Plasmid-mediated genomic recombination at the pilin gene locus enhances the *N*-acetyl-ᴅ-galactosamine-specific haemagglutination activity and the growth rate of *Eikenella corrodens*

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Eikenella corrodens belongs to a group of periodontopathogenic bacteria and forms unique corroding colonies on solid medium due to twitching motility. It is believed that an *N*-acetyl-ᴅ-galactosamine (GalNAc)-specific lectin on the cell surface contributes significantly to its pathogenicity and can be estimated by its haemagglutination (HA) activity. Recently, a plasmid, pMU1, from strain 1073 has been found; this plasmid affects pilus formation and colony morphology. To identify the gene involved in these phenomena, ORF 4 and ORFs 5–6 on pMU1 were separately subcloned into a shuttle vector, and the resultant plasmids were introduced into *E. corrodens* 23834. Transformants with the ORF 4 gene, which is identified to be a homologous gene of the type IV pilin gene-specific recombinase, lost their pilus structure and formed non-corroding colonies on a solid medium, whereas transformants with ORFs 5–6 exhibited the same phenotype as the host strain 23834. Southern analysis showed that the introduction of the ORF 4 gene into strain 23834 resulted in genomic recombination at the type IV pilin gene locus. The hybridization pattern of these transformants was similar to that of strain 1073. These results suggest that ORF 4 on pMU1 encodes a site-specific recombinase and causes genomic recombination of the type IV pilin gene locus. Furthermore, the introduction of ORF 4 into strain 23834 increased GalNAc-specific HA activity to a level equivalent to that of strain 1073. Although the morphological colony changes and loss of pilus structure are also observed in phase variation, genomic recombination of the type IV pilin gene locus did not occur in these variants. Moreover, an increase was not observed in the GalNAc-specific HA activity of these variants. These results suggested that the loss of pilus structure, the morphological change in colonies and the increase in HA activity due to plasmid pMU1 might be caused by a mechanism that differs from phase variation, such as a genomic recombination of the type IV pilin gene locus.

INTRODUCTION

Eikenella corrodens is a Gram-negative, facultatively anaerobic, rod-shaped bacterium; it is predominantly found in subgingival plaque in patients with advanced periodontitis (Tanner *et al.*, 1979). This bacterium can also be pathogenic, causing a variety of soft-tissue and wound infections (Chen & Wilson, 1992), such as endocarditis (Decker *et al.*, 1986) and other opportunistic infections. Monoinfection of germ-free or gnotobiotic rats with *E. corrodens* causes periodontal disease with severe alveolar bone loss (Listgarten *et al.*,

1978). Since *E. corrodens* is observed in the tooth-attached plaque area (Noiri *et al.*, 2001), it is believed that this bacterium may participate in the early stages of biofilm formation by specific coaggregation with Gram-positive and -negative bacteria in human periodontal pockets.

We have previously found that *E. corrodens* 1073 expresses a cell-associated *N*-acetyl-ᴅ-galactosamine (GalNAc)-specific *Eikenella corrodens* lectin-like substance (*Ec*LS) that mediates adherence to various host-tissue cell surfaces (Ebisu & Okada, 1983; Yamazaki *et al.*, 1981, 1988; Miki *et al.*, 1987). We have also reported that *Ec*LS mediates the coaggregation of *E. corrodens* with some strains of *Streptococcus sanguis* and *Actinomyces viscosus* that are predominant during the early

Abbreviations: GalNAc, *N*-acetyl-ᴅ-galactosamine; HA, haemagglutination.

stages of dental plaque formation (Ebisu et al., 1988). Since all these bacteria are thought to be the early colonizers in dental plaque (Kolenbrander et al., 2002), E. corrodens might play a key role in dental plaque formation. Moreover, we have reported that EcLS stimulates the mitogenic activity of B lymphocytes (Nakae et al., 1994). Furthermore, we have reported that E. corrodens 1073 induces KB cells (a human oral epidermoid carcinoma cell line) to secrete and express IL-6 and IL-8; a direct contact of E. corrodens with KB cells is not necessarily required for the stimulation of IL-6 and IL-8 secretion and expression (Yumoto et al., 2001). Thus, it is thought that EcLS contributes to the pathogenicity and virulence of E. corrodens and that its expression can be estimated by its haemagglutination (HA) activity.

