Studies on oral colonization of periodontopathogenic bacterium *Eikenella corrodens*

（歯周病原性細菌 *Eikenella corrodens* の口腔内定着に関する研究）

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2017

DEDICATED TO MY BELOVED PARENTS

CONTENTS

LIST OF ABBREVIATIONS

CE	Cell envelope
GalNAc	*N*-acetyl-$_D$-galactosamine
g	Gram
g/L	Gram/litre
HA	Hemagglutination
Δ*hlyA*	*hlyA*-deficient strain
H	hour
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
M	Molar
mM	Milimolar
min	Minute
mL	Mililitre
mg/mL	Milligram/mililitre
NaCl	Sodium chloride
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TSB	Tryptic soy broth

µL	Microlitre
µM	Micromolar
µF	Microfarad
µg/mL	Microgram/microlitre
%	Percentage
° C	Degree Celsius
Ω	Ohm

CHAPTER 1: GENERAL INTRODUCTION

A well-prepared army goes into battle with knowledge of the opposition, significant numbers of troops and excellent lines of communication. In some respects, bacterial pathogens are no different and in recent years it has become clear that bacterial cells are capable of exhibiting much more complex patterns of multicellular behavior than would perhaps be expected for simple unicellular micro-organisms. The ability of a single bacterial cell to communicate with its neighbors to mount a unified response that is advantageous to its survival in a hostile environment makes considerable sense. Such benefits may include improved access to complex nutrients or environmental niches, collective defense against other competitive micro-organisms or eukaryotic host defense mechanisms and optimization of population survival by differentiation into morphological forms better adapted to combating an environmental threat (Williams *et al.* 2000). There are approximately ten times as many bacterial cells in the human body as human cells (Cucchiara *et al.* 2009). However, most of the bacteria are harmless or beneficial to the host. For example, commensal bacteria provide infection resistance, increase the overall immunological capacity of the host and act as a colonization barrier towards pathogenic microorganism (Salminen *et al.* 1995; Tlaskalová-Hogenová *et al.* 2004; Liza *et al.* 2010)

Though, bacterial infections affect people worldwide, and they constitute major health problems with associated morbidity and mortality. Infectious diseases are among the top ten causes of death both in low income, middle income, and high income countries (WHO Fact sheet 2008; Kim and Amar 2006). Some common infectious diseases, caused by pathogenic bacteria, are peptic ulcers and gastritis caused by *Helicobacter pylori* (Aspholm-Hurtig *et al.* 2004), periodontitis caused by

Phorphyromonas gingivalis, *Aggregatibacter actinomycetemcomitans* and *Eikenella corrodens* (Asikainen *et al.* 2000; Hoyler *et al.* 2001) and dental caries associated with *Streptococci* and *Lactobacilli* (Marsh 1994).

Periodontal diseases include two general categories based on whether there is attachment or bone loss: gingivitis and periodontitis (Armitage *et al*. 2003). Gingivitis is induced by a bacterial plaque which forms on teeth at the gingival sulcus in the absence of effective oral hygiene **(**Page 1986**)**. The initial plaque accumulation is composed mainly of viridans streptococci and actinomyces (Loesche and Syed 1978; Moore *et al.* 1982). Subsequently, various gram-negative bacteria and spirochetes increase in number, and gingivitis develops. The continued presence of these bacteria and the associated gingivitis may lead to periodontitis, or loss of tooth attachment (Listgarten *et al.* 1986).

"Peri" means around, and "odont" refers to teeth. Periodontal diseases are infections of the structures around the teeth. These include the gums, the cementum that covers the root, the periodontal ligament and the alveolar bone. In the earliest stage of periodontal disease, gingivitis, the infection affects only the gums. In more severe forms of the disease, all of the supporting tissues are involved (Colgate, on line). Periodontal disease as a risk factor for the development of various systemic conditions, such as CVD, diabetes, adverse pregnancy outcomes, and osteoporosis, is a highly researched and debated topic **(**Kim and Amar 2006**)**.

In the past three decades, marked advances have occurred in our understanding of the infectious agents of periodontal disease. More than 700 different bacterial species are resident in the subgingival niche that are periodontopathogenic (Kuramitsu *et al.* 2007). The most frequently identified periodontal pathogens include three microaerophilic species (*Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*,

and *Eikenella corrodens*) and seven anaerobic species (*Porphyromonas gingivalis, Bacteroides forsythus, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Eubacterium*, and spirochetes). Socransky *et al.* (1998) divided the pathogens into two main clusters of microorganisms and deemed them the "red" and "orange" complexes. Furthermore, they defined "green", "yellow", and "purple" complexes as the bacterial colonies that formed on the tooth surface prior to the colonization of the "orange" and "red" complexes. The "red" complex consisted of three tightly related species: *T. forsythensis*, *P. gingivalis* and *T. denticola*. This complex is strongly related to pocket depth and bleeding on probing. Another complex ("orange" complex") included *F. nucleatum*/periodonticum subspecies, *P. intermedia*, *P. nigrescens*, *Peptostreptococcus micros*, *C. rectus*, *C. gracilis*, *C. showae*, *Eubacterium nodatum*, and *Streptococcus constellatus*, and seemed to precede colonization by species of the "red" complex. The "yellow" complex comprised six *Streptococcus* species: *Streptococcus sp.*, *S. sanguis*, *S. oralis*, *S. intermedius*, *S. gordonii*, and *S. mitis*, while *Capnocytophaga ochracea, Capnocytophaga gingivalis, Capnocytophaga sputigena, E. corrodens*, and *A. actinomycetemcomitans* serotype a made up the "green" complex. The fifth and final complex, the "purple" complex, consisted of *Veillonella parvula*, *Actinomyces odontolyticus*, *A. actinomycetemcomitans* serotype b, *Selenomonas noxia*, and *Actinomyces naeslundii* genospecies 2 (*Actinomyces viscosus*), but these did not constitute any cluster or ordination group.

Natural genomic transformation is present in a variety of bacterial organisms from different phyla and occupying various niches and although the mechanisms appear to be different between Gram-positive and Gram-negative organisms, the endpoint advantage is the same: to adapt to environmental stresses (Levine and Miller 1996;

Hofstad and Horn 1989) and by accelerating novel virulence traits (Marks *et al.* 2012). It is generally recognized that the colonization of bacteria on human mucosal tissues is an important step in the infectious process (Yamazaki *et al*. 1981). Bacterial pathogens have evolved a wide range of strategies to colonize and invade human body (Ribet and Cossart 2015). It has been reported that, Pneumococci lacking a gene (*pspC*) essential for colonization could repair its defect in the presence of a wild-type strain. In *Streptococcus pneumonia*, dual-strain carriage *in vivo* and biofilms formed *in vitro* can be transformed during colonization which can increase their pneumococcal fitness (Marks *et al.* 2012). Falush *et al.* provided direct evidence that, the panmictic population structure of *H. pylori* is caused by very frequent recombination during mixed colonization by unrelated strains resulting a highly flexible genome content and frequent shuffling of sequence polymorphisms throughout the local gene pool (Daniel *et al.* 2001). Previous studies of Skaar *et. al.* indicated that, inactivation and over expression of *irg* genes in the family of *Neisseria gonorrhoeae* have no role in pilin variation, DNA transformation or DNA repair (Eric *et al.* 2005). The results suggested that, colonization might have a correlation with the recombination of genomes. It has been reported that beta-hemolysin with sphingomyelinase activity from *S. aureus* damages keratinocytes, subsequently leading to colonization by microorganisms **(**Katayama *et al.* 2013**)**. It has also observed that colonization of *P. mirabilis* isolates recovered from the urine, kidney and bladder of CBA mice with UTI showed higher hemolytic activity (Mohr *et al.*1999). Therefore, hemolysin might have an effect on the colonization of several kinds of pathogenic microorganisms.

Eikenella corrodens is a member of the indigenous oral microbiota; however, it can also be an opportunistic pathogen. Under dysbiotic conditions, this bacterium can

be predominantly found in the subgingival plaque of patients with advanced periodontitis **(**Gully and Rogers 1995**;** Chen *et al.* 1996**)**, but has also been associated with a variety of non-oral human infections, like arthritis (Chang *et al.* 2005), brain abscess (Karunakaran *et al.* 2004), liver (Yamazaki *et al.* 1981) and placenta (Tønjum *et al.* 1985). Over the past 25 years, *E. corrodens* has increasingly been recognized for its pathogenic potential. It was first isolated by Eiken in 1958, it was originally named *Bacteroides corrodens* and classified as a corroding anaerobic bacterium (Eiken 1958).

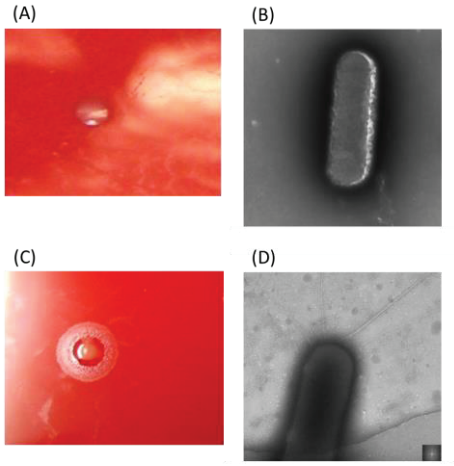

Fig. 1-1. Observation of *E. corrodens* strain 1073 (A and B) and strain ATCC23834 (C and D).
Colony morphologies were observed on sheep blood agar (A and C). Bacterial cells were observed by transmission electron microscope (C and D) . Strain 1073 forms corroding colonies, whereas strain ATCC23834 forms non-corroding colonies. The pilus structure was observed on the cell surface of strain ATCC 23834, whereas no pili was observed on that of strain 1073.

However, in1972, its current name was proposed when it was distinguished as a facultative anaerobe (Jackson and Goodman 1972; Hoyler *et al.* 2001). So far, its original description by Henriksen in 1948, but recently *Eikenella corrodens* has more gained recognition for its pathogenic potential in humans (Henriksen 1948; Brooks *et al.* 1974; Dorff *et al.* 1974). Since *E. corrodens* is detected in the plaque formed on teeth, it is thought to participate in the early stages of biofilm formation by specific co-aggregation with certain gram-positive and gram-negative bacteria present in human periodontal pockets. In addition, hemolysis is thought to be involved in many infections

caused by pathogenic bacteria by facilitating the growth of pathogenic bacteria due to ion acquisition from erythrocytes (Otto *et al.* 1992). But unfortunately, there have been few reports about the hemolysis of *E. corrodens*.

It has been reported that GalNAc-specific lectin (large molecule and is composed of several components including 25-, 45- and 300-kDa proteins) (Yumoto *et al.* 1996; Sporken *et al.* 1985) mediates the co-aggregation of *E. corrodens* with some strains of S*treptococcus sanguinis* and *Actinomyces viscosus*, which are predominant during the early stages of dental plaque formation and that it stimulates the mitogenic activity of B lymphocytes. Therefore, it can be said that the GalNAc-specific lectin contributes to the pathogenicity and virulence of *E. corrodens*, which can be estimated from hemagglutination (HA) activity (Azakami *et al.* 1996). Despite to the pathogenicity of *E. corrodens*, Tonjum *et al.* reported the transformation of *E. corrodens* with chromosomal DNA from an antibiotic resistant mutant of *E. corrodens* (Venkatarama *et al.* 1993). Based upon the results, *E. corrodens*, like *Neisseria* spp. have been characterized as natural competent for genetic transformation (Chan and McLaughlin 2000).

Rao and Progulske-Fox (1993) and Tønjum *et.al.* (1993) have been cloned 2 types IV pilin genes from *E. corrodens* 23834 and *E. corrodens* 31745. Previously, Azakami *et. al.* isolated a plasmid DNA (8.7 kb) from *E. corrodens* 1073 and designed as pMU1(Azakami *et al.* 2005). They found 7 open reading frames (ORFs) on pMU1 and an ORF4 which is homologous to the recombinase specific to the type IV pilin gene. *E. corrodens*1073 forms large non-corroding colonies on the solid media, whereas other strains of *E. corrodens* form small corroding colonies. Transformation of ORF4 gene using a shuttle vector into *E. corrodens* strain 23834 resulted in loss of pilus structure and the morphology of the transformants changed to corroding colonies from

non-corroding. Furthermore, the introduction of ORF4 into strain 23834 also resulted in the elevation of growth rate, hemolytic activity, biofilm formation etc (Azakami *et al.* 2006a). Therefore, it is considered that ORF4-induced genomic recombination may enhance the colonization of *E. corrodens* to the host cells.

