Two type IV pili of Vibrio parahaemolyticus play different roles in biofilm formation

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Vibrio parahaemolyticus; pili; biofilm; attachment; chitin.

Abstract
Vibrio parahaemolyticus RIMD2210633 has two sets of type IV-A pilus genes. One set is similar to that found in other Gram-negative bacteria, such as Pseudomonas aeruginosa, Vibrio cholerae (chitin-regulated pilus; ChiRP), and Vibrio vulnificus. The other is homologous to the genes for the mannose-sensitive hemagglutinin (MSHA) pilus. In this study, we analyzed the effects of the deletions in the pilin genes for each type IV pilus (the ChiRP and the MSHA pilus) on biofilm formation. Although the MSHA pilin mutant formed aggregates, the number of bacteria that attached directly to the coverslip was reduced, suggesting that this pilus contributes to the bacterial attachment to the surface of the coverslip. In contrast, the ChiRP mutant attached to the surface of the coverslip, but did not form aggregates, suggesting that ChiRP plays a role in bacterial agglutination during biofilm formation. These results suggest that the two type IV pili of V. parahaemolyticus contribute to biofilm formation in different ways. Both mutants showed a lower fitness for adsorption onto chitin particles than that of the wild type. Collectively, these data suggest that the use of two type IV pili is a refined strategy of V. parahaemolyticus for survival in natural environments.

Introduction
Vibrio parahaemolyticus, one of the human pathogenic vibrios, causes seafood-associated gastroenteritis (Honda & Iida, 1993). In most natural environments, microorganisms such as V. parahaemolyticus survive by forming communal structures known as biofilm (Costerton et al., 1995). Biofilm formation may provide bacteria with adaptive advantages (Kimberly, 2004), and is therefore important as a bacterial survival strategy. Despite its importance, there have been few reports of biofilm formation by V. parahaemolyticus (Lawrence et al., 1992; Enos-Berlage et al., 2005). Although Enos-Berlage et al. (2005) described the participation of many genes in the development of biofilms, it is still unclear how the products of these genes contribute to biofilm formation.

The production of a biofilm by Gram-negative microorganisms involves consecutive, discrete stages: (1) the planktonic stage, during which free-swimming bacteria approach the surface; (2) the monolayer stage, during which bacteria attach to the surface; and (3) when the bacteria move along the surface and associate with each other to form three-dimensional structures. Type IV pili (TFP), unique appendages on the bacterial surface, are reported to be important in biofilm development (Costerton et al., 1999).

The V. parahaemolyticus RIMD2210633 genome project (Makino et al., 2003) revealed that this organism has two sets of type IV-A pilus genes. In this study, we analyzed the contributions made by these two TFPs to biofilm formation by V. parahaemolyticus.

Materials and methods

Bacterial strains, plasmids, and culture conditions
Vibrio parahaemolyticus strain RIMD2210633 (Makino et al., 2003) was obtained from the Laboratory for Culture Collection, Research Institute for Microbial Diseases, Osaka University, and was used for the construction of deletion mutants and for functional studies. Escherichia coli strains DH5α and BW19851 were used for general genetic manipulations. Bacteria were cultured at 24 °C (assays for biofilm
formation and competition assays for chitin binding) or 37 °C (all other assays) in Luria–Bertani broth (LB), LB supplemented with 3% NaCl (for V. parahaemolyticus), in marine broth (for biofilm assays), or in M9 with 3% NaCl, 2 mM MgSO₄, and 0.5% maltose (for β-galactosidase and competition assays). Thiourea–citrate–bile-salt–sucrose (TCBS) agar was used to screen the mutant strains. Whenever necessary, antibiotics were used at the following concentrations: ampicillin, 100 μg mL⁻¹; kanamycin, 50 μg mL⁻¹; chloramphenicol, 5 μg mL⁻¹; gentamicin, 10 μg mL⁻¹; and nalidixic acid, 128 μg mL⁻¹.