Like several Gram-negative pathogens, including Neisseria gonorrhoeae, Neisseria meningitidis and Moraxella bovis, E. corrodens exhibits a phase variation resulting from the altered synthesis of type IV pili, which is reflected in the changes in colony morphology (Villar et al., 1999, 2001). Most type IV pili are composed primarily of type IV pilin, a protein that is approximately 150 to 165 amino acids long and is synthesized as a precursor form (prepilin) that contains a basic leader sequence of variable length (4–25 residues), with one exception: in N. gonorrhoeae (Wolfgang et al., 2000). Following translation, prepilin is cleaved at an atypical site by a cognate peptidase that simultaneously methylates the resultant amino-terminal amino acid, typically a phenylalanine residue. Following processing, the mature pilin is exported to the cell surface by a specific transport mechanism and assembled into pili (Strom & Lory, 1993). Type IV pili are thought to be involved in bacterial adhesion and to function as antigenic determinants.

On a solid medium, E. corrodens 1073 forms large, non-corroding colonies, whereas other strains form small, corroding colonies due to twitching motility. Rao & Progulske-Fox (1993) and Tonjum et al. (1993) have cloned two type IV pilin genes, from E. corrodens 23834 and E. corrodens 31745, respectively. However, in our previous electron microscopic study of E. corrodens 1073, we did not observe any pilus-like structure (Ebisu et al., 1981), although other investigators have suggested the presence of pili (Hood & Hirschberg, 1995). Therefore, the molecular mechanism(s) of phase variation in E. corrodens, including the formation of type IV pili, remains to be determined.

Recently, we isolated the plasmid pMU1 from E. corrodens 1073 and discovered seven ORFs on it (Azakami et al., 2005a). We demonstrated that the transformation of a derivative of pMU1 into strain 23834 resulted in loss of pilus structure from the cell surface and a morphological change in the colonies, i.e. from a corroding to a non-corroding form. In this study, we showed that the gene encoding the pilin gene-specific recombinase piv on plasmid pMU1 is involved in these phenomena. Moreover, we have suggested that the genomic recombination of the pilin gene locus of E. corrodens 23834 increases GalNAc-specific lectin activity.

METHODS

Bacterial strains, plasmids and media. E. corrodens 1073 was provided by S. S. Socransky (Forsyth Dental Center, Boston, MA, USA) and E. corrodens 23834 was obtained from the American Type Culture Collection (ATCC). Escherichia coli XL-1 Blue was used for cloning and sequencing. The Esch. coli/E. corrodens shuttle vector pLES2 (Stein et al., 1983) was obtained from the ATCC. E. corrodens cells were grown in tryptic soy broth containing 2 mg KNO$_3$ ml^{-1} and 5 mg haemin ml^{-1} or on sheep blood agar plates at 37 °C. Esch. coli XL-1 Blue was routinely cultured in LB broth or agar. Bacteria harbouring plasmids were cultured on a medium supplemented with 50 μg carbenicillin ml^{-1}.

Transformation of E. corrodens strains. E. corrodens was electrotransformed with 5–10 μg of plasmid DNA using a Gene Pulser electroporator (Bio-Rad). In brief, a 100 ml volume of bacteria 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. Next, a 39 μl volume of bacteria was mixed with 1 μl DNA and electroporated at 2·1 kV, 25 μF and 200 Ω. For transformations involving the broad-host-range shuttle vector pLES2 and its derivatives, cells were recovered by centrifugation after incubation for 12 h at 37 °C. After recovery, recombinant cells were cultured on sheep blood agar plates containing 50 μg carbenicillin ml^{-1} at 37 °C.

Curing experiment. Acridine orange was added at a final concentration of 100 μg ml^{-1} to a medium inoculated with approximately ten exponential-phase cells per millilitre. After 24 h incubation at 37 °C, the culture was diluted with the medium and plated out on sheep blood agar plates. The colonies on the plates were used for further experiments. Cured colonies were identified as those that were sensitive to carbenicillin.

Transmission electron microscopy. Drops of water (20 μl) were deposited on 24 h-old E. corrodens colonies on the sheep blood agar plates, and within 30 s, a 200 mesh Formvar-coated copper grid was floated on the surface of each drop for 30 s. The grids were removed, floated on a drop of 1 % phosphotungstic acid for 1·5 min, and the excess dye was then removed. Grids were dried and examined at 75 kV in a Hitachi H-7600 electron microscope.

