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Identification and characterization of a fibronectin-binding protein from *Granulicatella adiacens*

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SUMMARY

The interaction of microorganisms with fibronectin plays an important role in infective endocarditis. *Granulicatella adiacens* is a member of the oral microbiota, formerly known as nutritionally variant streptococci, and is often isolated from endocarditis patients. In the present study we identified a surface protein, designated Cha, which binds to fibronectin, by a plaque hybridization procedure using the *cshA* sequence as probe, which encodes a fibronectin-binding molecule of *Streptococcus gordonii* DL1. The *cha* sequence was highly homologous to *cshA* and encoded a product of 2351 amino acid residues. The protein comprised a unique sequence in the N-terminal half region. The C-terminal region contained nine complete, and one incomplete, 115-amino acid residue repeat blocks. Among eight strains of nutritionally variant streptococci, three *G. adiacens* strains and one *Abiotrophia defectiva* strain carried the *cha* gene. Heterologous expression studies suggested that Cha adhered to immobilized fibronectin, and that this function was located in the unique region. Recombinant Cha protein also adhered to immobilize fibronectin and partially inhibited adherence of *G. adiacens* to fibronectin in a dose-dependent manner. These results suggest that Cha is a cell surface protein that mediates adherence of *G. adiacens* to fibronectin.

INTRODUCTION

Nutritionally variant streptococci (NVS) were first described in 1961 as a new type of streptococci exhibiting satellitism around colonies of other bacteria (Frenkel & Hirsch, 1961). These organisms have been shown to grow in complex media supplemented with L-cysteine or pyridoxal hydrochloride (vitamin B_6) (Ruoff, 1991). NVS could be divided into two groups, *Streptococcus defectiva* and *Streptococcus adjacens* based on DNA-DNA hybridization (Brouqui & Raoult, 2001). In 1995, the genus *Abiotrophia* and the two species, *Abiotrophia defectiva* and *Abiotrophia adiacens*, were proposed on the basis of the genetic and phylogenetic analyses of the 16S rRNA sequence (Kawamura *et al.*, 1995). Since then, three new species have been added and were reclassified into the new genus *Granulicatella* (Collins & Lawson, 2000).

Granulicatella adiacens is part of the normal microbiota in the oral cavity (Mikkelsen *et al.*, 2000), and in the human genitourinary and intestinal tracts (Ruoff, 1991). In addition, the organism has been shown to be one of the most common within the oral NVS species (Kanamoto *et al.*, 1996). It has been reported that *G. adiacens* was isolated from human clinical specimens (Christensen & Facklam, 2001), endocarditis (Senn *et al.*, 2006a), central nervous system infection (Cerceo *et al.*, 2004), and osteomyelitis (Rosenthal *et al.*, 2002). In some cases of 'culture-negative' endocarditis, a possible pathogenic

agent was reported to be *Granulicatella* (Roggenkamp *et al.*, 1998).

In the first step of infection, bacterial adherence to host cells is a very important reaction. This is mediated by specific interactions between adhesins on the surface of the bacteria and receptors on the host cells. Fibronectin is one of the target substrates for *G. adiacens* as well as for other streptococci and staphylococci, and has been well characterized. Fibronectin is a 440-kDa dimeric glycoprotein, and is present in soluble and matrix forms in various fluids and tissues. It has multiple functional domains that interact with various substrates, such as fibrin, heparin, collagen, other components of the extracellular matrix, and a wide variety of eukaryotic and prokaryotic cells (Hamill, 1987).

Several fibronectin-binding proteins have been identified from various species of Gram-positive cocci, and their binding characteristics have been studied (Joh *et al.*, 1999). These fibronectin-binding proteins include FnbpA and FnbpB from *Staphylococcus aureus* (Collins & Lawson, 2000), SfbI and protein F1 from *Streptococcus pyogenes* (Talay *et al.*, 1992), FBP54 and PFBP from group A streptococci (Rocha & Fischetti, 1999), FnbA and FnbB from *Streptococcus dysgalactiae* (Lindgren *et al.*, 1993), GfbA from group G streptococci (Kline *et al.*, 1996) and CshA from *Streptococcus gordonii* (McNab *et al.*, 1996). The overall molecular architecture of these proteins is basically similar, except for FBP54, but their sizes vary considerably. Each carries a N-terminal signal sequence for transmembrane transport, and a C-terminal cell wall anchoring consensus sequence LPX[T,S,A]G followed by the hydrophobic cell wall- and membrane-spanning region and positively charged intracellular tail. The C-terminal portions of these proteins contain amino acid residue repeat blocks and these are associated with fibronectin-binding activity. The composition of other elements, such as proline-rich repeats and folded domains of known topology in the N-terminal region account for the specific characteristics of these proteins.

