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 motility, is a major component of dental plaque biofilm. Although this microorganism was first isolated from human periodontitis lesions, ensuing studies have also reported...
its presence in dental plaques from healthy periodontal sites\(^{10}\). Genome-wide transcriptome analysis of the subgingival microbiome in patients with periodontitis has revealed upregulation of putative virulence factors for \textit{C. ochracea}\(^{4}\). Little is known about the role of \textit{C. ochracea} in the pathogenesis and progression of periodontal disease, however\(^{2,11,15,19}\). Besides periodontitis, \textit{C. ochracea} has also been reported to cause several systemic infectious diseases such as sepsis and purpura fulminans\(^{3,20}\). The virulence of \textit{C. ochracea} in these diseases remains unclear, however.

\textit{Capnocytophaga ochracea} possesses a type-IX secretion system (T9SS), which has also been identified in \textit{Porphyromonas gingivalis} and other members of the phylum \textit{Bacteroidetes}\(^{14}\). The T9SS specifically exports proteins that have a gliding motility-associated C-terminal (CTD) domain. Several T9SS proteins have been identified in \textit{P. gingivalis}\(^{22,25}\) and \textit{Flavobacterium johnsoniae} (phylum \textit{Bacteroidetes})\(^{8,14}\). In \textit{P. gingivalis}, these proteins are essential for the secretion of major proteases\(^{22–25}\). In \textit{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 \textit{Tannerella forsythia}, the T9SS mutant increased biofilm formation compared with the wild-type strain\(^{17}\).

Genes orthologous to those that encode the T9SS proteins of \textit{F. johnsoniae} have also been found in the genome of \textit{C. ochracea}\(^{4}\), suggesting the possible involvement of T9SS-exported proteins in the gliding motility and biofilm formation of \textit{C. ochracea}. This group has previously identified three T9SS protein-encoding orthologous genes in \textit{C. ochracea}: \textit{gldK} and \textit{sprT}, which encode T9SS proteins; and \textit{sprB}, which encodes a gliding motility adhesin that is transported by T9SS to the cell surface\(^{9}\). Inactivation of each gene in \textit{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 annotated ‘hyalin’ have a conserved CTD domain in \textit{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 \textit{Salmonella enterica} serovar Enteritidis\(^{22}\), 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 \textit{C. ochracea}.

**Materials and Methods**

1. **Bacterial strains, plasmids, and growth conditions**

The \textit{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% N\(_2\), 10% H\(_2\), and 10% CO\(_2\)) 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 \textit{Escherichia coli} strain was grown in Luria-Bertani agar (Wako Pure Chemical Industries, Osaka, Japan) at 37°C under aerobic conditions.
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 plasmid 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 mediation and incubated overnight at 37°C under anaerobic conditions. The transformants

Table 1 Primers used for construction of TDE_0127 mutant strain.

| Primers   | Sequence (5'-3')                              | Description                                      |
|-----------|-----------------------------------------------|--------------------------------------------------|
| 0332-1F   | GGAATTCGATATCACCCCACGCTCAGTTTGATA TGGTGCCTT | For amplifying upstream region of Coch_0033      |
| 0332-1R   | GCTATCGGGGGTACCTAGTTAAACTATGGAATATAAAACT     | For amplifying upstream region of Coch_0033      |
| 0332-2EF  | GGTACCCCCGATAGCTTCCGCT                       | For amplifying ermF-ermAM from pVA2198           |
| 0332-2ER  | TCACCTTTACGCAGGGATCCCAGGCTGTC AGTAG           | For amplifying ermF-ermAM from pVA2198           |
| 0332-3F   | GCTGCGTAAGGTGATGAAATACGAGT                  | For amplification of the region of 938th to 170th in Coch_0033 |
| 0332-3R   | GCCGCGAATCTACAAAGGTTCCTCAAATCAG TCTACATTAG   | For amplification of the region of 938th to 170th in Coch_0033 |
were inoculated into tryptic soy agar containing 5 μg/ml hemin, 0.5 μg/ml menadione, 10 μg/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 previously. Briefly, 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 broth 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.

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...
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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 \( \text{ermF-ermAM} \) 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 and...
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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 genes. Certain surface proteins are important for attachment, interaction with other organisms, and biofilm formation. The T9SS secretes extracellular proteins and surface motility adhesins, and its components participate in biofilm formation in F. johnsoniae. Hyalin-like proteins were detected in bacteria belonging to the genus Capnocytophaga, but not other genera, by blast analysis using the NCBI database. 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. 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. It is known to bind to polysaccharides, which are abundant on most cell walls. 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...
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 use to clarify the molecular mechanisms by which the hyalin-like protein encoded by the Coch_0033 gene participates in gliding activity. This protein was also detected from Capnocytophaga canimorsus, which is a major pathogen in infections arising from bites\(^6\). 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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**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 CSM. Data are presented as mean ± SD, \(p<0.05\) compared with wild-type.
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