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Intermembrane rhomboid protease activity of *Rothia mucilaginosa*

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Abstract

Rothia mucilaginosa is a part of the normal oral microflora, which has been recognized as an endodontic pathogen and is also an emerging opportunistic pathogen in immune compromised hosts. In this study, we identified the rhomboid protease in *R. mucilaginosa* as a candidate virulence factor. A database search identified two rhomboid genes on the genome with open reading frames of 828 bp and 822 bp, respectively. The two genes are separated by 57 bp, suggesting that they constitute a bicistronic operon. Motifs conserved among rhomboid family proteins (HxxxN, GxSG, and GxxxG) were found in their deduced amino acid sequences. The two recombinant rhomboids and the rhomboid/liposome complexes (proteoliposomes) were prepared using a wheatgerm cell-free translation system, and their serine protease activities were assessed. The two rhomboids showed serine protease activity in the form of rhomboid/liposome complex, while the recombinant rhomboids alone showed little or no activity. On the supposition of host invasion, *R. mucilaginosa* was cultivated in medium with type I collagen, and the rhomboid mRNA levels were examined. Real-time reverse transcription polymerase chain reaction analysis revealed that the levels of the two rhomboid mRNAs increased slightly in culture with type I collagen, although the changes were not statistically significant. These results suggested that *R. mucilaginosa* possesses two active rhomboids with protease activity on the membrane, and the two rhomboids may be virulence factors involved in the response to host environmental conditions.

Keywords: *Rothia mucilaginosa*; Rhomboid protease; Endodontic pathogen; Virulence factor

1. Introduction

Rothia species are gram-positive cocobacilli present in the normal microflora of the oral environment and the upper respiratory tract. Although *Rothia* species were originally believed to be of low virulence, these microorganisms are now recognized as emerging pathogens involved in various systemic infections, including endocarditis [1, 2], meningitis [3], pneumonia [4], bacteremia [5], knee joint infection [6], and endophthalmitis [7]. Among the *Rothia* species, *Rothia mucilaginosa*, previously known as *Stomatococcus mucilaginosus*, has been frequently reported as an opportunistic pathogen involved in serious systemic infections. Immunocompromised individuals, such as patients with hematological malignancies undergoing chemotherapy or those with neutropenia, have been reported as target hosts of this microorganism [5, 8]. *R. mucilaginosa* has also been suggested to be involved in the pathogenesis of persistent periapical periodontitis [9]. Clinical isolates from persistent periapical lesions exhibit a dense meshwork-like structure on the cell surface, which is a component of their biofilm [9].

Periapical periodontitis is a disease caused by polymicrobial infection of oral bacteria in the tooth root canal. The root canal connected to the alveolar bone acts as reservoir of microorganisms to periapical lesions, and endodontic pathogens have been reported to cause serious systemic infections [10,11]. Periodontitis is linked to various systemic diseases, including cardiovascular disease, diabetes, and rheumatoid arthritis [12], and similar links between apical periodontitis and systemic disease have recently attracted attention [13–15]. *R. mucilaginosa* is a candidate pathogen

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that colonizes the root canals and causes apical periodontitis and systemic infectious diseases. However, its virulence factor has not been elucidated.

The rhomboid family of intermembrane serine proteases was initially identified in *Drosophila*, where they are required for growth factor signal generation by cleaving membrane-bound precursors [16]. The rhomboids are highly conserved among all kingdoms of life. It has been reported that the active sites of members of the rhomboid family are embedded in the lipid bilayer, where they cleave substrates in transmembrane domains [17]. The rhomboid family intramembrane active site has been shown to be accessible to water and capable of hydrolyzing an extramembrane peptide bond of the substrate [18]. Rhomboids have been reported to have a wide range of functions in eukaryotes, including roles in growth factor signaling [16], lipid metabolism [19], and apoptosis regulation [20]. The protease activity has also been reported to be involved in migration of cancer cells [21].

Analysis of bacterial genomes in the National Center for Biotechnology Information (NCBI) database showed that one or several copies of the rhomboid gene are present in a wide range of species. A number of detailed structural and theoretical studies of bacterial rhomboids have been reported to date, especially for GlpG of *Escherichia coli* and AarA of *Providencia stuartii* [22]. However, very little is known about the roles of bacterial rhomboids, including their substrates and associations with virulence. Rhomboids have been reported to be involved in the virulence of unicellular eukaryotes, such as protozoan or amebic parasites, through roles in immune evasion and invasion of host cells [23,24].

