Role of Hyalin-like Protein in Gliding and Biofilm Formation by *Capnocytophaga Ochracea*

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Abstract

Capnocytophaga ochracea possesses a type-IX secretion system that exports proteins which have a gliding motility-associated C-terminal (CTD) domain. This system is found in several species of the *Bacteroidetes* phylum. Hyalin, a large protein encoded by Coch_0033 in *C. ochracea* ATCC 27872, has a CTD domain and is posited to be involved in quorum sensing according to the database of the Kyoto Encyclopedia of Genes and Genomes. This suggests that it plays a role in biofilm formation *via* interbacterial communication. The aim of this study was to investigate the potential role of the hyalin-like protein coded by the Coch_0033 gene in gliding and biofilm formation of *C. ochracea*. A hyalin-like protein-deficient mutant strain of *C. ochracea*, designated mutant WR-1, was constructed through insertion of the *ermF-ermAM* cassette into the target gene. The spreading feature at the edge of the colony was lost in the mutant strain. Crystal violet and confocal laser scanning microscopy revealed no difference between the quantity of biofilm organized by the mutant and that organized by the wild-type strain. These data suggest that the hyalin-like protein encoded by the Coch_0033 gene is indeed involved in *C. ochracea* gliding activity.

Key words: *Capnocytophaga ochracea* — Hyalin-like protein —
Type-IX secretion system — Biofilm — Gliding

Introduction

Capnocytophaga ochracea, a Gram-negative rod-shaped bacterium with gliding motil-

ity^{13,21)}, is a major component of dental plaque biofilm. Although this microorganism was first isolated from human periodontitis lesions^{18,28)}, ensuing studies have also reported

its presence in dental plaques from healthy periodontal sites¹⁰⁾. Genome-wide transcriptome analysis of the subgingival microbiome in patients with periodontitis has revealed upregulation of putative virulence factors for *C. ochracea*⁴⁾. Little is known about the role of *C. ochracea* in the pathogenesis and progression of periodontal disease, however^{2,11,15,19)}. Besides periodontitis, *C. ochracea* has also been reported to cause several systemic infectious diseases such as sepsis and purpura fulminans^{3,20)}. The virulence of *C. ochracea* in these diseases remains unclear, however.

Capnocytophaga ochracea possesses a type-IX secretion system (T9SS), which has also been identified in *Porphyromonas gingivalis* and other members of the phylum *Bacteroidetes*¹⁴⁾. The T9SS specifically exports proteins that have a gliding motility-associated C-terminal (CTD) domain. Several T9SS proteins have been identified in *P. gingivalis*^{22,25)} and *Flavobacterium johnsoniae* (phylum *Bacteroidetes*)^{8,14)}. In *P. gingivalis*, these proteins are essential for the secretion of major proteases²²⁻²⁵⁾. In *F. johnsoniae*, T9SS proteins have been shown to facilitate the attachment of the cells to and their gliding across glass surfaces by exporting certain adhesins to the cell surface^{14,26)}. In *Tannerella forsythia*, the T9SS mutant increased biofilm formation compared with the wild-type strain¹⁷⁾.

Genes orthologous to those that encode the T9SS proteins of *F. johnsoniae* have also been found in the genome of *C. ochracea*¹⁴⁾, suggesting the possible involvement of T9SS-exported proteins in the gliding motility and biofilm formation of *C. ochracea*. This group has previously identified three T9SS protein-encoding orthologous genes in *C. ochracea*: *gldK* and *sprT*, which encode T9SS proteins; and *sprB*, which encodes a gliding motility adhesin that is transported by T9SS to the cell surface⁹⁾. Inactivation of each gene in *C. ochracea* affected gliding motility and biofilm formation, suggesting that the proteins exported by this system are key elements in the gliding motility and biofilm formation processes of this microorganism. Two proteins encoded by the Coch_0033 and _1336 genes and anno-

tated 'hyalin' have a conserved CTD domain in *C. ochracea*. This allows them to be translocated to the outside of the outer membrane by the T9SS. Blast analysis revealed that the identity and similarity of these two proteins was 31% and 46%, respectively. The functional ortholog of the protein encoded by the Coch_0033 gene was predicted to be a large surface protein, the protein of the *Salmonella enterica* serovar Enteritidis¹²⁾, which is involved in the quorum sensing pathway according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ontology database (https://www.genome.jp/kegg/kegg_ja.html). These results suggest a role for the Coch_0033 protein in quorum sensing in the process of biofilm formation. However, no information is available on this protein. The aim of the present study was to investigate the role of the hyalin-like protein encoded by the Coch_0033 gene in gliding activity and biofilm formation in *C. ochracea*.