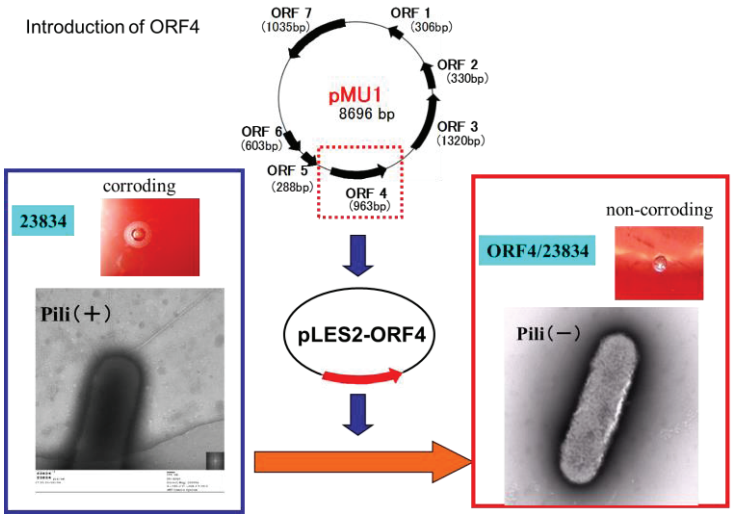

Fig. 1-2. Effect of the introduction of ORF4 into *E. corrodens* ATCC 23834 on its colony morphology and pilus structure. Introduction of ORF4 into strain 23834 resulted in loss of pilus structure and the morphology of the transformants changed to corroding colonies from non-corroding. Furthermore, the introduction of ORF4 into strain 23834 also resulted in the elevation of growth rate, hemolytic activity, biofilm formation.

In this study, I investigated the hemolysin and genomic recombination to clarify their relationships to the colonization of *E. corrodens* in the oral cavity. Therefore, the objective of this study is summarized as follows:

1. To analyze the relationship between pathogenicity and hemolysis of *E. corrodens*, I purified and identified the hemolysin from the periodontopathogenic bacterium *E. corrodens* 1073.
2. To investigate the effect of genomic recombination on the virulence factor of *E. corrodens*, I transformed specific gene from *E. corrodens* 1073 into some other strains of *E. corrodens* and observed the effect on the pathogenicity of various *E. corrodens* strains.

CHAPTER 2

Purification and Characterization of Hemolysin in the Periodontopathogenic Bacterium *Eikenella corrodens* Strain 1073

2.1 ABSTRACT

Eikenella corrodens 1073 was found to show hemolytic activity when grown on sheep blood agar. A high and dose-dependent hemolytic activity was detected in the cell envelope fraction, which was further purified by ion-exchange and gel-filtration chromatography. Consequently, a 65-kDa protein with hemolytic activity was obtained, suggesting that this protein might be a hemolysin. Its N-terminal amino acid sequence was nearly identical to that of X-prolyl aminopeptidase from *E. corrodens* ATCC 23834. To confirm that X-prolyl aminopeptidase functions as a hemolytic factor, I expressed the *hlyA* gene, encoding X-prolyl aminopeptidase, in *Escherichia coli*. After induction with isopropyl β-D-1-thiogalactopyranoside, a protein of about 65 kDa was purified on a Ni column, and its hemolytic activity was confirmed. Meanwhile, a strain with a disrupted *hlyA* gene, which was constructed by homologous recombination, did not show any hemolytic activity. These results suggested that X-prolyl aminopeptidase might function as a hemolysin in *E. corrodens*.

2.2 INTRODUCTION

Eikenella corrodens, a gram-negative, facultative anaerobic, rod-shaped bacterium, is predominantly found in subgingival plaque samples from patients with advanced periodontitis (Tanner *et al*. 1979). Monoinfection of germ-free or gnotobiotic rats with *E. corrodens* causes periodontal disease with severe alveolar bone loss (Listgrarten *et al.* 1978). Previously it has been found that *E. corrodens* 1073 contains a cell-associated, *N*-acetyl-D-galactosamine (GalNAc)-specific, lectin-like substance that mediates its adherence to cell surfaces of various host tissues (Ebisu *et al.* 1983; Yamazaki *et al.* 1981; Yamazaki *et al.* 1988; Miki *et al.* 1987). In addition, it has already been reported that the GalNAc-specific lectin mediates the coaggregation of *E. corrodens* with some strains of *Streptococcus sanguinis* and *Actinomyces viscosus* (Ebisu *et al.* 1988), which are predominant during early stages of dental plaque formation, and stimulates the mitogenic activity of B lymphocytes (Nakae *et al.* 1994). Furthermore, *E. corrodens* 1073 induces KB cells (a human oral epidermoid carcinoma cell line) to express and secrete interleukin (IL)-6 and IL-8, for which no direct contact of *E. corrodens* with KB cells is necessarily required is also reported (Yumoto *et al.* 2001). Therefore, it is believed that the GalNAc-specific lectin contributes to the pathogenicity and virulence of *E. corrodens*, and it can be evaluated by hemagglutination (HA) activity.

Previously, a plasmid of 8.7 kb in size in strain 1073 (Azakami *et al.* 2005) was found and investigated its relationship to pathogenicity. As a result, it has been suggested that the type IV pilin gene locus is recombined by a recombinase, which is encoded by this plasmid (Azakami *et al.* 2006a). Moreover, this genomic recombination enhances the GalNAc-dependent lectin activity in parallel with the hemolytic activity of *E. corrodens* ATCC 23834 was also reported (Azakami *et al.* 2006a; Matsunaga *et al.*

2011). However, the relationship between the GalNAc-dependent lectin activity and hemolytic activity of *E. corrodens* has not been understood yet.

In a healthy human body, the concentration of free iron should be maintained at 10^{-18} M (Bullen 1981). However, bacteria require iron concentrations of 0.05–0.5 μM for their growth (Martinez *et al.* 1990). It has been reported that the acquisition of iron for bacterial metabolism can be facilitated by hemolysis of host erythrocytes, which can release intracellular iron (Otto *et al.* 1992). Moreover, it has been reported that the crevicular fluid collected from periodontitis sites had a higher iron concentration compared to that collected from gingivitis sites (Mukherjee 1985). Furthermore, most hemolysins damage host tissue, causing lysis of erythrocytes to release hemoglobin by forming pores of varying diameters in the membrane (Goebel *et al.* 1988). Therefore, hemolysin is regarded as a virulence factor for many gram-positive and gram-negative pathogenic bacteria and might be vital for the *in vivo* survival of oral bacteria (Sato *et al.* 2012). Hemolysin is thought to be involved in many infections caused by pathogenic bacteria since it induces bone loss owing to its cytotoxic effect and facilitates the growth of pathogenic bacteria owing to iron acquisition from erythrocytes (Chan and McLaughlin 2000). It has been reported that as extracellular toxic proteins, hemolysins are produced by many gram-negative (e.g., *Escherichia coli*, *Serratia* spp., *Proteus* spp., *Vibrio* spp., *Pasteurella* spp., *Pseudomonas aeruginosa*) and gram-positive bacteria (e.g., *Streptococcus* spp., *Staphylococcus aureus*, *Listeria* spp., *Bacillus cereus*, *Clostridium tetani*), all of which possess a certain pathogenic potential (Al-Shammary *et al.* 2012). However, there have been few reports about hemolysin production by *E. corrodens*.

In this study, I purified and characterized a hemolytic factor from the periodontopathogenic bacterium *E. corrodens* 1073 to reveal the relationship between hemolysis and virulence.

2.3 MATERIALS AND METHODS

Bacterial strains and growth conditions:

E. corrodens 1073 was provided by Dr. S. S. Socransky (Forsyth Dental Center, Boston, MA, USA), and *E. corrodens* ATCC 23834 was obtained from the American Type Culture Collection (Rockville, MD, USA). I constructed *E. corrodens* 23834 (pMU4) in a previous study (Azakami *et al.*2005) *E. corrodens* cells were grown at 37 °C either in tryptic soy broth (TSB) containing 2 $mg \cdot mL^{-1}$ KNO_3 and 5 $\mu g \cdot mL^{-1}$ hemin or on sheep blood agar plates. Recombinant bacteria were cultured in TSB medium containing 30 $\mu L \cdot mL^{-1}$ kanamycin, tetracycline, or fusaric acid.

The *E. coli* cloning hosts, DH5α and BL21 (DE3), were grown at 37 °C with aeration in Luria–Bertani (LB) medium (10 $g \cdot L^{-1}$ tryptone, 5 $g \cdot L^{-1}$ yeast extract, and 5 $g \cdot L^{-1}$ NaCl). When necessary, LB medium was supplemented with carbenicillin (100 $\mu g \cdot mL^{-1}$) for plasmid selection and maintenance.

Preparation of a supernatant fraction of bacterial culture:

Cells in the late logarithmic phase of growth were harvested by centrifugation (8,000 × *g*, 10 min, 4 °C). The supernatant was sterile-filtered, dialyzed against 20 mM Tris–HCl buffer, and then lyophilized. After dissolving the pellet in 20 mM Tris–HCl buffer, the solution was used as a culture supernatant fraction for further investigation.

Preparation of a bacterial cell envelope fraction:

Cells in the late logarithmic phase of growth (3.6 L) were harvested by centrifugation (8,000 × *g*, 10 min, 4 °C), then resuspended in 20 mL of 20 mM Tris–HCl, pH 7.6, containing 0.1 mM phenylmethylsulfonyl fluoride, and sonicated for

30 min. After centrifugation (450 × *g*, 20 min, 4 °C) to remove intact cells, the supernatant was centrifuged at 10,000 × *g* for 40 min at 4 °C. The pellet was designated as a cell envelope (CE) fraction and used for further purification.

Purification of hemolysin:

1 g of the CE fraction was resuspended in 10 mL of 20 mM Tris–HCl, pH 7.6, containing 13.5 mM *n*-octyl-β-D-thioglucoside and then stirred overnight at 4 °C. After centrifugation at 40,000 × *g* for 60 min at 4 °C, the supernatant was dialyzed to remove the detergent and then diluted 30-fold with 20 mM Tris–HCl buffer (pH 7.6). Then, the solution was loaded onto a DEAE–Toyopearl column (Toyopearl® DEAE-650M, Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris–HCl, pH 7.6, and the column was washed with the same buffer until the wash solution was free of protein. The bound proteins were eluted with a linear gradient of 0–0.15 M NaCl at a flow rate of 0.3 $mL \cdot min^{-1}$. The protein content in each fraction was determined by measuring the absorbance at 280 nm. Each peak was collected and dialyzed against distilled water. After lyophilization and dissolution in 20 mM Tris–HCl buffer (pH 7.6), hemolytic activity of the fractions was measured. Fractions that showed hemolytic activity were loaded onto a Q-Sepharose column (Q Sepharose™ Fast Flow, GE Healthcare Life Sciences, Piscataway, NJ, USA) equilibrated with the same buffer and eluted with a linear gradient of 0–0.3 M NaCl at a flow rate of 0.3 $mL \cdot min^{-1}$. The collected fractions were tested for hemolytic activity after dialysis. Fractions that showed hemolytic activity were dissolved in 20 mM Tris–HCl buffer (pH 7.6), loaded onto a Sephadex G-75 gel filtration column (GE Healthcare Life Sciences) equilibrated with the same

buffer, and eluted with the same buffer at a flow rate of 0.3 $mL \cdot min^{-1}$. The fractions were tested for hemolytic activity after dialysis.

Amino acid sequencing:

After the transfer onto a polyvinylidene difluoride membrane, proteins were stained with Ponceau-S. The target protein was cut out from the membrane and applied to an automated protein sequencer (Shimadzu PPSQ-21A, Kyoto, Japan). The obtained sequence was analyzed using the BLAST program.

Hemolytic assay:

Samples (330 μL) were mixed with 560 μL of saline, 10 μL of 1 mM $CaCl_2$, and 100 μL of rabbit blood, which was pre-washed three times with saline, and incubated at 37 °C for 24 h. Blood cells incubated with distilled water served as a positive control, and those incubated with saline served as a negative control. Following centrifugation (4,000 × *g*, 15 min, 4 °C), the optical density of the supernatant was measured at 540 nm in a spectrophotometer, and the hemolytic activity was expressed as a percentage of the positive control.

Hemolysis was also evaluated by the observation of colonies on sheep blood agar plate. *E. corrodens* strains were grown on the blood agar plates at 37 °C until colonies formed. Then, I moved the plates into refrigerator and observed clear zone of hemolysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (Laemmli 1970) using a 10% or 15%

acrylamide separating gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 to visualize protein bands.

