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Genomic Recombination Enhances Pathogenic Factors in the Periodontopathogenic Bacterium *Eikenella corrodens*

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Abstract

We reported previously 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, we 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 a part of *E. corrodens*, which likely stimulates pathogenicity and virulence.

Keywords

Eikenella corrodens, Genomic Recombination, Biofilm, Hemolysis, Hemagglutination

1. Introduction

Eikenella corrodens, a facultative gram-negative anaerobic rod, is found predominantly in subgingival plaque samples of patients with advanced periodontitis [1]. The monoinfection of germ-free or gnotobiotic rats by *E. corrodens* causes periodontal disease with severe alveolar bone loss [2]. Given that *E. corrodens* is detected in dental plaque [3], 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.

We previously reported that *E. corrodens* 1073 presented a cell-associated *N*-ace tyl-$_D$-galactosamine (GalNAc)-specific lectin-like substance that enabled adherence to various host cell surfaces [4] [5] [6] [7]. Additionally, we reported that the GalNAc-specific lectin mediated coaggregation of *E. corrodens* with some strains of *Streptococcus sanguinis* and *Actinomyces viscosus* [8], which are predominant during the early stages of dental plaque formation, while also stimulating the mitogenic activity of B lymphocytes [9]. 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, we identified a DNA plasmid of 8.7 kb in strain 1073 [10] and designated it as pMU1. Upon investigating its relevance for *E. corrodens* pathogenicity, we 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, we confirmed that the introduction of the ORF4 gene into strain 23834 resulted in genomic recombination at the type IV pilin gene locus. Furthermore, we observed that this recombination event markedly enhanced GalNAc-specific lectin activity [11], as well as growth rate, biofilm formation, hemolytic activity, and adherence to epithelial cells [11] [12] [13].

In this study, we investigated the universal effect of genomic recombination in *E. corrodens* strains other than strain 23,834.

2. Materials and Methods

2.1. 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 [4]. *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 [14] 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. Bacterial growth was monitored spectrophotometrically by measuring optical density at 600 nm (OD_{600}).

2.2. 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.

2.3. Detection of Genomic Recombination at the Type IV Pilin Gene Locus

We designed primers A (GGGAAGAAAAGGGAAGTGCT) and B (TCTTCAGGTACC GTCAGCAAAA) based on the 16S rDNA sequence of *E. corrodens* (GenBank accession no. AF320620), and primers C (TTTTATCCGCAATGGGTATC) and D (TACAAATCT TTGCCCTTCAC) 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, we 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.

2.4. Hemagglutination Activity Assay

The HA activity assay was performed as previously described [4]. 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.

2.5. Adherence Assay for Quantitation of Biofilm Production

E. corrodens strains formed a macroscopically visible biofilm that was firmly at-

tached 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 [15]. 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.

2.6. Growth Rate Measurement

E. corrodens 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.

2.7. Statistical Analysis

In the detection of genomic recombination, hemagglutination assay, and growth rate measurement, the results are presented as mean values and standard deviations from triplicate measurements. In biofilm assay, the results are presented as mean values and standard deviations from at least 8 wells. The significance of intergroup differences was analyzed using Student's *t*-test (unpaired *t*-test).

3. Results and Discussion

3.1. Effect of ORF4 on Colony Morphology and Hemolysis of E. Corrodens

Previously, we reported that genomic recombination increased *E. corrodens* 23,834 pathogenicity [10] [11] [12] [13]. To examine the effect of genomic recombination in other strains, we introduced the recombinase-encoding ORF4 gene into seven clinical isolates, 261-2, 1080, 257-4, 612-L, L9B6, L8Ao3, and RV2. We checked the colony morphology of each transformant on solid agar plates. Whereas in the absence of ORF4, the strains formed corroding colonies (**Figure 1(a)**), colony morphology of strains 1080, L9B6, L8Ao3, and RV2 changed to non-corroding after introduction of ORF4 (**Figure 1(b)**). This change was not observed for strains 261-2, 612-L, and 257-4 (**Figure 1(b)**). Accordingly, strains were classified into two groups: A (strains 1080, L9B6, L8Ao3, and RV2), and B (strains 261-2, 612-L, and 257-4).

