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Antigenic group II chaperonin in *Methanobrevibacter oralis* may cross-react with human chaperonin CCT

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SUMMARY

***Methanobrevibacter oralis* is an archaeal species frequently isolated from sites of severe periodontitis. However, its pathogenic roles remain unclear. Here, we aimed to isolate group II chaperonin from *M. oralis* and examine its antigenicity. The genes encoding two chaperonin subunits (Cpn-1 and Cpn-2) were cloned from *M. oralis* using polymerase chain reaction and genome walking procedures. Recombinant proteins Cpn-1 and Cpn-2 were generated, and the reactivities of sera from patients with periodontitis were examined by Western immunoblotting. The open reading frames of Cpn-1 and Cpn-2 genes consisted of 1641 and 1614 base pairs, respectively. Putative ATP-binding domains conserved among the chaperonin family were observed in both genes. The deduced amino acid sequences of the two genes showed 28.8–40.0% identity to each of the subunits of human CCT (CCT1–8). Thirty and 29 of 36 patients' sera reacted with the recombinant Cpn-1 and recombinant Cpn-2, respectively. Western immunoblotting using antiserum against human CCT subunits indicated that anti-CCT3 and anti-CCT8 antibodies recognized recombinant Cpn-1. In addition, anti-CCT1, CCT3, CCT6, and CCT8 antibodies recognized an antigen of approximately 60 kDa in *M. oralis*. The results suggested that the chaperonin subunits of *M. oralis* were antigenic molecules that were recognized by periodontitis patients and that may cross-react with human chaperonin CCT.**

INTRODUCTION

Archaea are microorganisms classified as one of the primary domains distinct from bacteria and eukaryotes (Woese *et al.*, 1990). It is now clear that *Archaea* are ubiquitous organisms and are closely associated with plants and animals, including humans. *Methanobrevibacter* is one such major genus isolated from the human oral cavity (Belay *et al.*, 1988), gastrointestinal tract (Karlin *et al.*, 1982), and vagina (Belay *et al.*, 1990). Despite the ubiquity and close association with humans, no pathogenic *Archaea* have yet been identified, and there is controversy regarding whether any actually exist

(Cavicchioli *et al.*, 2003; Eckburg *et al.*, 2003; Jangid *et al.*, 2004).

Periodontitis is an inflammatory disease caused by polymicrobial infection of oral microorganisms in subgingival dental plaque. Some gram-negative anaerobic rods and spirochetes in the plaque are closely associated with the disease (Socransky *et al.*, 1998) and are generally referred to as periodontal bacteria. In addition to periodontal bacteria, *Methanobrevibacter* species have been identified in cases of periodontitis (Ferrari *et al.*, 1994; Kulik *et al.*, 2001; Vianna *et al.*, 2008). Lepp *et al.* (2004) reported a relation between relative abundance of the *Methanobrevibacter* population and the severity of chronic periodontitis. We have also reported isolation of *Methanobrevibacter oralis* and *M. oralis*-like phylotypes from sites of severe periodontitis (Yamabe *et al.*, 2008). Although no studies have conclusively identified *Archaea* as causative agents of periodontitis, these previous reports suggest the potential pathogenic role of *Archaea* in this disease.

Heat shock proteins (Hsp) are highly conserved through evolution and have essential roles as molecular chaperones that assist in the efficient folding of newly synthesized and stress-denatured polypeptide chains (Ellis & van der Vies, 1991; Hartl, 1996). Among the members of the Hsp family, those with an approximate molecular mass of 60 kDa are called chaperonins (Cpn) and are divided into two groups, group I and group II (Horwich & Willson, 1993; Kubota *et al.*, 1994). They are similar in architecture to the oligomeric ring complexes and function as molecular chaperones. The amino acid sequences of Cpn are similar within the group, but not between the groups (Kubota *et al.*, 1995). Group I Cpn are also termed Cpn60, Hsp60, or GroEL, and are generally found in the bacterial cytosol as well as in mitochondria and chloroplasts. Under conditions of stress, such as heat shock, the Cpn molecules also appear at the cell surface (Alard *et al.*, 2007). Group II Cpn are found in the archaeal and eukaryotic cytosol. The archaeal group II Cpn are also known as thermosomes, and most archaeal Cpn complexes consist of two subunit proteins. Group II Cpn of eukaryotes are known as CCT (chaperonin containing T-complex polypeptide) and the human CCT complex consists of eight subunit proteins (Phipps *et al.*, 1991; Frydman *et al.*, 1992; Kubota *et al.*, 1995). Eukaryotes and some *Archaea* possess both groups of Cpn (Klunker *et al.*, 2003).

In addition to the chaperone role, bacterial Hsp60 (group I) are known to be highly antigenic molecules (Zugel & Kaufman, 1999a,b). Hsp60 are common antigens among bacterial species and serve as major immunogens in protection from the pathogenesis of infectious diseases. On the other hand, several immune disorders, such as rheumatoid arthritis, are thought to be triggered by these molecules (Feige & van Eden, 1996; van Eden *et al.*, 1998; Zugel & Kaufman, 1999a,b). As a result of the considerably high degree of primary amino acid sequence identity (molecular mimicry), immune responses against the infection-derived Hsp60 occasionally target human Hsp60 (group I), and cross-reactivity has been implicated in autoimmune or inflammatory diseases (Kiessling *et al.*, 1991). In periodontitis, Hsp60 of *Porphyromonas gingivalis* is known to be antigenic (Maeda *et al.*, 1994, 2000) and has been reported to promote the pathogenesis of atherosclerosis (Choi *et al.*, 2004; Ford *et al.*, 2005, 2007). However, there have been no reports of antigenicity of group II Cpn in any microorganisms.

We have attempted to elucidate the pathogenic role of *M. oralis* through the host immune response, and reported that immunoglobulin G (IgG) antibodies against this microorganism were detected in sera from patients with periodontitis. The sera recognized antigenic bands of approximately 60–70 kDa (Yamabe *et al.*, 2008). In the current study, for the first step of elucidating the antigenic property of *M. oralis*, group II Cpn were isolated and the antigenicity was examined. This is the first report identifying an antigenic molecule from *Archaea* recognized by the human immune system.