Here, we hypothesized that the rhomboids represent potent virulence factors of *R. mucilaginosa* involved in the pathogenesis of apical periodontitis and systemic infections. As the first step to elucidate their roles, we isolated the rhomboids from *R. mucilaginosa* and examined their protease activity.

2. Material and methods

2.1. Bacterial strains and culture conditions

R. mucilaginosa DY-18 was cultivated on blood agar plates (BBL™ Microbiology Systems; Becton Dickinson and Company, Franklin Lakes, NJ, USA). Bacterial colonies on the agar plates were inoculated into tryptic soy broth (Becton Dickinson and Company) supplemented with 0.5% yeast extract (Becton Dickinson and Company) and were cultivated at 37°C with or without type I collagen (1 µg/ml) (Nitta Gelatin, Osaka, Japan). The cultivated cells were harvested by centrifugation and subjected to DNA extraction, RNA extraction, or sonication for gene cloning, real-time polymerase chain reaction (PCR), or protease assay, respectively. *E. coli* XL1-Blue was used for recombinant DNA procedures. *E. coli* were cultured aerobically in Luria-Bertani (LB) medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 37°C. Where required, ampicillin (50 µg/ml) was added to the medium.

2.2. Database search

A key word search was performed in the NCBI *R. mucilaginosa* genome database using the query term "rhomboid." Nucleotide and amino acid sequences of the two proteins annotated as rhomboid family intramembrane serine proteases were obtained from the database and used to clone the genes. Transmembrane protein structure prediction was performed using the bioinformatics tool TMHMM [25].

2.3. PCR and cloning of rhomboid protease genes

Primers for PCR were designed to amplify the whole length of the open reading frames (ORFs) of the two rhomboid proteases in *R. mucilaginosa* (Rrhom1 and Rrhom2). The primers (forward primer 5′-CCCCTCTCGAGGTGGAGAACTTTCCCAAC-3′ and reverse primer 5′- GGATTACTAGTCTAGTACCTGCCGTTTTG-3′) containing restriction sites (*Xho*I in the forward primer and *Spe*I in the reverse primer) were used for *Rrhom1* gene amplification. The primers (forward primer 5′-TCCTTGGATCCGTGGAGAACTTTCCCAAC-3′ and reverse primer 5′-GATTTACTAGTTCAGTACCTGCCGTTGAA-3′) with restriction sites (*Bam*HI in the forward primer and *Spe*I in the reverse primer) were used for *Rrhom2* gene amplification. Five extra nucleotides with a random sequence were added to the 5′ end of each primer for digestion of the PCR product with the restriction enzymes. PCR was performed in a volume of 25 µl using TaKaRa Taq™ HS Perfect Mix (Takara, Shiga, Japan) with 20 pmol of forward and reverse primers and approximately 200 ng of *R. mucilaginosa* genomic DNA. The thermal cycling program consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplicons of *Rrhom1* and *Rrhom2* genes were digested with the appropriate restriction enzymes and were cloned into the corresponding sites of the pEU-E01-GST-PS-MCS-N2 and pEU-E01-GST-PS-MCS-N3 expression vectors (CellFree Sciences, Ehime, Japan), respectively. The expressed recombinant proteins were used alone for the protease assay.

The *Rrhom1* and *Rrhom2* genes were also cloned into the pEU-E01-MCS expression vector (CellFree Sciences), and the expressed recombinant proteins were used for construction of rhomboid/liposome complexes (proteoliposomes). PCR primers with the restriction sites used for amplification of the rhomboid genes were as follows: (*Rrhom1* forward primer) 5′-CCCCTCTCGAG(*Xho*I)ATGGTGGAGAACTTTCCCAAC-3′, (*Rrhom1* reverse primer) 5′-GGATTGCGGCCGC(*Not*I)CTAGTACCTGCCGTTTTG-3′, (*Rrhom2* forward primer) 5′-TCCTTACTAGT(*Spe*I)ATGGTGGAGAACTTTCCCAAC-3′, (*Rrhom2* reverse primer) 5′-GATTTGGATCC(*Bam*HI)TCAGTACCTGCCGTTGAA-3′. The amplicons were digested with the appropriate restriction enzymes and cloned into the corresponding sites of the vector.