Next, we 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 (**Figure 1(a)**), strains 1080, L9B6, L8Ao3, and RV2 presented hemolytic activity after introduction of ORF4 (**Figure 1(b)**). No hemolytic activity was observed in strains 261-2, 612-L, and 257-4 after introduction of ORF4 (**Figure 1(b)**). These results were consistent with those relating to colony morphology, as hemolysis was detected

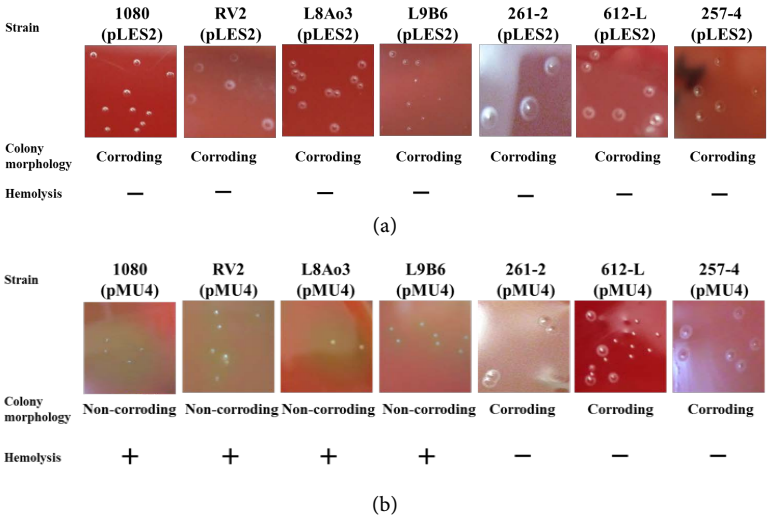

Figure 1. Colony morphology and hemolysis of *E. corrodens* strains. *E. corrodens* strains 1080, RV2, L8Ao3, L9B6 261-2, 612-L, and 257-4 were cultured on sheep blood agar at 37˚C. a) Strains transformed with empty vector pLES2; b) Strains transformed with ORF4 on pMU4. Colony morphology and hemolysis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

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 [16]. However, bacteria require an iron concentration of 0.05 - 0.5 μM for growth [17]. 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 [18]. Therefore, hemolysis is thought to be important for the pathogenicity of many in vading bacteria and increasing hemolytic activity through introduction of ORF4 might be vital for *in vivo* survival of oral bacteria.

3.2. Effect of ORF4 on Genomic Recombination at the Type IV Pilin Gene Locus

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

Given that the pili on the cell surface are involved in the cells' twitching motility as well as colony morphology [19] [20], 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.

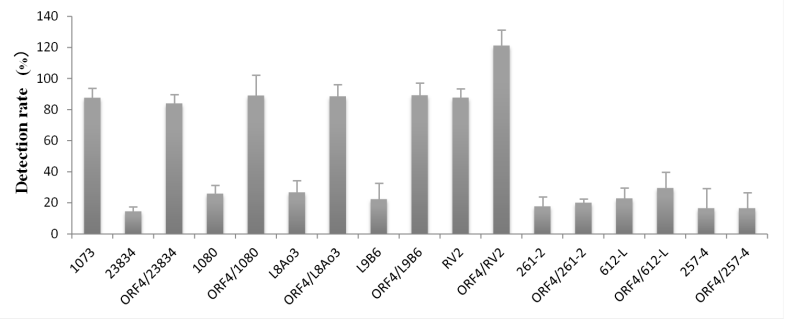

Figure 2. Detection of genomic recombination at the type IV pilin gene locus by real-time PCR using specific primers.

3.3. 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, we measured HA activity in seven *E. corrodens* strains. As shown in **Figure 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.