Southern blot analysis. After electrophoresis on agarose gels, DNA fragments were transferred onto a Hybond-N membrane (Amersham Bioscience). Hybridization of the membrane with a labelled probe and the detection of hybridized bands were conducted using a non-radioactive DNA labelling and detection kit (Amersham). The probe including the ecpAB region was amplified using genomic DNA of strain 23834 as a template and primers 5′-AAGCCTAGCCGGAAA-TGAAG-3′ and 5′-TTCGGAGGACAATATGCAGC-3′.

HA activity assay. The HA activity assay was performed as previously described (Ebisu & Okada, 1983). Erythrocytes that were obtained by the centrifugation of preserved rabbit blood were washed three times with saline before being suspended in PBS (pH 7·2) at a concentration of 2 %. The HA assay was performed in microtitre plates (Vdispo; Nalge Nunc International). Test preparations (50 μl) were serially diluted twofold in PBS and mixed for 2 min with equal volumes of the 2 % erythrocyte suspension. HA activity was examined after 1 h, and HA titres were expressed as the maximum dilution of the test preparation that exhibited HA.

RESULTS

ORF 4 irreversibly altered colony morphology

We have recently isolated a plasmid, pMU1, from E. corrodens 1073. As shown in Fig. 1(A), seven ORFs, which have
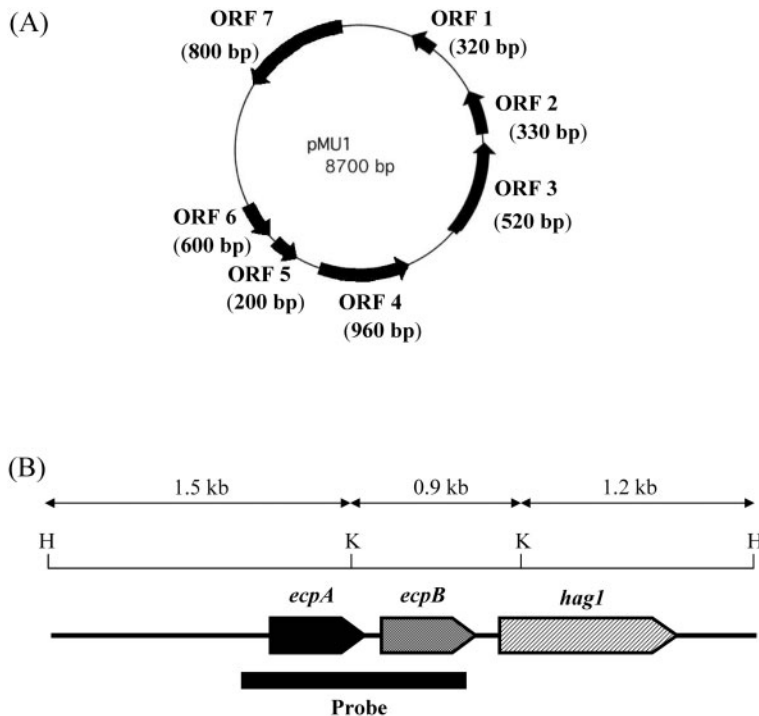
Fig. 1. (A) Physical map of plasmid pMU1. The arrows indicate the sizes and positions of ORFs 1–7. (B) Physical map of the *ecpAB* locus for *E. corrodens* 23834. The arrows indicate the sizes and positions of the ORFs. Flanking and internal restriction sites are shown. K, *Kpn*I; H, *Hind*III. The bar under the arrows indicates the probe used for Southern hybridization.

been designated ORF 1 to ORF 7, are found on the same strand of pMU1. Introduction of the fragments from ORF 4 to ORF 7 into strain 23834 causes irreversible changes in colony morphology (Azakami *et al.*, 2005a). Although strain 23834 forms corroding colonies on a solid medium, all the transformants carrying pMU1 were altered to non-corroding colonies. To identify the gene responsible for this effect, we subcloned ORF 4 and ORFs 5–6 into the *Esch. coli/E. corrodens* shuttle vector pLES2 and introduced the resultant plasmids pMU4 and pMU5–6 into *E. corrodens* 23834. As shown in Fig. 2, all transformants with pMU4 formed non-corroding colonies on the selection medium (panel B), whereas the transformants with pMU5-6 or pLES2 vectors formed corroding colonies similar to those of strain 23834 (panel A). Even after the elimination of pMU4 by acridine orange treatment, the transformants showed the non-corroding colony morphology (data not shown). These results suggest that ORF 4 on plasmid pMU1 irreversibly altered the colony morphology of *E. corrodens* from corroding to non-corroding.

