS and the bacterial RNA was extracted using a ZR Fungal/Bacterial RNA MicroPrep kit (Zymo Research, Orange, CA). The RNA samples were quantified, and 1 μg of each RNA sample was used for cDNA synthesis using RT PreMix (iNtRON, Sung-Nam, Korea) in a 20-μl reaction volume with incubation at 42°C for 1 h and 95°C for 5 min. Real-time PCR was performed to analyse intracellular *F. nucleatum* gene expression compared with *in vitro* expression. Complementary DNA was subjected to PCR for several target genes in 20-μl mixtures containing the appropriate forward and reverse primers (4 pmol each), 10 μl SYBR Premix Ex *Taq* (TaKaRa Bio Inc., Shiga, Japan), followed by PCR for 40 cycles with denaturation at 95°C for 15 s, annealing at 52–58°C for 15 s, and extension at 72°C for 33 s in an ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR products were subjected to a melting curve analysis to verify a single amplification product. The housekeeping gene encoding 16S ribosomal RNA was used as a reference to normalize expression levels and to quantify changes in gene expression between *F. nucleatum* grown *in vitro* and *in vivo*. The RT-PCR primer sequences are listed in Table 1.

RESULTS

Identification of *in-vivo*-induced *F. nucleatum* antigens

To identify *in-vivo*-induced antigens by IVIAT, we used pooled sera from 10 patients with periodontitis

Table 1 Primers used for RT-PCR analysis of the genes identified by IVIAT

Gene name	Gene ID	Primer sequence
16S rRNA		5′-AAGCGCGTCTAGGTGGTTATGT-3′ 5′-TGTAGTTCCGCTTACCTCTCCAG-3′
ABC transporter substrate binding protein	FN0659	5′-GTTGGAGCAACACCAGTTCC-3′ 5′-CCAAGTGGTTCAACATGCAC-3′
Hemin receptor	FN0831	5′- AGAGGACAAGGGGAAGGAAG-3′ 5′-GAACCACCACCAGGAATGAC-3′
Hemolysin-related protein	FN1486	5′-AGCAGGAGCGATAGGAACAA-3′ 5′- ATTGATTTCAGCGCCAGAAG-3′
Elongation factor-G	FN1546	5′-TGGTGTTCATGCTGAATTGG-3′ 5′-TCCTTTTTCAACAGCAGGAA-3′
Lys R family transcriptional regulator	FN0659	5′-TCACCCTTGAGATTTCCTTT-3′ 5′-GAAGTTGCAAAGGCTAAAAGC-3′
Outer membrane protein A	FN2059	5′-TTGCTTTCAGTAGGCATTGG-3′ 5′-GGGCATACAGACTCAATAGGAA-3′
Transposase	FN1676	5′-TTACTTGCATTTGCCGGTCT-3′ 5′-TGCAACATGACCAAGAGCAT-3′
Hemolysin	FN0132	5′-CCAATGCCACTGATGAACCT-3′ 5′-CAGCAGCTGAGACAGCATTG-3′
Conserved hypothetical protein	FN0579	5′-TTGAAACTTCTGCACCGAGA-3′ 5′-AACAGTTGCTGGTGCTTCAA-3′
Butyrate-acetoacetate CoA-transferase subunit B	FN1856	5′-TCTGCTGCTGTTGTTGCTTT-3′ 5′-GGGTGGAGCAATGGACTTAG-3′

adsorbed against *in-vitro*-grown *F. nucleatum* and *E. coli* BL21 (DE3). Immunoblotting analysis showed that the sera did not react with bacteria grown *in vitro*, indicating complete removal of antisera reacting with antigen expressed in the bacteria cultured *in vitro* (data not shown). Primary screening of approximately 30,000 recombinant clones of an *F. nucleatum* genomic expression library resulted in the identification of 243 reactive clones, and secondary screening identified 87 reproducibly reactive clones. Sequencing of the clone inserts revealed 32 unique open reading frames. The gene sequences were analysed using BLAST against the whole genome sequence of *F. nucleatum*. Gene identifications and functional classifications are presented in Table 2.

