RESEARCH ARTICLE

Highly expressed genes in a rough-colony-forming phenotype of *Aggregatibacter actinomycetemcomitans*: implication of a *mip*-like gene for the invasion of host tissue

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Abstract

Aggregatibacter actinomycetemcomitans, a potent pathogen of periodontitis, typically grows as a rough and adherent colony on primary isolated cultures. The colony transforms into a smooth phenotype during repeated subculture. In this study, we aimed to identify highly expressed genes in the rough-colony-forming phenotype for isolation of host-induced genes. Using a cDNA-subtractive hybridization technique, three genes, homologous to a macrophage infectivity potentiator gene (*mip*), peroxiredoxin gene (*prx*) and outer membrane protein gene (*ompA*), were identified. The expression levels of these genes in the rough-colony-forming phenotype were 4–10-fold higher as compared with the smooth-colony-forming phenotype. Attention was focused on the *mip*-like gene, and a recombinant protein and a deficient mutant were constructed. The recombinant protein reacted with sera from patients with periodontitis, suggesting the production of the Mip-like protein in periodontal lesions. Viable quantitative invasion assay demonstrated that the viable cell counts of the wild-type strain that invaded HeLa cells were more than fourfold as compared with the *mip*-deficient mutant. The expression of the *mip*-like gene, *prx*-like gene and *ompA*-like gene may be enhanced in the host, and the *mip*-like gene may play an important role in the infection of *A. actinomycetemcomitans*, especially in its invasion of the epithelium.

Introduction

Aggregatibacter (Actinobacillus) actinomycetemcomitans, a Gram-negative, nonmotile, facultative anaerobic cocobacillus, is one of the potent pathogens of periodontitis, especially localized juvenile periodontitis (Slots *et al.*, 1980; Meyer & Fives-Taylor, 1997). *Aggregatibacter actinomycetemcomitans* has also been associated with extraoral infections such as endocarditis (Anolik *et al.*, 1981), thyroid abscess (Burgher *et al.*, 1973), brain abscess (Martin *et al.*, 1967) and urinary tract infection (Townsend & Gillenwater, 1969). This microorganism has been shown to possess many virulence factors. They include a leukotoxin (Baehni *et al.*, 1979), immunosuppressive factors (Shenker *et al.*, 1982a, b), lipopolysaccharide (Kiley & Holt, 1980), a chemotactic inhibitor (Van Dyke *et al.*, 1982), cytotoxin (Shenker *et al.*, 1982a, b), and an ability to invade epithelial cells (Christersson *et al.*, 1987; Meyer *et al.*, 1991). However, the potential roles of these virulence factors in the pathogenesis of periodontitis and systemic infections are not well characterized. Phenotypic alteration of *A. actinomycetemcomitans* during *in vitro* subculture is one of the reasons for the difficulty in elucidating the virulence.

Aggregatibacter actinomycetemcomitans isolated from gingival sulcus initially produces rough, star-shaped colonies with irregular borders on solid media. In broth, the fresh clinical isolates autoaggregate and appear along the vessel wall, forming tenacious biofilms (Scanapieco *et al.*, 1987; Rosan *et al.*, 1988; Inouye *et al.*, 1990). This distinctive adherence property is lost within several *in vitro* subcultures. The phenotype transforms into a smooth, circular colony with a regular border. In broth, the cells transform into a

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turbid, homogeneous suspension without adherence to the vessel wall (Inouye *et al.*, 1990). Colonial morphology generally reflects the differential expression of components on the cell surface. The rough colony phenotypes (R-type) demonstrate abundant fimbriae, compared with the smooth colony phenotype (S-type) (Scanapieco *et al.*, 1987; Rosan *et al.*, 1988; Inouye *et al.*, 1990). Besides the cell surface molecules, elevated endotoxin and proteinase activities have been reported in the R-type (Fine *et al.*, 1999). In addition, the adherence and biofilm-forming abilities of *A. actinomycetemcomitans* increase resistance to antimicrobial agents and play an important role in colonizing the mouth (Fine *et al.*, 2001). These previous reports demonstrated the significant reduction of virulence during *in vitro* passage, and suggested the need to study *A. actinomycetemcomitans* in its clinical form (Fine *et al.*, 1999).

It is well documented that bacteria increase virulence in their host for survival. The rationale behind the current study was that identification of host- or host cell-induced genes should allow identification of the virulence factors of microorganisms. Although there was no strong evidence that this was an efficient strategy, some virulence genes were identified through this strategy (Lowe *et al.*, 1998). In this regard, isolation of genes highly expressed in the R-type strain (R-heg) of *A. actinomycetemcomitans* turns out to be an isolation of host-induced virulence genes. In the current study, we attempted to identify R-heg using the cDNA subtractive hybridization technique. Further, the role of an identified gene was investigated by constructing the deficient mutant.

Materials and methods

Bacterial strains and culture conditions

The isogenic pair of *A. actinomycetemcomitans* (R-type of 304-a and S-type of 304-b strains) isolated from a patient with aggressive periodontitis (Inoue *et al.*, 1998) was used in this study. A representative laboratory strain, ATCC 29523, was also used. These strains of *A. actinomycetemcomitans* were grown anaerobically on tryptic soy broth agar (Becton Dickinson and Company) supplemented with 0.5% yeast extract (Difco Laboratories) (TSBY). For the harvesting of the cells, the bacterial colony was transferred to liquid TSBY supplemented with 0.04% $NaHCO_3$, and was cultivated under anaerobic conditions at 37 °C. Where required, media were supplemented with spectinomycin (100 μg mL^{-1}).

Escherichia coli XL1-Blue (Stratagene) and BL21 Star™ (DE3) (Invitrogen) were used as the host for the recombinant DNA procedures and the construction of a recombinant protein. *Escherichia coli* cells were grown on a Luria–Bertani agar plate or broth. Where required, media were supplemented with ampicillin (50 μg mL^{-1}) and 1 mM isopropyl-β-D-thiogalactopyranoside.