Introduction of ORF 4 caused loss of pili

It has been reported that *E. corrodens* exhibits a phase variation that results from the altered synthesis of type IV pili, which is reflected by the changes in colony morphology (Villar *et al.*, 1999, 2001). Thus, it is believed that *E. corrodens* forms corroding colonies on solid medium due to mobility imparted by pili. As described above, we showed that ORF 4 on pMU1 was involved in morphologically changing the colony from corroding to non-corroding. To determine whether the morphological change induced by ORF 4 was caused by the loss of pili, we observed the cell surface structure of transformants with pMU4 by transmission electron microscopy. As shown in Fig. 3, although strain 23834 had the pilus structure on its cell surface (panel A), strain 1073 carrying pMU1 did not have any pilus (panel B). Pilus structure was not observed on the surface of strain 23834 (panel B), whereas strain 23834 (pMU5-6) and strain 23834 (pLES2) maintained the pili on the surface (panel A).
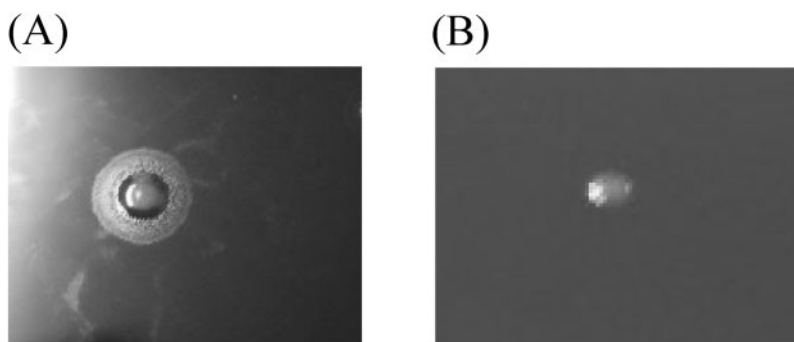
Fig. 2. Colony morphology of *E. corrodens* strains. Cells were cultured on sheep blood agar at 37 °C. (A) Corroding colony; (B) non-corroding colony.
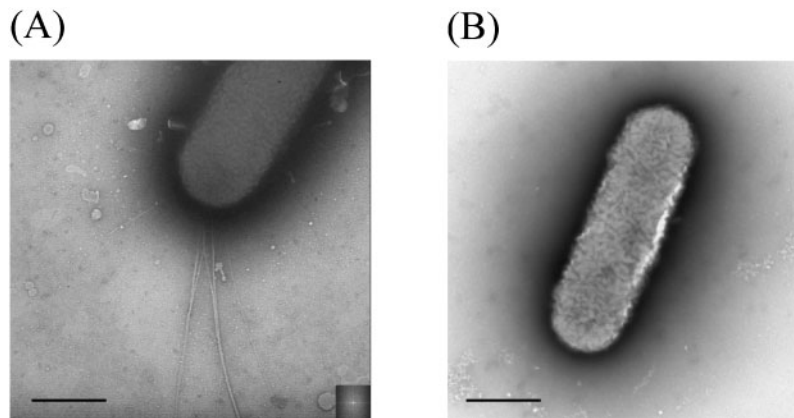
Fig. 3. Transmission electron micrographs of *E. corrodens* strains. Pilus structures were observed on the cell surface of 23834, 23834 (pLES2), 23834 (pMU5-6) and 23834 (phase variants) (panel A), but not on those of 23834 (pMU4) and 1073 (panel B). Negative staining was performed with 1% phosphotungstic acid. Bars, 0·5 μm.

These results suggest that ORF 4 is involved in the loss of pilus structure in strain 23834. Moreover, it is suggested that the morphological changes in the colonies due to ORF 4 might be caused by loss of pili.