Reactivity of the *ivi* antigens with individual periodontitis patient serum

To see how consistent the *ivi* antigens were in patients with periodontitis, sera of five individual patients were used to test the immunoreactivity to the clones expressing 32 individual antigens. As shown in Table 2, most of them were reactive to sera from at least three patients. However, protein translation elongation factor EF-G (FN1546) was

Table 2 *Fusobacterium nucleatum* genes identified by IVIAT and immunoreactive frequency of *ivi* genes

Functional classification	Gene ID	Gene name	Immunoreactive frequency in five individual sera[1]
Translation	FN0067	Isoleucyl-tRNA synthetase	3
	FN0070	Glycyl-tRNA synthetase beta chain	5
	FN0999	Glucanase/deblocking aminopeptidase	3
	FN1426	Serine protease	4
	FN1546	Elongation factor EF-G	0
	FN1641	30S ribosomal protein S19	3
Transcription	FN0503	LysR family transcriptional regulator	4
	FN2035	DNA-directed RNA polymerase	3
Transport	FN0504	Amino acid permease	3
	FN0659	ABC transporter, permease protein	5
	FN0831	Hemin uptake system, outer membrane receptor	1
Energy metabolism	FN0684	Prismane protein, hybrid-cluster protein	3
	FN1540	Iron-sulfur cluster-binding protein	3
	FN1586	Mandelate racemase/muconate lactonizing enzyme	3
	FN1765	Pyruvate kinase	5
Fatty acid and phospholipid metabolism	FN1856	Butyrate-acetoacetate CoA-transferase subunit B	5
Cell envelope	FN1458	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	5
	FN0558	TraT complement resistance protein precursor	3
	FN1980	Probable integral membrane protein	3
	FN2059	Outer membrane protein	3
Cellular processes	FN0132	Hemolysin	5
	FN1486	Hemolysin-related protein	3
Amino acid biosynthesis	FN1745	Cystathionine beta-lyases	3
DNA replication	FN0705	DNA polymerase I	5
Biosynthesis of cofactors	FN0941	Gamma-glutamyltranspeptidase	3
Other categories; Transposase	FN0599	Transposase, IS1167	3
	FN1676	Transposase	5
Other categories; Phage-related functions	FN1553	Possible abortive phage resistance protein	5
Unknown	FN0579	Conserved hypothetical protein (possible lipoprotein)	5
	FN1076	Conserved hypothetical protein	5
	FN1213	Hypothetical protein	3
	FN1805	Conserved hypothetical protein	4

[1]Number of immunoreactive sera to the *ivi* antigens among five individual sera.

