Genomic Recombination through Plasmid-Encoded Recombinase Enhances Hemolytic Activity and Adherence to Epithelial Cells in the Periodontopathogenic Bacterium *Eikenella corrodens*

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The periodontopathogenic bacterium *Eikenella corrodens* has an N-acetyl-d-galactosamine (GalNAc)-specific lectin, that contributes significantly to the pathogenicity of the bacterium. Recently, we reported that plasmid-mediated genomic recombination enhances the activity of this lectin. In this study, we investigated the effects of genomic recombination on certain virulence factors. Introduction of the recombinase gene resulted in hemolysis and significantly increased bacterial adhesion to epithelial cells. It was suggested that the enhanced adhesion was attributable to increased lectin activity due to genomic recombination, because it was inhibited by the addition of GalNAc. In contrast, invasion of the epithelial cells was remarkably reduced by genomic recombination. Although we assumed that this decrease in invasion resulted from a loss of type-IV pili, the phase variant did not show any decrease in invasion activity. This suggests that type-IV pili do not contribute to the invasive ability of *E. corrodens*. Our results suggest that genomic recombination enhances the pathogenicity of *E. corrodens*.

Key words: *Eikenella corrodens*; genomic recombination; hemolysis; bacterial adherence; periodontal disease

*Eikenella corrodens*, a facultative gram-negative anaerobic rod, is most often found in subgingival plaque samples of patients with advanced periodontitis.¹ Monoinfection of germ-free and of gnotobiotic rats by *E. corrodens* causes periodontal disease with severe alveolar bone loss.² Moreover, this bacterium is known to be associated with systemic human diseases, including atherosclerosis,³ endocarditis,⁴ and meningitis.⁵ Because *E. corrodens* is detected in the plaque formed on teeth,⁶ 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.

We have found that *E. corrodens* 1073 has a cell-associated N-acetyl-d-galactosamine (GalNAc)-specific lectin that mediates its adherence to various host-tissue cell surfaces.⁷–¹⁰ In addition, we have reported that the GalNAc-specific lectin mediates the co-aggregation of *E. corrodens* with some strains of *Streptococcus sanguinis* and *Actinomycetes viscosus*,¹¹ which are predominant during the early stages of dental plaque formation, and that it stimulates the mitogenic activity of B lymphocytes.¹² Therefore, it is believed that the GalNAc-specific lectin contributes to the pathogenicity and virulence of *E. corrodens*, which can be estimated from hemagglutination (HA) activity.

On a solid medium, *E. corrodens* 1073 forms large non-corroding colonies, whereas other strains form small corroding colonies due to twitching motility. Rao and Progulske-Fox³ and Tonjum et al.¹⁴ have cloned two type-IV pilin genes, one from *E. corrodens* 23834 and the other from *E. corrodens* 31745. However, in our previous electron microscopic study of *E. corrodens* 1073, we did not observe any pilus-like structures,¹⁵ although other investigators have suggested the presence of pilus.¹⁶ Like several gram-negative pathogens, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Moraxella bovis*, *E. corrodens* exhibits phase variation resulting from altered synthesis of type-IV pili, which is reflected in changes in colony morphology.¹⁷,¹⁸

We have identified a plasmid DNA of 8.7 kb in strain 1073,¹⁹ and we designated it pMU1. We investigated its relationship to the pathogenicity of *E. corrodens*. We found seven open reading frames (ORFs) on pMU1, and one of these (ORF4) was homologous to the recombinase specific to 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 a solid medium. Moreover, we confirmed that introduction of the ORF4 gene into strain 23834 results in genomic recombination at the type-IV pilin gene locus. Furthermore, we found that this genomic recombination remarkably enhances GalNAc-specific lectin activity,²⁰ which is thought to contribute to the pathogenicity of *E. corrodens* and its capacity for biofilm formation.²¹

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Abbreviations: GalNAc, N-acetyl-d-galactosamine; HA, hemagglutination; MEM, minimum essential medium; PBS, phosphate-buffered saline; CFU, colony-forming unit; ATCC, American Type Culture Collection
In this study, we investigated the effects of genomic recombination on certain virulence factors such as hemolysis and bacterial adherence to and invasion of epithelial cells.

Materials and Methods

Bacterial strains, plasmids, and media. E. corrodens 1073 was provided by S. S. Socransky (Forsyth Dental Center, Boston, MA), and E. corrodens 23834 was purchased from the American Type Culture Collection (ATCC). Escherichia coli XL-1 Blue was used for cloning and sequencing. E. coli/E. corrodens shuttle vector pLES2 was purchased from the ATCC. E. corrodens cells were grown in tryptic soy broth containing 2 mg/mL of KNO3 and 5 μg/mL of hemin, or on sheep-blood agar plates at 37 °C. Bacteria harboring plasmids were cultured on a medium supplemented with 50 μg/mL of carbenicillin.