2.4. Synthesis and purification of recombinant rhomboids

Synthesis and purification of the recombinant rhomboid proteins were performed by CellFree Sciences using a wheatgerm cell-free translation system [26]. Briefly, in vitro transcription and translation were performed using a CFS-TRI-1240G kit (CellFree Sciences) and the expression vector constructs described above. Transcription was carried out at 37°C for 6 h in the transcription buffer supplied with the kit with the recombinant expression vector (100 ng/µl), SP6 RNA polymerase (1 unit/µl), RNase inhibitor (1 unit/µl), and 2.5 mM nucleoside triphosphates (NTPs). Translation was carried out using 5.5 ml of SUB-AMIX (proprietary buffer containing all 20 amino acids), 250 µl of the transcription products, 1 µl of creatine kinase (20 mg/ml), and 250 µl of WEPRO1240G supplied with the kit for 16 h at 17°C. Glutathione sepharose 4B gel (GE Healthcare Biosciences, Rydalmere, NSW, Australia) was used for purification of the synthesized glutathione-*S*-transferase (GST)-tagged recombinant proteins.

2.5. Proteoliposome construction

Synthesis and purification of the two rhomboid proteoliposomes were performed by CellFree Sciences using the bilayer-dialysis method [27]. Briefly, 500 µl of reaction mixture containing 125 µl of wheatgerm extract, 125 µl of mRNA, 40 µg/ml creatine kinase, and 10 mg/ml asolectin liposome was overlaid with 2 ml of SUB-AMIX SGC solution (CellFree Sciences) in a 10-K MWCO Slide-A-Lyzer dialysis device (Thermo Fisher Scientific, Waltham, MA, USA), and the device was then immersed in 3.5 ml of SUB-AMIX SGC solution. The reaction was carried out at 15°C for 48 h with replacement of the dialysis solution after 24 h. Synthesized rhomboid protein/liposome complex was collected by centrifugation (20 000 × *g* for 10 min) at 4°C and washed three times with phosphate-buffered saline (PBS). After the washing step, the liposome complex was collected by centrifugation, and the pellet was resuspended in 0.5 ml of PBS for use as the proteoliposome sample for protease assay. The recombinant proteins and proteoliposome samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prior to the assay.

2.6. Protease activity assay

Serine protease activities of the recombinant proteins, proteoliposomes, and bacterial cells were assessed using an Amplite™ Universal Fluorimetric Protease Activity Assay Kit (AAT Bioquest, Pleasanton, CA, USA) according to the manufacturer's instructions. Briefly, 50 µl of each sample was added to 50 µl of substrate solution provided with the kit. The rhomboid samples in the proteoliposome form and the recombinant protein alone were used at a concentration of 25 µg/ml. Prior to the assay, bacterial cells were disrupted by sonication as described previously [28], and the protease activity was assessed at a concentration of 100 µg/ml. Trypsin (0.1 unit/µl) was used as a control. The fluorescence intensity of the solutions was monitored every 5 min for 6 h at 490/525 nm (Ex/Em) using a microplate reader (SpectraMax M5; Molecular Devices, Tokyo, Japan).

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from *R. mucilaginosa* under cultivation conditions with (1 µg/ml) or without type I collagen in exponential phase. A RiboPure™ RNA Purification-Bacteria Kit (Thermo Fisher Scientific) was used for extraction. Genomic DNA in the total RNA sample was digested with ezDNase™ (Thermo Fisher Scientific), and the RNA sample was subjected to reverse transcription (RT) reaction using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's instructions. The RT reaction mixture contained random hexamers (50 ng/µl) and 1 µg of total RNA. The synthesized cDNA was then used as the template for real-time PCR.

2.8. Real-time PCR

Real-time PCR was performed as described previously [29]. Primer sets were designed and used for quantitative detection of *Rrhom1* (forward: 5′-ACCTGGGCTCAATCGTCTT-3′, reverse: 5‘-TACACCGCCGATAAAGCCA-3′) and *Rrhom2* genes (forward: 5‘-GCGTGTACATCTTCGCCTACT-3′, reverse: 5‘-CCACTGACAAGACCGCCAAA-3′). PowerUP™ SYBRTM Green Master Mix (Thermo Fisher Scientific) was used for PCR with 50 ng of *R. mucilaginosa* cDNA. The PCR parameters included an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and

annealing plus extension at 60°C for 1 min. All amplification and detection steps were carried out using Step One Plus (Applied Biosystems, Foster City, CA, USA). The PCR product was detected by monitoring the increase in fluorescence of the dsDNA-binding SYBR Green dye. A standard curve was constructed using genomic DNA from aliquots of known *R. mucilaginosa* cell number. All data were analyzed using Step One software v2.3 (Applied Biosystems).