Cloning and sequencing of the gene encoding hemolysin:

Primers A (GAGTAAGATGAAGTGTGAAC) and B (AGGCTACCTGAAATT TCAGG) were designed based on the nucleotide sequence of the gene (*hlyA*) encoding X-prolyl aminopeptidase from *E. corrodens* ATCC 23834 (GenBank accession no. NZ_EQ973321). Using these primers, polymerase chain reaction (PCR) was performed with genomic DNA of *E. corrodens* 1073 as a template. The amplified gene was ligated into the pGEM-T vector (Promega, Madison, WI, USA) and then introduced into cells of *E. coli* DH5α by electroporation. DNA was sequenced by dideoxy chain termination using a BigDye® Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, CA, USA) and an ABI 3130 DNA sequencer (Thermo Fisher Scientific). The sequence of the *hlyA* gene from *E. corrodens* 1073 was deposited into the DDBJ/EMBL/GenBank databases under accession number LC208747.

Heterologous expression of hemolysin in *E. coli*:

The pET22b vector (Thermo Fisher Scientific) was used to construct a plasmid expressing the recombinant hemolysin protein with a 6 × His-tag sequence at its C-terminus. The hybrid plasmid was used to transform *E. coli* strain BL21 (DE3) (Thermo Fisher Scientific), which allows isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced expression of T7 RNA polymerase. To confirm overexpression of the target protein, 5-mL cultures were grown in an air shaker (250 rpm) at 37 °C in LB medium containing 100 $\mu g \cdot mL^{-1}$ calbenicillin. At an optical density of 0.5 at 600 nm,

IPTG was added to a final concentration of 1 mM to induce the T7 RNA polymerase-based protein expression. Finally, expression of the target protein was confirmed by SDS–PAGE. The expressed His-tagged protein was purified using a His60 Ni gravity column (Takara Bio, Kusatsu, Japan).

Construction of a hemolysin-deficient strain:

To construct a hemolytic factor-deficient strain, a kanamycin resistance gene was inserted into the *hlyA* gene, and this fragment was cloned into pBR322 carrying a tetracycline resistance gene. The resultant plasmid was introduced into *E. corrodens* 1073 by electroporation. Tetracycline-sensitive colonies were counterselected with fusaric acid, while kanamycin- and fusaric acid-resistant colonies were selected. Construction of the *hlyA*-deficient strain was confirmed using primers C (ATGAGTAATATTCACGCGCAACGTCTCGCC) and D (CATGGTAGCCATTGA GCCAAGCGGCTTCTT), which can amplify the *hlyA* gene, as well as primers E (CAACAAAGCCACGTTGTGTC) and F (AGCTGGCGTAATAGCGAAGA), which can amplify the kanamycin resistance gene.

Statistical analysis:

Results are shown as the mean values and standard deviation from triplicate measurements. The significance of intergroup differences was analyzed using the unpaired Student's *t*-test.

2.4 RESULTS AND DISCUSSION

Localization of the hemolytic factor in *E. corrodens* 1073:

Previously, I have been observed β-hemolysis surrounding colonies of *E. corrodens* 1073 on sheep blood agar media. Thus, I expected that the hemolytic factor(s) might be located on the bacterial cell surface or secreted into the extracellular space. To determine the location of the hemolytic factor(s) in *E. corrodens* 1073 cells, a bacterial culture was fractionated into two fractions (culture supernatant and CE fraction) as described in Materials and Methods, and a hemolytic assay was performed using each fraction. High hemolytic activity was detected in the CE fraction, whereas no activity was detected in the culture supernatant fraction (Fig. 2-1A). It was suggested that the hemolytic factor(s) might be located on the surface of bacterial cells. To further confirm the location of the hemolysin, the hemolytic assay was performed using different concentrations of the CE fraction. The hemolytic activity was increased with an increase of the CE concentration in a dose dependent manner (Fig. 2-1B). This result confirmed that the hemolytic factor is associated with the CE fraction of *E. corrodens*

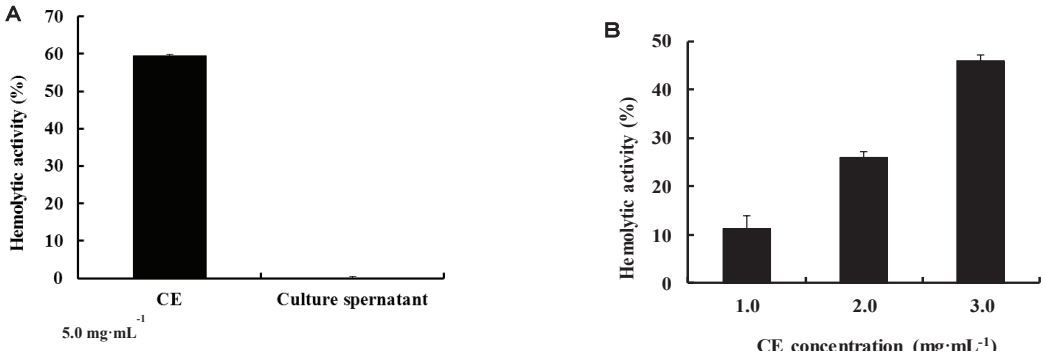

Fig. 2-1. Localization of the hemolytic factor in *E. corrodens* 1073.
Notes: (A) Bacterial culture of *E. corrodens* 1073 was fractionated into two fractions, a culture supernatant fraction and cell envelope (CE) fraction. Hemolytic activity of each fraction was assayed. (B) Hemolytic activity of the CE fraction was assayed at different concentrations. The assays were repeated at least three times. Mean values and standard deviations are indicated.

1073. Allaker *et al.* (1994) have reported that hemolytic activity was observed from culture supernatants of some clinical isolates of *E. corrodens.* However, we could not

observe any hemolytic activity from culture supernatant in this study. It may depend on the difference of assay conditions or the difference of used strains.

Purification of hemolysin from CE fraction:

To identify the protein(s) responsible for the hemolytic activity of *E. corrodens* 1073, purification of the hemolysin was performed from the CE fraction. After solubilization of CE with *n*-octyl-β-D-thioglucoside, the solubilized proteins were passed through a DEAE–Toyopearl column. After elution with a linear gradient of 0–0.15 M NaCl, an elution pattern shown in Fig. 2-2A was observed. We measured the

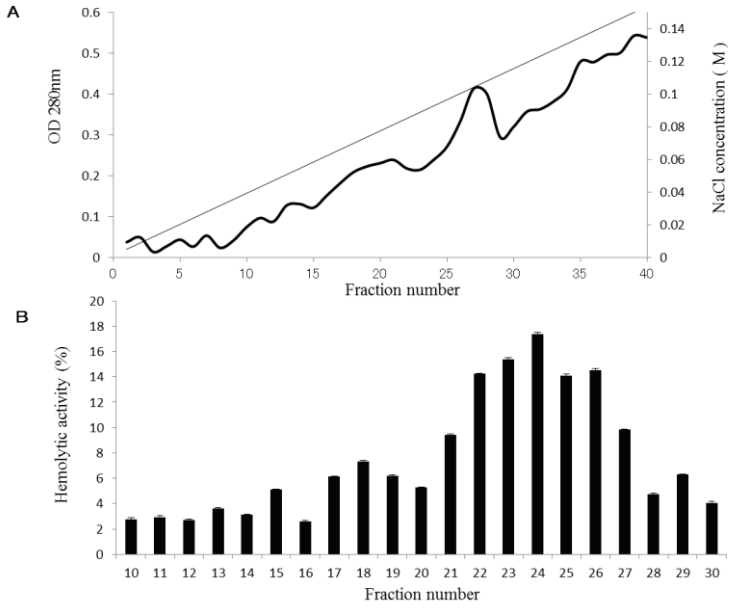

Fig. 2-2. Purification of the hemolytic factor by ion-exchange chromatography using a DEAE–Toyopearl column.
Notes: (A) After solubilization with a detergent, the CE fraction was passed through a DEAE–Toyopearl ion-exchange column. The bound proteins were eluted with a linear gradient of 0–0.15 M NaCl at a flow rate of 0.3 mL·min^{-1}. The protein content in each fraction was determined by measuring the absorbance at 280 nm. (B) Hemolytic activity of each eluted fraction was assayed. The assays were repeated at least three times. Mean values and standard deviations are indicated.

hemolytic activity of each fraction. Compared to other fractions, fractions 22–26 showed higher hemolytic activity (Fig. 2-2B). Thus, it was suggested that these fractions contained the hemolytic factor.

For further purification, these fractions were mixed together and passed through a Q-Sepharose fast-flow column. After elution with a linear gradient of 0–0.3 M NaCl, an elution pattern shown in Fig. 2-3A was observed. I measured the hemolytic activity of each fraction. As shown in Fig. 2-3B, fractions 32–38 showed higher hemolytic activity compared with that in other samples, suggesting that these fractions contained the hemolysin.

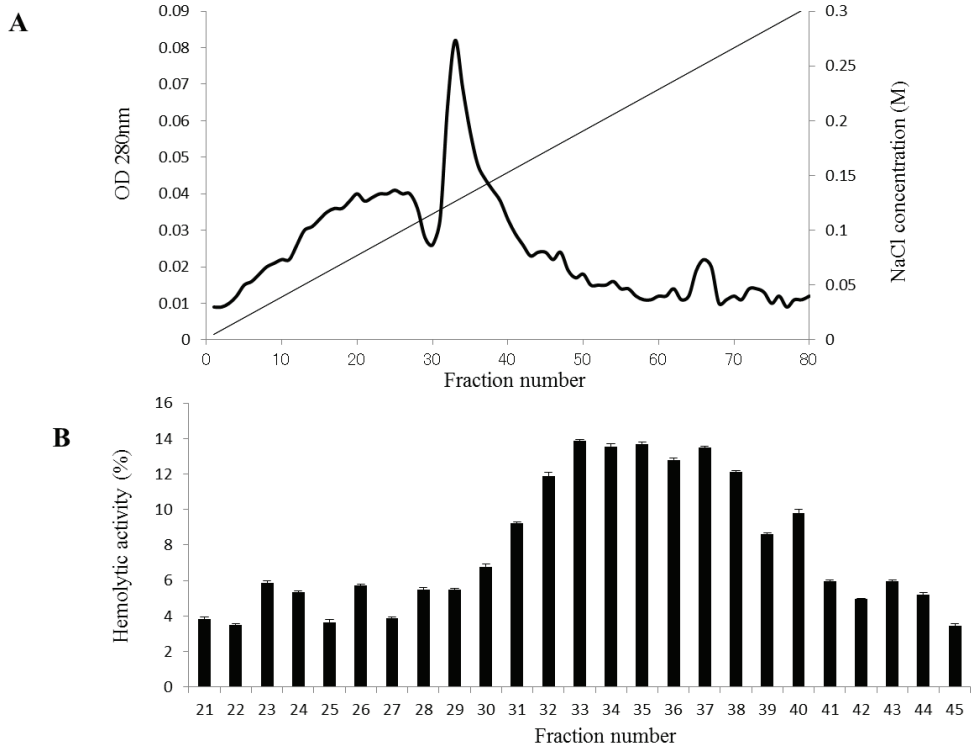

Fig. 2-3. Purification of the hemolytic factor by ion-exchange chromatography on a Q-Sepharose column.
Notes: (A) A fraction with a high hemolytic activity eluted after the DEAE ion-exchange chromatography was collected and passed through a Q-Sepharose$_1$ion-exchange column. The bound proteins were eluted with a linear gradient of 0–0.3 M NaCl at a flow rate of 0.3 mL·min . The protein content in each fraction was determined by measuring the absorbance at 280 nm. (B) Hemolytic activity of each eluted fraction was assayed. The assays were repeated at least three times. Mean values and standard deviations are indicated.

For further purification, these fractions were mixed together and separated by gel-filtration chromatography (Sephadex G-75) at a flow rate of 0.3 mL·min^{-1} (Fig. 2-4A). Hemolytic activity of each fraction was measured, and a high hemolytic activity was detected in fractions 43–46 (Fig. 2-4B).