Cell cultures. The KB cell line (derived from a human oral epidermoid carcinoma) was provided by T. Okamoto, Hiroshima University School of Dentistry. The Ca9-22 cell line (derived from a human gingival carcinoma) was purchased from the Japanese Cancer Research Resources Bank. KB cells and Ca9-22 cells were cultured in minimum essential medium (MEM) (Sigma, St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum (MP Biomedicals, Solon, OH) at 37 °C in a wafer-saturated atmosphere of 95% air and 5% CO2.

Adherence assay of epithelial cells. The adherence of the bacteria to epithelial cells was quantified by counting viable E. corrodens 1073 cells attached to KB or Ca9-22 monolayers, by procedures developed by Tokuda et al. with some modification.20) Approximately 105 KB cells or Ca9-22 cells in MEM were seeded in the wells of 24-well tissue culture plates and incubated until confluent monolayers developed. The bacteria were pelleted by centrifugation, washed twice in phosphate-buffered saline (PBS) (pH 7.2), and suspended in MEM at a concentration of 1.0 × 108 to 2.0 × 108 colony-forming units (CFU)/mL. Bacterial concentrations were determined spectrophotometrically according to standard curves. KB and Ca9-22 cell layers were washed 3 times with Hanks' balanced salt solution, inoculated with 500 μL of the microbial suspension, and incubated at 37 °C for 1 h. Non-adherent bacteria were removed by once-repeated washing with PBS, and cell-attached bacteria were quantified after lysis of the cell layers in 500 μL of distilled water and subsequent dilution with distilled water. Appropriate dilutions were spread onto tryptic soy agar plates, and CFUs were counted after culture on sheep-blood agar plates at 37 °C for 2 d. To determine the effects of the GalNAc-specific lectin on bacterial adherence to this cell line, GalNAc was added to the microbial suspension.

Invasion assay of epithelial cells. After infection at 37 °C for 3 h, non-adherent and adherent bacteria were removed by treatment with PBS supplemented with antibiotics. Invasive bacteria were quantified after lysis of the cell layers in 500 μL of distilled water and subsequent dilution with distilled water. Appropriate dilutions were spread onto tryptic soy broth plates, and CFUs were counted after culture on sheep-blood agar plates at 37 °C for 2 d.

Statistical analysis. The results of the various series of experiments are shown as the mean (SEM). The significance of intergroup differences among the adherent cells was analyzed by Student’s t test (unpaired t statistic).

Results and Discussion

Genomic recombination enhanced hemolysis of E. corrodens

Hillman et al. reported 12 different bacterial species belonging to five genera, Actinomyces, Streptococcus, Staphylococcus, Prevotella, and Actinobacillus, showing hemolytic activity in the subgingival plaque in periodontal patients.21) In addition, hemolytic activity has been reported to be elevated in diseased sites in the oral cavity. Hemolysis is thought to be involved in many infections caused by pathogenic bacteria, since it induces bone loss due to its cytotoxic effect and facilitates the growth of pathogenic bacteria due to ion acquisition from erythrocytes,22) but there have been few reports on hemolysis in E. corrodens. We investigated the effects of genomic recombination on the hemolytic activity in E. corrodens strains. As shown in Fig. 1, strains 23834 and 23834 (pLES2) showed no hemolytic activity on sheep-blood agar plates. However, the introduction of a recombinase gene into strain 23834 showed transparent β-type hemolysis around the colonies on the blood agar. These results suggest that genomic recombination in strain 23834 causes β-hemolysis of E. corrodens.

We have reported that E. corrodens cells adhered to human erythrocytes due to GalNAc-specific lectin and showed HA.11) In addition, recently we reported that genomic recombination in E. corrodens enhances HA activity.20) Moreover, many reports state that certain lectins that are capable of agglutinating erythrocytes of sheep and some other animals are also involved in hemolysis.26-28) Hence, it is suggested that the increased activity of GalNAc-specific lectin due to genomic recombination is involved in the increased hemolytic activity.

Otto et al. reported that acquisition of iron for bacterial metabolism can be facilitated by lysis of host erythrocytes through hemolysin, which releases intracellular iron.29) Hence, hemolysis is thought to be involved in the growth and survival of pathogenic bacteria. Recently, we reported that the growth of strain 23834 was improved by introducing the recombinase gene in TSB medium lacking sheep blood.20) Hence, it is suggested that E. corrodens takes advantage of both effects by genomic recombination to survive in oral cavities.