Endocarditis and bacteremia are the most frequently reported clinical diseases caused by *Abiotrophia* and *Granulicatella* species (Christensen & Facklam, 2001). The endocarditis caused by these organisms accounts for from 4.3 to 6% of streptococcal endocarditis (Brouqui & Raoult, 2001). In endocarditis, bacterial adhesion to fibronectin of the host cell is considered to play an important role (Hamill, 1987). We have also identified some specific characteristics that are correlated between fibronectin and *G. adiacens* (Ito *et al.*, 2004). In another study, we found that unlike fibronectin-binding of *S. aureus* or *S. pyogenes*, soluble fibronectin molecules did not bind to *G. adiacens* cells. In addition, *S. gordonii*, *Streptococcus sanguinis*, and *Streptococcus pneumoniae* bound to immobilized fibronectin but not to soluble fibronectin (McNab *et al.*, 1996). These results suggested that *G. adiacens* could be involved in infective endocarditis like some other streptococci, but no information is available on fibronectin-binding proteins from *G. adiacens* at the molecular level. In this study, we describe the gene cloning and initial characterization of a fibronectin-binding protein, Cha, on the surface of *G. adiacens*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Seven strains of genus *Granulicatella* and one *A. defectiva* were used. These included: *G. adiacens* strains ATCC 49175, G40 and HHP1; *Granulicatella para-adiacens* strains HTK1-1 and NMP2; *Granulicatella elegans* strains YTM1 and S1052-1. The *A. defectiva* strain was ATCC 49176. Two isolates of *G. adiacens* were originated from the blood or vegetations of endocarditis patients. The *G. para-adiacens* and *G. elegans* strains were isolates from the oral cavity of a healthy male. All strains were physiologically, serologically, and genetically characterized (Kanamoto *et al.*, 1996). They were grown in Todd-Hewitt broth containing 0.001% pyridoxal-HCl (vitamin B_6) anaerobically at 37°C overnight. *Streptococcus gordonii* DL1 was cultured in Todd-Hewitt broth. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates; antibiotic selection utilized ampicillin 100 μg ml^{-1}, kanamycin 50 μg ml^{-1} or erythromycin 500 μg ml^{-1}. *Lactococcus lactis* subsp. *cremoris* MG1363 was kindly provided by P. Moreillon, (Lausanne, Switzerland). The strain was grown in M17 broth (Difco, Detroit, MI) supplemented with 0.5% glucose (GM17) or on GM17 agar plates with selection by erythromycin at 5 μg ml^{-1}. Transformation was carried out according to the procedure described previously (Holo & Nes, 1989). The cultured bacteria were collected by centrifugation, washed twice and sus-

pended in a suitable buffer according to the experiment as described below.

Identification of a fibronectin-binding protein

Chromosomal DNA was isolated from all NVS strains as described previously (Yoshida *et al.*, 2002). To detect the gene encoding the fibronectin-binding protein in *G. adiacens*, Southern blot analysis was performed. DNA samples (1 μg per lane) were digested with restriction enzymes, *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sph*I or *Xho*I, and electrophoresed through 1.0% agarose. The separated DNA fragments were then transferred to a nylon membrane. Hybridization of the blots with a ^{32}P-labeled *cshA* gene fragment was performed under low stringency conditions. The blots were washed several times each for 30 min with 2× SSC (1× SSC: 150 mM NaCl and 15 mM $Na_3C_6H_5O_7$) containing 0.1% SDS at 65°C. The radiolabeled bands were detected by autoradiography. The *cshA* DNA was produced by PCR with F1 (FPA) and R1 (STV) primers using the DNA from *S. gordonii* DL1 as a template (Table 1).

A genomic library of *Eco*RI fragments of *G. adiacens* 49175 was constructed in the λZap Express vector using *E. coli* XL1-Blue MRF' cells as indicator cells (Stratagene, La Jolla, CA). The plaques were screened by hybridization with ^{32}P-labeled *cshA* DNA as probe. The positive phage clones were converted to a pBK-CMV phagemid by *in vivo* excision into *E. coli* XLOLR. The double-stranded nucleotide sequence of the inserts was determined using a primer-walking strategy with an ABI automated DNA sequencer.

The identification of the entire gene required the further extension of the 5′-area of the 5 kb *Eco*RI fragment in the isolated λZAP clone. The appropriate templates for the sequencing were generated by a series of inverse PCRs. Initially the chromosomal DNA was digested with *Alu*I, and the generated fragments were self-ligated with T4 DNA ligase, followed by inverse PCR with F2 and R2 primers. The PCR fragment obtained was inserted into pGEM-Teasy (Promega, Madison, WI) and sequenced. Similarly, an additional three inverse PCR approaches were carried out by *Msp*I with F3 and R3, and *Nla*III and *Hae*III with F4 and R4. The primers used are described in Table 1.

Screening for the gene encoding fibronectin-binding protein

In order to assess the distribution of the *cha* gene among NVS strains, PCR was carried out for the

Table 1 Primers used in this study

Primer	Sequence[1]	References
F1 (FPA)	5′-ACTCCCGGGCATTCCCAGCTGATTCGACT-3′	McNab *et al.* (1996)
R1 (STV)	5′-AAAGGTCGACGGAACTGTACTATCGATATGT-3′	McNab *et al.* (1996)
F2	5′-GTGACGCTTGATTCAGAAGG-3′	
R2	5′-TCTTTGTTTTCCTTGGGCAG-3′	
F3	5′-CCGTTTGATGAAAGTGATGCTC-3′	
R3	5′-ACTGCTGCTTGTTTCCCTGC-3′	
F4	5′-GGAAATCCAAAAGGAAGAGCAG-3′	
R4	5′-CCATACGTTTTCTATAGATTTC-3′	
F5	5′-TTACCCGGGATACTGTAGAAAGTACAGCGG-3′	
R5	5′-AAAAGTCGACTTTGGCGTAAACTTCACTTTTCC-3′	
F6	5′-CGACAACAGGAAAACAAGGTC-3′	
R6	5′-ATCCCCTGCTTCAAACTTAG-3′	
F7	5′-ATGGTAAACAAGTGGGAACG-3′	
R7	5′-ATCTGTCGGTTCTTTCGTTG-3′	
F8	5′-TGGATCCGTGGTTAGGTAGTGGTGAAAAAAGG-3′	
F9	5′-CTCTCGAGCCAACCGTATTACCAGTTCAACCA-3′	
F10	5′-CTCGATCGGTGATTCCTGGAGTTGGAACCTTC-3′	
R11	5′-CTCTCGAGCGCTGTACTTTCTACAGTATCAGC-3′	
F12	5′-CTCGATCGATTTGGCGTAAACTTCACTTTTCC-3′	
R13	5′-CTCTGCAGTTTGCAACGGCAAACAAAAAGCGC-3′	

[1]Restriction sites introduced into the primer sequences are underlined.