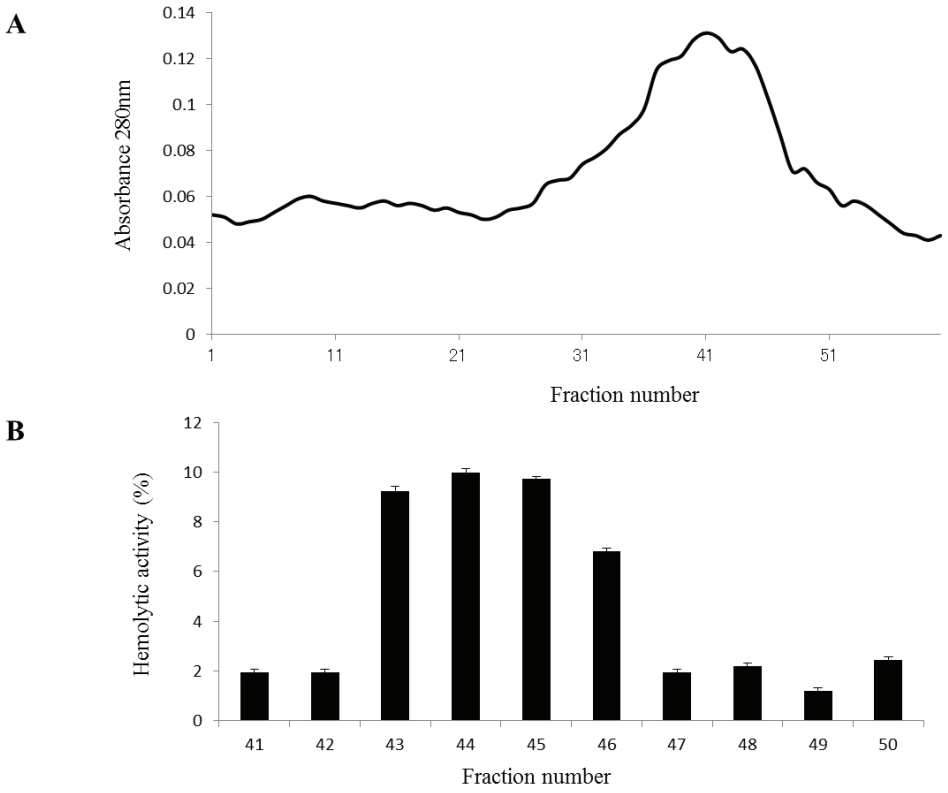

Fig. 2-4. Purification of the hemolytic factor by gel-filtration chromatography using a Sephadex G-75 column.

(A) A fraction with a high hemolytic activity eluted after the Q-Sepharose ion-exchange chromatography was collected and passed through a Sephadex G-75 column. The proteins were eluted with 20 mM Tris–HCl buffer (pH 7.6) at a flow rate of 0.3 $mL \cdot min^{-1}$. The protein content in each fraction was determined by measuring the absorbance at 280 nm. (B) Hemolytic activity of each eluted fraction was assayed. The assays were repeated at least three times. Mean values and standard deviations are indicated.

These samples were analyzed by 15% SDS–PAGE. As shown in Fig. 2-5, a single protein band was observed, with a molecular weight of about 65 kDa. Thus, it was suggested that the 65-kDa protein might be the hemolysin of *E. corrodens* 1073.

Identification of hemolysin protein:

Since it was suspected that the 65-kDa protein was the hemolysin, I determined its N-terminal amino acid sequence. The sequence (Ser–Asn–Ile–His–Ala–Gln–Leu–Leu–Ala–Ala) was nearly identical to that of X-prolyl aminopeptidase from *E. corrodens* ATCC 23834, except a substitution of leucine for arginine 7. Thus, it was

suggested that the hemolytic factor of *E. corrodens* 1073 might be X-prolyl aminopeptidase. As described later, I cloned and sequenced the gene encoding the X-prolyl aminopeptidase of *E. corrodens* 1073, which revealed that the substitution of leucine for arginine 7 was due to a misreading of the amino acid sequence.

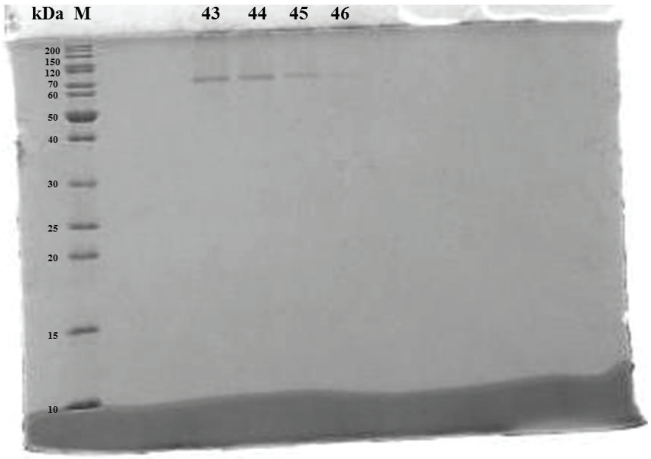

Fig. 2-5. SDS–PAGE analysis of the purified hemolytic factor.
Notes: After gel-filtration chromatography, proteins in fractions 43 to 46 were analyzed by SDS–PAGE. M, protein molecular marker, 10–200 kDa.

Heterologous expression of the hemolysin in *E. coli*:

I hypothesized that X-prolyl aminopeptidase might function as a hemolysin. To confirm this, the gene encoding X-prolyl aminopeptidase was cloned from *E. corrodens* 1073. Based on the nucleotide sequence of the gene encoding X-prolyl aminopeptidase from *E. corrodens* ATCC 23834, we designed primers A and B. Using these primers, the X-prolyl aminopeptidase gene, designated as *hlyA*, was amplified by PCR from the genomic DNA of *E. corrodens* 1073 (Fig. 2-6A). According to DNA sequencing of the *hlyA* gene, we found that amino acid sequence of HlyA of strain 1073 had 93.3% identity and 99.0% similarity to that of X-prolyl aminopeptidase of strain ATCC 23834.

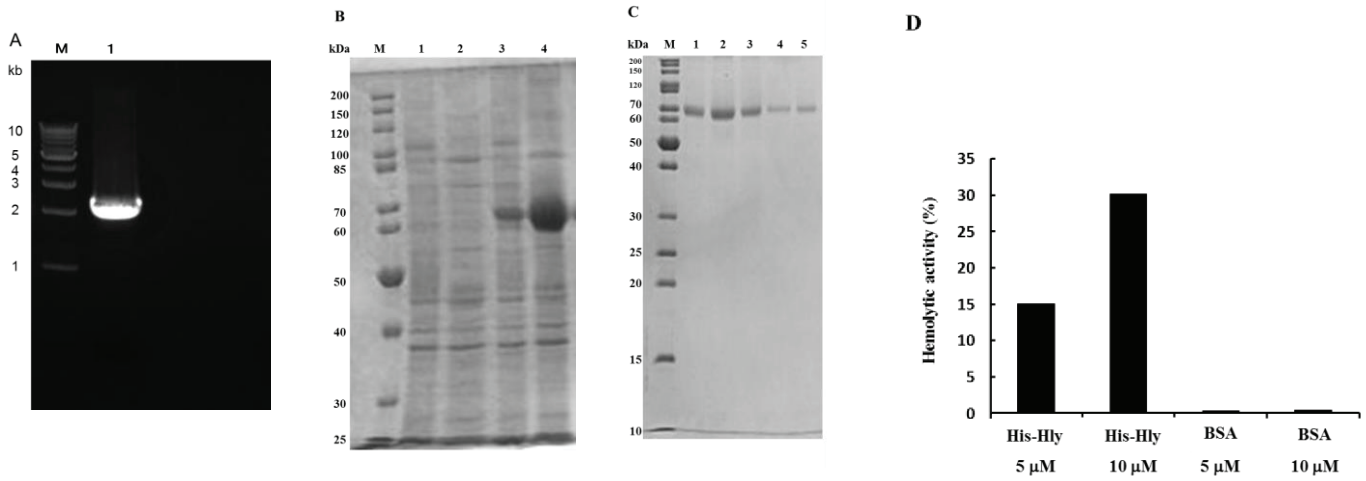

Fig. 2-6. Heterologous expression of the hemolysin in *E. coli.*
Notes: (A) The *hlyA* gene of *E. corrodens* 1073 was amplified by PCR using primers specific for the *hlyA* gene of *E. corrodens* ATCC 23834 and a band of about 2.3 kb was observed. (B) The pET22b vector was used to construct a plasmid expressing the recombinant HlyA protein with a C-terminal 6 × His-tag sequence. Overexpression of the HlyA protein was induced in *E. coli* BL21 (DE3) with IPTG. Expression of recombinant HlyA was analyzed by 10% SDS–PAGE. M, protein molecular marker; 1, crude extract of *E. coli* BL21 (DE3) (pET22b) without IPTG induction; 2, crude extract of *E. coli* BL21 (DE3) (pET22b) after induction with IPTG; 3, crude extract of *E. coli* BL21 (DE3) (pET22b/*hlyA*) without IPTG induction; 4, crude extract of *E. coli* BL21 (DE3) (pET22b/*hlyA*) after induction with IPTG. (C) After induction with IPTG, the overexpressed protein was purified by affinity chromatography using a His60 Ni gravity column. After binding of the crude extract of *E. coli* BL21 (DE3) (pET22b/*hlyA*), obtained after induction with IPTG, the target protein was eluted with 2 mL of an imidazole solution five times. Fractions 1 to 5, respectively, eluted with imidazole, were analyzed by SDS–PAGE. (D) Hemolytic activity of purified recombinant HlyA (5 and 10 μM) was assayed. Bovine serum albumin (BSA; 5 and 10 μM) was used as a negative control. The assays were repeated at least three times. Mean values and standard deviations are indicated.

(Fig. 2-7), only strain 1073 showed hemolytic activity. It was suggested that some substitution of the coding region of X-prolyl aminopeptidase might result in the difference of hemolytic activity between 2 strains. Alternatively, it was suggested that the mutation of its promoter region might result in the difference of expression of X-prolyl aminopeptidase.

The *hlyA* gene was ligated into the pET22b vector. The resultant plasmid was introduced into *E. coli* BL21 (DE3). After induction with IPTG, overexpression of a protein about 65 kDa was observed by SDS–PAGE (Fig. 2-6B). This protein was purified by affinity chromatography using a His60 Ni gravity column. The purified 65-kDa protein was observed as a single band in a fraction eluted with imidazole (Fig. 2-6C). The purified recombinant HlyA protein showed hemolytic activity, whereas no

activity was detected when using bovine serum albumin as a negative control (Fig. 2-6D). Moreover, the purified HlyA showed hemolytic activity in a dose dependent manner. Based on the data, it was suggested that the HlyA protein functions as a hemolytic factor.

Fig. 2-7. Amino acid homology of X-prolyl aminopeptidases from *E. corrodens* 1073 and ATCC23834.
Notes: Amino acid sequences of X-prolyl aminopeptidases from *E. corrodens* 1073 and ATCC23834 were aligned. | and . show identical amino acid and similar amino acid, respectively.

Δ*hlyA* mutation decreases hemolytic activity of *E. corrodens* 1073:

To further confirm HlyA involvement in hemolytic activity, a *hlyA*-deficient strain (Δ*hlyA*) was constructed. After selection of kanamycin- and fusaric acid-resistant colonies, disruption of the *hlyA* gene was confirmed by PCR (Fig. 2-8A). As shown in Fig. 2-8B, the wild-type strain of *E. corrodens* 1073 showed β-hemolysis around its colony on a sheep blood agar plate. However, the Δ*hlyA* strain showed no hemolysis (Fig. 2-8C) on a blood agar plate. Moreover, I measured hemolytic activities of both wild-type and Δ*hlyA* strains (Fig. 2-8D) and showed that the hemolytic activity of the Δ*hlyA* strain was lower compared with that of the wild-type strain. These results

suggested that X-prolyl aminopeptidase might function as a hemolytic factor in *E. corrodens* 1073.

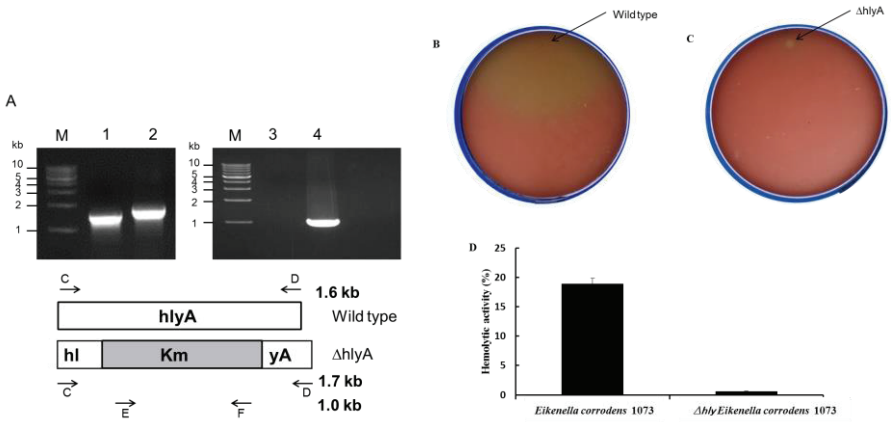

Fig. 2-8. Disruption of the *hlyA* gene abolished the hemolytic activity of *E. corrodens* 1073.
(A) Construction of a *DhlyA* strain of *E. corrodens* 1073. After selection of kanamycin- and fusaric acid-resistant colonies, disruption of the *hlyA* gene was confirmed by PCR. 1, PCR of genomic DNA of the wild-type strain with primers that can amplify the *hlyA* gene; 2, PCR of genomic DNA of the *DhlyA* strain with primers that can amplify the *hlyA* gene; 3, PCR of genomic DNA of the wild-type strain with primers that can amplify the kanamycin resistance gene; 4, PCR of genomic DNA of the *DhlyA* strain with primers that can amplify the kanamycin resistance gene. (B) (C) Hemolysis by the wild-type strain and the *DhlyA* strain of *E. corrodens* 1073. Colonies are shown by arrows. (D) Hemolytic activity of the wild-type and *DhlyA* strains of *E. corrodens* 1073 was assayed. The assays were repeated at least three times. Mean values and standard deviations are indicated.