Preparation of R-type and S-type cells

For the preparation of the R-type cells, the 304-a strain was cultured on a TSBY agar plate and a colony exhibiting the typical R-type form was transferred to the broth medium in a culture flask. For the removal of nonadherent cells that were considered to have transformed into the S-type, the broth was decanted and the flask wall was washed twice with phosphate-buffered saline [PBS(−)] (Invitrogen). The adherent cells on the vessel wall were harvested using a cell scraper (Techno Plastic Products AG) in PBS(−) and were used as the R-type. For the preparation of the S-type, a frozen stock of the 304-b strain was cultured on a TSBY agar plate, and a single S-type colony was subcultured in the broth medium. After several subcultures, a homogeneous suspension with nonadherent growth was confirmed, and the cells were used as the S-type.

RNA extraction

Total RNA was extracted from the 304-b strain in the exponential phase. Total RNA was also extracted from the 304-a strain when the adherent cells reached the confluent layer. For the extraction, RNAqueous™ (Ambion) was used according to the manufacturer's instructions. DNase I (Stratagene) was added to digest contaminated DNA, and the mRNA was further purified for subtractive hybridization using a MICROBExpress™ Bacterial mRNA Purification Kit (Ambion) according to the manufacturer's instructions.

Subtractive hybridization

The cDNA subtractive hybridization was performed using a PCR-Selected™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions, with a slight modification in cDNA synthesis. Briefly, the tester (R-type) and the driver cDNA (S-type) were synthesized from 2 μg of mRNA using AMV reverse transcriptase. Random primers (hexamer) were used instead of the oligo-dT primer. The cDNA of both the tester and the driver were double stranded using DNA polymerase I, RNase H and *E. coli* DNA ligase in the kit and were blunt-ended by RsaI digestion. The blunt-ended tester cDNA was then divided into two portions, and each was ligated with a different adaptor (AP-1 or AP-2) prepared in the kit. The AP-1- and AP-2-ligated tester cDNA were individually subjected to first hybridization with an excess amount (fivefold) of driver cDNA. The first hybridization was performed at 68 °C for 8 h after a denaturing step at 98 °C for 1.5 min. Solutions of the two hybridizations were immediately continued to the second hybridization without denaturing. In the second hybridization, the first hybridization solutions were mixed together, and fresh denatured driver cDNA was added. The second hybridization was performed at 68 °C overnight. Through the two

hybridization steps, differentially expressed sequences were expected to form a hybrid with different adaptors (AP-1 and AP-2) on their 5′ and 3′ ends.

Select-PCR amplification

R-heg was amplified by select-PCR according to the manufacturer's instructions of the PCR-Selected™ cDNA Subtraction Kit (Clontech). After two hybridization steps, the subtraction sample was used as a template for the first select PCR. An adaptor primer with the sequence common to AP-1 and AP-2 was used for the amplification. The nested PCR used two adaptor primers specific to AP-1 and AP-2, respectively, for the selective amplification of the hybrids with different adaptors at each end. The primers were prepared in the PCR-Selected™ cDNA Subtraction Kit (Clontech), and both PCRs were performed under conditions recommended by the manufacturer.

Quality control assay for PCR-selected subtraction

To evaluate the efficacy of the subtractive hybridization and the following select PCR, copy numbers of the 16S rRNA gene common to both the tester and the driver were monitored. Two hundred nanograms of the first PCR product, second PCR product and the original cDNA sample before the PCR were subjected to quantitative real-time PCR for the 16S rRNA gene of *A. actinomycetemcomitans* as described previously (Maeda *et al.*, 2003). A decrease in number of the 16S rRNA gene (from 1×10^8 to 10 copies ng^{-1}) was confirmed before the use of a subtraction product for library construction (data not shown).

Construction and screening of the subtraction library

A PCR-selected subtraction library was constructed by directly inserting the second PCR product into a TA cloning vector (pCR®2.1, Invitrogen). One microliter (approximately 100 ng of DNA) out of the 25 μL of the second PCR product was used for ligation with 50 ng of the vector. Then, 2 μL of ligation mixture (total ligation volume of 10 μL) was subjected to *E. coli* transformation. Recombinant clones were isolated from the library by blue–white selection, and the plasmids were purified. The recombinant plasmids were digested with EcoRI (Takara) to separate the inserts, and were electrophoresed on a 1.5% agarose gel. The digested plasmid clones were then transferred to a nylon membrane (Hybond™-N^+, GE Healthcare), and the membranes were subjected to hybridizations with the tester and driver cDNA probes. Whole populations of the tester and driver cDNA synthesized from 2 μg total RNA were labeled with [α-^{32}P] dCTP (GE Healthcare) using a Random Primer DNA Labeling kit (Takara) and were used as probes. Hybridization was performed in a buffer consisting of 6 × SSC (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt's solution (Nippon Gene), 0.5% sodium dodecyl sulfate (SDS) and 100 μg mL^{-1} salmon sperm DNA overnight at 65 °C with 2×10^6 c.p.m. mL^{-1} of the labeled probes. After hybridization, the membrane was washed for 0.5 h at 65 °C with a washing buffer containing 2 × SSC and 0.5% SDS, and then washed twice for 0.5 h at 65 °C with a washing buffer containing 0.2 × SSC and 0.1% SDS. Hybridization signals were detected and analyzed by a BAS 2000 Bio Imaging Analyzer (Fuji Film). Plasmid clones that hybridized to the 304-a cDNA probes, but not to 304-b cDNA probes, were selected as candidates for R-heg.

DNA sequencing and database search

DNA sequencing was performed using a BigDye® cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (3130xl Genetic Analyzer, Applied Biosystems). The sequence data were used to query GenBank, Oral Pathogen Sequence Databases (http://www.oralgen.lanl.gov/) and microbial genomes at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLAST sequence homology search program.