Introduction of the *piv* gene causes recombination of the *ecpAB* locus

A homology search revealed that ORF 4 is highly homologous to the gene encoding the type IV pilin gene-specific recombinase. Rao & Progulske-Fox (1993) have cloned two type IV pilin genes, *ecpA* and *ecpB*, from *E. corrodens* 23834 and have shown that they are located in tandem on its genome (Fig. 1B). To determine whether ORF 4 causes genomic recombination of pilin genes, we performed genomic Southern hybridization using the *ecpAB* region as a probe. We cloned a 1·2 kb fragment, including *ecpA* and *ecpB*, from strain 23834. Chromosomal DNAs were isolated from strains 23834, 23834 (pLES2), 23834 (pMU4) and 1073. These were digested with *Kpn*I and *Hin*dIII or with *Hin*dIII alone, and then hybridized to a 1·2 kb pilin gene fragment. As shown in Fig. 4(A), two bands of approximately 1·5 and 0·9 kb were detected in strains 23834 (lane 2)

and 23834 (pLES2; lane 4). However, the hybridized band was shifted to a single band of 2·7 kb in 23834 (pMU4; lane 5). The shifted band of 23834 (pMU4) was of the same size as that of strain 1073 (lane 6). These results suggested that ORF 4 might cause the recombination of the pilin gene locus on the chromosome, including the *ecpA* and *ecpB* genes. As shown in Fig. 4(B), similar genomic recombination was observed for the *Hin*dIII-digested genomic DNA. This result confirmed that ORF 4 might be involved in genomic recombination with the pilin gene locus.

Introduction of the *piv* gene enhances HA

Strain 1073 exhibits greater HA activity than the other *E. corrodens* strains. We felt that this depends on the ORF 4 gene; therefore, we assayed the HA activity of the strains transformed with pMU4. As shown in Table 1, 23834 (pMU4) exhibited strong HA activity (128-fold), whereas strains 23834 and 23834 (pLES2) exhibited weak activity (16-fold). The increased HA activity of strain 23834 (pMU4) was inhibited in the presence of GalNAc (data not shown). These results suggested that a GalNAc-specific lectin on the cell surface might be exposed by the loss of pilus structure.
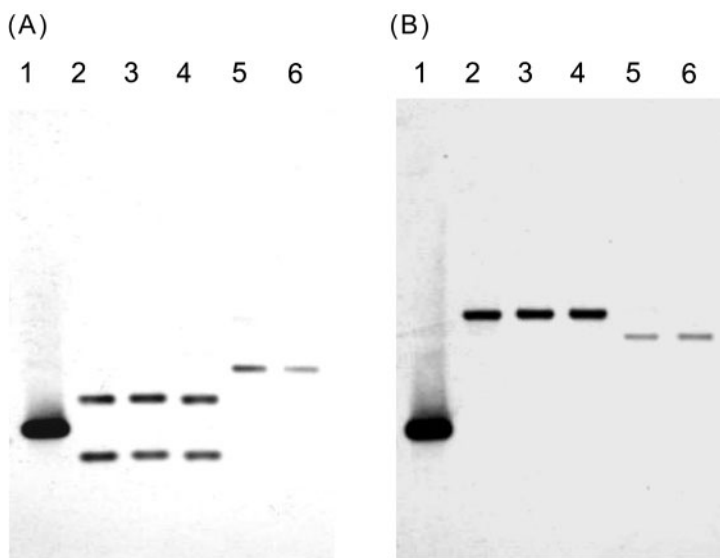
Fig. 4. Southern hybridization of (A) *Kpn*I- and *Hin*dIII-digested chromosomal DNA and (B) *Hin*dIII-digested chromosomal DNA from *E. corrodens* strains using a 1·2 kb *ecpAB* fragment as probe. (A) Total DNA was prepared from strain 23834 (corroding) (lane 2), strain 23834 (non-corroding phase variant) (lane 3), strain 23834 (pLES2) (lane 4), strain 23834 (pMU4) (lane 5), and strain 1073 (lane 6). PCR product including *ecpAB* was applied in lane 1 as a positive control in both panels.