unique and the repetitive regions, and for the repetitive unit. The PCR primers for detection of the *cha* gene were F5 and R5 for the unique region, F6 and R6 for the repetitive region and F7 and R7 for the repetitive unit. The amplified materials were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Preparation of recombinant Cha fragments

Recombinant Cha proteins were expressed from plasmids derived from pET-28a(+) (Novagen, Madison, WI). Two fragments of the *cha* gene from *G. adiacens* 49175 were amplified by PCR, and the fragments were cloned into a vector. The primers used for production of the unique region of Cha (rChaN) were F5 and R5. The PCR primer pair for production of the repetitive region (rChaR4) was F7 and R7. The rChaR4 polypeptide was designed to contain four repeat units and eliminate the C-terminal cell wall-anchoring region. *E. coli* BL21 cells were transformed with a vector or recombinant plasmids, and the fusion protein was induced by isopropyl-β-D-thiogalactopyranoside. His_6-Cha fusion polypeptides were purified by a HisTALON Superflow Cartridge Purification kit according to the instruction manual (Clontech, Palo Alto, CA). Imidazole-eluted material was dialyzed extensively against 10 mM phosphate buffer and 50 mM NaCl (pH 6.8) for 18 h at 4°C, followed by ultrafiltration for concentration. The protein preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Protein Assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard material.

Antisera

rChaN- and rChaR4-specific antisera were generated by using the individual recombinant proteins that were separated from the histidine-tag peptides after digestion of thrombin. Pathogen-free female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Seven-week-old mice were immunized subcutaneously with 15 μg protein per mouse in 500 μl emulsion with Freund's complete adjuvant (Difco). After 2 and 4 weeks, additional immunizations were carried out with Freund's incomplete adjuvant. The mouse handling and experimental procedures followed the institutional guidelines for animal care and use. Blood was collected 3 days after the last injection.

Fluorescence microscopy

Bacterial cells were applied to an objective slide, and incubated for 20 min with 100-fold dilutions of either anti-rChaN antiserum, anti-rChaR4 antiserum or the corresponding preimmune sera. Cells were washed three times with phosphate-buffered saline (PBS) and incubated for 20 min with 100-fold diluted Alexa Fluor 488 goat anti-mouse IgG (H + L) conjugate (Molecular Probes, Eugene, OR). Cells were examined on a Leica TCS SP5 upright microscope with a 64× objective. Alexa Fluor tagged signals were visualized following illumination with a mercury vapor lamp that yields excitation and emission optima of 488 and 535 nm, respectively. Photographs were taken with a digital camera and analyzed with the installed Leica image analyzing software (version 1.6.0, Leica, Wetzlar, Germany).

Heterologous expression of Cha

All constructs for heterologous expression were established using *E. coli* as an intermediate host. PCR was carried out to acquire each component using chromosomal DNA of *G. adiacens* 49175 as a template. Fragments were cloned into pGEM-T Easy vector prior to digestion with appropriate restriction enzymes. Sets of digested fragments were then ligated to the corresponding recipient vector, pOri23 (provided by P. Moreillon). Constructs were established in *E. coli*, followed by transformation of *L. lactis* by electroporation as described elsewhere (Que *et al.*, 2000). Specific transformants were isolated by erythromycin selection.

Bacterial adherence to immobilized fibronectin

Fibronectin-adherence activity was determined as described previously with minor modifications (Ito *et al.*, 2004; Yamaguchi, 2004). Briefly, *G. adiacens* 49175 and *L. lactis* and strains harboring either pOri23 or the rsCha expression plasmid were radiolabeled with [*methyl*-^{3}H] thymidine (300 kBq ml^{-1}; 3.3 TBq mmol^{-1}; NEN, Boston, MA), followed by washing and suspension in adherence buffer (10 mM phosphate buffer, 50 mM NaCl, 0.05% Tween20, pH 6.8). The average specific activities were 3.0×10^{-5} cpm per cell for

G. adiacens 49175 and 3.6×10^{-4} cpm per cell for *L. lactis*. A flat-bottom microtiter plate was coated with 100 μl per well of fibronectin in PBS. The concentration of fibronectin as coating agent was 5 μg ml^{-1} unless otherwise stated. The plate was kept at 4°C for 16 h. The blocking procedure was carried out by the addition of 200 μl PBS containing 0.2% Tween 20 to each well at room temperature for 1 h. The wells were then washed twice with 200 μl of the adherence buffer, and 50 μl aliquots of the mixture of recombinant Cha protein and radiolabelled bacterial cells (approximately 1000 cpm) in the adherence buffer were incubated at 37°C for 2 h. After washing four times, 100 μl of a solution containing 1% SDS and 0.4 N NaOH was added and then the mixture was incubated for a further 2 h at 37°C. The contents of the wells were then transferred to scintillation vials, mixed with 3 ml of Clear-sol II (Nacalai Tesque, Kyoto, Japan), and tested for radioactivity. The rate of bacteria binding was calculated by comparison to the initial applied radioactivity. Assays were done in duplicate and repeated three times.

Binding of recombinant Cha proteins to immobilized fibronectin

Flat-bottom wells in a plastic microtiter plate were coated with 100 μl of 5 μg ml^{-1} of human fibronectin or human fibrinogen as a control protein (BioPur AG, Bubendorf, Switzerland). The plate was blocked and washed as described above. Recombinant ChaN and ChaR4 proteins were two-fold diluted with adherence buffer, and 100 μl aliquot was applied to each well and incubated for 1 h. The plate was washed with the adherence buffer three times, and 100 μl of anti-ChaN mouse serum or anti-ChaR4 mouse serum diluted with buffer (1 : 2000) was added and incubated for 1 h at room temperature. Following three washes, 100 μl of diluted HRP conjugated anti-mouse IgG (1 : 2000) was added. After 30 min, the plate was washed three times and 200 μl of ABTS substrate solution (Zymed, South San Francisco, CA) was added and incubated at room temperature. Optical density at 405 nm was measured after 1 h. The assay was performed three times.