Materials and Methods

1. Bacterial strains, plasmids, and growth conditions

The *C. ochracea* wild-type strain ATCC 27872 was used for the construction of a mutant strain (designated WR-1) deficient in the hyalin-encoding gene, Coch_0033. Both strains were routinely maintained in tryptic soy agar containing 5 µg/ml hemin, 5 µg/ml menadione, and 10% defibrinated horse blood, without (ATCC 27872) or with (WR-1) 10 µg/ml erythromycin (Sigma-Aldrich, St. Louis, MO, USA), under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂) in an anaerobic chamber at 37°C. These strains were also grown in tryptic soy broth (Becton Dickinson, Sparks, MD, USA) supplemented with 5 µg/ml hemin and 0.5 µg/ml menadione at 37°C under anaerobic conditions at 37°C. The plasmid-transformed *Escherichia coli* strain was grown in Luria-Bertani agar (Wako Pure Chemical Industries, Osaka, Japan) at 37°C under aerobic conditions.

Table 1 Primers used for construction of TDE_0127 mutant strain.

Primers	Sequence (5'-3')	Description
0332-1F	GGAATTCGATATCACCCACGCTCAGTTTGATA TGGTGCGTT	For amplifying upstream region of Coch_0033
0332-1R	GCTATCGGGGGTACCTAGTTATTAACTATGGT ATATAAACT	For amplifying upstream region of Coch_0033
0332-2EF	GGTACCCCCGATAGCTTCCGCT	For amplifying <i>erm-FermAM</i> from pVA2198
0332-2ER	TCACCTTTACGCAGCGGATCCCCGAAGCTGTC AGTAG	For amplifying <i>erm-FermAM</i> from pVA2198
0332-3F	GCTGCGTAAAGGTGATGAAATACAGAT	For amplification of the region of 938th to 1970th in Coch_0033
0332-3R	GCCGCGAATTCATAACAGGTTCCCACTACG TCTACCATTAG	For amplification of the region of 938th to 1970th in Coch_0033

2. Construction of hyalin-encoding gene (Coch_0033)-deficient mutant

To construct the mutant strain, the inactivated Coch_0033 gene was incorporated into wild-type *C. ochracea* ATCC 27872 cells *via* electroporation-mediated double cross-over recombination with a DNA fragment containing a part of the gene interrupted by the *ermF-ermAM* cassette.

The genomic sequence of *C. ochracea* ATCC 27872 was obtained from the GenBank database (Accession No. NC_013162), while its hyalin protein sequence (Coch_0033) was obtained from the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>). The primers used in this study are listed in Table 1. To construct the hyalin-like protein (Coch_0033)-encoding gene mutant, a 1,100-bp fragment upstream of the Coch_0033 gene, the *ermF-ermAM* cassette, and a 1033-bp fragment of the Coch_0033 gene (938th to 1970th) were amplified by means of PCR from chromosomal DNA of *C. ochracea* ATCC 27872 and the pVA2198 plasmid⁵⁾ with the primer pairs 0332-1F/0332-1R, 0332-2EF/0332-2ER, and 0332-3F/0332-3R, respectively (Fig. 1A). The *ermF-ermAM* cassette was inserted into the upstream and downstream fragments of the target genes and cloned into plasmid pGEM-

T Easy (Promega, Tokyo, Japan) in a single step by using the In-Fusion cloning system (Takara Bio Inc. Shiga, Japan). The obtained plasmids were transformed into *E. coli* One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). The *E. coli* transformants were selected on Luria-Bertani agar plates containing 100 µg/ml ampicillin. The recombinant plasmid DNA was then isolated from the transformants and linearized with *Not* I and the fragments subsequently introduced into *C. ochracea* ATCC 27872 by means of electroporation. In brief, *C. ochracea* ATCC 27872 cells in the mid-logarithmic-phase were harvested from 100 ml culture medium, washed 3 times in ice-cold distilled water, and suspended in 0.2 ml of 10% glycerol. Then, 10 µg linearized DNA fragments was mixed with 40 µl cells and the mixture incubated on ice for 1 min before being transferred to a 0.1-cm electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Electroporation was performed using a Gene Pulser II device (Bio-Rad) with the following settings: 1.8 kV voltage, 25 µF capacitance, and 250 Ω resistance. Immediately after electroporation, the cells were suspended in 1 ml tryptic soy broth containing 5 µg/ml hemin and 0.5 µg/ml menadione and incubated overnight at 37°C under anaerobic conditions. The transformants

Fig. 1 Schematic representation and confirmation of inactivation of Coch_0033.