METHODS

Archaeal strains

M. oralis DSM 7256 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The purchased cells in liquid medium were pelleted and washed twice with phosphate-buffered saline (Invitrogen, Carlsbad, CA). The washed cells were subjected

directly to DNA extraction or were used as antigens for Western immunoblotting without subculture.

DNA extraction and amplification of genomic DNA

Genomic DNA was extracted from *M. oralis* by the method described by Stauffer *et al.* (Stauffer *et al.*, 1981). The extracted DNA was amplified using a REPLI-g Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amplified genomic DNA was used for genome walking and polymerase chain reaction (PCR) as described below.

Detection and cloning of chaperonin genes

We attempted to amplify partial fragments of both group I and group II Cpn genes from genomic DNA of *M. oralis* by PCR. The PCR for amplification of the group I Cpn gene was performed as described previously (Goh *et al.*, 1996; Hill *et al.*, 2004) using a set of universal primers based on the common sequence of the genes. For amplification of the group II Cpn genes, primers were designed based on two registered sequences of group II Cpn subunits of *Methanobrevibacter smithii* (accession number: NC_009515, regions: 209884–211539 and 784085–785704). Two primer sets were designed from one of the Cpn genes (region: 209884–211539) and a set of primers was designed from another gene (region: 784085–785704). The nucleotide sequences of the primers for group II Cpn are shown in supplementary Figs S1 and S2. The contents of the PCR mixtures (50 μl) were 2.5 units of Ampli*Taq* Gold, 1 × buffer (Applied Biosystems, Foster, CA), 2.5 mm $MgCl_2$, 0.4 μm of forward and reverse primers, 0.2 mm deoxynucleotide triphosphates, and 100 ng of *M. oralis* genomic DNA. The PCR parameters included an initial incubation at 94°C for 9 min to activate the Ampli*Taq* Gold DNA polymerase, followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR products were separated on 1.5% agarose gels and were purified from the gels using a QIAEX®II Gel Extraction Kit (Qiagen, Hilden, Germany). The purified fragments were then cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) for sequencing.

Genome walking

To obtain the full sequence of the Cpn genes, a PCR-based genome walking method (Siebert *et al.*, 1995) was performed. For the genome walking, a Universal GenomeWalker Kit (Clontech Laboratories, Mountain View, CA) was used according to the manufacturer's instruction manual. Briefly, amplified genomic DNA of *M. oralis* was digested with *Dra*I. Following digestion, the GenomeWalker adaptor provided with the kit was ligated to each end of the endonuclease-digested DNA for construction of the adaptor-ligated GenomeWalker library. For PCR of gene walking in each of 5′-flanking and 3′-flanking regions, two gene-specific primers were designed based on the nucleotide sequences of the cloned partial fragments of Cpn genes (shown in Figs S1 and S2). The primary PCR contained the GenomeWalker library as a template with the outer gene-specific primers and the first adaptor primer provided with the kit. The primary PCR product was then used as a template for nested PCR with the inner gene-specific primer and the second adaptor primer. Both PCR amplifications were performed using Advantage Genomic Polymerase Mix (Clontech Laboratories) under the conditions recommended by the manufacturer. The amplified DNA fragments were cloned to the pCR2.1 vector (Invitrogen, Carlsbad, CA) and were sequenced.

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 and microbial genomes held at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) using the blast sequence homology search program. Per cent sequence identities between *M. oralis* Cpn and other homologues of Cpn were analysed using Genetyx software (version 8; Genetyx, Tokyo, Japan).

Construction of recombinant protein

The recombinant Cpn of *M. oralis* (rCpn) was constructed using a pET Directional TOPO® Expression Kit (Invitrogen, Carlsbad, CA) according to the

manufacturer's instructions. Briefly, a partial open reading frame of the Cpn gene of *M. oralis* was amplified by PCR and inserted into the plasmid vector pET101/D-TOPO® (Invitrogen). The sequences of primers and the positions in the Cpn genes are shown in Figs S1 and S2. For directional cloning, four nucleotides (cacc) were added to the 5′ end of the forward primers, and the reverse primers were designed to allow the PCR product in frame with the histidine (His)-tag in the vector. *Escherichia coli* Rosetta™ 2 strain (DE3) (Novagen, Darmstadt, Germany) was transformed with the recombinant plasmid and was cultivated in Luria–Bertani broth supplemented with 50 μg ampicillin per ml and 1 mm isopropylthio-β-d-galactoside. The recombinant protein was purified from *E. coli* using a Qiagen Ni-NTA Fast Start Kit (Qiagen, Hilden, Germany) based on His-tag technology. Fractions eluted from the Ni-NTA columns were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the gel region corresponding to rCpn was excised. The recombinant proteins in the excised gel fragment were then eluted and recovered from the gel using an Electro Eluter model 422 (Bio-Rad, Hercules, CA), and subjected to SDS–PAGE and Western immunoblotting.

SDS–PAGE and Western immunoblotting

SDS–PAGE and Western blotting analysis were performed as described previously (Kokeguchi *et al.*, 1989). Sera from patients with periodontitis were used at a final dilution of 1 : 200 with 5% (weight/volume) skimmed milk in Tris-buffered saline (10 mm Tris–HCl buffer, pH 7.5, 0.9% NaCl; M-TBS). Anti-human CCT1 (Abnova Co, Taipei, Taiwan), CCT3, CCT5, CCT6, and CCT8 polyclonal antibodies (Proteintech Group Inc.) were used at a dilution of 1 : 500 in M-TBS. Anti-penta·His-tag antibody (Qiagen) was used at a dilution of 1 : 1000 in TBS containing 3% bovine serum albumin. *E. coli* transformed with the Cpn genes of *M. oralis*, rCpn, recombinant human CCT1 subunit (Abnova Co, Taipei, Taiwan), human gingival fibroblasts, or *M. oralis* were used as antigens. Whole-cell lysates of *E. coli*, human gingival fibroblasts, and *M. oralis* were prepared as described previously (Maeda *et al.*, 2000; Yamabe *et al.*, 2008). Horseradish peroxidase-conjugated goat anti-human IgG antibody (Chemicon International, Inc.), goat anti-mouse IgG antibody (Millipore) or donkey anti-rabbit IgG antibody (GE Health Care, Pittsburgh, PA) were used at a dilution of 1 : 5000 with M-TBS for detection.