Table 1. Haemagglutination of rabbit erythrocytes by cell cultures of *E. corrodens* strains

Haemagglutination titres were expressed as the maximum dilution of the test preparation that still showed haemagglutination. Values shown are means of results from three independent experiments. +GalNAc: to determine the effect of the GalNAc-specific lectin on haemagglutination, GalNAc (1 mM) was added to the assays.

Strain	Haemagglutination titre	
	− GalNAc	+ GalNAc
1073	128	0
23834	16	0
23834 (non-corroding variant)	16	0
23834 (pLES2)	16	0
23834 (pMU4)	128	0
23834 (pMU5–6)	16	0

The increased activity of strain 23834 (pMU4) was equivalent to that of strain 1073.

Morphological colony changes and loss of pili resulting from the presence of ORF 4 are caused by a mechanism that differs from phase variation

Most *E. corrodens* strains form small corroding and non-corroding colonies on solid medium. The non-corroding variants arise irreversibly from corroding variants at a frequency much greater than that of mutation rates (Villar *et al.*, 1999). To investigate whether morphological changes in the colonies and loss of pili resulting from the presence of ORF 4 are caused by phase variation, we compared the properties of strain 23834 (pMU4) with those of phase variants from strain 23834. The phase variation of strain 23834 from corroding to non-corroding did not alter the pilus structure on the cell surface (Fig. 3) or the HA activity (Table 1). Moreover, in phase variants of strain 23834, no genomic recombination was observed in the *ecpAB* region (Fig. 4, lane 3). These results suggested that the increase in HA activity resulting from the presence of plasmid pMU1 might be due to genomic recombination of the pilin gene

locus. Furthermore, it is suggested that the loss of pili might be a result of this recombination and might not contribute directly to the increase in HA activity.

Introduction of the *piv* gene improves the growth of *E. corrodens*

E. corrodens 23834 grows at a slower rate than strain 1073 (Fig. 5). Cultures of strain 23834 reached stationary phase at $OD_{600} = 0.4$, whereas strain 1073 grew until $OD_{600} = 0.7$. Moreover, the lag phase of strain 23834 was longer than that of strain 1073. We compared the growth curve of strain 23834 (pMU4) with that of strain 23834. Interestingly, the growth curve of strain 23834 (pMU4) was nearly the same as that of strain 1073.

DISCUSSION

In a previous study, we found a plasmid, pMU1, from *E. corrodens* 1073 that affects colony morphology and pilin expression (Azakami *et al.*, 2005a). In this study, we have demonstrated that ORF 4 was responsible for both the changes (Figs 2 and 3). These results suggested that a product of ORF 4 might be related to the loss of pilus structure from the cell surface of *E. corrodens*. It has been reported that the pili on a cell surface are involved in the twitching motility of cells as well as colony morphology (McMichael, 1992; Villar *et al.*, 2001). Thus, we considered that the morphological change in the colonies from corroding to non-corroding occurred due to a loss of pili. Since the introduction of pMU4 into strain 23834 increased GalNAc-specific HA activity (Table 1), we felt that a GalNAc-specific lectin was exposed due to the loss of the pilus structure from the cell surface. However, this hypothesis was refuted because phase variants from strain 23834 exhibited similar HA activity (Table 1), but formed non-corroding colonies and had lost their pili in a similar manner to strain 23834 (pMU4) (Figs 2 and 3). This was confirmed by the finding that no genomic recombination was observed in the phase variants (Fig. 4). Although most of the *E. corrodens* 23834 formed corroding colonies, non-corroding variants arose irreversibly from corroding variants at a considerable frequency. Therefore, we concluded that GalNAc-specific lectin activity was increased due to genomic recombination within
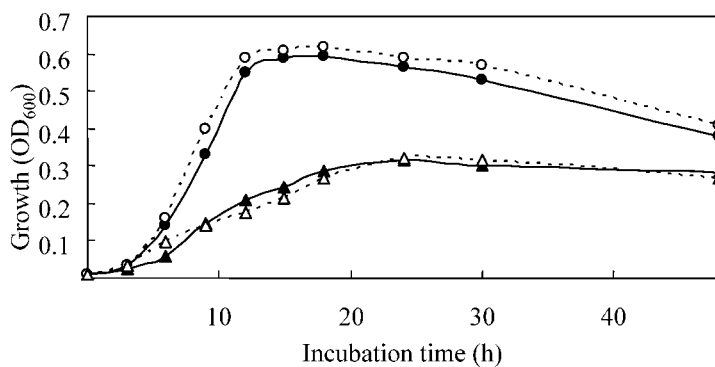
Fig. 5. Comparison of growth curves for *E. corrodens* strains 1073 (●), 23834 (corroding) (▲), 23834 (pMU4) (○), and 23834 (non-corroding) (△). Bacterial growth was monitored spectrophotometrically by OD_{600}.