Construction of pilA and mshA mutant strains

Chitin-regulated pilus (ChiRP) pilin gene (pila) and mannose-sensitive hemagglutinin (MSHA) pilin gene (msha) single-deletion mutants were created by overlap PCR (primary sequences are shown in Table 1), as described previously (Park et al., 2004). The final PCR products, containing deletions in the pilA or msha genes, were cloned into the pT7Blue T-vector (Novagen Inc.), and then removed with the appropriate restriction enzymes (BamHI and Sphi for the pilA mutant, or BamHI and PstI for the msha mutant). They were then cloned into the suicide vector pYAK1 (Kodama et al., 2002). The resulting plasmids were introduced into E. coli BW19851 before conjugation with V. parahaemolyticus RIMD2210633. TCBS agar containing chloramphenicol was used to screen the deletion mutants, which were then selected on LB plates supplemented with 10% sucrose. pilA and msha double mutants were created by the same method using the pilA deletion mutant.

Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Oligonucleotide sequence (5' → 3')*</th>
</tr>
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<tbody>
<tr>
<td>Deletion of pilA</td>
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<tr>
<td>729-01</td>
<td>GGATCCACGCAGAAACCGGAGTCG</td>
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<tr>
<td>729-02</td>
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<td>GTGGTAGCCTACGACGCTGTCGTAACG</td>
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<td>729-04</td>
<td>CGTATGATCCTCCAGGCTCTAACCGAATTACG</td>
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<tr>
<td>Deletion of msha</td>
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<td>227-01</td>
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<tr>
<td>323-01</td>
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<tr>
<td>227-03</td>
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<tr>
<td>227-04</td>
<td>CGTACGCTAAACGAGAACCCGGCTGCTAAGGCCC</td>
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<td>Cloning of pilA promoter region</td>
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<tr>
<td>611-01</td>
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<td>611-02</td>
<td>GAATTCCTCATTACAGTGAAACCTCG</td>
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<tr>
<td>Complementation of pilA</td>
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<td>1116-01</td>
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<td>Complementation of msha</td>
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<td>704-02</td>
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<td>RT-PCR of pilA</td>
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<tr>
<td>1115-01</td>
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<tr>
<td>Recombinant PilA</td>
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<tr>
<td>902-02</td>
<td>GTCGACGTACGCAATCTGAGAGAGG</td>
</tr>
</tbody>
</table>

*Underlined nucleotides represent engineered restriction enzyme sites.

Complementation of V. parahaemolyticus type IV pilus mutant strains

A DNA fragment encompassing the pilA promoter and pilA was amplified by PCR (the primer sequences are shown in Table 1). The amplicon was cloned into the pT7Blue T-vector, and then subcloned into pSA19CP-MCS (Nomura et al., 2000). This plasmid was then introduced into the pilA deletion mutant strain A34 by electroporation with the Gene Pulser apparatus (Bio-Rad), as previously described (Hamasima et al., 1990). TCBS agar containing chloramphenicol was used to screen for the complemented strain. To express msha in the complemented strain, the promoter of the structural gene for thermostable direct hemolysin (tdhA) was introduced by PCR upstream from the msha gene and was used to test for expression of MshA (the primer sequences are shown in Table 1) (Okuda & Nishibuchi, 1998).

Antibody production

The following synthetic peptides were used for antibody production: CGDLKKLDAGNT for MshA and CDADSIDGG for PilA. The peptides were synthesized and used to immunize rabbits to raise polyclonal antibodies directed against MshA or PilA, respectively (Sigma Genosys). Recombinant PilA protein was prepared with the E. coli pET system (Novagen). The DNA fragment containing pilA was generated by PCR using primers 902-05 and 902-02 (Table 1). This fragment was cloned into the pET28a expression vector. The plasmid was then introduced into E. coli BL21. Recombinant PilA protein expressed in E. coli was used to raise polyclonal antiserum, as described previously (Cantarelli et al., 2002).