Nucleotide sequence accession number

The sequence of the cloned *cha* DNA described here has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB375984.

RESULTS

Identification and characterization of Cha

Southern blot analysis under low stringency conditions was performed on genomic DNA from a comparable *G. adiacens* 49175 strain in order to detect a homologous candidate of CshA, which was one of the fibronectin-binding proteins of *S. gordonii* (ABV09381.1) (Fig. 1A). The sequence of the probe DNA was designed as a part of the repetitive region that was located in the C-terminal half, and contained four units of tandem repeat composed of 345 nucleic

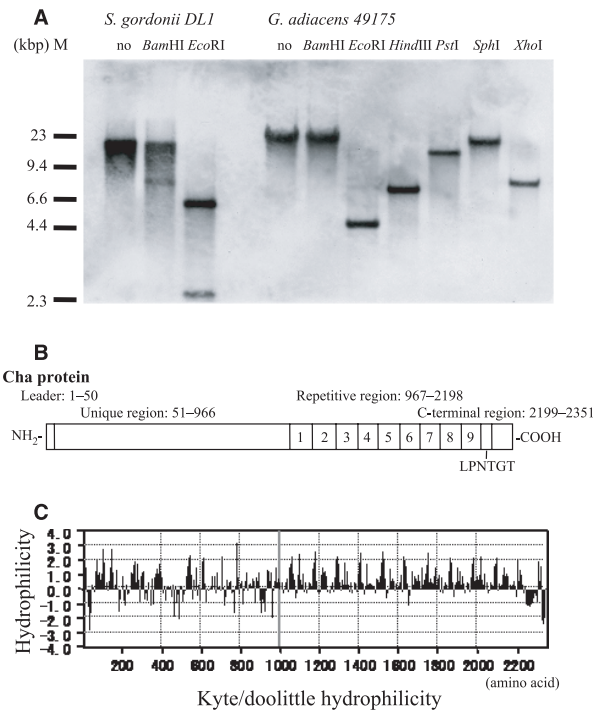

Figure 1 Identification of the *cha* gene from the *G. adiacens* ATCC 49175. (A) Southern blot hybridization of *S. gordonii* DL1 and *G. adiacens* 49175 DNA with a *cshA* probe. DNA (1 μg per lane) was digested with each indicated enzyme and hybridized with the ^{32}P-labelled probe under low stringency conditions. Probe DNA was produced by PCR designed to amplify the repetitive region of *cshA* in *S. gordonii*. M, the pattern of lambda DNA digested with *Hind*III as molecular weight markers. (B) Schematic representation of the Cha polypeptide feature is shown. (C) Kyte-Doolittle hydrophilicity analysis of the Cha polypeptide is shown with a sliding window of nine amino acid residues. The positive and negative indices represent the hydrophilic and hydrophobic regions, respectively.

acids each. Under low stringency conditions, the labeled probe was hybridized with DNA specimens from not only *S. gordonii* DL1 but also *G. adiacens* 49175 (McNab *et al.*, 1994). In the case of *G. adiacens,* only one band was observed for each six kinds of restriction enzyme used and their sizes varied depending on the enzymes. Among these enzymes, *Eco*RI digestion produced an approximately 5 kb hybridizing fragment and the enzyme was used for making a lambda phage-based library to isolate the *cshA*-like gene in *G. adiacens*. In contrast, no positive hybridized bands were detected in the DNA specimens derived from *G. adiacens* under high stringency conditions (data not shown).

To isolate the complete gene, a lambda phage library of genomic DNA was screened by plaque hybridization. The conditions of this hybridization were the same as for the Southern hybridization. The isolated clone, λZAP-*cha*, contained an approximately 5 kb *Eco*RI fragment. A large open reading frame with the stop codon at the 3′-end was detected in the fragment, but the 5′-region was truncated. For 5′ extension to isolate the entire reading frame, a series of inverse PCRs was carried out. In another approach, rescreening of the lambda library was carried out to identify the fragment upstream of the 5 kb fragment. The probe used was the *Alu*I fragment produced by the inverse PCR. The isolated clone, λZAP-*cha*5, contained an approximately 4 kb fragment on the 5′ side.

The total length of the gene was 7053 bp, and the deduced protein consisted of 2351 amino acid residues (Fig. 1B). Here, we tentatively designated this protein as Cha (cell-surface high molecular weight adhesin of *G. adiacens*). The N-terminal region of the Cha molecule possessed a putative leader sequence that consisted of 50 amino acid residues. The mature protein sequence of Cha was orthologous to the *S. gordonii* CshA sequence. Alignment of the amino acid sequences revealed an overall 41% identity and 56% similarity between the Cha protein and CshA protein in the N-terminal half. The C-terminal half of the Cha protein contained nine units and an incomplete unit of the semi-conserved sequence of 115 amino acid residues. Just after the repetitive region, a consensus motif for the cell wall anchoring of the Gram-positive bacteria (LPNTGT) is present, followed by a hydrophobic segment (trans-membrane) and a hydrophilic tail (cytoplasmic). Kyte-Doolittle hydrophilicity analysis revealed that the N-terminal leader region and C-terminal cell wall anchoring region were typically hydrophobic (Fig. 1C).

