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SUMMARY OF DOCTORAL THESIS

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Title: 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)

Eikenella corrodens, a facultative anaerobic Gram-negative rod, is predominantly found in sub-gingival plaque samples of patients with advanced periodontitis. Monoinfection of germ-free or gnotobiotic rats with *E. corrodens* causes periodontal disease with severe alveolar bone loss. 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. 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*, which are predominant during early stages of dental plaque formation, and stimulates the mitogenic activity of B lymphocytes. 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. 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.

In a healthy human body, the concentration of free iron should be maintained at 10^{-18} M. However, bacteria require iron concentrations of 0.05–0.5 μM for their growth. 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. Therefore, hemolysin is regarded as a virulence factor for many pathogenic bacteria and might be vital for the *in vivo* survival of oral bacteria. 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, a plasmid of 8.7 kb in size in strain 1073 was found and investigated its relationship to pathogenicity. Moreover, Azakami *et al*. 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. 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. Moreover, this genomic recombination enhances the GalNAc-dependent lectin activity in parallel with the hemolytic activity of *E. corrodens* ATCC 23834 was also reported. However, the relationship between the GalNAc-dependent lectin activity and hemolytic activity of *E. corrodens* has not been understood yet. 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. I determined the localization of hemolysin in *E. corrodens* cells. Presence of high hemolytic activity was observed from the cell envelope (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. Thus, I expected that hemlysin was localized in CE fraction. 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. I determined the N-terminal amino acid sequence of this protein. The results suggested that this protein is almost similar with the X-prolyl aminopeptidase in *E. corrodens* 23834. To confirm whether X-prolyl aminopeptidase is responsible for hemolysin in *E. corrodens*, I cloned the gene (*hlyA*) coding X-prolyl aminopeptidase from *E. corrodens* 1073. The recombinant HlyA protein was expressed in *Escherichia coli* after induction by IPTG and purified it using Ni column. The hemolytic activity was detected from the purified recombinant HlyA protein. Moreover, Δ*hlyA* gene strain was constructed by homologous recombination. The construction of mutant strain was confirmed by PCR using *hlyA* gene-specific primers. Δ*hlyA* strain did not show any hemolytic activity. Finally I investigated the relationship between hemolytic activity and lectin activity in *E. corrodens*. It was suggested that hemolysis by HlyA might require lectin activity by which bacterial cells can approach blood cells.These results suggested that X-prolyl aminopeptidase might function as hemolysin in *E. corrodens*1073.

Previously, Azakami *et al*. reported that the genomic recombination by the plasmid-encoded recombinase gene enhanced some pathogenic factors in *E. corrodens* strain 23834. In chapter 3, to determine whether these enhancements were common in this bacterium, we introduced the recombinase gene ORF4 into seven clinically isolated strains. I could introduce the recombinase gene into these strains successfully. Next I developed the novel method to detect the genomic recombination at the type IV pilin gene locus by real-time PCR using specific primers. As a result, genomic recombination was observed in strains 1080, L9B6, L8Ao3, and RV2 (group A), but not in strains 261-2, 612-L, and 257-4 (group B). Given that the pili on the cell surface are involved in the cells' twitching motility as well as colony morphology, 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. Similarly, group A strains displayed changed colony morphology following loss of type IV pili, which was not observed in group B. 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.Group A strains showed also enhanced HA activity, growth rate, hemolytic, activity and biofilm formation. 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.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. 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.