3. Results

3.1. Gene structure

Figure 1 shows the structure around *Rrhom1* and *Rrhom2* genes on the chromosome of *R. mucilaginosa*. The ORFs of the *Rrhom1* and *Rrhom2* genes consisted of 828 bp and 822 bp, respectively. The deduced amino acid sequences of the two rhomboids showed 77.8% sequence identity. The two ORFs were separated by 57 bp, suggesting that the two genes probably constitute a bicistronic operon.

Figure 1 Genetic map around the two rhomboid genes (Rrhom1 and Rrhom2) of R. mucilaginosa. The Rrhom1 and Rrhom2 genes were located side by side on the chromosome of R. mucilaginosa with an interval of 57 bp and probably constitute a bicistronic operon. Peptidylprolyl isomerase and shikimate 5-dehydrogenase genes were located in the upstream and downstream regions of the two genes, respectively

The transmembrane structures of Rrhom1 and Rrhom2 were analyzed using the deduced amino acid sequences from the genomic data and TMHMM software. The amino acid sequences of the two rhomboids of *R. mucilaginosa* and the transmembrane helix regions are illustrated in Fig. 2. Both rhomboids were predicted to contain six transmembrane domains. The N-termini and C-termini of the rhomboids were predicted to be located on the cytoplasmic side of the membrane. The HxxxN, GxSG, and GxxxG motifs were conserved in both Rrhom1 and Rrhom2. The L1 loop consisted of 34 amino acid residues and was located outside the membrane.

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Rrhom1:MENFPNYAGYGQTPTVCVNHPNRVSYAQCKRCNRTVCGECQVALDVGMVCPDCYRDLTGG 60
Rrhom2:MENFPNYMNYGQSPTVCVNHPDRVSYAQCKRCNRTVCGECQTAQGAGVVCPDCYRELNGG 60
       *******  *** ******** ******************* *    * ******* * **

Rrhom1:SSKAQGSKAQGSFLARHWHITYTLILINVAMYGLQQIIPNRWMVNHGMMWWPYVQHGEYY 120
Rrhom2:S-----SKAKGSFLARHWHITYTLILINVAMYGLQQIIPNRWMVNHGMMWWPYVQHGEYY 115
       *     *** **************************************************

Rrhom1:RLISYGFLHSQNDPMHLVWNMIYLFIFGVSIERMMGRWKFLVVYLGSIVFGAFGVHVLTP 180
Rrhom2:RLISYGFLHSQNDPMHLVWNMIYLFIFGVSIERMMGRWKFLFVYMAATVGAGLSVYIFAY 175
       ***************************************** **    *     *

Rrhom1:STSVAGASGGVYGLYGAFLVILILRKQKDTARLFMLLIGIGVVQSFFNPNISHACHAGGF 240
Rrhom2:YRGAVGASGGVYGLYGAFFVILLIRRQKDTARLFILLIGIGVVQNLFTPHISHAGHFGGL 235
            ************* ****** ******** *********  * * **** * **

Rrhom1:IGGVLLTLLFVPFVKKP---ESSDPPQQSPQQWQNGRY 275
Rrhom2:VSGALATFLILPFVKKPESSESSEPPQQGPRRWFNGRY 273
         * * * *  ******   *** **** *  * ****
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Figure 2 Amino acid sequences of Rrhom1 and Rrhom2. Rrhom1 and Rrhom2 consisted of 275 and 273 amino acids, respectively. Identical amino acids are indicated by asterisks, and the two rhomboids showed 77.8% sequence identity. The transmembrane structures were analyzed using TMHMM software [25], and the predicted transmembrane domains are indicated by boxes. The domains inside and outside the membrane are indicated by single underlines and broken underlines, respectively. The predicted L1 loop and the conserved motifs (HxxxN, GxSG, and GxxxG) among rhomboid family proteins are indicated by double underline and arrows, respectively

3.2. Recombinant protein construction

PCR-amplified *Rrhom1* and *Rrhom2* genes were cloned into the appropriate sites of the pEU expression vector. Prior to construction of the recombinant proteins, the nucleotide sequences of the DNA inserts were confirmed to be identical to those in the genome database (data not shown).