Western blot analysis

Bacteria were grown overnight, and diluted 100-fold in marine broth, or M9 broth supplemented or not supplemented with GlcNAc mixture. Whole-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel (16%) electrophoresis. Following electrophoretic separation, the proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with anti-MshA or anti-PilA antiserum (Sigma Genosys), and
gentamicin was used to screen for the transformant. RIMD2210633 by electroporation. TCBS agar containing plasmid was introduced into the region was amplified by PCR (the primer sequences are shown in Table 1). Purified RNA from the tetracycline-resistance gene from an RNA LA PCR kit (AMV, ver. 1.1) was used as the positive control for RT-PCR analysis.

**RNA purification and reverse transcription-PCR (RT-PCR) analysis**

The RNeasy Mini Kit (Qiagen) was used to extract total RNA from the wild type strain grown in marine or LB broth. The RNA was treated with DNase I (TaKaRa) to eliminate residual genomic DNA and then used to generate cDNA with random primers (nonamers) and AMV Reverse Transcriptase XL (TaKaRa). The cDNA was examined by PCR for the presence of transcripts from the pilA gene (the primer sequences are shown in Table 1). Purified RNA from the tetracycline-resistance gene from an RNA LA PCR kit (AMV, ver. 1.1) was used as the positive control for RT-PCR analysis.

**β-Galactosidase assay**

A promoterless β-galactosidase reporter vector, pHRP309 (Parales & Harwood, 1993), was used to estimate the transcription level of the pilA gene. The pilA promoter region was amplified by PCR (the primer sequences are shown in Table 1) and cloned into pHRP309. The reporter plasmid was introduced into *V. parahaemolyticus* RIMD2210633 by electroporation. TCBS agar containing gentamycin was used to screen for the transformant.

The transcription level of the pilA gene was estimated from the activity of β-galactosidase. Briefly, overnight cultures were inoculated into M9 minimal medium (100-fold dilution) supplemented with 3% NaCl, 2 mM MgSO4, and 0.5% maltose, and grown until log phase (OD600 nm = 0.28). Then, 0.6 mM N-acetylglucosamine (GlcNAc) containing (GlcNAc)2–6 (chito-oligosaccharide mixture; Seikagaku Corporation) was added to the culture, which was further incubated for 0, 1, or 5 h (Meibom et al., 2004). The cultures were assayed for β-galactosidase activity, as described previously (Miller, 1972).

**Biofilm formation assays**

Overnight marine broth cultures were diluted 100-fold with fresh medium. An aliquot (200 μL) was transferred to a glass tube and incubated at the indicated temperature for 9 h. Biofilm formation was quantified as described previously (Watnick et al., 1999).

**Light microscopy (LM)**

Overnight bacterial cultures in marine broth were diluted 100-fold with fresh medium and 2 mL was transferred to each well of a six-well plate containing a glass coverslip. At the indicated end-point, the coverslips were rinsed twice with phosphate-buffered saline (PBS). Cells were fixed with 100% methanol for 5 min and then stained with 20% Giemsa solution. The preparations were observed by LM (× 300 magnification) using an Eclipse TE 2000-U microscope (Nikon) equipped with a charge-coupled device camera (DXM 1200; Nikon). A computer equipped with NIH image software was used for image acquisition, processing, and quantification.

**Scanning electron microscopy (SEM)**

Coverslips with attached bacteria were prepared as for LM. The coverslips were prefixed in 2% glutaraldehyde in 0.1 M PBS (pH 7.2) for 1 h (Giron et al., 1991) and postfixed in 1% osmium tetroxide in 0.1 M PBS (pH 7.2) for 1 h. The coverslips were dehydrated by stepwise replacement of the water with ethanol from 20% to 99%, and dried with t-butylalcohol in an ES-2030 freeze dryer (Hitachi). Processed coverslips were then coated with gold palladium and examined using a scanning electron microscope (S-4500, Hitachi).