Distribution of the *cha* gene in different strains of NVS

Several fibronectin-binding proteins have been described in a variety of streptococci and staphylococci, but it has not been reported that NVS strains have fibronectin-binding proteins. Therefore, we determined the distribution of the *cha* gene in strains of the genera *Granulicatella* and *A. defectiva*. For this purpose, three sets of primer pairs were constructed (Fig. 2A). For the unique region, the forward primer was designed just downstream of the leader sequence and the reverse primer was designed upstream of the repetitive region. For the repetitive region, the forward primer was located within the unique region and the reverse primer was located within the C-terminal hydrophobic trans-membrane area. For the repetitive unit, both primers were designed within one repetitive unit, and the reaction generated one, two or more copy units. Chromosomal DNAs from eight strains were subjected to PCR by each region-specific primer pair of the *cha* gene. The *cha* gene was detected in *G. adiacens* G40*, G. adiacens* HHP1, and *A. defectiva* 49176 as well as *G. adiacens* 49175. In these four strains, the *cha* gene was well conserved for the unique region and repetitive unit. In contrast, the amplicons of the repetitive region were heterologous among these strains (Fig. 2B–D). We suggested that the heterogeneity was dependent on the copy number of repetitions and/or on the variation of the shifting area between the unique area and the repeat region. The *cha* gene was not detected in any of the strains of *G. para-adiacens* and *G. elegans*.

The Cha protein is present at the bacterial surface

Immunofluorescence microscopy analyses were performed in order to assess the expression and the location of the Cha protein on *G. adiacens* 49175 cells. Two parts of the *cha* gene were subcloned into the pET-28a(+) expression vector and the expressed His6-fused Cha proteins were purified with a HisTALON Superflow Cartridge (Fig. 3A, B). When antiserum specific for the recombinant Cha unique region was used as the primary anti-

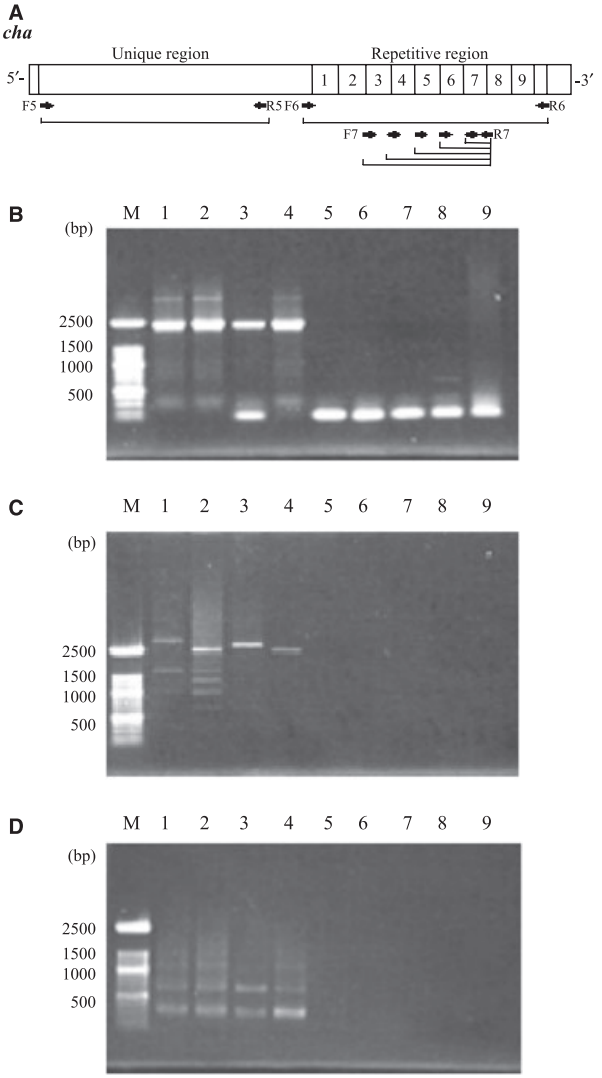

Figure 2 Distribution of the *cha* gene among NVS strains. (A) A structural feature of the *cha* gene in *G. adiacens* 49175 is indicated. The arrows indicate oligonucleotide primers constructed for the PCR analysis. (B) The primer pair for the unique region of *cha* was F5 and R5 to produce 2547 bp *G. adiacens* 49175 fragment. (C) The primer pair for the repetitive region of *cha* was F6 and R6 (3862 bp). (D) The primer pair for the repetitive unit of *cha* was F7 and R7 (345 bp). The sequences of the primers used are described in Table 1. Lanes: 1, *G. adiacens* 49175; 2, *G. adiacens* G40; 3, *G. adiacens* HHP1; 4, *A. defectiva* 49176; 5, *G. para-adiacens* HKT1-1; 6, *G. para-adiacens* NMP2; 7, *G. elegans* YTM1; 8, *G. elegans* S1052-1; 9, no template; M, molecular weight markers.

body, Alexa Fluor-tagged signals were strongly detected on the surface of the bacteria. The experiment with Cha repetitive unit-specific antiserum yielded almost the same results. In contrast, no labeling was observed when pre-immune serum was used (Fig. 3C).

Expression of the unique region or the repetitive region of the Cha protein on the surface of *L. lactis* and demonstration of the fibronectin-binding function of these regions

To further characterize the biological activity of the Cha protein as a fibronectin-binding molecule, the heterologous expression of this protein was assessed by *in vitro* assays. For this experiment, the nonpathogenic bacterium *L. lactis,* which has a low-fibronectin-binding background, was used as host strain. The unique region or the repetitive region of the Cha protein was expressed on the surface of *L. lactis* as a fusion protein connected with the endogenous anchor region of the Cha protein (rsChaN and rsChaR). Those strains that expressed the objective protein heterologously were selected by erythromycin (Fig. 4A).

In order to confirm the expression of the fusion proteins on the surface of the recombinant organisms, an immunofluorescence staining was carried out. The recombinant strain harboring the pOri-chaN plasmid showed clear reactivity when an anti-rChaN specific antibody was used. This indicated the presence of the fusion protein on the surface of the strain (Fig. 4B). When pre-immune serum or anti-rChaR4-specific serum was used, the strain was not stained. Similarly, the recombinant strain harboring the pOri-chaC plasmid was reacted with anti-rChaR4 serum but not pre-immune serum or anti-rChaN-specific serum. In contrast, the *L. lactis* strain transformed with pOri23 showed no reaction with the pre-immune serum, anti-rChaN antiserum or anti-rChaR4 antiserum. These results clearly demonstrated that the objective region of the Cha protein was expressed as a cell surface molecule on the recombinant *L. lactis* strains.