Genomic recombination enhanced the adherence of E. corrodens to epithelial cells

We have reported that E. corrodens adheres to oral epithelial cells via GalNAc-specific lectin.5) Moreover, we reported that genomic recombination enhances
GalNAc-specific lectin activity in *E. corrodens*. Hence, in order to investigate the effect of genomic recombination on adherence to oral epithelial cells, we performed an adherence assay using KB cells, cells of an oral epithelial cell line. As shown in Fig. 2A, the adhesion activity of strain 23834 to the KB cells was low, approximately 10–20% that of strain 1073. The introduction of pLES2 vector into strain 23834 caused only a slight increase in adhesion to KB cells, but introduction of the recombinase gene into strain 23834 resulted in a significant increase in adhesion to KB cells. Moreover, the increased adherence activity was inhibited by the presence of 2 mM GalNAc (Fig. 2B). These results suggest that the GalNAc-specific lectin activity enhanced by the genomic recombination contributes to the increase in the adhesion of epithelial cells.

Because KB was found to be contaminated with HeLa cells at the time it was established, we performed an adherence assay using another cell line, human gingival epithelial cells Ca9-22. As shown in Fig. 2C, introduction of the recombinase gene into strain 23834 increased adhesion to the Ca9-22 cells as well as to the KB cells. Moreover, the increased adherence activity was inhibited by the presence of 2 mM GalNAc (Fig. 2B). These results suggest that the GalNAc-specific lectin activity enhanced by the genomic recombination contributes to the increase in the adhesion of epithelial cells.

**Genomic recombination reduced invasion by *E. corrodens***

In general, invasion of host cells by pathogenic bacteria allows pathogens to evade host defenses and facilitates their dissemination both within and across cellular barriers. However, Han *et al.* reported that *E. corrodens* strain 23834 appeared to be weakly invasive or noninvasive as compared to other periodontopathogenic bacteria, such as *Porphyromonas gingivalis*. To determine whether *E. corrodens* can invade oral epithelial cells, we performed an invasion assay using KB cells.

As shown in Fig. 3, strains 1073 and 23834 (pMU4) invaded the KB cells, but the invasion activity was much lower than the adherence activity. The invasion activity of strain 1073 was as low as that of the strain that underwent genomic recombination. Moreover, introduction of the recombinase gene into strain 23834 reduced the invasion activity of the strain into the KB cells, but increased adhesion to oral epithelial cells. Because we have reported that introduction of the recombinase gene into strain 23834 resulted in genomic recombination within the type-IV pilin gene locus and caused loss of pili on its surface, we assumed that type-IV pili are involved in the invasion by *E. corrodens* of oral epithelial cells.
epithelial cells. In fact, there are many reports stating that pilus structures are involved in invasion by many bacteria, including some periodontopathogenic bacteria. Njoroge et al. reported that P. gingivalis major fimbriae are required for adherence to and invasion of oral epithelial cells. Moreover, Yilmaz et al. reported that fimbriated P. gingivalis cells induce the formation of integrin-associated focal adhesion, with subsequent remodeling of the actin and tubulin cytoskeleton for internalization.

To determine whether type-IV pili are involved in invasion by E. corrodens of oral epithelial cells, we performed an invasion assay using a phase variant of strain 23834. It has been reported that E. corrodens exhibits phase variation resulting from a loss of type-IV pili, which is reflected in irreversible morphological changes in colonies, from corroding to non-corrod-ing.17,18) Recently, we found that the colony morphology of phase variants of strain 23834 changed from corroding to non-corroding, and that the variants lost their pilus structure without genomic recombination.20) As shown in Fig. 3, the phase variant of strain 23834 did not show any decrease in the invasion of KB cells. Moreover, the phase variant strain showed no hemolytic activity (Fig. 1) and no significant increase in adhesion activity (Fig. 2A). These results suggest that the presence of type-IV pili on the strain’s surface does not contribute to the invasion of oral epithelial cells by E. corrodens. Furthermore, it was suggested that the invasion activity was reduced by factors other than type-IV pili, which were altered due to the genomic recombination.

We have found that plasmid-mediated genomic recombination enhances GalNAc-specific HA activity and the ability of E. corrodens to form biofilms.20,21) We suggest that genomic recombination in E. corrodens enhances hemolysis and bacterial adherence to oral epithelial cells. However, we also suggest that the genomic recombination reduced bacterial invasion of epithelial cells. Because GalNAc-specific lectin is thought to contribute to the pathogenicity of E. corrodens and the invasion activity of E. corrodens is negligible compared to its adhesion activity,24) it is suggested that the decrease in invasion activity does not contribute to the pathogenicity of E. corrodens. Hence, we conclude that plasmid-mediated genomic recombination enhances the periodontopathogenicity of E. corrodens by increasing GalNAc-specific lectin activity.

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