Northern hybridization

The mRNA accumulations of selected genes (candidates for R-heg) from the subtraction library were examined by Northern hybridization. Total RNA (2.5 μg) was electrophoresed and was transferred to a nylon membrane (Hybond™-N^+, GE Healthcare). The selected genes were labeled with [α-^{32}P]dCTP using Random Primer DNA Labeling kits (Takara) and were used as probes. Hybridization and washing were performed using a Northern Max™ Kit (Ambion) according to the manufacturer's instructions. The intensities of the hybridization signals of the R-heg were analyzed using NIH IMAGE version 1.61 (National Institutes of Health) and were normalized by the intensities of 16S rRNA gene detected on the ethidium bromide-stained gel. The intensities of the R-heg candidates were compared between 304-a and 304-b strain.

Construction of recombinant macrophage infectivity potentiator (Mip)-like protein

The recombinant Mip-like protein of *A. actinomycetemcomitans* was constructed using a pET Directional TOPO® Expression Kit (Invitrogen) according to the manufacturer's instructions. Briefly, the entire ORF of the *mip*-like gene (AA01517 in the genome sequence of *A. actinomycetemcomitans* at Oral Pathogen Sequence Databases) was amplified from ATCC 29523 by PCR using a forward (5′-caccatgt taaaaatgaagaaaatttcagt-3′) and a reverse primer (5′-

tttcgccgggggtgacgtccaatacttcaat-3′). For the directional cloning, four nucleotides (cacc) were added to the 5′ end of the forward primer and the reverse primer was designed to allow the PCR product in frame with the histidine (His)-tag and a short peptide termed V5 epitope in the vector. The amplified *mip*-like gene was then inserted into the plasmid vector pET101/D-TOPO® (Invitrogen). The *E. coli* BL21 Star™ (DE3) was transformed with the recombinant plasmid, and the transformed *E. coli* was disrupted by sonication. The recombinant protein was purified from the sonicated sample using Ni-NTA superflow columns (Qiagen). The sonicated samples and the purified samples were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and Western blot analysis.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis were performed as described previously (Kokeguchi *et al.*, 1989). Protein profiles of the *E. coli* transformed with the *mip*-like gene and the purified recombinant Mip-like protein were analyzed by SDS-PAGE. The reactivity of sera from patients with periodontitis against the recombinant Mip-like protein was examined by Western immunoblotting. Sera from patients and healthy subjects were used as the first antibody at a final dilution of 1 : 200. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) antibody (INC Biochemicals) was used as the second antibody for detection.

Serum samples

Eight patients' sera were selected from the serum sample collection of Okayama University Hospital of Medicine and Dentistry with the permission of the patients after obtaining informed consent. The serum IgG antibody titer to the sonic extracts of *A. actinomycetemcomitans* ATCC 29523 had been examined previously by an enzyme-linked immunosorbent assay (ELISA) for the clinical diagnosis of periodontitis (Murayama *et al.*, 1988). The eight sera selected in this study demonstrated elevated IgG antibody titers (above 400 ELISA units) to the extracts (Murayama *et al.*, 1988), and the strong reactivity to the whole-cell lysate of *A. actinomycetemcomitans* was confirmed by Western blot analysis before use (data not shown). Eight periodontally healthy subjects were also examined as controls. The use of human subjects in this study was approved by the Okayama University Hospital Ethics Committee (Approved no. 624).

Construction of a *mip*-like gene deficient mutant

The deficient mutant of the *mip*-like gene was constructed using the *A. actinomycetemcomitans* ATCC 29523 strain. Inactivation of the *mip*-like gene was accomplished by homologous recombination of a spectinomycin resistance gene (*spc*) (LeBlanc *et al.*, 1991) into the chromosomal locus. The entire *mip*-like gene was amplified by PCR as described above and was cloned into a TA cloning vector (pCR2.1®, Invitrogen). The *spc* was amplified by PCR using forward (5′-cggaccggtaacgtaacgtgactg-t-3′) and reverse primers (5′-cggaccggttaaataataaaacaaa-3′) combined with the AgeI site. The amplified *spc* was digested with AgeI (New England Biolabs) and was inserted into the ORF (AgeI site) of the cloned *mip*-like gene. The TA cloning vector (3 μg) containing the *mip*-like gene with the *spc* was linearized with SacI (Takara) digestion and was introduced by electroporation into *A. actinomycetemcomitans* cells (10^6–10^7 cells) suspended in ice-cold 10% glycerol. The electroporation was performed in a Gene Pulser (Bio-Rad) set to 12.5 kV cm^{-1}, and the cells were incubated in TSBY liquid medium for 2 h. After the incubation, the cells were plated on TSBY agar containing spectinomycin (100 μg mL^{-1}). Colonies on the plate were retrieved, and the insertional mutagenesis of the *mip*-like gene was confirmed by Southern hybridization.

Southern hybridization

Southern hybridization was performed as described by Southern (1975), with slight modifications (Miyamoto *et al.*, 1991). Genomic DNA was purified from *A. actinomycetemcomitans* strains using the method outlined by Stauffer *et al.* (1981), and was digested with endonuclease BglI or DraI (Takara). The whole length of the *mip*-like gene was used as a probe.

Growth rate of the *mip*-deficient mutant

The growth kinetics was compared between the *mip*-deficient mutant and its wild type (WT). The mutant and WT were precultured to the early stationary phase in TSBY medium. One hundred and fifty microliters of precultured cells were transferred to 4 mL of fresh TSBY, and the $OD_{570\,nm}$ of the medium was monitored every 2 h.