Serum samples

Sera from 36 patients were selected from the serum sample collection of the Okayama University Hospital of Medicine and Dentistry. Serum IgG antibody titer to the periodontal pathogens had been examined previously for clinical diagnosis (Murayama *et al.*, 1988). The sera selected in this study had elevated IgG antibody titers (greater than the mean + 2SD of the healthy controls) to the sonication extract of *P. gingivalis*, a potent pathogen in periodontitis, suggesting that patients had considerable periodontitis lesions (Murayama *et al.*, 1988). Six periodontally healthy subjects were also examined as controls. The use of human subjects in this investigation was approved by Okayama University Hospital Ethics Committee (approved no. 624).

RESULTS

Cloning and sequencing of *M. oralis* Cpn genes

PCR using universal primers for the group I Cpn gene yielded no amplification products (data not shown). However, PCR using primers designed from group II Cpn genes of *M. smithii* successfully amplified the genes, yielding reaction products of approximately 900, 1000, and 1450 base pairs (bp) (data not shown).

The three PCR products were cloned and sequenced. The nucleotide sequences of the two PCR products of 900 and 1000 bp overlapped each other by 602 bp; i.e. PCR using two primer sets designed from the same Cpn gene of *M. smithii* amplified the identical gene from *M. oralis* (Cpn-1 gene: Fig. S1). The remaining PCR product of 1450 bp had a distinct sequence (Cpn-2 gene: Fig. S2). By genome walking, DNA fragments containing 5′-flanking and 3′-flanking regions of the cloned partial Cpn-1 and Cpn-2 genes were amplified, and the complete nucleotide sequences of the Cpn-1 and Cpn-2 genes were determined (Figs S1 and S2). The open reading frames of the Cpn-1 and Cpn-2 genes consisted of 1641 bp (encoding 546 amino acids) and 1614 bp (encoding 537 amino

acids), respectively. The deduced amino acid sequences of both Cpn-1 and Cpn-2 genes contained four putative ATPase domains (Kubota *et al.*, 1995). The nucleotide sequences of the genes have been registered with the DNA Data Bank of Japan (*Cpn-1* gene: AB376229; *Cpn-2* gene: AB455150).

Homology search

A blast sequence homology search did not find any sequences identical to Cpn-1 or Cpn-2 genes in GenBank or Microbial Genomes at NCBI. Among the registered sequences in the database, Cpn-1 and Cpn-2 showed the highest levels of amino acid identity to each of the Cpn genes of *M. smithii* from which PCR primers were designed for amplification. The identity of Cpn-1 to one of the *M. smithii* Cpn was 83.9%, and Cpn-2 showed 91.1% identity to another *M. smithii* Cpn. Cpn-1 and Cpn-2 showed amino acid identities of 17.6% and 21.5% to human Hsp60 (group I), respectively. The identities to *E. coli* GroEL were 21.6% (Cpn-1) and 20.7% (Cpn-2). Amino acid identities of Cpn-1 to each of the human CCT subunits ranged between 28.8 and 37.8%, while Cpn-2 showed amino acid identities ranging from 31.4 to 40.0% to the CCT subunits (Table 1). CCT3 and CCT4 showed the greatest levels of sequence identity with Cpn-1 and Cpn-2 among the human CCT subunits, respectively. The amino acid sequences of CCT3 and CCT4 were aligned with Cpn-1 and Cpn-2, respectively, as shown in supplementary Figs S3 and S4.

Amino acid sequence identity between *M. oralis* Cpn and other Cpn homologues in *Archaea* ranged from approximately 40 to 90%. Representative results of the homology search in *Archaea* are shown in Table 2.

Construction and purification of recombinant proteins

A partial Cpn-1 gene fragment (849 bp from the start codon) and Cpn-2 gene fragment (780 bp from the start codon) were cloned into the expression vector (Figs S1 and S2). Protein profiles of *E. coli* transformed with the expression vector and rCpn during the purification steps were shown in Fig. 1. On SDS–PAGE analysis, no significant differences were found between control *E. coli* and those transformed with either the Cpn-1 or Cpn-2 gene. However, Western blotting analysis using anti-penta·His-tag antibody detected the recombinant protein in both transformants. *E. coli* transformed with the Cpn-1 gene expressed proteins of 42 kDa, 35 kDa, and 42 kDa that were reactive with anti-penta·His-tag antibody, while those transformed with the Cpn-2 gene expressed a protein of 40 kDa. The recombinant proteins of 42 kDa (rCpn-1) and 40 kDa (rCpn-2) expressed in *E. coli* were purified by Ni-NTA columns and gel elution.

Table 1 Amino acid sequence identity between *Methanobrevibacter oralis* chaperonin (Cpn) and human CCT subunits (%)

Cpn subunit	CCT1	CCT2	CCT3	CCT4	CCT5	CCT6	CCT7	CCT8
Cpn-1	36.6	33.1	37.8	37.4	36.4	28.8	34.1	35.1
Cpn-2	37.4	33.4	38.4	40.0	39.0	31.4	38.1	33.3

Accession numbers: *M. oralis* Cpn-1 (AB376229), Cpn-2 (AB455150), CCT1 (CAA37064), CCT2 (NP-006422), CCT3 (NP-005989), CCT4 (NP-006421), CCT5 (NP-036205), CCT6 (NP-001009186), CCT7 (NP-006420), CCT8 (NP-006576).