**Competition assays for chitin binding**

Competition experiments were performed as described previously, with modifications (Meibom et al., 2004; Kirn et al., 2005). Chitin beads (New England Biolabs) were washed five times in PBS. Bacteria that had been grown in M9 supplemented with a GlcNAc mixture were also washed twice in PBS. Bacterial suspensions (mutant and wild type) were mixed in a 1:1 ratio, and added to the beads. The mixture was incubated at room temperature for 1 h with gentle shaking. The beads were washed three times and vigorously suspended in PBS by vortex agitation. The suspension was plated and the bacteria were quantified by viable plate counting. To differentiate the two strains, a spontaneous nalidixic-acid-resistant derivative of the wild

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**Table 2. Bacterial strains examined in the biofilm formation assay**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIMD2210633</td>
<td><em>Vibrio parahaemolyticus</em> O3:K6 tdh&lt;sup&gt;+&lt;/sup&gt; wild type</td>
</tr>
<tr>
<td>S8A</td>
<td>Spontaneous nalidixic-acid-resistant derivative of RIMD2210633</td>
</tr>
<tr>
<td>A34</td>
<td>pil&lt;sup&gt;A&lt;/sup&gt; (ChiRP pilin gene)-deleted derivative of RIMD2210633</td>
</tr>
<tr>
<td>M3</td>
<td>mshA (MSHA pilin gene)-deleted derivative of RIMD2210633</td>
</tr>
<tr>
<td>W15</td>
<td>pil&lt;sup&gt;A&lt;/sup&gt;- and mshA-deleted derivative of RIMD2210633</td>
</tr>
<tr>
<td>CAA34</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;, pSA19 carrying a fragment containing mshA and its promoter (pSA19-pili/A) was introduced into A34</td>
</tr>
<tr>
<td>CPA34</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;, pSA19 only was introduced into A34</td>
</tr>
<tr>
<td>CMM3</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;, pSA19 carrying a fragment containing mshA and tdh promoter (pSA19-mshA-tdh) was introduced into M3</td>
</tr>
<tr>
<td>CPm3</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;, pSA19 only was introduced into M3</td>
</tr>
</tbody>
</table>

then the proteins were detected with an ECL system (Amersham Biosciences).

The role of two type IV pili of *V. parahaemolyticus* (Parales & Harwood, 1993), was used to estimate the transcription level of the pilA gene. The pilA promoter region was amplified by PCR (the primer sequences are shown in Table 1). Purified RNA from the tetracycline-resistance gene from an RNA LA PCR kit (AMV, ver. 1.1) was used as the positive control for RT-PCR analysis.

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type was selected by previously reported selection methods (Johnson et al., 2005).

The fitness (\(w\)) of the deletion mutants relative to that of the marked wild type was calculated as:

\[
w(\text{tested strain}, \, T_S; \, \text{nalidixic-acid-resistant strain}, \, S8A) = \frac{\ln(TS_F/TS_0)}{\ln(S8A_F/S8A_0)}
\]

where \(TS_0\) and \(S8A_0\) are the initial CFUs, and \(TS_F\) and \(S8A_F\) are the CFUs at the end of the experiment.

**Results**

Two sets of genes for the biogenesis of type IV-A grouped pili were identified from the completed genome sequence of *V. parahaemolyticus* strain RIMD2210633 (Makino et al., 2003). One of these has significant homology to the TFP of *Pseudomonas aeruginosa* (Nunn et al., 1990), the third TFP of *Vibrio cholerae* (Fullner & Mekalanos, 1999), and the TFP of *Vibrio vulnificus* (Paranjpye & Strom, 2005). We tentatively refer to this TFP as the new type IV pilus, following *V. vulnificus* (Paranjpye & Strom, 2005). We tentatively refer to this TFP as the new type IV pilus, following (Fullner & Mekalanos, 1999). The other TFP has significant homology to the MSHA pilus of *V. cholerae* and *V. vulnificus* (Marsh & Taylor, 1999; Chen et al., 2003), which is peculiar to vibrios.