Invasion assay

A quantitative invasion assay using HeLa cells was performed as described by Meyer *et al.* (1991), with slight modifications. Briefly, approximately 1×10^5 HeLa cells were incubated in a 24-well tissue culture plate (Corning) containing Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) for 16 h. The *mip*-deficient mutant and the WT strains cultured to the mid exponential phase were inoculated to the semi-confluent monolayers of HeLa cells. The bacterial cell numbers were adjusted to obtain a multiplicity of infection of 1000 bacteria to 1 HeLa cell. After coincubation for 4 h, extracellular, unattached bacteria were removed by

washing with PBS(–) (Invitrogen). The HeLa cells were further incubated for 2 h in the presence of gentamicin (100 μg mL^{-1}) and ampicillin (100 μg mL^{-1}) to kill the remaining extracellular bacteria. The monolayer cells were then washed twice with PBS(–), and the cell layer was removed by PBS(–) containing 0.05% trypsin and 0.02% EDTA. The detached cells were washed again with PBS(–) and were plated on TSBY agar plates. The TSBY agar plates were incubated under anaerobic conditions for 2 days at 37 °C.

Results

Screening the subtraction library and database analysis (homology search)

Approximately 150 white colonies were randomly selected from the PCR-selected subtraction library, and the plasmids were purified from each *E. coli* clone. The size of the insert DNA was determined by EcoRI digestion and agarose gel electrophoresis, and 105 clones with inserts longer than 500 bp were initially selected.

The 105 plasmid clones (clone numbers 1–105) were subjected to hybridization screening using the whole cDNA probes of 304-a and 304-b strains. Through the screening, 23 clones that hybridized to the 304-a cDNA probes, but not to the 304-b cDNA probes, were selected as candidates for R-heg. The insert DNA of the candidate clones were sequenced, and a homology search was performed. The result of the homology search is shown in Table 1. Ten clones contained the genes that showed amino acid identity (57–100%) with certain sequences in the database, while the remaining 13 clones did not exhibit significant homology with any registered sequences and appeared as hypothetical proteins in the genome database of *A. actinomycetemcomitans* (http://www.oralgen.lanl.gov/). Three clones in the candidates contained an identical sequence similar to the gene for peptidyl-prolyl *cis–trans* isomerase of *Haemophilus influenzae*, which was also known as a gene for *mip* among intracellular parasites such as *Legionella* or *Chlamydia*. The deduced amino acid sequence of Mip-like protein was aligned with the sequences of the peptidyl-prolyl *cis–trans* isomerase of *H. influenzae* and *Legionella* Mip, and is shown in Fig. 1. Three clones carrying the outer membrane protein gene (*ompA*) were also included in the 23 candidates. In addition to these, homologues of the sugar fermentation stimulation protein gene, peroxiredoxin gene (*prx*), the gene for D-lactate dehydrogenase and the σ 32 factor were identified. Among the 13 clones encoding hypothetical proteins, two clones showed an identical sequence, and therefore 18 distinct genes were selected as candidates for R-heg.

The nucleotide sequences of the *prx*-like gene and the *mip*-like gene have been registered with the DNA Data Bank of Japan (*prx*-like gene: AB126578, *mip*-like gene: AB048691).

mRNA accumulations of the R-heg candidates

The mRNA accumulations of the candidates for R-heg were compared between the 304-a and the 304-b strain by Northern hybridization. The intensity of the hybridization signal was normalized with the 16S rRNA gene detected on the ethidium bromide-stained gel. The mRNA level of the *mip*-like gene in the 304-a strain was approximately fourfold higher than that in the 304-b strain. The mRNA accumulations of the *ompA* and the *prx*-like gene in the 304-a strain were approximately 9- and 10-fold higher than that in the 304-b strain, respectively (Fig. 2a). These three genes were identified as R-heg. The mRNA levels of the other candidates were almost the same between the 304-a and the 304-b strains (Fig. 2b).

Table 1. Homologous molecules of R-heg candidates

Clone number*	Homologous molecule (bacterial species)	Accession no. (region†)	Sequence‡ identity (%)
11, 86, 94§	Peptidyl-prolyl *cis/trans* isomerase (*Haemophilus influenzae*)	NZ_AADO01000002 (363553–364278)	56.7
	Macrophage infectivity potentiator (Mip) (*Legionella pneumophila*)	NC_002942 (866844–867551)	32.9
5, 7, 100§	Outer membrane protein (OmpA) (*A. actinomycetemcomitans*)	BAA75215	100
2	Sugar fermentation stimulation protein (*H. influenzae*)	NC_009567 (1858143–1858859)	71.3
92	Peroxiredoxin (Prx) (*H. influenzae*)	NZ_AADP01000002 (440921–441646)	95.9
58	D-Lactate dehydrogenase (*H. influenzae*)	NC_007146 (89625–90620)	81.7
79	RNA polymerase σ 32 factor (*H. influenzae*: H 10269)	NC_009566 (320441–321286)	71.4
24, 61§	Hypothetical protein¶	–	–
12, 28, 32, 35, 36, 41, 78, 82, 84, 88, 99	Hypothetical proteins¶	–	–

*Twenty-three R-heg candidates selected from 105 clones in the subtraction library.
†In the case of the genome sequence.
‡Amino acid sequence.
§Multiple clones with an identical sequence.
¶On the genome sequence of *Aggregatibacter actinomycetemcomitans* in Oral Pathogen Databases.