Table 2 Amino acid sequence identity (%) of chaperonin (Cpn)[1] between *Methanobrevibacter oralis* and other *Archaea* species

Archaeal species	*M. smithii*	*M. thermautotrophicus*	*Methanosphaera stadtmanae*	*Archaeoglobus fulgidus*	*Thermococcus kodakarensis*	*Haloarcula marismortui*
M. oralis Cpn-1	84 and 58	60 and 59	57 and 45	52 and 51	53 and 53	51 and 46
M. oralis Cpn-2	92 and 58	81 and 70	79 and 45	64 and 63	65 and 63	61 and 55

[1]Two Cpn subunits were compared with each other.

Accession numbers: *M. oralis* Cpn-1 (AB376229), Cpn-2 (AB455150), *Methanobrevibacter smithii* (NC-009515), *Methanobrevibacter thermautotrophicus* (NC-000916), *M. stadtmanae* (NC-007681), *A. fulgidus* (NC-000917), *T. kodakarensis* (NC-006624), *H. marismortui* (NC-006396).

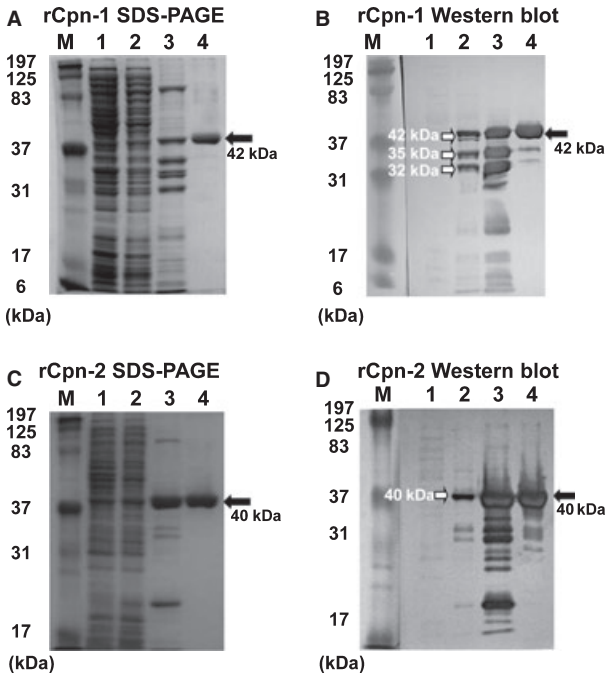

Figure 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analysis of recombinant chaperonin 1 (rCpn-1) and rCpn-2. (A) and (B) showed the results of SDS–PAGE and Western blotting analysis of rCpn-1 during the purification steps, respectively. (C) and (D) show the results of SDS–PAGE and Western blotting analysis of rCpn-2, respectively. Distinct expression bands of rCpn-1 and rCpn-2 were not seen in protein profiles of the transformant *Escherichia coli* on SDS–PAGE, while anti-penta·His-tag antibody detected the recombinant proteins in each *E. coli* (white arrows). *E. coli* transformed with the Cpn-1 gene expressed proteins of approximately 32 kDa, 35 kDa, and 42 kDa that reacted with anti-penta·His-tag antibody (B). The 42-kDa recombinant protein was purified as rCpn-1. *E. coli* transformed with the Cpn-2 gene expressed a recombinant protein of approximately 40 kDa (white arrow in D) and was purified as rCpn-2. Final purification samples of rCpn-1 and rCpn-2 are indicated by black arrows. Lane M, molecular weight marker; lane 1, control *E. coli* (5 μg); lane 2, *E. coli* transformed with either the Cpn-1 or Cpn-2 gene (5 μg); lane 3, Ni-NTA column-purified fraction (1 μg); lane 4, final purification sample (500 ng).

Reactivity of sera from patients against rCpn

The reactivities of sera from patients with periodontitis against rCpn-1 and rCpn-2 were examined by Western immunoblotting (Fig. 2). Sera from 30 and 29 of the total of 36 patients reacted with rCpn-1 and rCpn-2, respectively. Two and three of six control sera showed weak reactivity to rCpn-1 and rCpn-2, respectively.

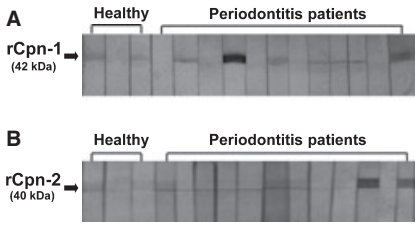

Figure 2 Serum reaction to *Methanobrevibacter oralis* chaperonin (Cpn). Reactivities of sera from patients against recombinant Cpn-1 (rCpn-1) (A) and rCpn-2 (B) were examined by Western immunoblotting. Sera from 36 patients with periodontitis and six healthy subjects were tested, and the representative results are shown. Two of six healthy controls and 30 of 36 patients showed reactivity to rCpn-1. Sera from three of the six controls and 29 of the 36 patients showed reactivity against rCpn-2. Each membrane strip contained approximately 50 ng rCpn-1 or rCpn-2.

Cross-reactivity between *M. oralis* Cpn and human CCT subunits

Cross-reactivity between *M. oralis* Cpn and human CCT was examined using anti-human CCT1, CCT3, CCT5, CCT6, and CCT8 polyclonal antibodies and rCpn of *M. oralis*. Western blotting analysis indicated that anti-human CCT3 and CCT8 antibodies reacted with rCpn-1 (Fig. 3), whereas none of the anti-human CCT antibodies reacted with rCpn-2 (data not shown).