(A) Inactivation of Coch_0033 gene using *ermF-ermAM* cassette. Sequences flanking 5'-end of Coch_0033 gene and fragment in Coch_0033 gene were amplified with primers 0332-1F and 0332-1R, and primers 0332-3F and 0332-3R, respectively. *ermF-ermAM* cassette was inserted in between amplified fragments and cloned. Circular plasmid was then linearized and introduced into *C. ochracea* ATCC 27872 by electroporation. Resultant hyalin protein: *ermF-ermAM* strain was named WR-1.

(B) Confirmation of inactivation of Coch_0033 by PCR. Insertion of *ermF-ermAM* cassette was confirmed by amplification of insertion fragment from *C. ochracea* WR-1 genomic DNA. Lane M: Size marker; lane 1: fragment amplified with primers 0332-1F and 0332-3R; lane 2: fragment amplified with primers 0332-2EF and 0332-2ER; lane 3: fragment amplified with primers 0332-1F and 0332-2ER; lane 4: fragment amplified with primers 0332-2EF and 0332-3R.

(C) Confirmation of inactivation of protein coded by Coch_0033 gene by SDS-PAGE. Lanes; 1: *C. ochracea* ATCC 27872; 2: *C. ochracea* mutant WR-1; M: molecular size marker. Density of band around 500 kDa (arrow) showed significant decrease in WR-1 (TDE_0033 deficient mutant).

were inoculated into tryptic soy agar containing 5 μ g/ml hemin, 0.5 μ g/ml menadione, 10 μ g/ml erythromycin, and 10% defibrinated horse blood and incubated for 7 days at 37°C under anaerobic conditions.

3. Confirmation of gene deletion by PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Correct gene replacement in the erythromycin-resistant mutants was confirmed by

means of PCR. Briefly, the fragments were amplified with primers 0332-1F and 0332-3R, 0332-2EF and 0332-2ER, 0332-1F and 0332-2ER, and 0332-2EF and 0332-3R from genomic DNA of *C. ochracea* strain WR-1. The length of the fragments confirmed by electrophoresis through 1% agarose.

The transformed *C. ochracea* cells were grown to the early stationary phase at 37°C in tryptic soy broth. The cells were then harvested by centrifugation at 8,000g for 20 min at 4°C, after which they were washed twice in

phosphate buffered saline (PBS; pH 7.4). The cells were disrupted by sonication and the supernatant collected by centrifugation at 8,000g for 20 min at 4°C, after which they were subjected to SDS-PAGE. The supernatants were mixed with NuPAGE LDS sample buffer (Invitrogen) and reducing agent (Invitrogen) and incubated at 70°C for 10 min. The samples (10 µg of protein) were separated by NuPAGE Tris-Acetate Gel (3–8% gels; Invitrogen) under reducing conditions. After electrophoresis, the separated protein bands were detected by silver staining.

4. Microscopic observation of colony morphology on solid agar

The colony spreading morphology of *C. ochracea* colonies on solid agar surfaces was examined under a stereomicroscope (Stemi 508, Carl Zeiss MicroImaging Inc., Göttingen, Germany) and laser microscope (LEXT OSL 4000, Olympus, Tokyo, Japan). In brief, cells in the early stationary phase were suspended in fresh medium to an OD₆₆₀ of 1.0. The cell suspensions were then spotted onto tryptic soy agar (agar content, 3%) supplemented with hemin (5 µg/ml), menadione (0.5 µg/ml), and 0.1% yeast extract (Becton Dickinson). After incubation for 5 days at 37°C under anaerobic conditions, colony spreading was observed under a stereomicroscope and laser microscope equipped with a digital camera.

5. Crystal violet biofilm formation assay

The mass of biofilms formed by the *C. ochracea* wild-type strain and mutant WR-1 were examined in 96-well polystyrene plates (Sumitomo Bakelite, Tokyo, Japan) as described previously⁷. Briefly, the *C. ochracea* wild-type strain and mutant WR-1 were pre-cultured for 2 days and then adjusted to an OD₆₆₀ of 0.01 with tryptic soy broth containing 5 µg/ml hemin and 0.5 µg/ml menadione. They were then inoculated into a 96-well polystyrene cell culture plate and incubated for 1 day or 2 days under anaerobic conditions at 37°C. After removal of planktonic cells by washing in distilled water, the biofilm orga-

nized on the bottom of the well was stained with 50 µl of 0.1% crystal violet for 15 min. After the crystal violet solution was removed and each well washed with distilled water, the biomass-associated crystal violet was extracted with 200 µl of 99.5% ethanol. The extracted biomass-associated crystal violet (100 µl/well) was transferred to a new microtiter plate, and the OD₅₉₅ measured using a spectrophotometer (Spectramax M5e; Molecular Device, Sunnyvale, CA, USA).