To examine the expression of the pilins of these TFPs in *V. parahaemolyticus* RIMD2210633, the bacteria were grown in marine broth, or M9 broth supplemented or not supplemented with GlcNAc mixture. The growth medium (marine broth, or M9 supplemented or not supplemented with GlcNAc mixture) did not affect the expression of MshA. In contrast, when we used polyclonal antiserum raised against PilA (the new type IV pilin), we observed no specific signal on Western blot analysis (data not shown). We also prepared recombinant PilA protein using the *E. coli* pET expression system (Novagen), and raised polyclonal antiserum as described previously (Can-tarelli et al., 2002). The resulting antiserum reacted with PilA protein expressed in *E. coli*, but no specific signal was obtained with this antiserum on *V. parahaemolyticus* grown in marine broth, or M9 supplemented or not supplemented with GlcNAc mixture. It has previously been reported that *V. vulnificus* expresses PilA at low levels under *in vitro* conditions (Paranjpye & Strom, 2005). We concluded that the production of PilA protein by *V. parahaemolyticus* under *in vitro* conditions might be too low to be detected with Western blotting. Therefore, we examined PilA expression at the transcriptional level by RT-PCR analysis. The presence of PCR products (361 bp) confirmed the transcription of pilA in RIMD2210633 grown in either marine or LB broth, confirming that pilA is transcribed in *V. parahaemolyticus* (data not shown).
Fig. 3. LM analysis of the biofilm development of the wild type and representative mutant strains on the surface of the coverslips. Strains were incubated in marine broth at 24 °C for 9 h. (a) A, RIMD2210633 (wild type); B, W15, pilA and mshA double mutant; C, M3, mshA mutant; D, CMM3, M3(pSA19-mshA); E, CPM3, M3(pSA19 only); F, A34, pilA mutant G, CAA34, A34(pSA19-pilA); and H, CPA34, A34(pSA19 only). Bar = 50 μm. Areas indicated by arrows are magnified. Experiments were repeated at least three times and representative images are shown here. (b) Total numbers of surface-attached cells. NIH image software was used for quantification.
Meibom et al. (2004) reported that the expression of the new type IV pilus by *V. cholerae* is induced by chitin. Therefore, using a β-galactosidase assay, we analyzed whether the expression of *pilA* encoding the new type IV pilus was also induced by chitin (GlcNAc mixture), in *V. parahaemolyticus* strains carrying a plasmid with a *pilA* promoter–*lacZ* fusion. The assays confirmed the induction of *pilA* by the GlcNAc mixture (Fig. 1), suggesting that *pilA* expression can also be induced by chitin. After this result, we renamed the new type IV pilus ‘ChiRP (chitin-regulated pilus)’, following a previous report (Meibom et al., 2004).

To determine the functions of these expressed TFPs, *V. parahaemolyticus* mutants with deletions in the pilin genes were constructed. The respective genes were also used to generate complemented strains (see Table 2 for a list of the strains used in this study). Mutations in the pilin genes did not affect the growth rates of these strains (data not shown).

To characterize biofilm formation, we first examined the extent of biofilm formation by the wild type strain (RIMD2210633). The accumulation of biomass on the walls of glass tubes was quantified using crystal violet, as described previously (Watnick et al., 1999). *Vibrio parahaemolyticus* formed more biofilm during culture in marine broth than in LB broth or M9 low-nutrient medium (data not shown). More stable biofilms were formed at 24 °C than at 37 °C or 30 °C (data not shown). Consequently, all biofilm formation assays were carried out in marine broth at 24 °C.

Investigation of the kinetics of biofilm development by the wild type strain grown in marine broth suggested that 9 h of incubation was suitable to observe the early stage of biofilm development (data not shown).