The reactivities of anti-human CCT antibodies against the whole-cell lysate of *M. oralis* and human

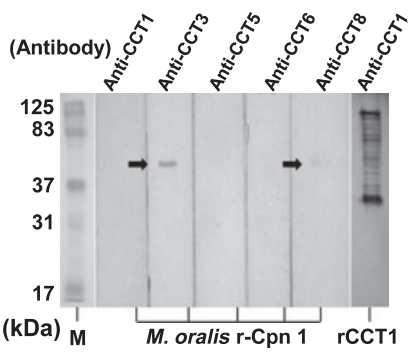

Figure 3 Reactivity of anti-human chaperonin (CCT) antibodies against recombinant chaperonin 1 (rCpn-1). Commercially available antibodies against human CCT1, CCT3, CCT5, CCT6, and CCT8 were used for Western immunoblotting to examine the reactivity against rCpn-1 and rCpn-2. Anti-CCT3 and anti-CCT8 antibodies reacted to rCpn-1, whereas none of the antibodies reacted to rCpn-2 (data not shown). Each lane contained approximately 50 ng rCpn-1. Recombinant human CCT1 (rCCT1) was loaded in the right lane, and the reactivity of anti-human CCT1 antibody was tested as a control. Lane M, molecular weight marker.

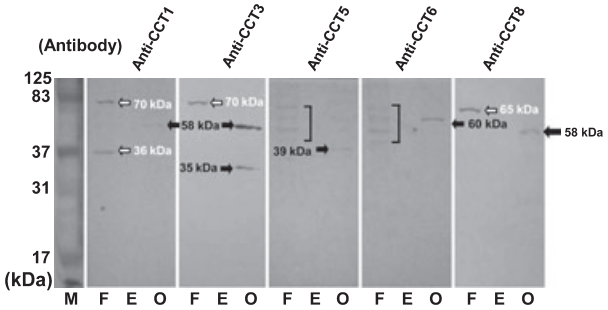

Figure 4 Reactivity of anti-human chaperonin (CCT) antibodies against *Methanobrevibacter oralis* cell lysate. The reactivities of anti-human CCT antibodies (anti-CCT1, anti-CCT3, anti-CCT5, anti-CCT6, and anti-CCT8) against whole-cell lysate of *M. oralis* (lane O) and human fibroblasts (lane F) were examined by Western immunoblotting. Anti-CCT1, anti-CCT3, anti-CCT6, and anti-CCT8 antibodies reacted to antigens of approximately 60 or 58 kDa of *M. oralis*. Anti-CCT3 antibody also recognized a 35-kDa antigen in *M. oralis*, and anti-CCT5 antibody recognized an antigen of molecular mass 39 kDa distinct from *M. oralis* Cpn. The lysate of human fibroblasts was used as a control containing CCT subunits. Anti-CCT1 and anti-CCT3 antibodies reacted to a fibroblast antigen of approximately 70 kDa. Anti-CCT1 antibody also recognized a 36-kDa antigen, and anti-CCT8 antibody reacted to an antigen of approximately 65 kDa in fibroblasts. Anti-CCT5 and anti-CCT6 antibodies recognized multiple fibroblast antigens at around 60 kDa (brackets). Each lane contained approximately 2 μg of protein. Lanes E and M, empty lane and molecular weight marker, respectively.

fibroblasts were examined by Western immunoblotting (Fig. 4). Each of the anti-human CCT antibodies reacted with the human fibroblast antigens (control) at around 60–70 kDa. In addition to the antigen of approximately 70 kDa, anti-human CCT1 antibody also recognized a 36-kDa fibroblast antigen. Anti-CCT5 and CCT6 antibodies detected multiple fibroblast antigens at around 60 kDa. In the reaction against *M. oralis*, anti-CCT1, CCT3, and CCT8 antibodies recognized an antigen of approximately 58 kDa in *M. oralis*. In addition, anti-CCT6 antibody recognized an antigen of approximately 60 kDa in *M. oralis*. In addition to the 58-kDa antigen, anti-CCT3 antibody detected another antigen of approximately 35 kDa. Anti-CCT5 antibody recognized only a 39-kDa antigen in *M. oralis*.

DISCUSSION

Methanobrevibacter species were identified in cases of periodontitis as early as 1988 (Belay *et al.*, 1988). However, their pathogenicity and influence on the pathogenesis of periodontitis have not been clarified. Recently, Lepp *et al.* (Lepp *et al.*, 2004) reported that *M. oralis* and similar species were the predominant microorganisms in microflora of sites of severe periodontitis. They also demonstrated that the relative abundance of *Treponema* species was significantly lower in sites with methanogenic *Archaea* than in those sites without methanogenic *Archaea*. Lepp *et al.* (2004) explained the pathogenic role of methanogenic *Archaea* through their influence on the microbial communities of subgingival plaque. Apart from their hypothesis, we are trying to determine the pathogenicity through antigenicity and host immune responses. We have recently reported that sera from patients with periodontitis recognized components of *M. oralis* including major 60–70 kDa antigens (Yamabe *et al.*, 2008). As Cpn with a molecular mass of 60–70 kDa are predicted to be highly expressed in *Archaea* (Karlin *et al.*, 2005), we aimed to identify the Cpn from *M. oralis* and characterize the antigenic property through the serum reaction of patients and the cross-reactivity with human CCT.

In the present study, two group II Cpn genes were identified in *M. oralis* but group I Cpn was not identified. Although some archaeal species possess both groups of Cpn, most *Archaea* possess only group II Cpn (Klunker *et al.*, 2003). Two genes for the group II Cpn subunits were found in the genome of *M. smithii* (NC_009515), and most group II Cpn of *Archaea* consist of two subunits (Kubota *et al.*, 1995). These findings, taken together, suggest that *M. oralis* has group II Cpn consisting of two subunits, but not group I Cpn.

We determined the complete nucleotide sequences of the two group II Cpn genes of *M. oralis*. The sequence identities between *M. oralis* Cpn and human CCT subunits ranged from 28.8 to 40.0% (Table 1), and conserved peptides (three to nine amino acids) were found throughout the length of the sequence (Figs S3 and S4). These findings of structural analysis suggest the possibility of immunological cross-reactivity based on molecular mimicry between human and *M. oralis* Cpn. In addition, Cpn homologues show high levels of sequence identity among archaeal species (Table 2: approximately 40–90%). *Archaea* are ubiquitous organisms prevalent in animals, including humans. Cpn may have the potential to be common antigens among archaeal strains similar to Hsp60 among bacterial strains.