6. Confocal laser scanning microscopic analysis of biofilms

The *C. ochracea* wild-type strain and mutant WR-1 were precultured for 2 days and diluted to OD₆₆₀ of 0.01 with tryptic soy agar containing 5 µg/ml hemin and 0.5 µg/ml menadione. Then 2 ml diluted cell suspension was inoculated into a glass base dish (35-mm) and incubated for 2 days at 37°C under anaerobic conditions. Following the removal of the medium, the dish was washed with PBS to remove non- and weakly-adhered cells. The biofilms on the bottom of the dish were treated with the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. The biofilms were incubated in the dark at room temperature for 15 min, washed twice with PBS, and finally observed by means of a confocal laser scanning microscope (LSM5 DUO; Carl Zeiss MicroImaging Inc., Göttingen, Germany) with a 40×/1.2 water immersion objective. The biofilms were scanned in increments using excitation wavelengths of 488 (labelling live cells) and 543 nm (dead cells). The volume of the biofilms was calculated at an intensity of 1024×1024 pixels and with the step set at 0.5 µm in the Z-stack from bottom to top; density was calculated at an intensity of 512×512 pixels and with a central 10 Z-stack using Zen 2009 (Carl Zeiss MicroImaging Inc.) and Imaris 7.0.0 (Bitplane AG, Zurich, Switzerland) software.

7. Statistical analysis

The data were analyzed using a Student's *t*-test. All statistical analyses were performed

Fig. 2 Colony morphology of *C. ochracea* wild-type and mutant strains on solid agar surface.

Effects of Coch_0033 gene deletion on spread of colonies on solid agar surface. A: Representative photographs under stereomicroscope of wild-type *C. ochracea* ATCC 27872 and mutant WR-1 are shown. B: Colony edge of *C. ochracea* ATCC 27872; *C. ochracea* cells (arrow) were spread out from edge of colony. C: Colony edge of *C. ochracea* mutant WR-1; No *C. ochracea* cells were observed around colony.

using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All data were considered significant at $p < 0.05$.

Results

1. Confirmation of inactivation of hyalin-encoding gene (Coch_0033) using PCR and SDS-PAGE

Correct gene replacement in the erythromycin-resistant mutants was confirmed by means of PCR and SDS-PAGE analyses. As shown in Fig. 1B, the *ermF-ermAM* cassette had been inserted in the hyalin-encoding gene in the mutant WR-1 (Fig. 1B). The deduced molecular mass of the protein encoded by the Coch_0033 gene was 425376. In the SDS-PAGE, the density of the band around 500 kDa showed a significant decrease in the mutant WR-1 (Fig. 1C). The size was almost the same as that deduced from the amino acid sequence (Coch_0033).

2. Comparison of morphological characteristics of colonies between *C. ochracea* wild-type and mutant

Almost no difference was observed in growth rates between that of the wild-type strain and that of the mutant WR-1 (data not shown). To investigate the effects of Coch_0033 gene-deficiency on the biofilm

formation of the *C. ochracea* wild-type and WR-1 strains, the colony morphology of *C. ochracea* ATCC 27872 strain and WR-1 on a solid agar surface after 1-week culture was examined under a stereomicroscope (Fig. 2A). The wild-type strain formed colonies that exhibited marked spreading across the solid agar surface, whereas the mutant strain formed colonies without spreading. Bacteria-like structures were observed on the outside of the colony in the wild-type strain (Fig. 2B), whereas no such structure was observed in the mutant strain (Fig. 2C). Repeated investigation revealed no colony spread with the mutant strain until 3 weeks. This result indicates that the protein encoded by the Coch_0033 gene is crucial for the gliding activity of *C. ochracea* across solid surfaces.

3. Analysis of biofilm characteristics

To investigate differences between the biofilms formed by the *C. ochracea* wild-type and mutant WR-1 strains, the biomass produced by each was examined by crystal violet staining, and the volume and density of each biofilm examined by confocal laser scanning microscopy (CLSM). No difference was observed in the biomass of the biofilm formed in the 96-well plate by *C. ochracea* WR-1 and ATCC 27872 at 24 or 48 hr (Fig. 3). Under CSLM, the calculated volume of biofilm showed no difference between ATCC 27872

Fig. 3 Effects of Coch_0033 deficiency on mass of biofilm organized by *Capnocytophaga ochracea*.