Recombinant Rrhom1 and Rrhom2 proteins were successfully expressed in the wheatgerm cell-free translation system (CellFree Sciences). The recombinant proteins with the GST-tag were purified by affinity chromatography. The protein profiles during purification are shown in Fig. 3. The recombinant proteins of Rrhom1 and Rrhom2 with the GST-tag both showed the expected molecular mass of approximately 50 kDa. The protein profiles of the proteoliposome samples are also shown in Fig. 3. The recombinant proteins of Rrhom1 and Rrhom2 used for proteoliposome construction did not contain the GST-tag and appeared at molecular masses of approximately 26 kDa and 25 kDa, respectively.

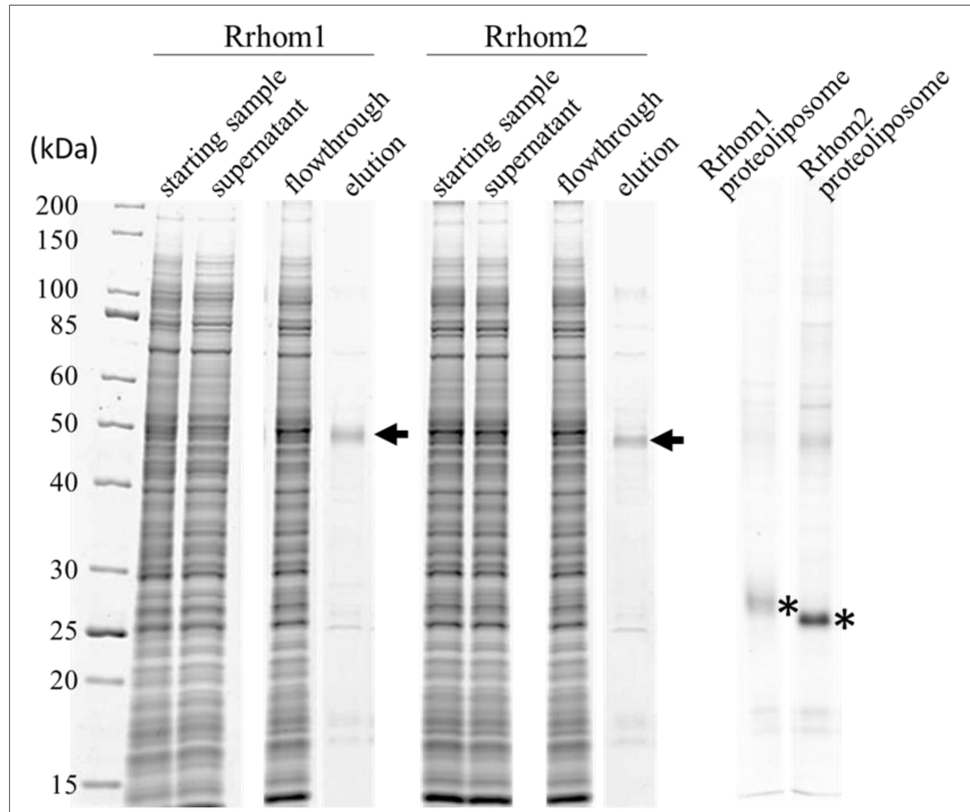

Figure 3 Protein profiles of the recombinant protein during purification. Pooled fractions of the starting samples, supernatant samples after centrifugation (20 000 × g for 10 min), flowing through samples from the glutathione sepharose 4B gel and the eluted samples were analyzed by SDS-PAGE. Purified GST-tagged recombinant rhomboid proteins (Rrhom1 and Rrhom2) of R. mucilaginosa are indicated by arrows. Purified rhomboid proteoliposomes are also shown by asterisks. Aliquots of approximately 500 ng of purified protein were loaded onto the gel

3.3. Protease activity

The proteoliposomes of Rrhom1 and Rrhom2 both showed serine protease activity, while the recombinant proteins alone had no activity. Representative kinetic curves of the samples are shown in Fig. 4. The fluorescence intensity was normalized relative to PBS with the substrate and shown in relative fluorescence units (RFU). Reaction mixtures of the recombinant proteins of Rrhom1 and Rrhom2 without liposomes showed high fluorescence intensity at the start point for unknown reasons, which decreased rapidly at the basal line. The curves of proteoliposome reaction reached a plateau at a reaction time of ~4 h.