To examine the antigenicity of *M. oralis* Cpn, rCpn-1 and rCpn-2 were constructed. As distinct bands of rCpn were not seen on SDS-PAGE analysis of the transformant *E. coli*, the levels of expression were estimated to be low. Both Cpn genes of *M. oralis* contained codons rarely used in *E. coli*, such as AUA and CUA. The Rosetta strain supplied with tRNA genes for these codons was used as the *E. coli* host strain (Novy *et al.*, 2001). However, efficient expression of the recombinant protein may still be difficult because of the codon usage of the Cpn genes of *M. oralis*. The Cpn-1 gene-transformed *E. coli* expressed recombinant proteins of 32, 35, and 42 kDa that reacted with anti-penta·His-tag antibody. The molecular mass of rCpn-1 was estimated to be about 42 kDa, and the His tag was fused to the C-terminus of rCpn-1. Therefore, the 32-kDa and 35-kDa recombinant proteins were probably truncated at the N-terminus. These molecules may be translated from the downstream methionine codons.

Most of the sera from patients reacted with rCpn-1 and rCpn-2, suggesting that Cpn-1 and Cpn-2 of *M. oralis* are highly antigenic molecules. Among them, five sera showed very strong reactivity to either or both of the rCpn (three of them were representatively shown in Fig. 2). These patients were possibly exposed to *M. oralis* and Cpn in periodontal lesions. Interestingly, all these high responders had severe periodontal lesions (bone loss score: 26.4–54.2%). A study for relations between serum reactivity to the Cpn and the clinical status of patients will be a good strategy for elucidation of *M. oralis* on the pathogenesis of periodontitis. Since an elevated IgG antibody titer against *P. gingivalis* was a criterion for the serum selection, the relations could not be cleared in the current study. Quantitative analysis of the serum reaction and various categories of patients will be required for the elucidation. In previous studies, membrane lipids of methanogens were shown to have strong adjuvant activity (Conlan *et al.*, 2001; Krishnan *et al.*, 2001). However, there have been no previous reports of antigenic molecules from *Archaea*. Our recent report indicated that sera from patients with periodontitis recognized the components of *M. oralis* (Yamabe *et al.*, 2008), and one of the antigenic molecules was identified in the current study. Although definite roles are still under elucidation, *Archaea* with strong adjuvant and antigenic molecules are probable modifier of inflammation in periodontal lesion. Weak reactions were seen in healthy controls. These reactions may have been to the result of exposure to other *Archaea*, which resulted in cross-reactivity among their Cpn. *M. smithii* in the gut is the most likely candidate for the source of cross-reactive Cpn.

Human CCT consists of eight subunit proteins. In the present study, cross-reactivity between *M. oralis* Cpn and human CCT subunits was examined using rCpn and five commercially available antibodies against each human CCT (subunits 1, 3, 5, 6, and 8). These antibodies were produced by immunizing animals with the recombinant CCT subunits (whole or approximately half-length). Anti-CCT 2, 4, and 7 antibodies were also obtained from commercial sources. However, they were produced by immunizing animals with short CCT peptides that did not include homologous sequences between *M. oralis* Cpn and human CCT. As the recognition epitopes were limited in the short sequences, anti-CCT 2, 4, and 7 antibodies without the possibility of cross-reactivity were excluded in the present study. Western blotting analysis suggested that CCT3 and CCT8 were cross-reactive antigens of *M. oralis* Cpn-1 with molecular mimicry. In contrast, none of the anti-CCT antibodies examined reacted with rCpn-2. Cpn-2 may not cross-react with human CCT subunits 1, 3, 5, 6, and 8. However, as neither of the rCpn examined here included the C-terminal regions, cross-reactivity in the truncated regions should be examined in future studies. In addition, polyclonal antibodies against CCT2, 4, and 7 are required to examine cross-reactivity of the uninvestigated subunits in the present study. CCT4, which showed the highest level of sequence identity to Cpn-2 (40.0%), is the most promising target for such studies.

In addition to rCpn, the reactivities of anti-human CCT antibodies against whole-cell lysate of *M. oralis* were examined. Each of the antibodies against the CCT subunits detected the cross-reactive antigens in *M. oralis.* As anti-CCT3 and anti-CCT8 antibodies reacted with rCpn-1, the 58-kDa antigen detected by these antibodies was thought to be Cpn-1 in *M. oralis*. The 58-kDa and 60-kDa antigens detected by anti-CCT1 and anti-CCT6 antibodies were not definitely identified as Cpn of *M. oralis*. However, because of the molecular weights and sequence homology between the CCT subunits and *M. oralis* Cpn, the

detected antigens were suggested to be *M. oralis* Cpn. In addition to CCT3 and CCT8, CCT1 and CCT6 may cross-react with *M. oralis* Cpn. Taken together with the reactivity to rCpn, anti-CCT1 and anti-CCT6 antibodies may recognize C-terminal regions of either Cpn-1 or Cpn-2. The 35-kDa and 39-kDa antigens detected by anti-CCT3 and anti-CCT5 antibodies with the distinct molecular mass of Cpn may have molecular mimicry with the human CCT subunit. Whole-cell lysate of human fibroblasts was used as control antigen including human CCT subunits. Anti-CCT1, CCT3, and CCT8 antibodies recognized antigens of approximately 65–70 kDa in fibroblasts, suggesting that they were very specific antibodies. In contrast, anti-CCT5 and CCT6 antibodies recognized multiple antigen bands at around 60 kDa. As the human CCT subunits show approximately 30% amino acid sequence identity with each other, anti-CCT5 and anti-CCT6 antibodies may cross-react with other CCT subunits. The 36-kDa fibroblast antigen detected by anti-CCT1 antibody may have cross-reactive epitopes with human CCT1.