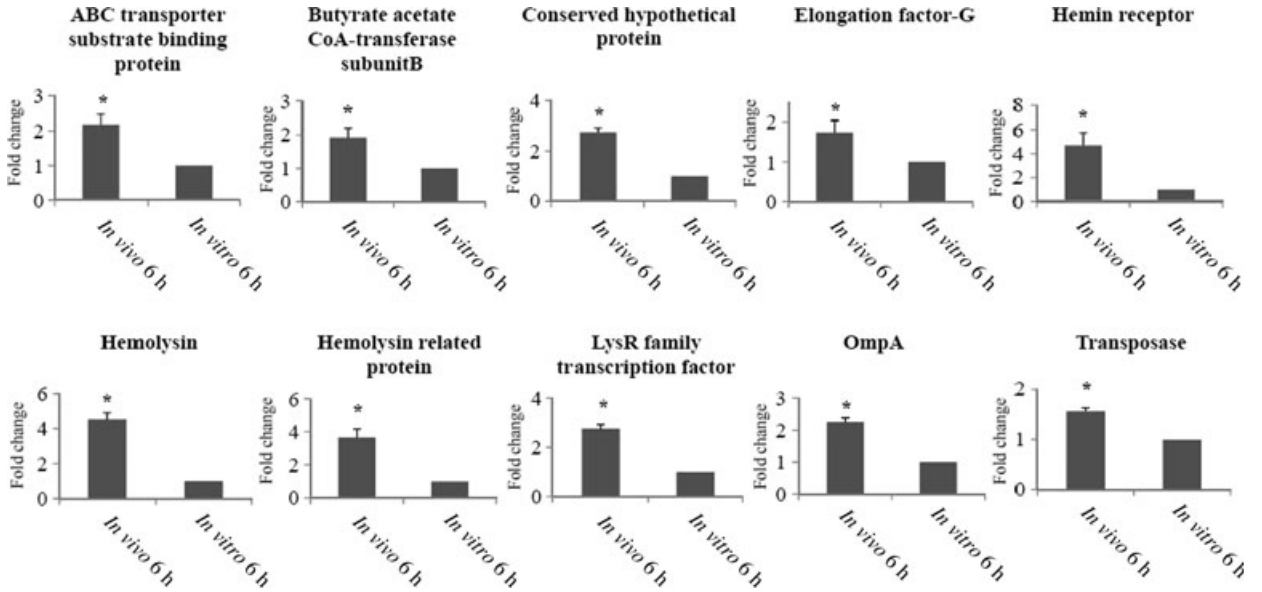

Figure 1 Messenger RNA expression of 10 selected *Fusobacterium nucleatum* genes from infected HOK-16B cells compared with bacteria grown *in vitro*. HOK-16B cells were infected with *F. nucleatum* for 6 h and the mRNA expression of 10 genes detected by IVIAT was analysed by real-time RT-PCR. Gene expression is presented as the relative ratio compared with that of bacteria grown *in vitro*. Asterisk indicates statistical significance at $P < 0.05$.

reactive with none of the sera and hemin receptor (FN0831) was reactive with only one patient serum, indicating the high degree of the host variability of these two antigens.

Verification of *in vivo* induction of IVIAT-identified genes by real-time RT-PCR

To verify *in vivo* induction of IVIAT-identified genes of *F. nucleatum*, we performed real-time RT-PCR of the selected genes encoding putative virulence factors using intracellular *F. nucleatum* RNA and compared with those of bacteria grown in broth without exposure to host cells. Ten IVIAT-identified genes were selected: elongation factor EF-G (FN1546), butyrate-acetoacetate CoA-transferase subunit B (FN1856), ABC transporter substrate binding protein (FN0659), hemolysin-related protein (FN1486), hemolysin (FN0132), transposase (FN1676), outer membrane protein A (FN2059), hemin receptor (FN0831), LysR family transcriptional regulator (FN0503) and conserved hypothetical protein (FN0579). As shown in Fig. 1, gene expression of *F. nucleatum* grown *in vivo* was significantly increased compared with bacteria grown *in vitro*.

DISCUSSION

Identification of *in-vivo*-induced genes provides insight into gene regulation of pathogens during infection. In the present study, IVIAT was applied to select protein antigens that are specifically expressed during *F. nucleatum* infection in patients with periodontitis and we identified 32 immunogenic proteins of *F. nucleatum* by screening with sera from patients with periodontitis. In addition, most of the *ivi* antigens showed reactivity to sera from at least three of five patients, verifying the *in vivo* induction of these genes. However, protein translation elongation factor EF-G and hemin receptor belonged to the less common *ivi* antigens, indicating a high degree of host variability. Many genes known to have house-keeping functions like translation, transcription, DNA replication and cell envelope structure were detected by IVIAT as shown in other reports (Yoo *et al.*, 2007; Gu *et al.*, 2009) and we speculate that they exert some function that is not required during *in vitro* growth. To develop a rapid and simple *in vitro* infection model for characterizing *ivi* genes we infected *F. nucleatum* in oral epithelial cell line HOK-16B cells and evaluated the expression of 10 selected *ivi* genes by

real-time RT-PCR. Although the regulation of the remaining genes needs to be analysed and genes that were not reactive in *ivi* gene screening need to be included as negative controls, the same induction pattern of 10 genes detected by IVIAT and real-time RT-PCR suggests that *F. nucleatum* gene regulation occurs in epithelial cells, the first target cell for this bacterium and the upregulated gene products lead to a host immune response to elicit antibodies. Therefore, the functional roles like inflammatory response, quorum sensing and cell lysis of the *ivi* genes could be further characterized in a cell culture model. However, the results obtained using an *in vitro* cell culture model must be interpreted with caution because the *in vitro* model represents only a limited condition of human infection. Real-time RT-PCR reflects the transcriptional level and therefore protein regulation needs to be evaluated in the cell culture infection model.