Heterologous expression assays provided the initial demonstration that the recombinant Cha protein on *L. lactis* interacted specifically with immobilized fibronectin in a saturable, dose-dependent manner (Fig. 4 C). Heterologous expression of ChaN on the surface of *L. lactis* induced a four-fold increase in fibronectin adherence compared to *L. lactis* harboring pOri23 at the maximum point (3.2 μg ml^{-1} of fibronectin), while parallel experiments with ChaR increased *L. lactis* fibronectin binding by 207% at the maximum point (3.2 μg ml^{-1} of fibronectin). These data suggested that both the unique and the repetitive regions possessed important fibronectin-binding properties demonstrable in the context of the intact bacterial surface.

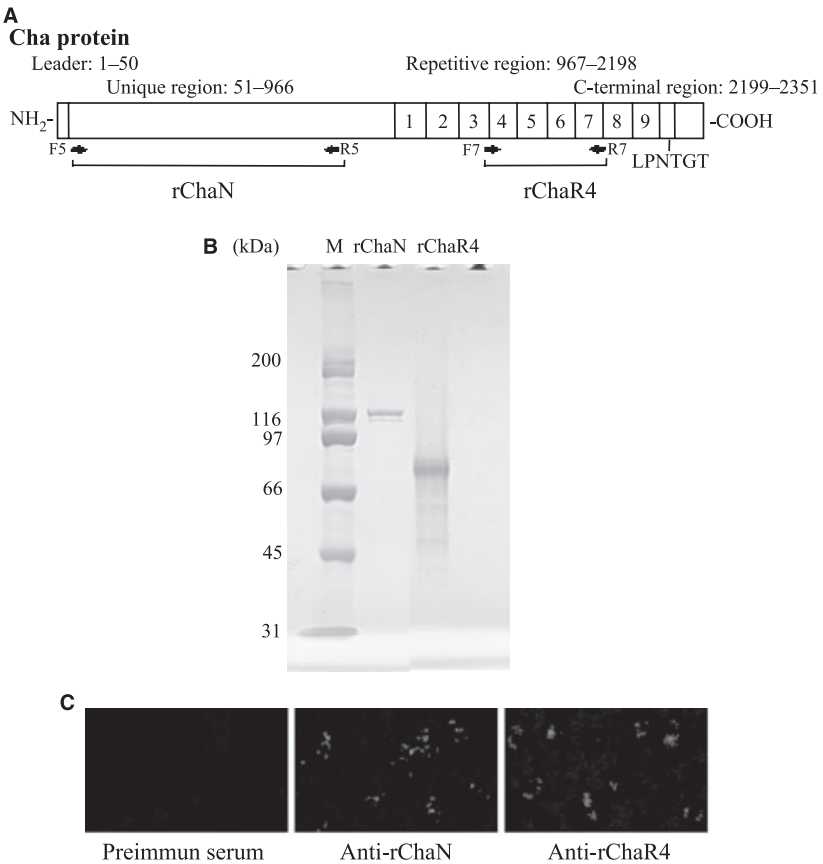

Figure 3 Expression and surface localization of the Cha protein on *G. adicens* 49175. (A) The recombinant proteins rChaN and rChaR4 are indicated in the structural feature of the Cha protein in *G. adiacens* 49175. Arrows show nucleotide primers for the construction of DNA fragments coding each recombinant Cha protein. Specific antisera were established in mice by immunization of each recombinant protein separated from the tag peptide. (B) SDS-PAGE analysis confirmed the recombinant ChaN and ChaR4 proteins after affinity column purification. M, molecular weight markers. (C) Immunofluorescence microscopic analyses detected the Cha on the surface of *G. adiacens* pretreated with rChaN- or rChaR4-specific serum. Pre-immune mouse serum was used as a negative control.

Role of recombinant Cha proteins in adherence of *G. adiacens* cells to fibronectin

In order to determine the domains that account for binding to immobilized fibronectin, two kinds of experiment were performed in the use of recombinant ChaN protein, and recombinant ChaR4 protein, which was composed of four repeat units from the C-terminal half. It was demonstrated that recombinant ChaN and ChaR4 proteins bound to immobilized fibronectin in a dose-depended fashion in an ELISA system using specific antiserum for each protein (Fig. 5A). Generally optical density by ChaN was higher than ChaR4. The almost maximum optical density was detected at the condition of 1.2 mg ml^{-1} of ChaN. No significant reaction was detected for binding of both ChaN and ChaR4 proteins when fibrinogen was used as coating agent. Next we tested their ability to inhibit the adherence of *G. adiacens* cells competitively (Fig. 5B). Both the rChaN and rChaR4 polypeptides resulted in a considerable reduction of binding in a dose-dependent fashion. The binding bacteria accounted for approximately 14% of the applied *G. adiacens* 49175 cells when the concentration of recombinant ChaN protein was 5 mg ml^{-1} (250 μg in 50 μl). The inhibition began to plateau when the concentration reached 1.2 mg ml^{-1} of the protein (60 μg in a 50 μl volume). While the recombinant ChaR4 protein showed a similar level of inhibition at the plateau, slightly weaker inhibition was detected for the fibronectin adherence of *G. adicens*. The inhibition began to plateau when the concentration of the protein reached 2.5 mg ml^{-1}. These results suggest that the unique region might be more essential for cell