C. ochracea ATCC27872 and mutant WR-1 were incubated in tryptic soy broth containing 5 µg/ml hemin and 0.5 µg/ml menadione (A) for 24 h, (B) for 48 h, in 24-well plate under anaerobic conditions. Biomass of biofilms was then assayed by crystal violet staining. Data are presented as mean $\pm$ SD (n = 10).

and mutant WR-1 (Fig. 4A), whereas the density of the biofilm organized by mutant WR-1 was slightly lower than that of ATCC 27872 ($p < 0.05$).

Discussion

In the present study, inactivation of the Coch_0033 gene led to a decrease in the gliding activity of *C. ochracea*. The T9SS, a novel protein secretion system, was recently identified in *P. gingivalis* and other members of the phylum *Bacteroidetes*. Other bacteria, including major periodontal pathogens, have also

been found to harbor T9SS-related genes¹⁴. Certain surface proteins are important for attachment, interaction with other organisms, and biofilm formation¹. The T9SS secretes extracellular proteins and surface motility adhesins, and its components participate in biofilm formation in *F. johnsoniae*²⁶. Hyalin-like proteins were detected in bacteria belonging to the genus *Capnocytophaga*, but not other genera, by blast analysis using the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This suggests that the hyalin-like protein encoded by the Coch_0033 gene is an essential component in *Capnocytophaga*, although further analysis of the gliding mechanism of *C. ochracea* is required to confirm this.

In the present study, the biofilm formation of the mutant WR-1 showed only a slight difference from that of the wild-type. An earlier study by this group indicated that inactivation of one T9SS protein, SprB, affected biofilm formation. This protein is involved in gliding motility, which is likely involved in biofilm formation by *C. ochracea*⁹. In the present study, although gliding activity was clearly affected by inactivation of Coch_0033 in the mutant WR-1, biofilm formation was not. Almost no difference was observed in growth rate between the wild-type strain and the mutant WR-1 (data not shown). This indicates that gliding activity is not a major factor in biofilm formation. The SprB protein is propelled through a left-handed helical loop along the surface of the cell, with gliding being produced by its attachment to the hard surface^{16,22}. It is known to bind to polysaccharides, which are abundant on most cell walls²⁷. On the surface of the mutant WR-1, SprB may be involved in adherence to polysaccharides on the walls of other cells.

Information from *in silico* analysis using the NCBI and KEGG databases on the function of the protein coded by the Coch_0033 gene is scarce. In the 3958-amino acid sequence of this protein, only 2 regions show similarity to other proteins (CTD domain: 88 amino acids; and hyalin: 72 amino acids). Other regions showed no similarity to any other domain or

Fig. 4 Effects of Coch_0033 gene deficiency on structure of biofilm organized by *Capnocytophaga ochracea*.

Confocal laser scanning microscopic analysis of *C. ochracea* biofilms.

A: Confocal laser scanning microscopic analysis of *C. ochracea* ATCC 27872 and mutant WR-1 biofilms. Two *C. ochracea* strains were incubated for 48 h at 37°C under anaerobic conditions and then stained with Live/Dead BacLight Bacterial Viability Kit. Images are presented as each x-y image (upper) and each x-z reconstruction (lower). B: Calculated volume and density of biofilms from CSLM. Data are presented as mean $\pm$ SD *, $p < 0.05$ compared with wild-type.

protein. The results of the present study and blast analysis suggest that the protein encoded by the Coch_0033 gene is exported across the outer membrane *via* T9SS, and that while it plays a role in the gliding activity of this microorganism, it does not play a major role in biofilm formation. The function clarified in the present study comprises an additional function to that predicted in the KEGG database (quorum sensing). Further analysis concerning its location, structure, and interaction with other molecules is required.

Additional approaches are currently being used to clarify the molecular mechanisms by which the hyalin-like protein encoded by the Coch_0033 gene participates in gliding activ-

ity. This protein was also detected from *Capnocytophaga canimorsus*, which is a major pathogen in infections arising from bites⁶⁾. Therefore, further clarification of the role of this T9SS-transported protein in this microorganism is important in clarifying its virulence. Understanding this will provide the key to controlling the development of the microbiome of periodontitis lesions.

Conclusion

The results of the present study suggest that hyalin-like protein plays a role in the gliding ability of *C. ochracea*.

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