ABC transporters are multi-domain membrane proteins. They are responsible for many biological processes, including cell division and efflux/influx regulation across cellular membranes. Al-Haroni *et al.* (2008) reported that the ABC transporter is related to antibiotic resistance in *F. nucleatum*. In addition, an ABC transporter is also known to be involved with autoinducer 2 uptake, which may play a pivotal role in *F. nucleatum* quorum sensing, which regulates bacterial gene expression in response to cell density. Quorum-sensing bacteria synthesize and secrete extracellular signaling molecules called autoinducers, and the ABC transporter plays an important role in uptake of these molecules (Fong *et al.*, 2001; Taga *et al.*, 2001; Xavier & Bassler, 2005; Rezzonico & Duffy, 2008). EF-G participates in protein synthesis by peptidyl tRNA translocation and, together with ribosomal recycling factor, promotes the release of deacylated tRNA and mRNA from the ribosome and splits the ribosome into two subunits (Laurberg *et al.*, 2000). Although the virulence of EF-G in fusobacteria has not been clearly reported, EF-G has been proposed as a candidate for use as a vaccine, because the EF-G of *Fusobacterium varium* was shown to react with sera from patients with ulcerative colitis (Minami *et al.*, 2009). LysR family transcriptional regulators are highly conserved in structure and are known to function in bacteria to regulate the genes involved in virulence, metabolism, quorum sensing, toxin production, attachment and secretion (Maddocks & Oyston, 2008). The LysR-type transcriptional regulator gene *ilvY* of *V. vulnificus* is an *in vivo*-induced gene that forms an operon with its regulatory target gene *ilvC* and positively regulates *ilvC* transcription. A LysR-type quorum-sensing transcriptional regulator was detected in *E. coli* O157:H7 by IVIAT and is known to participate in the regulatory cascade that controls the expression of virulence factors via quorum sensing. Butyrate-acetoacetate CoA-transferase subunit B plays an important role in production of butyrate. Butyrate produced by *F. nucleatum* is correlated with oral malodor and inhibits gingival fibroblast proliferation, preventing wound healing. It is also present in elevated levels in plaque-associated periodontitis and may allow bacteria to penetrate the epithelium (Bolstad *et al.*, 1996). Hemolysin, a secreted protein and transposable element, is considered to be one of the major virulence factors involved in fusobacterial infections and similar to the hemolysin open reading frame of *Burkholderia cepacia* (Kapatral *et al.*, 2002). Fusobacterial hemolysin caused lysis of erythrocytes, producing iron, and created an anaerobic environment by reducing the oxygen supply to the site of infection (Miao *et al.*, 2010). In addition to the genes mentioned above, hemin uptake receptor, hemolysin-related protein, outer membrane protein A, transposase, and a highly conserved hypothetical protein were confirmed to be upregulated in intracellular bacteria, and many groups have reported that they are either directly or indirectly involved in bacterial pathogenesis (Takahashi *et al.*, 2000; Kapatral *et al.*, 2002; Love, 2007). Serine protease is a bacterial virulence factor that degrades the extracellular matrix proteins of host cells and leads to periodontal tissue damage as well as degradation of IgA, which may help *F. nucleatum* to evade the host immune system (Bachrach *et al.*, 2004).

IVIAT has also been used to study periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* (Song *et al.*, 2002; Cao *et al.*, 2004; Richardson *et al.*, 2005; Yoo *et al.*, 2007). The identified genes fell into several different functional groups, including microbe–host cell interactions, colonization, acquisition of metals, regulation of other genes, and surface associated molecules. Two *in vivo*-induced genes identified in the present study have also been detected in other bacteria by IVIAT. A LysR family transcriptional regulator was detected in *A. actinomycetemcomitans*, *V. vulnificus*, and

E. coli O157 (Kim *et al.*, 2003; Cao *et al.*, 2004; John *et al.*, 2005). An ABC transporter was detected in *E. coli* O157, *B. anthracis*, *S. enterica*, Group A *Streptococcus* and *S. suis* serotype 2 ((John *et al.*, 2005; Salim *et al.*, 2005; Harris *et al.*, 2006; Rollins *et al.*, 2008; Gu *et al.*, 2009).

In summary, we identified 32 *in-vivo*-induced immunogenic proteins of *F. nucleatum* that are reactive with pooled human sera from patients with periodontitis. IVIAT-identified *F. nucleatum* genes in this study may accelerate the elucidation of *F. nucleatum*-mediated molecular pathogenesis and the *in-vivo*-induced gene profile could be used as an infection model to screen antibacterial and anti-inflammatory compounds. In further studies, constructing isogenic mutants and comparing them with the wild-type for virulence will determine the functional role of these *in vivo*-induced genes.

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