R. mucilaginosa and *E. coli* cell components both showed similar serine protease activity and kinetic curves. The bacterial cells (100 μg/ml) showed almost identical levels of protease activity to trypsin (0.1 unit/μl).

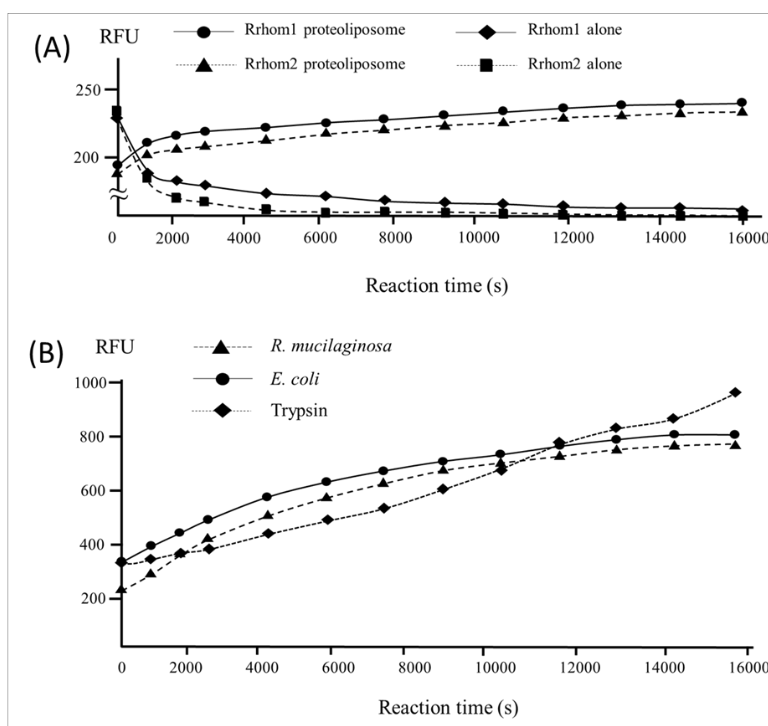

Figure 4 Kinetics of protease activity of the recombinant rhomboids (A) and sonicated cells of *R. mucilaginosa* and *E. coli* (B). RFU denotes the fluorescence intensity resulting from the degradation of the substrate and is shown after normalization relative to the negative control. The recombinant rhomboids (Rrhom1 and Rrhom2) were used alone or embedded in the lipid bilayer (proteoliposome). Trypsin (0.1 unit/μl) was used as a positive control

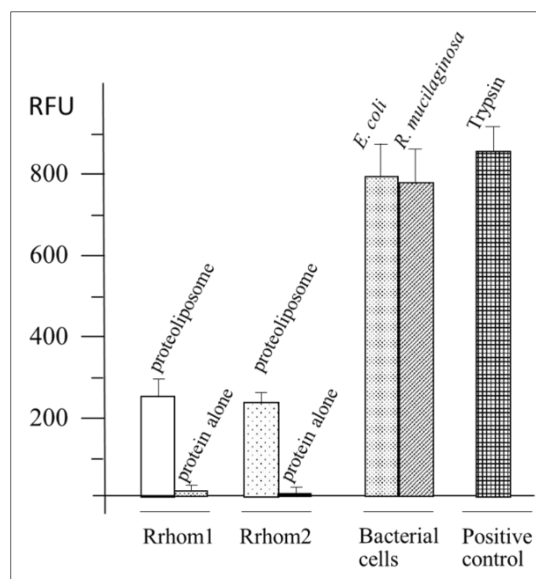

Figure 5 Protease activity of the recombinant proteins and sonicated bacterial cells at 4-h reaction time. The recombinant rhomboids (Rrhom1 and Rrhom2) were used alone or embedded in the lipid bilayer (proteoliposome). Trypsin (0.1 unit/μl) was used as a positive control. The recombinant protein alone showed almost no protease reactivity

Measurements were made in triplicate and performed twice, and the RFU of each reaction after 4 h are shown in Fig. 5. Rrhom1 and Rrhom2 in proteoliposome form showed almost identical levels of protease activity. The RFU of Rrhom1/liposome complex and that of the Rrhom2/liposome complex were 240.6 ± 45.5 and 233.3 ± 39.5,

respectively. The recombinant Rrhom1 and Rrhom2 proteins without liposomes showed almost no reactivity (4.4 ± 11.3 and 0.6 ± 12.2 RFU, respectively). *R. mucilaginosa* and *E. coli* cell components showed almost identical protease activity with RFU of 766.7 ± 92.6 and 799.6 ± 83.5, respectively, which were almost identical to the activity of trypsin at 0.1 unit/μl (845.5 ± 76.3 RFU).