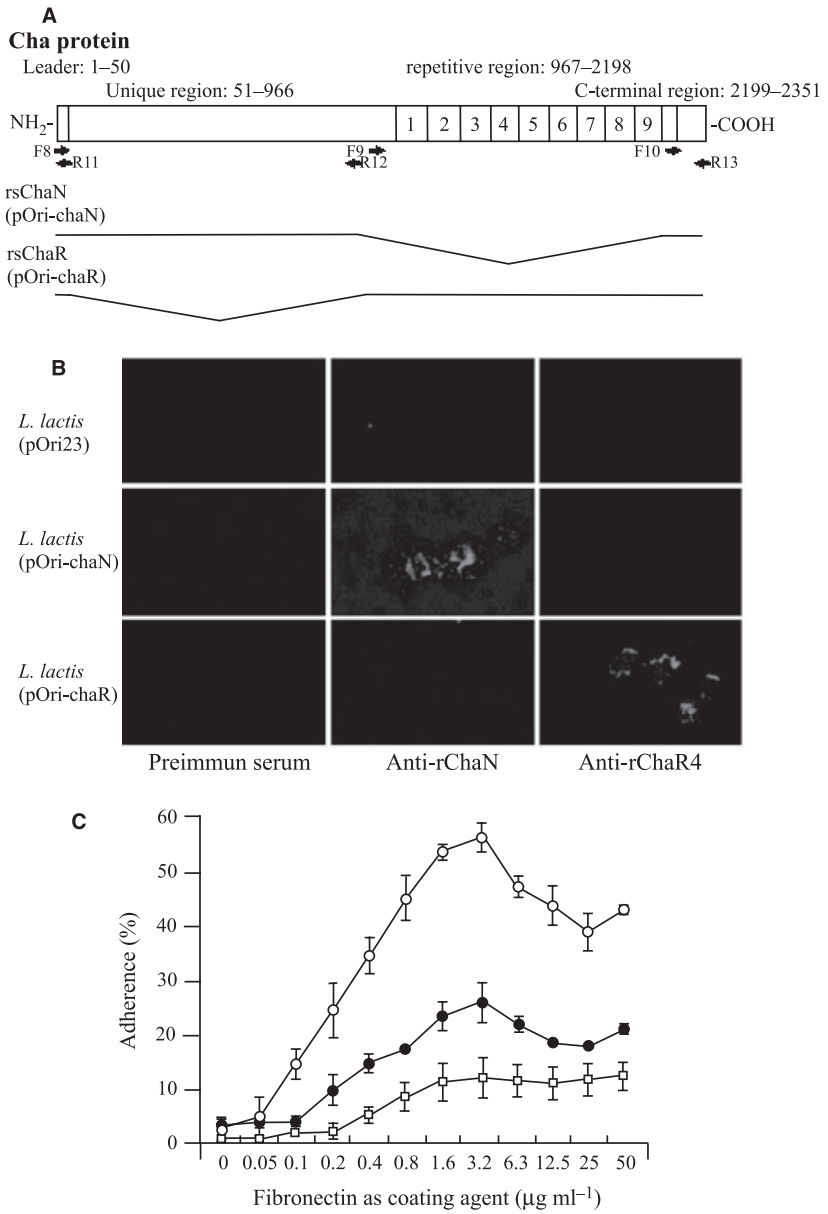

Figure 4 Adherence to immobilized fibronectin of recombinant Cha proteins expressed heterologously on *L. lactis*. (A) Recombinant cell surface proteins rsChaN and rsChaR are indicated in the structural feature of the Cha protein in *G. adiacens* 49175. Arrows indicate the nucleotide primers used to construct DNA fragments coding each rsCha protein. Parentheses indicate the plasmids used to transform *L. lactis*. (B) Detection of recombinant proteins on the surface of *L. lactis* strains by immunofluorescence. Pre-immune mouse serum was used as a negative control. (C) Fibronectin-binding activity of the cell surface Cha proteins on *L. lactis* transformants harboring pOri-chaN (○), pOri-chaR (•) or pOri23 (□). Microtiter plate wells were coated with increasing amounts of fibronectin. The bacterial inoculum was approximately 1000 cpm in 50 μl per well for all experiments. The results are presented as a percentage of adherence compared with the original bacteria applied. Values shown represent the means ± standard deviation for duplicate assays from three independent experiments.

adhesion of *G. adiacens* to fibronectin than the repetitive region. The results obtained with recombinant Cha proteins were consistent with the characteristics of *L. lactis* strains expressing Cha proteins heterologously.

DISCUSSION

The adhesion of pathogens to host tissue is a critical early step in the process of infection. Fibronectin plays a vital role in a variety of normal physiological