So far, it has been reported that various bacteria produce hemolytic factors such as a pore-forming toxin (Los FCO *et al.* 2013), sphingomyelinase, and phospholipase (Flores-Diaz *et al.* 2016). The pore-forming toxin disrupts host cells by forming pores in the cell membrane. Sphingomyelinase and phospholipase disrupt host cells by enzymatically hydrolyzing the lipid bilayer of the cellular membrane. It has also been reported that some proteins containing an α-helix structure function as hemolysins by sticking out of the cellular membrane. Although there has been no report showing that any aminopeptidase acts as a hemolytic factor, it is suggested that the X-prolyl aminopeptidase from *E. corrodens* 1073 might contain an α-helix structure that can function as a hemolysin. I analyzed the amino acid sequence of X-prolyl aminopeptidase using secondary structure prediction software by Chou and Fasman (1974) and found an α-helix structure is located at its N-terminus. However, a further

study is necessary, since it is unknown whether X-prolyl aminopeptidase activity is required for hemolytic activity or a part of this protein is required.

Allaker *et al.* (1994) have reported that many strains of *E. corrodens* produce high levels of proline aminopeptidase and thiol-dependent hemolysin. However, they have not identified the proteins responsible for these activities and have not clarified the relationship between proline aminopeptidase and hemolysin in *E. corrodens*. It suggests that the hemolysin found in this study was different from other reported proteins. Furtheremore, they suggested that reduced conditions are necessary for *E. corrodens* hemolytic activity. In this study, *E. corrodens* strains were grown in aerobic condition without shaking for aeration. In our assay, I added the calcium chloride into reaction mixture, because little activity was detected in the absence of calcium chloride in our early study. It was suggested that the reducing power of calcium ion might affect hemolytic activity of *E. corrodens*.

Relationship between GalNAc-dependent lectin activity and hemolytic activity:

Previously, we have reported that genomic recombination by a plasmid-mediated recombinase enhances the GalNAc-dependent lectin activity in parallel with the hemolytic activity in *E. corrodens* ATCC 23834 (Azakami *et al* 2006a; Matsunaga *et al.* 2011). These data suggested that the hemolytic activity is correlated with the lectin activity. To elucidate this, I measured the hemolytic activity of recombinant HlyA in the absence and presence of 3 mM GalNAc. As shown in Fig. 2-9A, the hemolytic activity was decreased by the addition of GalNAc. Since it has been reported that the lectin activity of *E. corrodens* 1073 was inhibited by GalNAc (Azakami *et al.* 2006b), this result suggested that the decrease in hemolytic activity might be due to the inhibition of

lectin activity. However, the lectin activity was detected in the Δ*hlyA* strain at the same level as in the wild-type strain (Fig. 2-9B). These results suggested that hemolysis by HlyA might require lectin activity by which bacterial cells can approach blood cells. It is suggested that X-prolyl aminopeptidase might contribute to the pathogenicitiy of *E. corrodens*.

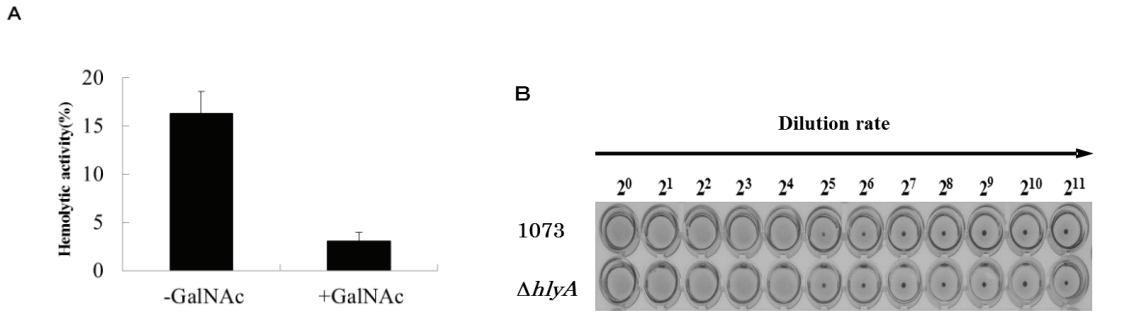

Fig. 2-9. GalNAc inhibits hemolytic activity of *E. corrodens* 1073.
Notes: (A) Hemolytic activity of purified recombinant HlyA (5 mM) was assayed in the absence and presence of 3 mM GalNAc. The assays were repeated at least three times. Mean values and standard deviations are indicated. (B) Hemagglutination (HA) activity of the wild-type and *DhlyA* strains of *E. corrodens* 1073 was assayed. HA titers were expressed at the maximum dilution of the preparation that still showed HA. A representative image of three independent experiments is shown.

CHAPTER 3

Genomic recombination enhances pathogenic factors in the periodontopathogenic bacterium *Eikenella corrodens*

3.1 ABSTRACT

Previously it has been reported that plasmid-mediated genomic recombination at the pilin gene locus increased hemagglutination activity, growth rate, biofilm formation, hemolytic activity, and adherence to epithelial cells in *Eikenella corrodens* 23834. To determine whether these enhancements were common in this bacterium, I introduced the recombinase gene ORF4 into seven clinically isolated strains. Genomic recombination at the type IV pilin gene locus was observed in strains 1080, L9B6, L8Ao3, and RV2 (group A), but not in strains 261-2, 612-L, and 257-4 (group B). Similarly, group A strains displayed changed colony morphology following loss of type IV pili, which was not observed in group B. Group A strains showed also enhanced hemagglutination activity, growth rate, hemolytic, activity and biofilm formation. These results suggest that ORF4-induced genomic recombination at the pilin gene locus is a general phenomenon in *E. corrodens,* which likely stimulates pathogenicity and virulence.

3.2 INTRODUCTION

E. corrodens, a facultative gram-negative anaerobic rod, is found predominantly in subgingival plaque samples of patients with advanced periodontitis (Tanner *et al.* 1979). The monoinfection of germ-free or gnotobiotic rats by *E. corrodens* causes periodontal disease with severe alveolar bone loss (Listgarten *et al.* 1978). Given that *E. corrodens* is detected in dental plaque (Noiri *et al.* 2001), it is thought that the bacterium may participate in the early stages of biofilm formation by specific coaggregation with certain gram-positive and gram-negative bacteria present in human periodontal pockets.

Previously it has been found that *E. corrodens* 1073 secreted cell-associated GalNAc-specific lectin-like substance that enabled adherence to various host cell surfaces (Ebisu and Okada1983; Yamazaki *et al*. 1981; Yamazaki *et al.* 1988; Miki *et al.* 1987). Additionally, it has found that the GalNAc-specific lectin mediated coaggregation of *E. corrodens* with some strains of *Streptococcus sanguinis* and *Actinomyces viscosus* (Ebisu *et al.* 1988), which are predominant during the early stages of dental plaque formation, while also stimulating the mitogenic activity of B lymphocytes (Nakae *et al*. 1994). Therefore, GalNAc-specific lectin is thought to contribute to the pathogenicity and virulence of *E. corrodens*, and this property can be estimated by hemagglutination (HA) activity.

On solid medium, *E. corrodens* 1073 forms large, non-corroding colonies, whereas other strains form small, corroding colonies due to twitching motility. Previously, Azakami *et al.* (2005) identified a DNA plasmid of 8.7 kb in strain 1073 and designated it as pMU1. Upon investigating its relevance for *E. corrodens* pathogenicity, I identified seven ORFs on pMU1, one of which (ORF4) was

homologous to the recombinase specific for the type IV pilin gene. Transformants with pMU4, in which the ORF4 gene was subcloned into a shuttle vector, lost their pilus structure and formed non-corroding colonies on solid medium. Moreover, I confirmed that the introduction of the ORF4 gene into strain 23834 resulted in genomic recombination at the type IV pilin gene locus. Furthermore, it has been observed that this recombination event markedly enhanced GalNAc-specific lectin activity (Azakami *et al.* 2006a), as well a growth rate, biofilm formation, hemolytic activity, and adherence to epithelial cells (Azakami *et al.* 2006a; Matsunaga *et al.* 2011; Azakami *et al.* 2006b). In this study, I investigated the universal effect of genomic recombination in *E. corrodens* strains other than strain 23834.

3.3 MATERIALS AND METHODS

Bacterial strains, plasmids, and media:

E. corrodens 1073 and 1080 were provided by S. S. Socransky (Forsyth Dental Center, Boston, MA, USA) and *E. corrodens* 23834 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). *E. corrodens* 612-L, 257-4, and 261-2 were isolated clinically from human supragingival plaque (Ebisu and Okada 1983). *E. corrodens* L8Ao3, L9B6, and RV2 were kindly provided by Dr. Giuseppe Valenza (University of Würzburg, Würzburg, Germany). *Escherichia coli* XL-1 Blue was used for cloning and sequencing. The *E. coli*/*E. corrodens* shuttle vector pLES2 (Stein *et al.* 1983) was obtained from the ATCC. *E. corrodens* cells were grown in tryptic soy broth (TSB) containing 2 mg/mL KNO_3 and 5 μg/mL hemin, or on sheep blood agar plates at 37°C. Bacteria harboring plasmids were cultured on a medium supplemented with 50 μg/mL carbenicillin.

Transformation of *E. corrodens* strains:

E. corrodens was electrotransformed using a Gene Pulser electroporator (Bio-Rad, Hercules, CA, USA) with 5 to 10 μg plasmid DNA. Briefly, a 100-mL bacterial culture was grown for 12 h, washed three times in solution A (272 mM sucrose, 1 mM $MgCl_2$ pH 7.4), and resuspended in 100 μL solution A. A 39-μL aliquot of the bacterial suspension was mixed with 1 μL DNA and electroporated at 2.1 kV, 25 μF, 200 Ω. For transformations involving the broad-host-range shuttle vector pLS88 and its derivatives, cells were recovered by being spun for 12 h at 37°C. After recovery, recombinant cells were cultured on sheep blood agar plates containing 50 μg/mL carbenicillin at 37°C.

Detection of genomic recombination at the type IV pilin gene locus:

I designed primers A (GGGAAGAAAAGGGAAGTGCT) and B (TCTTCA GGTACCGTCAGCAAAA) based on the 16S rDNA sequence of *E. corrodens* (GenBank accession no. AF320620), and primers C (TTTTATCCGCAATGGGTATC) and D (TACAAATCTTTGCCCTTCAC) based on the type IV pilin gene sequence of *E. corrodens* 23834 (GenBank accession no. Z12609). Primers A and B allow the detection of all *E. corrodens* strains; whereas primers C and D are specific for those strains, in which genomic recombination occurred at the type IV pilin locus. To determine the occurrence of genomic recombination, I used these primers in combination with real-time PCR.

Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) as follows: 23 μL master mix was added to 96-well PCR plates containing genomic DNA and primers. The plates were sealed with a clear plastic sheet and placed in an ABI 7300 Sequence Detector (Applied Biosystems). During the course of 40 cycles (94°C, 15 s and 60°C, 1 min), data were collected through optical cables connected to each well. Ct values were calculated by the ABI 7300 software and genomic recombination was estimated from the following formula: (Ct value by primers C and D)/(Ct value by primers A and B) = Detection rate of genomic recombination.

Hemagglutination activity assay:

The HA activity assay was performed as previously described (Ebisu and Okada 1983). Erythrocytes that were obtained by the centrifugation of preserved rabbit blood were washed three times with saline before being suspended in

phosphate-buffered saline (PBS, pH 7.2) at a concentration of 2%. The HA assay was performed in microtiter plates (Vdispo; Nalge Nunc International, Roskilde, Denmark). Test preparations (50 µL) were serially diluted two fold in PBS and mixed for 2 min with equal volumes of the 2% erythrocyte suspension. HA activity was examined after 1 h, and HA titers were expressed as the maximum dilution of the test preparation that exhibited HA activity.

Adherence assay for quantitation of biofilm production.