3.4. Quantitative detection of rhomboid mRNA

The mRNA expression levels of *Rrhom1* and *Rrhom2* genes of *R. mucilaginosa* were quantified by real-time PCR (Fig. 6). Under culture conditions with type I collagen, *Rrhom1* and *Rrhom2* genes showed expression levels of 763.9 ± 300.1 and 763.1 ± 105.5 copies/50 ng cDNA, respectively, which were reduced to 594.7 ± 110.2 and 695.0 ± 94.0 copies/50 ng cDNA, respectively, under culture conditions without type I collagen. Although not statistically significant, the rhomboid mRNA levels were slightly higher under culture conditions with than without type I collagen (*t* test: $P = 0.25$ and 0.27 for *Rrhom1* and *Rrhom2*, respectively). The two genes showed similar levels of expression under the same culture conditions.

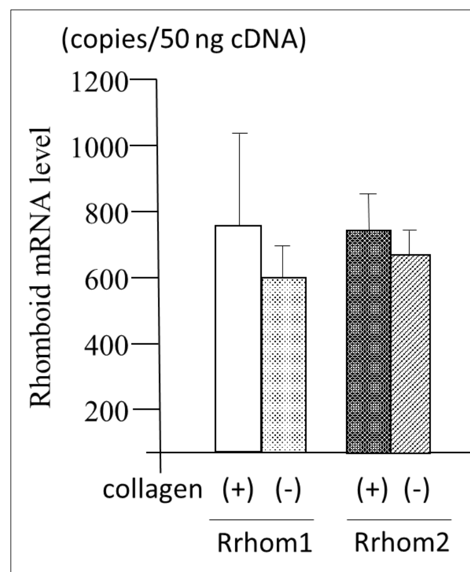

Figure 6 Rhomboid mRNA expression quantified by real-time RT-PCR. Total RNA was extracted from *R. mucilaginosa* cultivated with or without type I collagen and reverse transcribed. Although not statistically significant, the average rhomboids mRNA expression level was increased slightly by addition of type I collagen

4. Discussion

We identified two rhomboid genes in the *R. mucilaginosa* genome. The rhomboid family genes are widespread among both eukaryotes and prokaryotes. Many eukaryotes carry several copies of rhomboid family genes, usually 7–15 copies, while the genomes of prokaryotes contain several copies of one homologue [30]. The two rhomboids of *R. mucilaginosa* showed 77.8% identity at the amino acid sequence level, suggesting that they are homologues with similar functions. The two rhomboid genes of *R. mucilaginosa* were located in tandem with an intervening interval of 57 bp, strongly suggesting that the two genes constitute a bicistronic operon. The rhomboids of *Enterobacteriaceae*, including *E. coli*, are organized in an operon [31]. The *glpG* gene, a rhomboid gene of *E. coli*, is organized within the *glpEGR* operon [31]. The *glpE* gene encodes a transferase that transfers sulfate from thiosulfate to cyanine and glycerol [32]. It has also been reported that the *glpR* repressor regulates the *glp* regulon in the absence of glycerol [33]. In *Bacillus subtilis*, the rhomboid gene was shown to be organized in an operon with the glucokinase gene [34]. Distinct from these bacteria, two rhomboids are organized in an operon in *R. mucilaginosa*, and the results suggest the cooperative functions of the two rhomboids.