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Aa:MLKMKKISVPVLILSAVVSSGVLAESKTDAKFTD-DSSYAVGVLFGSDLQGLINAQKGMI 59
Hi:MLKIQKLSIAALMVSAVISSQVFAE---DNTFDEKAASYAVGTLMGSQMKDLVDSHKEVI
Lp:-------MFMKMKLVTAAVMGLAMSTAMAATDATSLATDKDKLSYSIGADLGKNFKNQGI
   ...  . .   . .... .... ..   ....    ..... . ..    . .  .  *

Aa:DYNNAKVIAGISDVLNGKVKLEGNKEIASTLQGINAKLKAENEKRVAVMVKKAEEEGKKF 119
Hi:KYDNVRILDGLKDALEGKVDVRKDEKIQKTLESIEAKLVAASKAKAEAIAKQAKEEGDKF
Lp:DVNPEAMAKGMQDAMSGAQLALTEQQMKDVLNKFQKDLMAKRTAEFNKKADENKVKGEAF
   ....     *  *.. *..       .  .*  . ..* *   .     .. ....* .*

Aa:TAEFAKKDGVKKTASGLLYRVEKEGSGDAIKAADVVKVHYTGKLPDGTVFDSSRERGTPA 179
Hi:RAEFAKGKDVKTTQSGLMYKIESAGKGDTIKSTDTVKVHYTGKLPNGKVFDSSVERGQPV
Lp:LTENKNKPGVVVLPSGLQYKVINSGNGVKPGKSDTVTVEYTGRLIDGTVFDSTEKTGKPA
    .*.... .*. . *** *...  * *. ..  *.*.*.***.*.*.*.***. ..* *.

Aa:EFPLNQVIKGWTEGLQLVKKGGQIELVIPADLGYGEDGAGDKIPPHSTLYFDIEVLDVTP 239
Hi:EFQLDQVIKGWTEGLQLVKKGGKIQLVIAPELGYGEQGAGASIPPNSTLIFDVEVLDVNP
Lp:TFQVSQVIPGWTEALQLMPAGSTWEIYVPSGLAYGPRSVGGPIGPNETLIFKIHLISVKK
   .*.. **.*.***.***...*. ......  *.**. ..*  *.*..**.*......* .

Aa:AK--
Hi:KSEK
Lp:SS--
    .
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Fig. 1. Sequence comparison of *Aggregatibacter actinomycetemcomitans* Mip-like protein (Aa), peptidyl-prolyl *cis–trans* isomerase of *Haemophilus influenzae* (Hi) and *Legionella pneumophila* Mip (Lp). The amino acid sequence of the Mip-like protein of *A. actinomycetemcomitans* (Aa) showed 32.9% identity with the sequence of *L. pneumophila* Mip and 56.7% identity with the peptidyl-prolyl *cis–trans* isomerase of *H. influenzae*. The conserved amino acid sequences among the homologues were located at their C-terminus (Tradler *et al*., 1997) and were indicated by boxes. The identical sequences among the three proteins are shown by asterisks. The black dots indicate the common sequences among two of the three homologue proteins. Numbers on the right-hand side indicate the amino acid position of the Mip-like protein of *A. actinomycetemcomitans*. Accession number: Mip-like protein of *A. actinomycetemcomitans* (AB048691), peptidyl-prolyl *cis–trans* isomerase of *H. influenzae* (NZ_AADO01000002, region: 363553–364278), *L. pneumophila* Mip (NC_002942, region: 866844-867551).

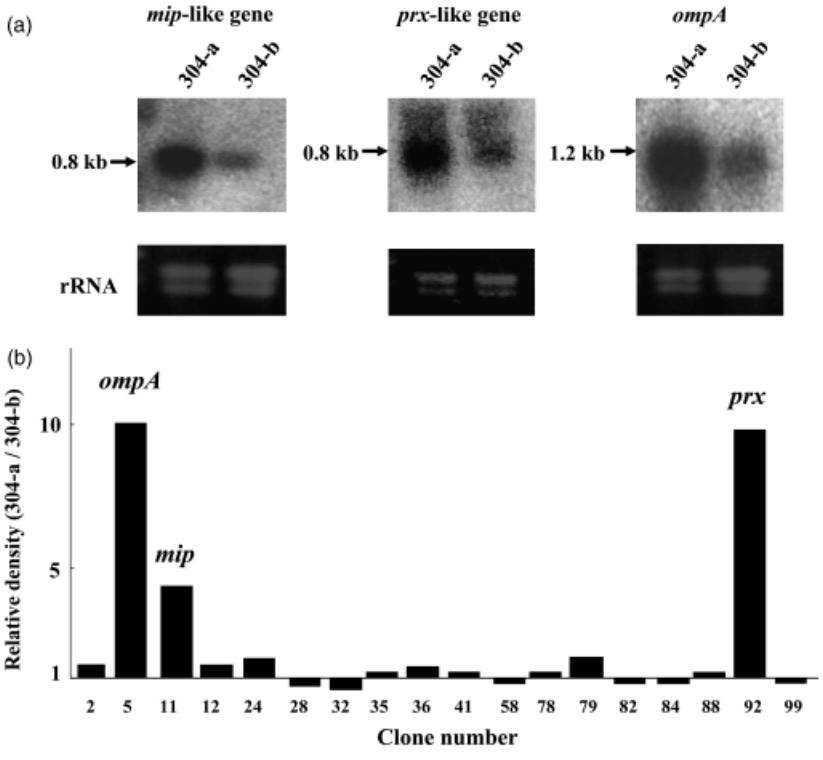

Fig. 2. The mRNA accumulation of R-heg candidates. Eighteen genes were selected as candidates for R-heg through subtractive hybridization and the following hybridization screening. The mRNA accumulations of the candidates were examined by Northern hybridization. Total RNA (2.5 μg) was extracted from the 304-a and 304-b strains and was probed with the candidate genes. The mRNA accumulations of the *mip*-like gene, *prx*-like gene and *ompA* were enhanced in the 304-a strain compared with that of the 304-b strain. Results of the three genes are representatively shown in (a). The intensities of the signals were analyzed using the NIH IMAGE and were normalized with the 16S rRNA gene detected on the ethidium bromide-stained gel. The signal intensity was compared between 304-a and 304-b, and the ratio of the signal intensity (304-a/304-b) is shown in (b). The mRNA levels of the *mip*-like gene, *prx*-like gene and *ompA* in the 304-a strain were 4–10-fold higher compared with the 304-b strain. The numbers under the horizontal axis are clone numbers of each gene corresponding to the numbers in Table 1.