E. corrodens strains formed a macroscopically visible biofilm that was firmly attached to the wells of 96-well tissue culture plates (non-treated polystyrene, flat-bottom with lid; BD Bioscience, San Jose, CA, USA), and biofilm production was determined as described previously (Djordjevic *et al.* 2002). The assay measured the primary attachment and accumulation of multilayered cell clusters, and subsequent biofilm production on the polystyrene surface. Briefly, after growth in TSB for 36 h at 37°C, plates were gently washed four times with PBS, and adherent bacterial cells were fixed with methanol followed by staining with crystal violet. The optical density of the stained adherent bacterial biofilms was measured at 595 nm (OD_{595}) with a spectrophotometer.

Statistical analysis:

Results are presented as mean values and standard deviations from triplicate measurements. The significance of intergroup differences was analyzed using Student's *t*-test (unpaired *t*-test).

3.4 RESULTS AND DISCUSSION

Effect of ORF4 on colony morphology and hemolysis of *E. corrodens*:

It has been reported that genomic recombination increased *E. corrodens* 23834 pathogenicity (Azakami *et al.* 2005; Azakami *et al.* 2006a; Matsunaga *et al.* 2011; Azakami *et al.* 2006b). To examine the effect of genomic recombination in other strains, I introduced the recombinase-encoding ORF4 gene into seven clinical isolates, 261-2, 1080, 257-4, 612-L, L9B6, L8Ao3, and RV2. I checked the colony morphology of each transformant on solid agar plates. Whereas in the absence of ORF4, the strains formed corroding colonies (Fig. 3-1A), colony morphology of strains 1080, L9B6, L8Ao3, and RV2 changed to non-corroding after introduction of ORF4 (Fig. 3-1B). This change was not observed for strains 261-2, 612-L, and 257-4 (Fig. 3-1B). Accordingly, strains were classified into two groups: A (strains 1080, L9B6, L8Ao3, and RV2), and B (strains 261-2, 612-L, and 257-4).

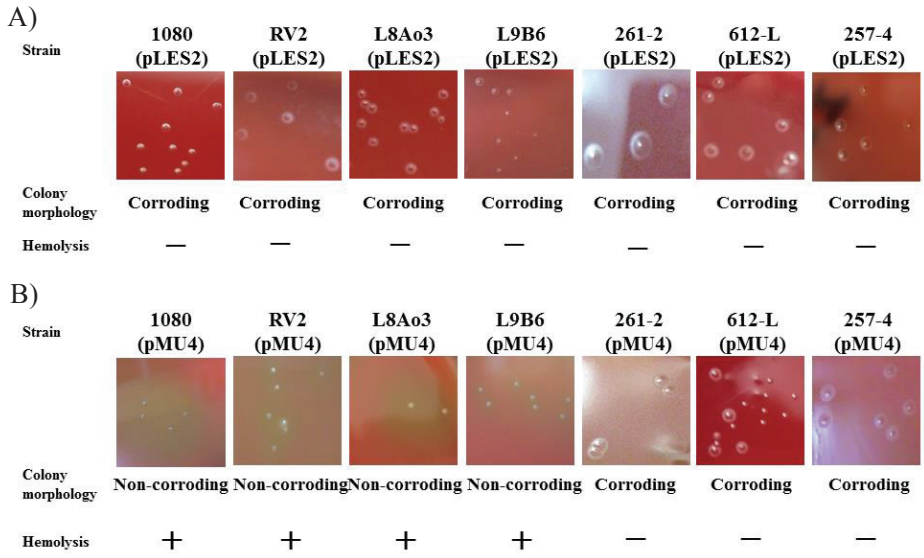

Fig. 3-1. Colony morphology and hemolysis of *E. corrodens* strains.
E. corrodens strains 1080, RV2, L8Ao3, L9B6 261-2, 612-L, and 257-4 were cultured on sheep blood agar at 37°C. A) Strains transformed with empty vector pLES2. B) Strains transformed with ORF4 on pMU4. Colony morphology and hemolysis were observed on agar plates.

Next, I assessed hemolysis of *E. corrodens* strains on sheep blood agar media. Hemolysis can be observed as a transparent zone surrounding the colony. Although no hemolysis was observed in the absence of ORF4 (Fig. 3-1A), strains 1080, L9B6, L8Ao3, and RV2 presented hemolytic activity after introduction of ORF4 (Fig. 3-1B). No hemolytic activity was observed in strains 261-2, 612-L, and 257-4 after introduction of ORF4 (Fig. 3-1B). These results were consistent with those relating to colony morphology, as hemolysis was detected in all strains whose colony morphology was altered by introduction of ORF4.

In a healthy human body, the concentration of free iron should be maintained at 10^{-18} M (Bullen JJ 1981). However, bacteria require an iron concentration of 0.05–0.5 μM for growth (Martinez *et al.* 1990). To this end, bacteria can satisfy their metabolic needs and acquire iron by applying hemolytic factors that lyse host erythrocytes and cause the release of intracellular iron (Sato *et al.* 2012). Therefore, hemolysis is thought to be important for the pathogenicity of many invading bacteria and increasing hemolytic activity through introduction of ORF4 might be vital for *in vivo* survival of oral bacteria.

Effect of ORF4 on genomic recombination at the type IV pilin gene locus:

I showed previously that introduction of ORF4 into strain 23834 resulted in genomic recombination at the type IV pilin locus. Here, I investigated whether the same occurred in other *E. corrodens* strains. In strains 261-2, 612-L, and 257-4, I could not detect any increased genomic recombination compared to the non-transformed strains (Fig. 3-2). Instead, introduction of ORF4 resulted in enhanced genomic recombination in strains 1080, L9B6, L8Ao3, and RV2 (Fig. 3-2). These results were consistent with

those pertaining to colony morphology and hemolysis. Accordingly, group A strains showed both colony morphology changes and genomic recombination at the type IV pilin locus, whereas group B strains displayed none of the above.

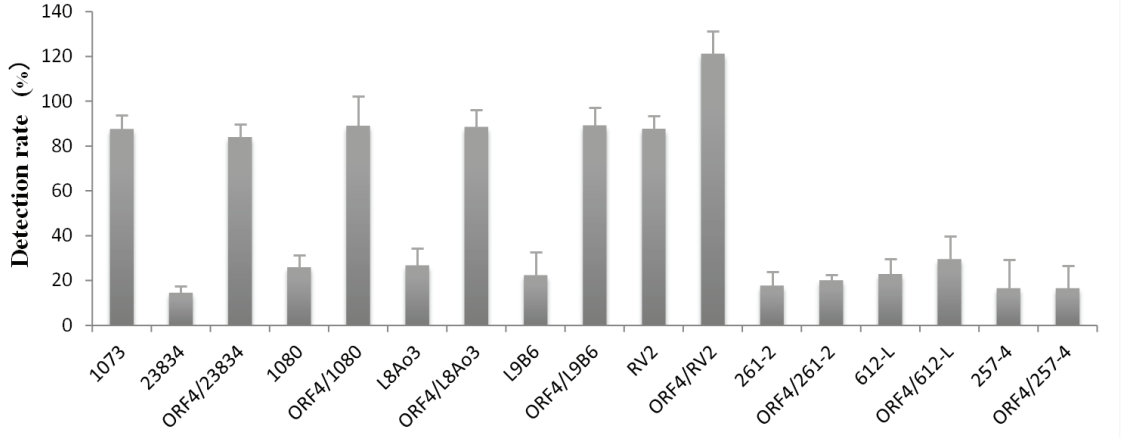

Fig. 3-2. Detection of genomic recombination at the type IV pilin gene locus by real-time PCR using specific primers. Ct values were calculated by specialized software and genomic recombination was evaluated based on the following formula: Ct value by primers specific for genomic recombination/Ct value by primers specific for *E. corrodens* = Detection rate of genomic recombination. Assays were performed at least three times. Mean values and standard deviations are reported.

Given that the pili on the cell surface are involved in the cells' twitching motility as well as colony morphology (McMichael 1992; Villar *et al.* 2001), it is likely that the change from corroding to non-corroding colonies reflected a loss of pili following genomic recombination at the type IV pilin locus.

Effect of ORF4 on hemagglutination activity:

It is believed that the GalNAc-specific lectin contributes to the pathogenicity and virulence of *E. corrodens*. To assess the effect of introducing ORF4 on pathogenicity, I measured HA activity in seven *E. corrodens* strains. As shown in Fig. 3-3, HA activity was high in group A strains following introduction of ORF4, but did not change in group B strains even after introduction of ORF4.

I reported previously that genomic recombination by plasmid-mediated recombinase stimulated simultaneous GalNAc-dependent lectin activity and hemolytic activity in *E. corrodens* 23834(Azakami *et al.* 2006a; Matsunaga *et al.* 2011). Recently, I demonstrated that hemolytic activity decreased upon addition of GalNAc as described in chapter 1. Here, I show that the introduction of ORF4 enhanced both hemolytic activity and GalNAc-dependent lectin activity (Figs. 3-1 and 3-3). These findings suggest that hemolytic activity correlates with lectin activity. Moreover, I recently isolated the hemolytic factor from *E. corrodens* 1073 and demonstrated that in its absence lectin activity was the same in this as in the wild-type strain as described in chapter 1. It has been suggested that hemolysin and lectin are not the same protein, because absence of hemolysin does not have any effect on lectin. It is thought that once lectin mediates adhesion to the blood cell, the latter becomes susceptible to attack by hemolysins present on the bacterial surface. Therefore, enhancement of hemolytic activity following introduction of ORF4 may depend on increased lectin activity.

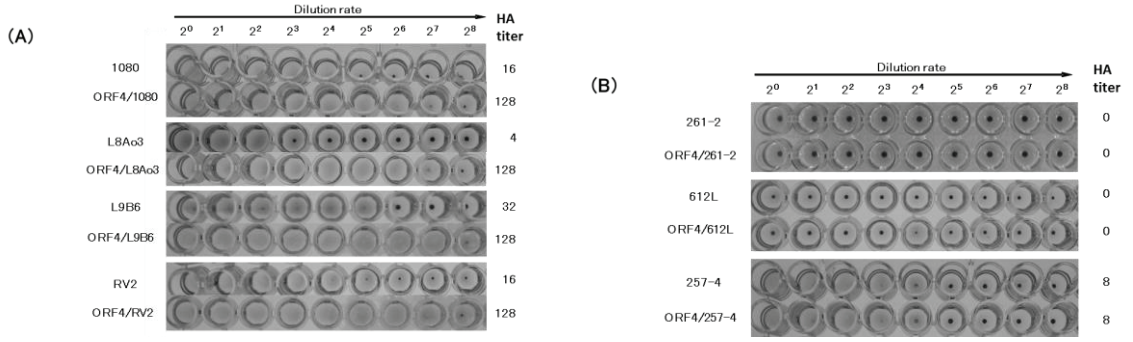

Fig. 3-3. Hemagglutination of rabbit erythrocytes by cell cultures of *E. corrodens*.
Hemagglutination titers were expressed as the maximum dilution of the preparation that still showed hemagglutination. Values shown represent means derived from three independent experiments. (A) Strains 1080, L8Ao3, L9B6, and RV2 transformed or not transformed with ORF4 on pMU4. (B) Strains 261-2, 612-L, and 257-4 transformed or not transformed with ORF4 on pMU4.

Effect of ORF4 on biofilm formation:

Previously, I reported that introduction of ORF4 into strain 23834 resulted in increased biofilm formation (Azakami *et al.* 2006a). Here, I investigated whether the

same occurred in other *E. corrodens* strains. As shown in Fig. 3-4, biofilm formation increased in group A strains following introduction of ORF4 on pMU4, but not in group B strains.

I suggested earlier that the GalNAc-specific lectin and other factors contributed additively to biofilm formation in some strains of *E. corrodens* (Azakami *et al.* 2006a). In this study, I demonstrate that the introduction of ORF4 enhanced both GalNAc-specific lection activity and biofilm formation. Accordingly, the GalNAc-specific lectin might be involved in biofilm formation by *E. corrodens*.