X-ray crystallography of *E. coli* GlpG has advanced our knowledge of the structural characteristics of rhomboids. The analysis revealed an aqueous channel below the membrane surface that is continuous with the active site [35]. The transmembrane structures of the two rhomboids of *R. mucilaginosa* were predicted using TMHMM [25]. Both rhomboids were shown to possess six transmembrane domains, and the deduced amino acid sequences were shown to include HxxxN, GxSG, and GxxxG motifs. Four different topological classes of rhomboid-like proteins have been reported,

with the basic class consisting of a six-transmembrane core [36]. GlpG of *E. coli* and some eukaryotic rhomboids, such as *Saccharomyces cerevisiae* Rbd2, belong to this class. The rhomboids of *R. mucilaginosa* were suggested to belong to the basic topological class. The conserved motif HxxxN in the second transmembrane domain and GxSG motif near the N-terminus of the 4th transmembrane domain have been reported to be essential elements of the active site of the enzyme [37]. The GxxxG motif in the 6th transmembrane domain plays an important role in tight packing between the 4th transmembrane domain and the 6th domain required for maintenance of its conformation [37]. The conserved motifs of *R. mucilaginosa* rhomboids probably play the same structural and enzymatic roles.

Although the rhomboid family proteins are universally present in all kingdoms of life, not all of them show enzymatic activity [36]. We constructed recombinant *R. mucilaginosa* rhomboids and confirmed that they showed enzymatic activity. As rhomboids are embedded in the membrane, the recombinant rhomboids/liposome complexes (proteoliposomes) were prepared in addition to the recombinant rhomboid proteins alone. Interestingly, the two *R. mucilaginosa* rhomboids showed serine protease activity in the proteoliposome form, but neither recombinant protein alone showed any activity. The two molecules showed almost the same protease activity. These observations showed that the two rhomboids of *R. mucilaginosa* are active enzymes with serine protease activity, and also suggested that the conformation of the rhomboids in the lipid bilayer is required for the protease activity. Rhomboid active sites are embedded in the lipid bilayer where they cleave substrates within the membrane. In addition to the protease activity within the membrane, Maegawa *et al*. [18] demonstrated that their intramembrane active site was accessible to water and hydrolyzed an extramembrane peptide bond of the substrate. The results of protease assay in the present study support this previous observation, and suggest that the rhomboids are accessible to substrates other than membrane proteins.

The results of real-time RT-PCR demonstrated that the two rhomboid genes of *R. mucilaginosa* were transcribed and probably expressed in the cells. The mRNA expression levels of the *Rrhom1* and *Rrhom2* genes were quantified separately, and shown to be similar for the two genes. The results suggested that the two genes constitute an operon. Type I collagen was added to the culture medium to mimic the infectious environment of dentin and other host tissues, and its influence on rhomboid gene expression was examined. Although not significant, the mRNA levels of the two rhomboids were increased slightly by addition of type I collagen. The expression of rhomboids may be enhanced in response to environmental conditions in the host, although the relation to virulence is not yet clear.

The substrates and roles of *R. mucilaginosa* rhomboids have not been elucidated. In eukaryotes, rhomboid family proteins have been implicated in a variety of processes, including epidermal growth factor signaling in *Drosophila melanogaster*, lipid metabolism, energy production, chloroplast development, apoptosis regulation, endoplasmic reticulum protein trafficking, etc. [18]. The relevance and function of rhomboid proteases in bacteria remain poorly investigated, with only a single substrate of a bacterial rhomboid reported to date. It has been reported that AarA, a rhomboid of *P. stuartii*, cleaved TatA, an essential component of the twin-arginine translocation system and was involved in quorum sensing [38]. Most studies of bacterial rhomboids involved the creation of rhomboid-null mutants, and prediction of functions. *E. coli glpG*-deficient mutants showed enhanced resistance to cefotaxime and reduced intestinal colonization in a murine model [39]. In *Mycobacteria*, rhomboid deletion mutants showed altered colony morphology, impaired biofilm formation, and increased antibiotic sensitivity [40]. Another recent study identified new roles of *Shigella sonnei* GlpG and Rhom7, which are involved in quality control of membrane orphan proteins of respiratory complexes [41]. Although the molecular mechanisms underlying these phenotypes are still unknown, the rhomboid family proteins in bacteria may have multiple functions, and may be involved in the pathogenesis of infectious diseases. The rhomboids of *R. mucilaginosa* identified in the present study may have similar functions to other bacterial rhomboids described in these previous reports.

5. Conclusion

We isolated genes encoding two rhomboids from *R. mucilaginosa*. The two rhomboids showed the typical conformation with six transmembrane domains and serine protease activity. These molecules may have important biological roles and potentially act as virulence factors.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare.

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