Using these conditions, we compared the biofilms formed by the TFP mutants with that formed by the wild type strain. Our results show that biofilm formation by the TFP mutant strains was partially defective (Fig. 2). Biofilm formation by all the TFP mutants (A34, *pilA* deletion mutant; M3, *mshA* deletion mutant; and W15, *pilA* and *mshA* deletion mutant) was reduced to two-thirds of that of the parent strain. Complementation of these genes increased the formation of the biofilms (Fig. 2). These data suggest that both ChiRP and MSHA pili contribute to biofilm formation by *V. parahaemolyticus*.

Next, we investigated how each TFP contributes to biofilm formation. The detailed biofilm architectures of the wild type and mutants were observed by microscopic analysis (Fig. 3a and b). The wild type strain formed microcolonies as the biofilm progressed (Fig. 3a-A). The mutant strain W15 did not form any aggregates and only a few cells were attached to the glass surface (Fig. 3a-B). The difference between the wild type and the TFP double mutant indicates that the TFPs are required for biofilm formation. Then, we observed the biofilm formed by each mutant with a single gene deletion to identify the functions of the two TFPs.

Although the MSHA mutant, M3, formed aggregates, the number of bacteria directly attached to the coverslip was greatly reduced (Fig. 3a-C). When the *mshA* gene was complemented in *trans*, direct bacterial attachment to the glass surface was restored (Fig. 3a-D). These results suggest that MSHA pili contribute to the attachment of *V. parahaemolyticus* to the glass surface, which is in accordance with the data reported for the MSHA pili of *V. cholerae* (Watnick & Kolter, 1999; Moorthy & Watnick, 2004).

Interestingly, the ChiRP mutant, A34, attached to the entire surface of the coverslip, but did not form aggregates (Fig. 3a-F). Complementation of A34 with *pilA* restored microcolony formation (Fig. 3a-G). This change in phenotype suggests that ChiRP may contribute to bacterium–bacterium interactions during biofilm formation. We observed this change of phenotype in greater detail using SEM. Thick microcolonies composed of numerous cells were observed with the wild type (Fig. 4a). In contrast, A34 demonstrated no microcolony formation. The bacterial cells did not aggregate with each other, but were attached individually to the glass surface (Fig. 4b). These results suggest that ChiRP contributes to the attachment of the bacteria to each other. We also observed the MSHA mutant, M3, by SEM. Very few aggregates were observed, and almost no bacteria were attached to the glass surface (data not shown). We speculate that this was
Table 3. Competitive assays for chitin binding

<table>
<thead>
<tr>
<th>Assay</th>
<th>Compared strains</th>
<th>Relative fitness (w*)</th>
<th>P value compared with assay 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A34/S8A</td>
<td>0.346 ± 0.127</td>
<td>0.004</td>
</tr>
<tr>
<td>2</td>
<td>M3/S8A</td>
<td>0.386 ± 0.200</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>RIMD2210633/S8A</td>
<td>0.907 ± 0.200</td>
<td></td>
</tr>
</tbody>
</table>

A34, pilA mutant; M3, mshA mutant; RIMD2210633, wild type; S8A, spontaneous nalidixic-acid-resistant derivative of RIMD2210633.

Values represent the means of five separate experiments.

*w (tested strain, TS/nalidixic acid-resistant strain, S8A) = ln(TSF/TS0)/ln(S8A/S8A0), where TSO and S8A0 are initial CFUs and TSI and S8AI are the CFUs at the end of the experiment.

due to the procedure used for SEM, which includes more stringent rinsing of the coverslips than is used for LM. Therefore, the results of SEM may more strongly reflect the reduced capacity of M3 for direct attachment to the coverslip surface than do the results of LM, as can be seen in Fig. 3A-c. Microscopic analysis suggests that both types of TFP in V. parahaemolyticus play a role in biofilm formation, with each type of pilus contributing to a distinct step in the process.