Table 2. Comparison of characteristics of *E. corrodens* strains

Strain	Colony morphology*	Pili†	Haemagglutination activity	Genomic recombination‡	Growth rate
1073	N	−	High	+	High
23834	C	+	Low	−	Low
23834 (phase variant)	N	−	Low	−	Low
23834 (pLES2)	C	+	Low	−	Low
23834 (pMU4)	N	−	High	+	High

*Cells were cultured on sheep blood agar at 37 °C. N, Non-corroding colonies; C, corroding colonies.

†Pilus structure on the cell surface was observed by transmission electron microscopy.

‡Genomic recombination in the *ecpAB* locus was detected by Southern hybridization using an *ecpAB* fragment as a probe.

the *ecpAB* locus. Since it is believed that GalNAc-specific lectin contributes very significantly to the pathogenicity and virulence of *E. corrodens*, these organisms may require plasmid pMU1 to survive in the oral environment. We have summarized the characteristics of the *E. corrodens* strains in Table 2.

Previous homology database examination has revealed that ORF 4 is homologous to the *piv* gene encoding the pilin gene-specific recombinase of other pathogenic bacteria, such as *Mor. bovis* and *N. meningitidis* (Azakami *et al.*, 2005a). For example, the genes encoding the major pilin subunits of *Mor. bovis* are located on an invertible segment of DNA that allows alternate expression of *tfpQ* or *typI* (type IV pilin; Fulks *et al.*, 1990). The *piv* gene, located immediately adjacent to the invertible segment, encodes the site-specific DNA invertase. The expression of the type IV pili in *Mor. bovis* is regulated by a site-specific DNA inversion system by Piv (Heinrich & Glasgow, 1997). Thus, we assumed that the loss of pili following transformation of ORF 4 might be due to genomic recombination in the pilin genes. In this study, it is suggested that transformation of ORF 4 results in genomic recombination within the *ecpAB* locus, although the recombination sites and the events following recombination are yet to be determined (Fig. 4). Moreover, the shifted band of strain 23834 with the introduced ORF 4 gene was of the same size as that of strain 1073 carrying pMU1. This finding suggests that strains 1073 and 23834 might be derived from the same origin and that strain 1073 might have evolved from strain 23834 by horizontal transfer of a plasmid. A homology search has revealed that gene clusters homologous to pMU1 are located in other bacterial genomes (Azakami *et al.*, 2005a). Genes homologous to all the ORFs on pMU1 are found in the genome of *N. meningitidis*. Moreover, genes homologous to ORFs 1, 3, 6 and 7 of pMU1 are found on a plasmid, pJTPS1, of *Ralstonia solanacearum*. These gene clusters might also be transferred horizontally in the environment. Following the acquisition of pMU1, strain 1073 may have acquired the ability to adhere in an oral environment, and this may have contributed to its high periodontal pathogenicity.

As shown in Fig. 5, the growth of strain 23834 was improved by the introduction of pMU4. Although the reason for this effect is not yet clear, it is thought that this might occur as the result of the genomic recombination by ORF 4. Moreover, we have found that *E. corrodens* forms a biofilm on polystyrene surfaces and that the biofilm formation of strain 23834 is increased remarkably after the introduction of pMU4 (Azakami *et al.*, 2005b). Although pili are required for the initial attachment process in most bacteria, it is thought that the lectin on the cell surface plays an important role in *E. corrodens*. Therefore, it is suggested that biofilm formation by *E. corrodens* depends on the lectin rather than pili. It is believed that biofilm formation is a process by which pathogenic bacteria attach to a solid surface and thereby enhance their pathogenicity (Kolenbrander *et al.*, 2002). Therefore, along with an increase in growth, increased biofilm formation caused by pMU4 might also be one of the strategies by which *E. corrodens* survives in the oral cavity and enhances its pathogenicity and virulence.

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