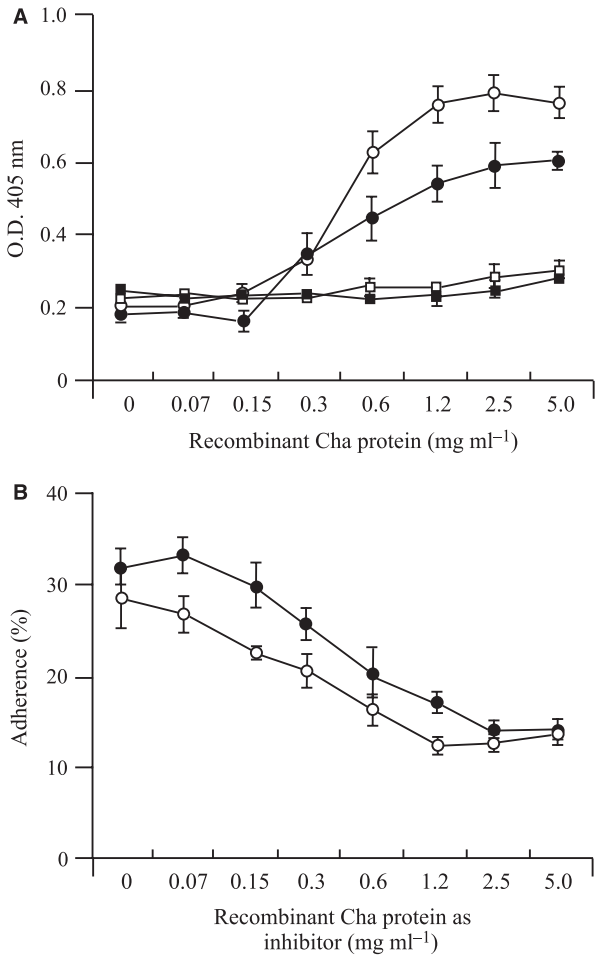

Figure 5 Adherence of recombinant Cha proteins to immobilized fibronectin. (A) Binding to immobilized fibronectin was estimated. Dilution of rChaN protein (○) or rChaR4 protein (•) were applied to wells coated with fibronectin. Protein binding was measured by anti-ChaN serum or anti-ChaR4 serum respectively. For control reactions, the same concentrations of rChaN protein (□) or rChaR4 protein (■) were applied to wells coated with human fibrinogen. Measurements were performed as above. Values shown represent the mean ± standard deviation for duplicate assays from three independent experiments. (B) Inhibition of bacterial adherence to immobilized fibronectin was estimated. Radioactively labeled *G. adiacens* 49175 cells were mixed with the appropriate dilution of rChaN protein (○) or rChaR4 protein (•) and applied to wells coated with fibronectin. The results are presented as percentage adherence of the numbers of bacteria applied. Values shown represent the means ± standard deviation for duplicate assays from three independent experiments.

processes (Knodler *et al.*, 2001), and is considered to be a specific receptor for bacterial colonization and virulence. For many pathogens, the ability to bind to fibronectin is an important characteristic for the establishment, maintenance and dissemination of infection (Joh *et al.*, 1999). For example, fibronectin plays a role in endovascular bacterial adhesion during the formation of vegetations in infective endocarditis. The adherence of oral NVS to immobilized fibronectin, which may be exposed or deposited at sites of endothelial damage, is suggested to be an important step in the pathogenesis of culture-negative endocarditis (van de Rijn, 1985). A relationship between the fibronectin-binding capacity of *G. adiacens* and its endovascular infectivity in catheterized rats was previously demonstrated (Okada *et al.*, 2000).

The genome sequences of many bacterial strains have been completed over the past decade. Many fibronectin-binding proteins have been reported in streptococci and staphylococci, but such proteins have not yet been described in NVS strains such as the genus *Granulicatella* and *Abiotrophia* species. Therefore, we screened the genomic library of a type strain of *G. adiacens*, and found Cha encoding a fibronectin-binding protein, which exhibited the typical characteristics found in some of the streptococcal fibronectin-binding proteins already reported.

The analysis of the adherence of NVS strains to extracellular matrix proteins including fibronectin, laminin and fibrinogen, demonstrated a close relationship between adherence and endocardial infectivity(Okada *et al.*, 2000). Such a universal extracellular matrix-binding ability of NVS strains is strongly suggested to be involved in the virulence of this species. *G. adiacens,* a highly virulent pathogen, adhered to immobilized proteins more readily than did the *G. para-adiacens* or *G. elegans* strains, which are less virulent pathogens. *A. defectiva,* a moderately virulent pathogen, showed less affinity to fibronectin than *G. adiacens*, but considerable affinity to other components. Therefore, the fibronectin-binding ability of cells may be an important factor involved in the endovascular infectivity of NVS species. Another report demonstrated that *A. defectiva* strains had higher binding affinity to a crude extracellular matrix preparation, which was assumed to be rich in laminin, than *G. adiacens* (Tart & van de Rijin, 1991). Yet another report showed the strong attachment of *A. defectiva* strains to fibronectin, whereas *Granulicatella* spp. strains were not adhered (Senn *et al.*, 2006b). These results may be attributable to differences in the experimental conditions, which could have modified the binding of the bacteria. In the pres-

ent study, three kinds of PCR analyses were carried out to assess the distribution of the *cha* gene among the genera *Granulicatella* and *A. defectiva*. Interestingly, all strains of *G. adiacens* and *A. defectiva* tested were *cha* positive, but all the strains of *G. para-adiacens* and *G. elegance* were *cha*-negative. This result supported the idea that the Cha protein was associated with the fibronectin-binding capacities of *Granulicatella* and *A. defective*, and their virulence for bacterial endocarditis. Moreover, the presence of *cha* could possibly be used as a marker gene for discrimination among NVS species, although further studies using more NVS strains will be needed to obtain consistent information.

In the fibronectin-binding protein CshA of *S. gordonii*, the N-terminal unique region bound to fibronectin (McNab *et al.*, 1996). Since the activity was localized in the repetitive region of other fibronectin binding proteins in the groups G and A streptococci, the Cha/CshA structure may be unique (Schwarz-Linek *et al.*, 2006). Because of the high similarity between proteins Cha and CshA, the fibronectin-binding activity was suggested to be in the N-terminal unique region of Cha but not in the repetitive region. We investigated which regions of Cha bound to fibronectin, by using two kinds of experiments. The results showed that Cha has fibronectin-binding activity in the repetitive region as well as in the unique region, and the affinity of the unique region was higher than that of the repetitive region. The recombinant ChaN protein inhibited the adherence a little more strongly than the recombinant Cha repetitive protein. Because the sequence of the repetitive unit of Cha had significant homology with CshA of *S. gordonii* but not with that of fibronectin-binding proteins of the groups G and A streptococci, the mechanism of bacterial adherence to fibronectin of the Cha and CshA proteins might differ from that of these groups. Moreover the multiple redundant adhesins on the bacterial cell surface make the analyses of interactions with specific host receptors complex (Jakubovics *et al.*, 2009).

Since Cha is suggested to be as important a factor for the adherence and virulence of bacteria as Csh in *S. gordonii*, further investigation of its structure and function will facilitate the future applications of this protein in the prevention of infectious diseases, including endocarditis caused by NVS strains. In addition, this report provided a novel candidate for the vaccination of bacterial infective endocarditis.

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