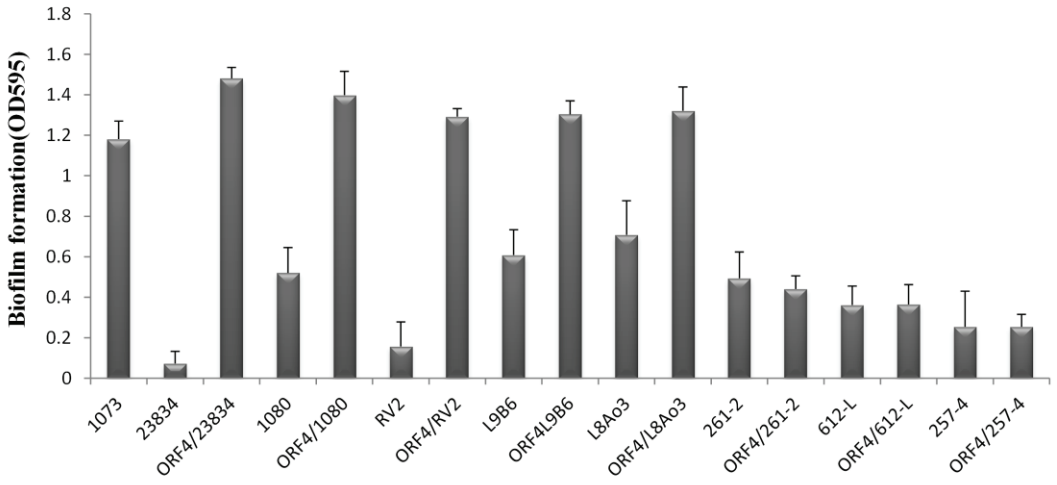

Fig. 3-4. Biofilm formation by *E. corrodens* strains.
Strains 1073, 23834, 1080, RV2, L9B6, L8Ao3, 261-2, 612-L, and 257-4 transformed or not transformed with ORF4 on pMU4 were grown aerobically in TSB medium containing hemin and KNO_3 at 37°C using polystyrene microtiter plates. Biofilm formation was quantified by measuring optical density at 595 nm (OD_{595}). Assays were performed in at least 8 wells; mean values and standard deviations are shown.

Effect of ORF4 on the growth rate of *E. corrodens*:

Given that in our previous study, the introduction of ORF4 stimulated growth rate (Azakami *et al.* 2006a), I investigated whether the same occurred in other *E. corrodens* strains. As shown in Fig. 3-5, growth rate in group A strains increased after introduction of ORF4 on pMU4, whereas that in group B strains did not change. Biofilm formation is

thought to be a means by which pathogenic bacteria fix to a solid surface and enhance their pathogenicity (Kolenbrander *et al.* 2002). In this light, increased growth and biofilm formation following transformation with pMU4 might represent one of the strategies by which *E. corrodens* can survive in the oral cavity and, at the same time, promote pathogenicity and virulence.

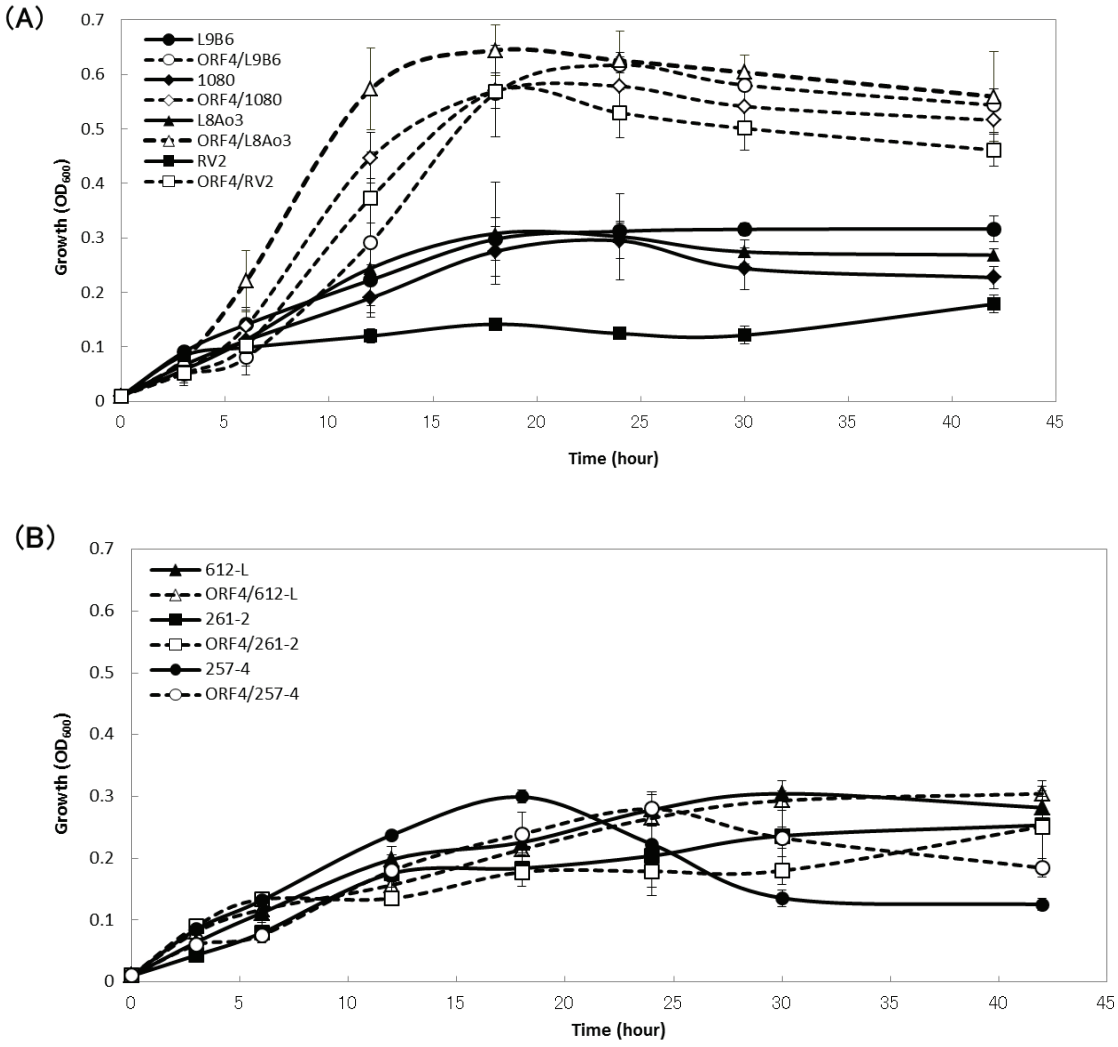

Fig. 3-5. Growth rates of *E. corrodens* strains.
A) Strains L9B6, 1080, L8Ao3, and RV2 transformed or not transformed with ORF4 on pMU4. B) Strains 612-L, 261-2, and 257-4 transformed or not transformed with ORF4 on pMU4. Strains were grown aerobically in TSB medium containing hemin and KNO_3 at 37°C. Bacterial growth was monitored spectrophotometrically by measuring optical density at 600 nm (OD_{600}). Assays were performed at least three times. Mean values and standard deviations are reported.

CHAPTER 4: GENERAL CONCLUSION

Periodontal diseases are disorders of supporting structures of the teeth (example: gingivae, periodontal, ligament and supporting alveolar bone). The disease is due to an inflammatory process that results in destruction of supporting tissue of the teeth which is a result of mixed microbial infection (Haffajee *et al.* 2004; Kim and Amar 2006; Darveau 2010; Hasan and Palmer 2014). It has already been reported to affect up to 90% of the world's population (Petersen *et al*. 2005; Borojevic 2012; Mobeen *et al*. 2008). In addition, evidence has suggested for a potential role of periodontal infections in more serious systemic diseases including cardiovascular disease, respiratory infections, diabetics and low-birth weight complications (Li *et al.* 2000; Igari *et al.* 2014). *E. corrodens* which is a fastidious facultative anaerobic gram negative bacillus, present as a part of the normal human oral flora (Jackson *et al*. 1984). Previous research has revealed that the monoinfection of germ free or gnotobiotic rats by *E. corrodens* causes severe periodontal disease, suggesting that *E. corrodens* is one of periodontopathogenic bacteria (Listgarten *et al.* 1978).

Otto *et al.* (1992) reported that addition of iron for bacterial metabolism may be facilitated by lysis of host erythrocytes through hemolysin, which release intracellular iron. Thus, hemolysis might be involved in the growth and survival of pathogenic bacteria. In chapter 2, I tried to purify and characterize the hemolysin produced by *Eikenella corrodens* 1073 to understand its involvement in pathogenicity. Labischinski *et al.* (1985) reported that the envelope of Gram-negative bacteria has a well-defined structure: a classical protein lipid bilayer constituting the inner or cytoplasmic membrane and an outer membrane composed of an unusual asymmetric

bilayer, separated by a narrow aqueous compartment, the periplasm. In this study, I observed the presence of hemolysin was higher from CE fraction of *E. corrodens* 1073 by performing hemolytic assay. It was suggested that the hemolytic factors might be located on the surface of bacterial cells. Because I could not find any signal sequence to secrete from cytoplasm in the amino acid sequence of X-prolyl aminopeptidase, it remains to be seen how it localize on the bacterial cell surface.

To identify the protein responsible for the hemolytic activity of *E. corrodens* 1073, hemolysin from CE fraction was purified by using three types of column (DEAE-Toyopearl column, Q-sepharose fast flow column and gel filtration column). After purifying the samples by column, 65kDa protein was observed in 15% SDS-PAGE, which might be responsible for hemolytic activity of *E. corrodens* 1073. As it was suspected that the purified protein was hemolysin, to confirm it we determined amino acid sequence of this protein. As a result, its sequence was almost similar with the X-prolyl aminopeptidase in *E. corrodens* 23834 except for the substitution of seventh arginine for leucine. The tiny difference was observed in the sequence. However, I cloned and sequenced the gene encoding the X-prolyl aminopeptidase of *E. corrodens* 1073, revealing that the substitution of leucine for arginine 7 was due to a misreading of the amino acid sequence. Thus, I suggested that hemolysin of *E. corrodens* 1073 might be function as X-prolyl aminopeptidase. To confirm this hypothesis, DNA sequencing of *hlyA* gene were performed and found that amino acid sequence of HlyA of *E. corrodens* 1073 had 93.3% identity and 99.0% similarity to that of X-prolyl aminopeptidase of *E. corrodens* 23834. Although there is much more similarity between hemolysin of both strains, but the reason why only *E. corrodens* 1073 showed hemolytic activity remains unknown. It was suggested that there might be some

substitution of the coding region or presence of any mutation in promoter region of X-prolyl aminopeptidase that might cause differences between the hemolytic activities between both strains.

To further clarify whether X-prolyl aminopeptidase of *E. corrodens* 1073 function as hemolysin, I expressed hemolysin in *E. coli* and the expressed protein was purified by affinity chromatography using His60 Ni gravity column. Purified 65kDa protein was observed as a single band and also hemolytic activity was detected from the purified recombinant HlyA. Moreover, purified HlyA showed hemolytic activity in a dose dependent manner which supported that HlyA protein functions as hemolytic factor. For further confirmation, *hlyA*-deficient strain (Δ*hlyA*) was constructed and compare with the wild type strain of *E. corrodens* 1073. As a result, Δ*hlyA* strain showed no hemolysis on blood agar plate. Moreover, when hemolytic activities were measured for both strains, hemolytic activity of Δ*hlyA* strain was decreased as compared with the wild strain. These results suggested that X-prolyl aminopeptidase function as hemolytic factor in *E. corrodens* 1073.

Although there has been no report showing that aminopeptidase can act as a hemolytic factor, it is suggested that the X-prolyl aminopeptidase from *E. corrodens* 1073 might contain an α-helix structure that can function as a hemolysin. Since it is unknown whether X-prolyl aminopeptidase is required for hemolytic activity or not, it has been reported that extra cellular products (high levels of extracellular proline aminopeptidase and a thiol-dependent hemolysin) were found in different strains of *E. corrodens* of this nature may be central to pathogenicity, as indicated for a number of other virulent organisms (Makinen and Makinen 1987; Goebel *et al*. 1988; Allaker *et al*.

1994), which supported that X-prolyl aminopeptidase might contribute to the pathogenicitiy of *E. corrodens*.

Previously, it has been reported that genomic recombination by a plasmid-mediated recombinase enhances the GalNAc-dependent lectin activity in parallel with the hemolytic activity in *E. corrodens* 23834 (Matsunaga *et al.* 2011). These data suggested a relationship between the activity of hemolysin and lectin. To elucidate this, I measured the hemolytic activity of recombinant HlyA in the absence and presence of 3 mM GalNAc. The result suggested that the decrease in hemolytic activity might be due to the inhibition of lectin activity. Moreover, I recently isolated the hemolytic factor from *E. corrodens* 1073 and demonstrated that absence of lectin activity was the same in this as in the wild-type strain. It has been hypothesized that hemolysin and lectin are not the same protein, because absence of hemolysin does not have any effect on lectin activity. It is thought that once lectin mediates adhesion to the blood cell, the latter becomes susceptible to attack by hemolysins present on the bacterial surface.