We reported previously that genomic recombination by plasmid-mediated recombinase stimulated simultaneous GalNAc-dependent lectin activity and hemolytic activity in *E. corrodens* 23834 [11] [12]. Recently, we demonstrated that hemolytic activity decreased upon addition of GalNAc [21]. Here, we show that the introduction of ORF4 enhanced both hemolytic activity and GalNAc-dependent lectin activity (**Figure 1** and **Figure 3**). These findings suggest that hemolytic activity correlates with lectin activity. Moreover, we 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 [21]. 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.

3.4. Effect of ORF4 on Biofilm Formation

Previously, we reported that introduction of ORF4 into strain 23834 resulted in increased biofilm formation [11]. Here, we investigated whether the same occurred in other *E. corrodens* strains. As shown in **Figure 4**, biofilm formation increased in group A strains following introduction of ORF4 on pMU4, but not in group B strains.

We suggested earlier that the GalNAc-specific lectin and other factors contributed additively to biofilm formation in some strains of *E. corrodens* [11]. In this study, we demonstrate that the introduction of ORF4 enhanced both GalNAc-specific lectin activity and biofilm formation. Accordingly, the GalNAc-specific lectin might be involved in biofilm formation by *E. corrodens*.

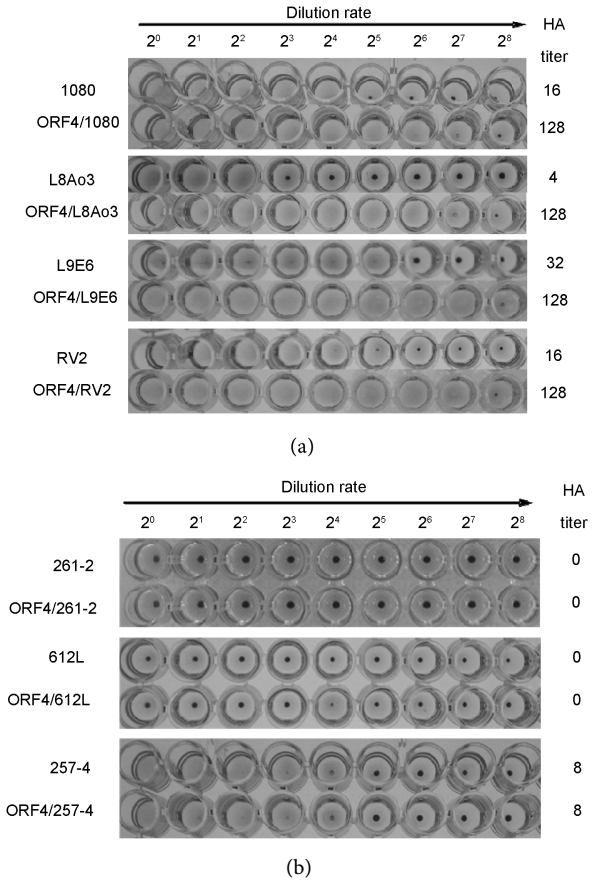

Figure 3. Hemagglutination of rabbit erythrocytes by cell cultures of E. corrodens. (a) Strains 1080, L8Ao3, L9B6, and RV2 transformed or not transformed with ORF4 on pMU4; (b) Strains 261-2, 612-L, and 257-4 transformed or not transformed with ORF4 on pMU4.

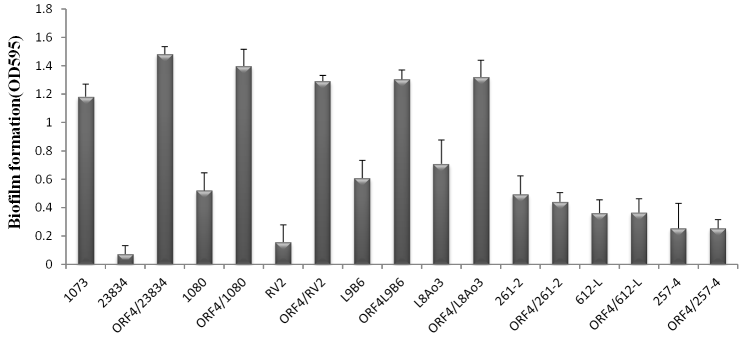

Figure 4. Biofilm formation by *E. corrodens* strains. Strains 1073, 23834, 1080, RV2, L9B6, L8Ao3, 261-2, 612-L, and 257-4 transformed or not transformed with ORF4 on pMU4 were grown aerobically in TSB medium containing hemin and KNO_3 at 37˚C using polystyrene microtiter plates.