The relation between autoimmune diseases and group I Cpn has been the subject of a great deal of discussion (Kiessling *et al.*, 1991; Wu & Tanguay, 2006). With regard to periodontal disease, it has been reported that Hsp60 from periodontal pathogens cross-react with human Hsp60 and may cause an autoimmune reaction to human Hsp60 (Choi *et al.*, 2004; Mandal *et al.*, 2004). The autoimmune reaction against Hsp60 was suggested to be involved in the pathogenesis of periodontitis and atherosclerosis (Ueki *et al.*, 2002; Ford *et al.*, 2005, 2007). In contrast, there have been very few studies regarding the autoimmune reaction against group II Cpn (human CCT), although human CCT was reported to be an immune target in patients with autoimmune disease or cancer (Yokota *et al.*, 2000; Schmits *et al.*, 2002). Yokota *et al.* (2000) reported that there were greater differences between sera from autoimmune disease patients and controls in CCT-reactive antibodies than in anti-group I Cpn (Hsp60) antibodies, suggesting that CCT may be a useful diagnostic antigen with which to detect the autoantibodies prevalent in patients with rheumatic disease. They hypothesized that infection with bacteria may cause an immune response to Hsp60 and result in the production of antibodies against epitopes structurally related to human CCT, and showed that the CCT-reactive autoantibodies recognized conformational epitopes conserved among CCT and Hsp60 (group I) members. Despite the low level of amino acid sequence identity (15–20%), bacterial Hsp60 was considered to be a cross-reactive antigen to human CCT because of their similar three-dimensional architecture. *Archaea* with group II Cpn have not yet been accepted as pathogens exposed to the host immune system, and the antigenicity of their Cpn has not been reported previously. However, recent reports of *Archaea* in patients with periodontitis (Lepp *et al.*, 2004; Yamabe *et al.*, 2008) and the antigenic properties of *M. oralis* Cpn revealed in the present study strongly suggest that *M. oralis* was prevalent in periodontal lesions and that the Cpn were cross-reactive antigens of human CCT recognized by the host immune system.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Nucleotide and deduced amino acid sequence of the Cpn-1 gene.
Figure S2. Nucleotide and deduced amino acid sequence of the Cpn-2 gene.
Figure S3. Sequence alignment of Cpn-1 with human CCT subunits.
Figure S4. Sequence alignment of Cpn-2 with human CCT4.

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REFERENCES

Alard, J.E., Dueymes, M., Youinou, P. and Jamin, C. (2007) Modulation of endothelial cell damages by anti-Hsp60 autoantibodies in systemic autoimmune diseases. *Autoimmun Rev* **6**: 438–443.

Belay, N., Johnson, R., Rajagopal, B.S., de Macario, E.C. and Daniels, L. (1988) Methanogenic bacteria from human dental plaque. *Appl Environ Microbiol* **54**: 600–603.

Belay, N., Mukhopadhyay, B., Conway, D.M., Galask, R. and Daniels, L. (1990) Methanogenic bacteria in human vaginal samples. *J Clin Microbiol* **28**: 1666–1668.

Cavicchioli, R., Curmi, P.M., Saunders, N. and Thomas, T. (2003) Pathogenic archaea: do they exist? *BioEssays* **25**: 1119–1128.

Choi, J.I., Chung, S.W., Kang, H.S. *et al.* (2004) Epitope mapping of *Porphyromonas gingivalis* heat-shock protein and human heat-shock protein in human atherosclerosis. *J Dent Res* **83**: 936–940.

Conlan, J.W., Krishnan, L., Willick, G.E., Patel, G.B. and Sprott, G.D. (2001) Immunization of mice with lipopeptide antigens encapsulated in novel liposomes prepared from the polar lipids of various Archaeobacteria elicits rapid and prolonged specific protective immunity against infection with the facultative intracellular pathogen *Listeria monocytogenes*. *Vaccine* **19**: 3509–3517.

Eckburg, P.B., Lepp, P.W. and Relman, D.A. (2003) *Archaea* and their potential role in human disease. *Infect Immun* **71**: 591–596.

van Eden, W., van der Zee, R., Paul, A.G. *et al.* (1998) Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? *Immunol Today* **19**: 303–307.

Ellis, R.J. and van der Vies, S.M. (1991) Molecular chaperones. *Annu Rev Biochem* **60**: 321–347.

Feige, U. and van Eden, W. (1996) Infection, autoimmunity and autoimmune disease. *EXS* **77**: 359–373.

Ferrari, A., Rrusa, T., Rutili, A., Canzi, E. and Biavati, B. (1994) Isolation and characterization of *Methanobrevibacter oralis* sp.nov. *Curr Microbiol* **29**: 7–12.

Ford, P.J., Gemmell, E., Hamlet, S.M. *et al.* (2005) Cross-reactivity of GroEL antibodies with human heat shock protein 60 and quantification of pathogens in atherosclerosis. *Oral Microbiol Imunol* **20**: 296–302.

Ford, P.J., Gemmell, E., Timms, P., Chan, A., Preston, F.M. and Seymour, G.J. (2007) Anti-*P. gingivalis* response correlates with atherosclerosis. *J Dent Res* **86**: 35–40.

Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J.S., Tempst, P. and Hartl, F.U. (1992) Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. *EMBO J* **11**: 4767–4778.

Goh, S.H., Potter, S., Wood, J.O., Hemmingsen, S.M., Reynolds, R.P. and Chow, A.W. (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *J Clin Microbiol* **34**: 818–823.

Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature* **381**: 571–580.

Hill, J.E., Penny, S.L., Crowell, K.G., Goh, S.H. and Hemmingsen, S.M. (2004) cpnDB: a chaperonin sequence database. *Genome Res* **14**: 1669–1675.

Horwich, A.L. and Willson, K.R. (1993) Protein folding in the cell: functions of two families of molecular chaperone, hsp 60 and TF55-TCP1. *Philos Trans R Soc Lond B Biol Sci* **339**: 313–325.