Recombinant Mip-like protein and the reactivity of patients' sera

Recombinant Mip-like protein was constructed, purified and subjected to Western immunoblotting using sera from patients with periodontitis. Protein profiles of the transformed *E. coli* and fraction samples of Ni-NTA column chromatography are shown in Fig. 3a and b, respectively. An approximately 32-kDa protein was expressed in the *E. coli* transformed with the *mip*-like gene. The purified protein showed a single band at the expected size of 32 kDa. In the Western blot analysis, six out of eight patients' sera reacted with the purified recombinant protein, while two out of eight healthy control sera showed week reactivity (Fig. 3c).

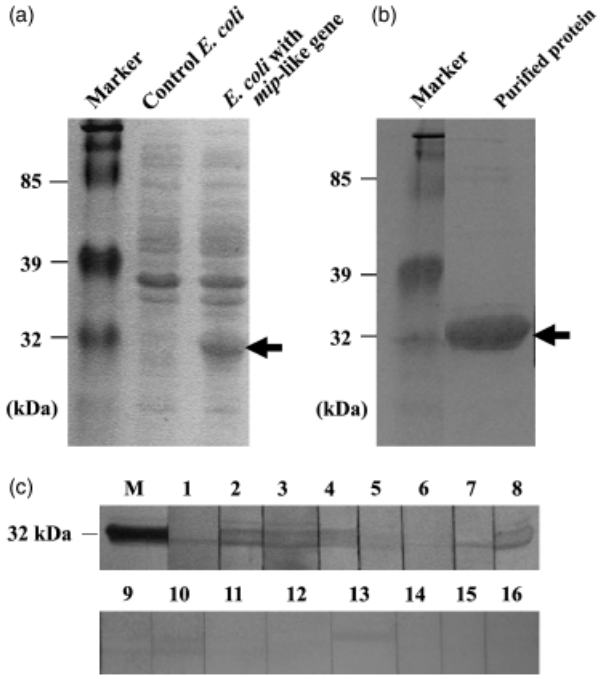

Fig. 3. Recombinant Mip-like protein and the reactivity of patients' sera. Recombinant Mip-like protein was constructed and subjected to Western immunoblotting against patients' sera. Protein profiles of transformed *Escherichia coli* (3 μg) with the *mip*-like gene and purified recombinant Mip-like protein (500 ng) are shown in (a) and (b), respectively. The transformed *E. coli* with the *mip*-like gene expressed an approximately 32-kDa protein under the induced condition with 1 mM isopropyl-β-D-thiogalactopyranoside. The control *E. coli* contained the expression vector without insert DNA. The expressed protein was purified by Ni-NTA column chromatography, and the purified sample appeared at 32 kDa on Coomassie brilliant blue-stained SDS-PAGE gel. The arrows indicate the recombinant Mip-like protein. (c) The reactivity of sera to the recombinant Mip-like protein. Six out of eight patients' sera showed reactivity to the recombinant protein, while two sera from healthy subjects showed weak reactions in lanes 10 and 13. Each strip contained approximately 50 ng of the purified protein. Lane M, recombinant Mip-like protein stained with amido black; lane 1–8, reactions of patients' sera; lane 9–16, reactions of healthy controls.

Construction of the *mip*-deficient mutant

The *spc* was used as a marker of homologous recombination. Three colonies of *A. actinomycetemcomitans* transformants on the TSBY plate containing the spectinomycin were randomly selected, and genomic DNAs were purified. Genomic DNA was also prepared from the WT strain (ATCC 29523). These genomic DNAs were then digested with DraI or BglI, and the digested DNA fragments were subjected to Southern hybridization with the ^{32}P-labeled probe of the *mip*-like gene. In the BglI-digested genomic DNA fragments of the WT strain, the hybridization signal was detected at approximately 2.0 kb, while the signal increased in size to around 2.7 kb in the three selected transformants because of the insertion of a 0.7-kb *spc* gene. When the genomic DNAs were digested with DraI, a single 1.5-kp band was detected in the WT strain, while two bands at around 1.3 and 0.9 kb were detected in the three transformants because the *spc* contained a DraI site (Fig. 4). One of the three transformants was used as the *mip*-deficient mutant for the invasion

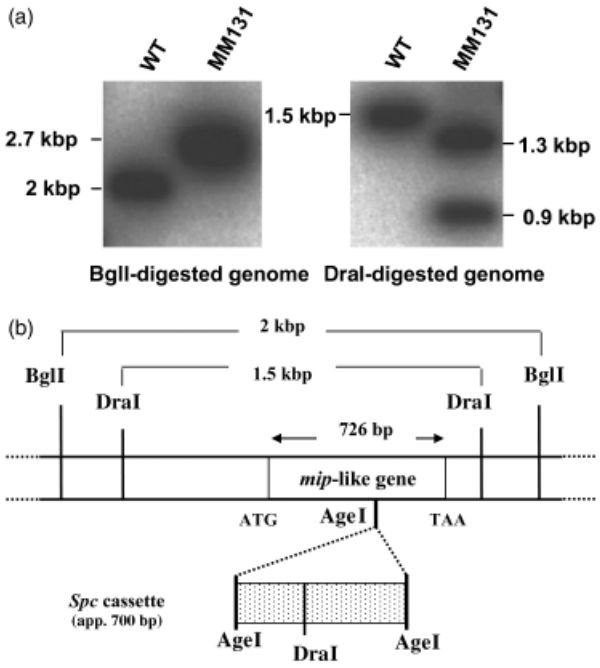

Fig. 4. Southern blot analysis of the *mip*-deficient mutant. The integration event of the *spc* cassette (0.7 kbp) into the *mip*-like gene on the chromosomal DNA was analyzed by Southern blot analysis (a). Restriction map around the *mip*-like gene is shown in (b). ORF of the *mip*-like gene was interrupted by the insertion of the *spc* cassette into the AgeI site. The chromosomal DNA from WT and the *mip*-deficient mutant (MM131) were digested with BglI or DraI, and were probed with the *mip*-like gene. The hybridization signal of BglI-digested DNA from MM131 was approximately 0.7 kbp longer than that of WT. A single 1.5-kbp band was detected from the DraI-digested DNA from WT, while two signals were detected at 1.3 and 0.9 kbp from the MM131. The *mip*-like gene and the *spc* contained no restriction site of BglI, and the *spc* cassette contained a single DraI site.