3.5. Effect of ORF4 on the Growth Rate of *E. corrodens*

Given that in our previous study, the introduction of ORF4 stimulated growth rate [11], we investigated whether the same occurred in other *E. corrodens* strains. As shown in **Figure 5**, growth rate in group A strains increased after in-

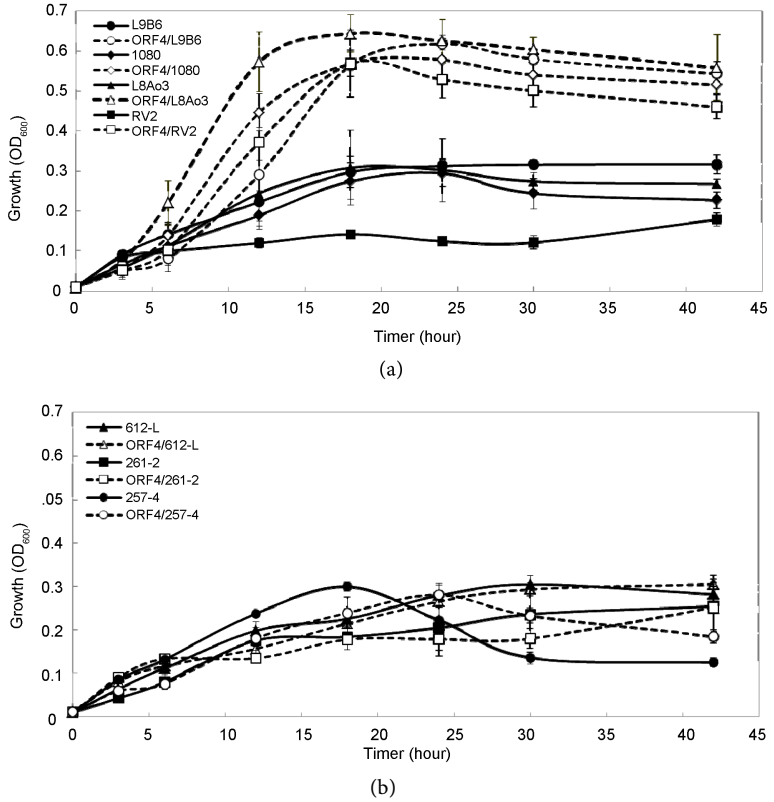

Figure 5. Growth rates of *E. corrodens* strains. a) Strains L9B6, 1080, L8Ao3, and RV2 transformed or not transformed with ORF4 on pMU4; b) Strains 612-L, 261-2, and 257-4 transformed or not transformed with ORF4 on pMU4.

troduction 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 [22]. 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.

3.6. Conclusions

In the present study, colony morphological change, genomic recombination at the pilin gene locus, and enhanced GalNAc-specific lectin activity, hemolytic activity, biofilm formation, and growth rate were observed simultaneously following introduction of ORF4 into *E. corrodens* in four of the seven clinical isolates tested. These results suggest that the changes recorded in the four strains might be attributed to the same mechanism. Given that ORF4 is homologous to the gene encoding, a recombinase specific for the type IV pilin gene, genomic recombination at the pilin locus is likely caused by introduction of ORF4. Moreover, changes to colony morphology may be associated to the loss of pili derived from genomic recombination. However, it is presently unknown why enhancement of biofilm formation, lectin activity, hemolytic activity, and growth rate was observed following introduction of ORF4. One possibility is that the intro-

duction of ORF4 may cause genomic recombination at multiple loci. Furthermore, as we did not observe any changes in three of the strains, it may be that the target sequences for genomic recombination is not located in their genomes. It remains to be seen how and where ORF4-mediated genomic recombination occurs, and how it promotes pathogenicity and virulence of *E. corrodens*.

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