Jangid, K., Rastogi, G., Patole, M.S. and Shouche, Y.S. (2004) *Methanobrevibacter*: is it a potential pathogen? *Curr Sci* **86**: 1475–1476.

Karlin, D.A., Jones, R.D., Stroehlein, J.R., Mastromarino, A.J. and Potter, G.D. (1982) Breath methane excretion in patients with unresected colorectal cancer. *J Natl Cancer Inst* **69**: 573–576.

Karlin, S., Mrázek, J., Ma, J. and Brocchieri, L. (2005) Predicted highly expressed genes in archaeal genomes. *Proc Natl Acad Sci USA* **102**: 7303–7308.

Kiessling, R., Grönberg, A., Ivanyi, J. *et al.* (1991) Role of hsp60 during autoimmune and bacterial inflammation. *Immunol Rev* **121**: 91–111.

Klunker, D., Haas, B., Hirtreiter, A. *et al.* (2003) Coexistence of group I and group II chaperonins in the archaeon *Methanosarcina mazei*. *J Biol Chem* **278**: 33256–33267.

Kokeguchi, S., Kato, K., Kurihara, H. and Murayama, Y. (1989) Cell surface protein antigen from *Wolinella recta* ATCC 33238. *J Clin Microbiol* **27**: 1210–1217.

Krishnan, L., Sad, S., Patel, G.B. and Sprott, G.D. (2001) The potent adjuvant activity of archaeosomes correlates to the recruitment and activation of macrophages and dendritic cells *in vivo*. *J Immunol* **166**: 1885–1893.

Kubota, H., Hynes, G., Carne, A., Ashworth, A. and Willison, K. (1994) Identification of six Tcp-1-related genes encoding divergent subunits of the TCP-1-containing chaperonin. *Curr Biol* **4**: 89–99.

Kubota, H., Hynes, G. and Willison, K. (1995) The chaperonin containing t-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur J Biochem* **230**: 3–16.

Kulik, E.M., Sandmeier, H., Hinni, K. and Meyer, J. (2001) Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis. *FEMS Microbiol Lett* **196**: 129–133.

Lepp, P.W., Brinig, M.M., Ouverney, C.C., Palm, K., Armitage, G.C. and Relman, D.A. (2004) Methanogenic

Archaea and human periodontal disease. *Proc Natl Acad Sci USA* **101**: 6176–6181.

Maeda, H., Miyamoto, M., Hongyo, H., Nagai, A., Kurihara, H. and Murayama, Y. (1994) Heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*: molecular cloning and sequence analysis of its gene and purification of the recombinant protein. *FEMS Microbiol Lett* **119**: 129–135.

Maeda, H., Miyamoto, M., Kokeguchi, S. *et al.* (2000) Epitope mapping of heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*. *FEMS Immunol Med Microbiol* **28**: 219–224.

Mandal, K., Jahangiri, M. and Xu, Q. (2004) Autoimmunity to heat shock proteins in atherosclerosis. *Autoimmun Rev* **3**: 31–37.

Murayama, Y., Nagai, A., Okamura, K., Nomura, Y., Kokeguchi, S. and Kato, K. (1988) Serum immunoglobulin G antibody to periodontal bacteria. *Adv Dent Res* **2**: 339–345.

Novy, R., Drott, D., Yaeger, K. and Mierendorf, R. (2001) Overcoming the codon bias of *E. coli* for enhanced protein expression. *Innovations* **12**: 1–3.

Phipps, B.M., Hoffmann, A., Stetter, K.O. and Baumeister, W. (1991) A novel ATPase complex selectively accumulated upon heat shock is a major cellular component of thermophilic archaebacteria. *EMBO J* **10**: 1711–1722.

Schmits, R., Cochlovius, B., Treitz, G. *et al.* (2002) Analysis of the antibody repertoire of astrocytoma patients against antigens expressed by gliomas. *Int J Cancer* **98**: 73–77.

Siebert, P.D., Chenchic, A., Kellogg, D.E., Lukyanov, K.A. and Lukyanov, S.A. (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res* **23**: 1087–1088.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. and Kent, R.L. Jr (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**: 134–144.

Stauffer, G.V., Plamann, M.D. and Stauffer, L.T. (1981) Construction and expression of hybrid plasmids containing the *Escherichia coli glyA* genes. *Gene* **14**: 63–72.

Ueki, K., Tabeta, K., Yoshie, H. and Yamasaki, K. (2002) Self-heat shock protein 60 induces tumour necrosis factor-α in monocyte-derived macrophage: possible role in chronic inflammatory periodontal disease. *Clin Exp Immunol* **127**: 72–77.

Vianna, M.E., Holtgraewe, S., Seyfarth, I., Conrads, G. and Horz, H.P. (2008) Quantitative analysis of three hydrogenotrophic microbial groups, methanogenic *Archaea*, sulfate-reducing bacteria, and acetogenic bacteria, within plaque biofilms associated with human periodontal disease. *J Bacteriol* **190**: 3779–3785.

Woese C.R., Kandler O. and Wheelis M.L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA* **87**: 4576–4579.

Wu, T. and Tanguay, R.M. (2006) Antibodies against heat shock proteins in environmental stresses and diseases: friend or foe? *Cell Stress Chaperones* **11**: 1–12.

Yamabe, K., Maeda, H., Kokeguchi, S. *et al.* (2008) Distribution of *Archaea* in Japanese patients with periodontitis and humoral immune response to the components. *FEMS Microbiol Lett* **287**: 69–75.

Yokota, S., Hirata, D., Minota, S. *et al.* (2000) Autoantibodies against chaperonin CCT in human sera with rheumatic autoimmune diseases: comparison with antibodies against other Hsp60 family proteins. *Cell Stress Chaperones* **5**: 337–346.

Zugel, U. and Kaufman, S.H. (1999a) Immune response against heat shock proteins in infectious diseases. *Immunobiology* **201**: 22–35.

Zugel, U. and Kaufman, S.H. (1999b) Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin Microbiol Rev* **12**: 19–39.