In *Edwardsiella tarda*, *ethR* which is a transcription regulator of *ethB* (one of the hemolysin system), exhibited a positive effect on *luxS* expression and AI-2 activity by binding to the *luxS* promoter region (Wang *et al.* 2009). Previously in my lab, AI-2 was purified from *E. corrodens* 1073 (Karim *et al.* 2013a). It was hypothesized that the *luxS*-dependent signal plays key role in biofilm formation of oral cavity (Azakami *et al*. 2006b) and AI-2 enhanced biofilm formation directly on *E. corrodens* 1073 (Karim *et al.* 2013b). In this current study, hemolysin was purified from *E. corrodens* 1073. However, further research is required to understand the underlying relationship between hemolysin with AI-2 and *luxS* in *E. corrodens* 1073.

Recombination is thought one of the crucial evolutionary processes for microorganisms. Azakami *et al*. (2005) reported about the presence of a plasmid DNA in *E. corrodens* 1073 and investigated its relationship with the pathogenicity of *E. corrodens.* The presence of 7 open reading frames (ORFs) were found in this plasmid and the introduction of ORF4 gene into strain 23834 resulted in genomic recombination at the type IV pilin gene locus. Moreover, the transparent β-type hemolysis was observed around the colonies of transformants on blood agar (Matsunaga *et al.* 2011).

Previously, Azakami *et al.* have reported that the introduction of ORF4 into *E. corrodens* 23834 can elevated several virulence factors. In chapter 3, to observe the pathogenic effect of genomic recombination in other strains, I introduced the ORF4 gene into seven other strains of *E. corrodens*: 261-2, 1080, 257-4, 612-L, L9B6, L8Ao3, and RV2. I checked colony morphologies and hemolytic activities of each strain on sheep blood agar plates. Colony morphologies of strains 1080, L9B6, L8Ao3, and RV2 (strain A group) was changed from corroding to non-corroding after introduction of ORF4, while the change was not observed for strains 261-2, 612-L, and 257-4 (strain B group). It has been found that bacteria can satisfy their metabolic needs and acquire iron by applying hemolytic factors that lyse host erythrocytes and cause the release of intracellular iron (Otto *et al.* 1992). Therefore, hemolysis is thought to be necessary for the pathogenicity of many invading bacteria and increasing hemolytic activity through introduction of ORF4 might be vital for *in vivo* survival of oral bacteria.

To observe the effect of genomic recombination into GalNAc-specific lectin, HA activities of same strains were measured. The result was similar to that of colony morphology and hemolysin. High HA activity was found for group A strains after ORF4 transformation, whereas no change was observed in group B strains. These

findings suggested that hemolytic activity correlates with the lectin activity. Biofilm formation was also performed with those transformation strains. Increased biofilm formation following transformation of ORF4 might represent one of the strategies by which *E. corrodens* to survive and also to promote pathogenicity and virulence in the oral cavity. This result also consistent with those relating to colony morphology and hemolysis was detected in all strains whose colony morphology was altered by introduction of ORF4. However, mechanism for enhancement of several virulence factors for group A strains is still unknown. One possibility is that the introduction of ORF4 may cause genomic recombination at multiple loci. Therefore, further research is required to understand the mechanism for the pathogenicity of *E. corrodens*.

However, despite some limitations, taken all together, these observations suggested that colonization has a correlation with the pathogenicity of *E. corrodens*. It is certainly one of the important strategies for *E. corrodens* to survive in the host environment. Understanding this relationship could give an idea about the role of *E. corrodens* in a complex ecological niche and new treatment of periodontal diseases.

SUMMARY

Eikenella corrodens, a facultative anaerobic Gram-negative rod, is predominantly found in sub-gingival plaque samples of patients with advanced periodontitis. It is thought that hemolysin contributes to not only the acquisition of iron for the bacterial growth but also bacterial colonization to the host cells in most pathogenic bacteria. Previously, Azakami *et al*. (2006a) have reported that the introduction of recombinase gene (ORF4), which is found from the plasmid DNA in *E. corrodens* 1073, into *E. corrodens* 23834 resulted in genomic recombination. Moreover, they have reported that it enhances some pathogenic factors involved in bacterial colonization, such as hemolytic activity, hemagglutination activity, growth rate and biofilm formation. In this research, I aimed to identify and analyze the hemolysin from *E. corrodens* 1073 to clarify its relationship to the colonization to host cells. Furthermore, I tried to investigate the universal effect of ORF4 introduction on the bacterial colonization to host cells.

In chapter 1, I described the general introduction and purpose of the thesis.

In chapter 2, I aimed to purify the hemolysin from *E. corrodens* 1073 and identify it. Presence of high hemolytic activity was observed from CE fraction of *E. corrodens* 1073 compare to the cell supernatant fraction and the activity was detected from CE fraction in a dose-dependent manner. Next, purified of hemolysin was performed by using DEAE ion exchange chromatography, Q-sepharose chromatography and gel filtration chromatography. As a result, 65kDa protein was purified and which might be the hemolysin protein. N-terminal amino acid sequence of this protein suggested that this protein is almost similar with the X-prolyl aminopeptidase in *E. corrodens* 23834.

After purification of this protein using Ni column, hemolytic activity was detected from the purified protein. Moreover, Δ*hlyA* gene strain was constructed by homologous recombination. Δ*hlyA* strain did not show any hemolytic activity which suggested that X-prolyl aminopeptidase might function as hemolysin in *E. corrodens*1073.

To determine whether these enhancements were common in this bacterium, I introduced the recombinase gene ORF4 into seven clinically isolated strains. Genomic recombination at the type IV pilin gene locus was observed in strains 1080, L9B6, L8Ao3, and RV2 (group A), but not in strains 261-2, 612-L, and 257-4 (group B). Similarly, group A strains displayed changed colony morphology following loss of type IV pili, which was not observed in group B. Group A strains showed also enhanced hemagglutination activity, growth rate, hemolytic, activity and biofilm formation. These results suggest that ORF4-induced genomic recombination at the pilin gene locus is a general phenomenon in *E. corrodens,* which likely stimulates pathogenicity and virulence.

From these results, I suggest the following two conclusions: (1) X-prolyl aminopeptidase might function as hemolysin in *E. corrodens*1073. (2) The enhancement of pathogenicity by ORF4-induced genomic recombination is general phenomenon in *E. corrodens.* The various factors including hemolysin, adhesion factor, and biofilm-related factor are involved in colonization of periodontopathogenic bacterium *E. corrodens* in the oral cavity. Understanding of these factors may provide critical information for the prevention and treatment of periodontal diseases.

和文要約

Eikenella corrodens は、グラム陰性の通性嫌気性桿菌で、重度歯周病患者の病変部から頻繁に分離される。溶血素は多くの病原性細菌において生育のための鉄の獲得のみならず、病原因子としても寄与することが知られており、宿主への定着において重要な役割を果たすと考えられているまた。以前、阿座上らは *E. corrodens* 1073 株より発見されたプラスミド由来のリコンビナーゼ遺伝子(ORF4)の 23834 株への導入がゲノムの再編を引き起こし、宿主への定着に関与する溶血活性や赤血球凝集活性、バイオフィルム形成能など増加させることを示した。本研究では、宿主への定着に関与する溶血素を同定し解析を行った。また、ORF4 の導入による宿主への定着能の増加が *E. corrodens* において普遍的に起こるかどうかを調べた。

第 1 章では、本論文のバックグラウンドと目的について述べた。

第 2 章では、*E. corrodens* 1073 株から溶血因子を精製し、その同定および解析を行った。1073 株を培養後、細胞外被（CE）画分と培養上清画分を得た。CE 画分から、用量依存的な溶血活性が観察された。次に、溶血因子の精製を DEAE イオン交換クロマトグラフィー、Q-セファロースクロマトグラフィー、ゲル濾過クロマトグラフィーによって行った。その結果、溶血因子と思われる 65 kDa のタンパク質が精製された。このタンパク質の N 末端アミノ酸配列から、このタンパク質は *E. corrodens* 23834 株の X-プロリルアミノペプチダーゼとほぼ同様であることが示唆された。このタンパク質を大腸菌で発現後、Ni カラムにより精製したところ、精製タンパク質から溶血活性が検出された。さらに、相同的組換えによって作成した X-プロリルアミノペプチダーゼ遺伝子(*hlyA*)の欠損株

（Δ*hlyA* 株）では、溶血活性が見られなかった。これらのことから、*E. corrodens* 1073 株において X-プロリルアミノペプチダーゼが溶血因子として機能することが示唆された。

第 3 章では、ORF4 を 7 株の臨床分離株（261-2 株、257-4 株、612-L 株、1080 株、L9B6 株、L8Ao3 株および RV2 株）に導入し、ゲノム再編による宿主定着能の増加が *E. corrodens* において普遍的にみられるかどうかを調べた。ORF4 の導入後、1080 株、L9B6 株、L8Ao3 株および RV2 株（以後、Ａグループ株と呼ぶ）ではタイプ 4 線毛遺伝子領域でのゲノムの組換えが観察されたが、261-2 株、612L 株および 257-4 株（以後、Ｂグループ株と呼ぶ）では組換えは見られなかった。また、Ａグループ株では corroding から non-corroding へのコロニー形状の変化が起こったが、Ｂグループ株では変化は観察されなかった。さらに、赤血球凝集活性、増殖速度、溶血活性およびバイオフィルム形成能の増加がＡグループ株で観察されたが、Ｂグループ株では変化は見られなかった。これらの結果は、ORF4 が誘導する線毛遺伝子領域の組み換えが *E. corrodens* において一般的な現象であり、これにより本菌の感染性や病原性を増加させることが示唆された。

これらの結果より、著者は以下のことを示唆した。（１）*E. corrodens* 1073 株において X-プロリルアミノペプチダーゼが溶血因子として機能することが示唆された。（２）ORF4 が誘導する線毛遺伝子領域の組み換えが *E. corrodens* において一般的な現象であり、これにより本菌の感染性や病原性を増加させることが示唆された。歯周病原性細菌 *E. corrodens* の口腔内への定着には、溶血因子や付着因子、バイオフィルム関連因子など様々な因子が関与している。これらの理解は、歯周病の予防や治療への重要な情報を与えるかもしれない。

ACKNOWLEDGEMENTS

First and foremost all praises belong to the most merciful, Almighty Allah. It would not have been possible to write this doctoral thesis without the helpful cooperation of the kind people around me, to only some of them I particularly mention here.

The completion of this study could not have been possible without the expertise of my supervisor of this work, Prof. Hiroyuki Azakami, PhD, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan, for his kind guidance, advice, patient and encouragement during the entire period of my study. I am deeply thankful to him for providing me support and various supplies that facilitated my comfortable study and stay in Yamaguchi.

In addition, I would like to thank my co-supervisor, Prof. Shin-ichi Ozaki, PhD, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan and Prof. Tadanori Aimi, PhD, Faculty of Agriculture, Tottori University, Japan, for their kind suggestions. The completion of my work and this dissertation was impossible indeed without their valuable suggestions. I am also extremely indebted to Dr. B. L. Bassler for kindly providing *Vibrio harveyi* BB170.

I also feel great pleasure in expressing my gratitude to all of my teachers in the Department of Biological Chemistry, Yamaguchi University, and Faculty of United Graduate School of Agricultural Sciences, Tottori University for their generous help whenever I need.

A special thanks to Dr. M. Minnatul Karim for his personal support, endless encouragement and mental support throughout my study and staying in Japan. I also would like to thank Ms. Ayako Nagao for her providing me useful knowledge about

experimental set-up. I wanted to thank our laboratory research members, Ms. Sari Takahara, Ms. Natsumi Morishige, Mr. Masafumi Shimatani, Mr. Ryohei Iida and all other members of Laboratory of Food Chemistry for their cordial helps to finish this work.

I am obliged to the Japanese Ministry of Education, Culture, Sports, Science, and Technology for providing me the Monbukagakusho Scholarship.

Finally, I am truly thankful to my parents, my brother and other family members for their encouragement and mental support.

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LIST OF PUBLICATIONS

(1) **Fariha Jasin Mansur**, Sari Takahara, Mihoko Yamamoto, Masafumi Shimatani, Mohammad Minnatul Karim, Yuichiro Noiri, Shigeyuki Ebisu and Hiroyuki Azakami, Purification and characterization of hemolysin from periodontopathogenic bacterium *Eikenella corrodens* strain 1073. Bioscience, Biotechnology, and Biochemistry 81: 1246-1253 (2017) (Published online: 28 February 2017)

(This paper covers **Chapter 2** in the thesis)

(2) **Fariha Jasin Mansur**, Kazunori Yamada, Natsumi Morishige, and Hiroyuki Azakami, Genomic Recombination Enhances Pathogenic Factors in the Periodontopathogenic Bacterium *Eikenella corrodens*. Advances in Microbiology 7: 231-240 (2017) (Published online: 14 April 2017)

(This paper covers **Chapter 3** in the thesis)