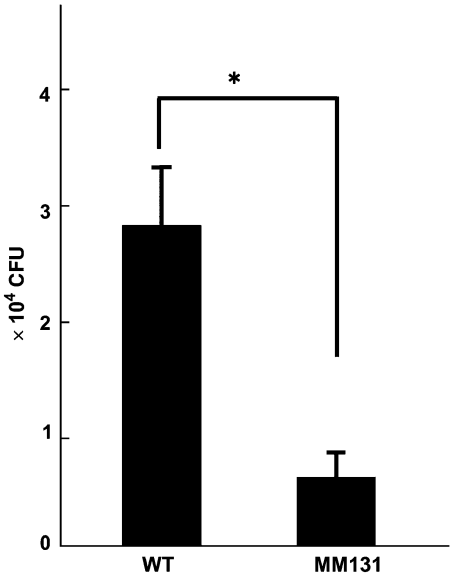

Fig. 5. Viable cell counts of the *mip*-deficient mutant in HeLa cells. The *mip*-deficient mutant (MM131) and the parent WT strain were recovered 6 h after they were incubated with HeLa cells. Triplicate measurements were carried out individually three times. Data are represented as CFU per well of culture plate (viable *Aggregatibacter actinomycetemcomitans* in 10^5 HeLa cells). Error bars denoted the SDs. A significant reducing ability of intracellular survival was demonstrated in MM131. An asterisk indicates a *P*-value of < 0.01 (Mann–Whitney test).

assay designated as MM131. No significant difference was seen in the growth kinetics between the WT and the MM131 strains (data not shown).

Invasion assay

Triplicate measurements were carried out individually three times for the invasion assay using the WT and the MM131 strains. Appropriate dilutions were spread on the TSBY plates, and CFU per well of a tissue culture plate (each well had initially contained 1×10^8 bacterial cells and 1×10^5 HeLa cells) was determined 6 h after the coincubation of *A. actinomycetemcomitans* and HeLa cells. Figure 5 demonstrates the CFU per well of invaded WT and MM31 strains in HeLa cells. The CFU of the WT strain was about fourfold higher than that of MM131.

Discussion

The morphological differences between the S-type and the R-type strains are obvious, and the differential expressions of the components such as fimbriae or membrane proteins on their surface structures have already been reported (Rosan *et al.*, 1988; Haase *et al.*, 1999; Mintz & Fives-Taylor, 2000). However, besides the surface components, the expression levels of a variety of genes were considered to be regulated in response to the environmental change from the oral cavity to the *in vitro* culture. Supporting this, Fine *et al.* (1999) demonstrated the reduction of virulence of *A. actinomycetemcomitans* such as proteinase activity through the *in vitro* passage. The rationale behind the current study is that the genes expressed in freshly isolated *A. actinomycetemcomitans* should contain virulence factors for its survival and adaptation to the environmental stresses in the host. To isolate the host-induced virulence genes, the cDNA subtractive hybridization technique was used.

We have succeeded in isolating three R-heg from 150 clones in the subtraction library. Colony hybridization preliminary performed for screening the library did not work well because of the high level of background interference (data not shown). This might be caused by hybridizations of whole population cDNA probes of *A. actinomycetemcomitans* that included homologous genes with *E. coli*. Therefore, the plasmids needed to be purified before screening. From 150 plasmid clones, 23 clones were initially selected by the hybridization screening. The number of the 16S rRNA gene was exponentially decreased through the subtraction procedure, and the plasmid clones that hybridized to 304-a cDNA probes were selected. However, most of the selected genes demonstrated almost the same levels of mRNA accumulations in both the 304-a and the 304-b strain, except for the three R-heg due to an unknown reason. In addition, the gene for fimbriae (*flp*) was not identified by the screening, although the presence of fimbriae is one of the characteristics of the R-type strain (Inoue *et al.*, 1998). The cut-off limit of the insert size (500 bp) might be the reason for the concealment of *flp*. As a consequence, a virulence (invasion)-associated gene, *mip*-like gene, was included in the R-heg, and therefore, isolation of R-heg was a good strategy for isolation of host-induced or virulence genes. However, because the screening capacity is limited, it seems to be difficult to cover the entire population of R-heg by the cDNA subtraction procedure.

Genes for OmpA, peroxiredoxin-like and Mip-like proteins were revealed to be R-heg, which might be induced in the host. The OmpA of *A. actinomycetemcomitans* has been reported to be a highly antigenic protein associated with the pathogenesis of periodontitis (Wilson, 1991). The OmpA family has multifunctions such as phage receptor (Datta *et al.*, 1977) or porin activity (Nikaido, 1987). Notably, associations of this molecule with the structural integrity of the cell membrane (Sonntag *et al.*, 1978) and invasion of brain microvascular endothelial cells have been reported (Prasadarao *et al.*, 1996). Although the definitive role of OmpA for *A. actinomycetemcomitans* has not been elucidated, this molecule is considered to be associated with the morphological change and virulence of *A. actinomycetemcomitans* similar to other OmpA homologues.