Occurrences of V. parahaemolyticus are significantly associated with zooplankton. This fact suggests that bacterial adsorption onto the chitinous exoskeletons of zooplankton may aid the survival of V. parahaemolyticus (Kaneko & Colwell, 1975). We were interested in the functions of the TFPs in this adsorption onto zooplankton in the aquatic environment. Therefore, we used competition experiments to compare the fitness of the mutant and wild type bacteria for adsorption onto chitin. The competition experiments were performed as described previously, with modifications (Meibom et al., 2004; Kirn et al., 2005). Nearly equal numbers of the wild type and each TFP mutant strain were combined and incubated with chitin beads for 1 h. The chitin beads were then rinsed and the bacteria that were associated with the beads were detached from them and quantified by viable plate counting. To differentiate the two strains, we used S8A, a spontaneous nalidixic-acid-resistant derivative of the wild type strain (see Materials and methods). Differences in viable plate counts between the marked wild type and the mutants were expressed as relative fitness values (Table 3), in which values of < 1 indicated a defect in the competitiveness of the designated strain (see Materials and methods). The relative fitness value of the ChiRP mutant strain was 0.346 ± 0.127 and that of the MSHA pilus mutant was 0.386 ± 0.200 (Table 3). These low fitness values were not caused by the spontaneous nalidixic acid-resistant mutation, because the spontaneous nalidixic acid-resistant derivative, S8A, was also competed against the unmarked wild type (Table 3); similar relative fitness values were obtained regardless of which strain had the marker. These results suggest that both ChiRP and MSHA pilus were involved in the bacterial adsorption onto the chitin beads.

Discussion

Vibrio parahaemolyticus is known to attach to environmental surfaces, such as those of algae and zooplankton, and to form biofilms (Kaneko & Colwell, 1975; Kumazawa et al., 1991). Therefore, the study of biofilm formation by V. parahaemolyticus is important for our understanding of the lifestyle of this organism in natural environments. Many factors, including flagella and exopolysaccharide, are involved in biofilm formation (Watnick et al., 1999). Among these factors, TFP is also important (Costerton et al., 1999). We focused on the TFPs of V. parahaemolyticus and investigated their roles in biofilm formation. From the observations of the biofilms formed by wild type and pilin-deficient strains, we demonstrated that the two TFPs contribute to biofilm formation in different ways, via distinct processes. Several reports have demonstrated the contribution of TFP to biofilm formation in vibrios (Watnick et al., 1999; Enos-Berlage et al., 2005; Paranjpye & Strom, 2005), but the distinct roles played by the two kinds of TFP in one organism have not been reported to date.

In this study, we also confirmed that a chitin derivative, a GlcNAc mixture, regulates the expression of PilA of the ChiRP, as has been reported for V. cholerae (Meibom et al., 2004), but does not regulate MshA expression. From all these results, we hypothesize the following scenario. In the natural environment, V. parahaemolyticus first attaches to a surface through the MSHA pilus. If the surface substance contains chitin, V. parahaemolyticus hydrolyzes it to GlcNAc with chitinases. The GlcNAc produced then enhances the expression of ChiRP, which plays a role in bacterium–bacterium interactions. Therefore, biofilms are formed efficiently on chitin-containing surfaces, and the bacterium can obtain nutrients in a stable manner. In contrast, if V. parahaemolyticus attaches to a substrate that does not contain chitin, the expression of ChiRP is not upregulated. Thus, the attachment of the bacteria to a nonchitinous substrate is not stable, and the bacteria are easily dispersed. Consequently, V. parahaemolyticus is free to swim to the next substrate to obtain nutrients. We suggest that the use of the two types of TFP, which contribute to biofilm formation in different ways in distinct steps, is a refined strategy of V. parahaemolyticus for its survival in natural environments poor in nutrients, such as the marine environment.

We propose that GlcNAc has an important role in the ecology of V. parahaemolyticus in our hypothesized scenario. GlcNAc, a chitin monomer, is a common modification of glycoproteins and lipids present on the intestinal epithelium (Bjork et al., 1987; Finne et al., 1989). Therefore, the two TFPs (especially ChiRP) may function like GlcNAc-binding protein A (GbpA) in the presence of GlcNAc, in the intestinal environment as well as in the marine environment (Kirn et al., 2005).
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