Peroxiredoxins, a family of peroxidases, have been reported to use thioredoxin, alkyl hydroperoxide reductase subunit F (AhpF), cyclophilin A or tryparedoxin for the decomposition of hydroperoxides and H_2O_2 (Chae *et al.*, 1994; Li Calzi & Poole, 1997; Nogoceke *et al.*, 1997; Lee *et al.*, 2001), and are responsible for the antioxidant defense in bacteria (Hofmann *et al.*, 2002). Recently, a new isoform of peroxiredoxin, which uses glutaredoxin, has been reported (Rouhier *et al.*, 2001). Following this, a chimeric enzyme, consisting of the peroxiredoxin region at the N-terminus and glutaredoxin region at the C-terminus (PGdx), has been identified in *H. influenzae* (Pauwels *et al.*, 2003). The *prx*-like gene of *A. actinomycetemcomitans* demonstrated a significant sequence identity with this fusion gene (95% identity in amino acid sequence). The *prx*-like gene of *A. actinomycetemcomitans* possibly possesses functions similar to the PGdx for survival of the cells in the host under oxidative environmental stresses.

The *ompA*- and *prx*-like genes demonstrated high homology with previously published sequences. Compared with these, the homology of the *mip*-like gene to the *Legionella mip* or the peptidyl-prolyl *cis–trans* isomerase of *H. influenzae* was not so high as to definitely identify it as their homologue. However, because conserved amino acid sequences of these homologues (Tradler *et al.*, 1997) were seen (Fig. 1), the Mip-like protein of *A. actinomyctemcomitans* might have functions similar to these molecules. Interestingly, on the genome sequence of *A. actinomycetemcomitans* at Oral Pathogen Databases, two distinct *mip*-like genes are located. The *mip*-like gene identified in the current study is registered with a gene ID of AA01517, while another *mip*-like gene is registered as AA00282. The deduced amino acid sequence from another *mip*-like gene showed 21.9% identity to *Legionella* Mip (data not shown). The sequence identity was lower than that of the identified *mip*-like gene in the current study. However, another *mip*-like gene will be an interesting subject for elucidating the virulence of *A. actinomycetemcomitans*, especially its ability to invade.

Mip is a 24-kDa protein with the enzymatic property of a peptidyl-prolyl *cis/trans* isomerase, homologous to the FK 506-binding protein, and contributes to the intracellular survival of *Legionella* or *Chlamydia* species (Engleberg *et al.*, 1989; Lundemose *et al.*, 1992). Among periodontal pathogens, *A. actinomycetemcomitans* and *Porphyromonas gingivalis* are known to have an ability to invade the host cells (Christersson *et al.*, 1987; Meyer *et al.*, 1991; Lamont *et al.*, 1992). Therefore, in the current study, attention was focused on the role of the *mip*-like gene as one of the virulence factors of *A. actinomycetemcomitans* and the *mip*-deficient mutant (MM131) was constructed for the analysis.

The invasion assay demonstrated that viable cell counts of the MM131 in HeLa cells were approximately fourfold fewer than that of the parent strain. The Mip null mutants of *Legionella* species are even more dramatically impaired in their ability to infect macrophages and protozoa. It has been reported that there were 10–1000-fold differences in their infection abilities between the mutants and the parent strains (Cianciotto *et al.*, 1989; Cianciotto & Fields, 1992). The *Legionella* mutant was also impaired in its ability to infect lung epithelia (Cianciotto *et al.*, 1995). The role of Mip is not considered to be specific to phagocytic cells. The Mip protein is localized on the surface of *Legionella* and is suggested to be involved in uptake into host cells (Helbig *et al.*, 2001). Recently, it has been reported that Mip also has an extracellular function in the pathogenesis of *Legionella* with a binding property to collagens (Wagner *et al.*, 2007). The impaired ability of MM131 to invade HeLa cells and the presence of a conserved sequence strongly suggest that the *mip*-like gene identified in the current study belongs to the *mip* homologue and is involved in the host cell invasion of *A. actinomycetemcomitans*. The role of the *mip*-like gene in the mechanisms of invasion and intracellular survival of *A. actinomycetemcomitans* remain to be elucidated. However, further characterization of the Mip-like protein will facilitate the study of the virulence of *A. actinomycetemcomitans*.

The recombinant Mip-like protein was constructed to analyze its antigenicity. Six patients' sera out of eight reacted with the recombinant protein. This suggests that the Mip-like protein of *A. actinomycetemcomitans* is expressed in the periodontal lesion and is a target of the humoral immune response. On the other hand, two sera did not show reactivity although all patients' sera demonstrated a high antibody titer against the whole cell sonic extract of *A. actinomycetemcomitans*. Because members of the Mip protein are homologous to the eukaryotic FK 506-binding protein (Fischer *et al.*, 1992), its antigenicity may be low due to immunologic tolerance. Weak reactions of two control sera may suggest the cross-reactivity of the Mip-like protein with the homologues of other microorganisms. Taken together with the unimpaired ability of MM131 to grow in bacteriologic medium, the *mip*-like gene seems to play a role in its intracellular survival rather than being a highly antigenic protein or a crucial molecule for normal growth of *A. actinomycetemcomitans*. However, recently, Bas *et al.* (2008) reported that the Mip protein of *Chlamydia* was suggested to be a toll-like receptor ligand. Besides the invasion, role of the Mip-like protein in proinflammatory cytokine production will also be an interesting subject.

The current study identified three R-heg of *A. actinomycetemcomitans*. They are the *ompA*, the *prx*-like gene and the *mip*-like gene, which are thought to be a highly antigenic protein, an antioxidant molecule and a possible potentiator for the invasion of host cells, respectively. Analysis of the isogenic strain is considered to represent an effective strategy for elucidating the pathogenesis of *A. actinomycetemcomitans*. Recent advances in molecular biology techniques, such

as DNA microarray or proteome analysis, will pave the way for future studies.

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