Biochemical characterization of different types of adherence of \textit{Vibrio} species to fish epithelial cells

X. H. Wang and K. Y. Leung

\textbf{INTRODUCTION}

\textit{Vibrio} species are Gram-negative bacteria that cause a systemic infection in fish called vibriosis. The authors previously demonstrated that internalization and cytotoxicity are important virulence mechanisms in vibrio–fish epithelial cell interactions. Adherence is a prerequisite for successful internalization. In this study, the adherence capability of two invasive strains [\textit{V. anguillarum} 811218–5W and G/Virus/5(3)] was compared with that of two non-invasive strains [\textit{V. damselae} ATCC 33539 and \textit{V. anguillarum} S2/5/93(2)] using adherence assays in three different types of fish cells (epithelioma papillosum of carp, EPC; grunt-fin tissue, GF; and fat-head minnow epithelial cells, FHM). For all four strains there was no significant difference ($P > 0.05$) in the adherence to the different cell lines. \textit{V. anguillarum} 811218–5W exhibited the highest adherence, followed by G/Virus/5(3) and S2/5/93(2); \textit{V. damselae} ATCC 33539 showed the lowest adherence. The super-adherence characteristic of \textit{V. anguillarum} 811218–5W on EPC cells was not affected by inhibitors, sugars, low temperature (4°C) incubation, or non-biological surfaces such as glass coverslips. The galactose-linked adherence characteristic of \textit{V. anguillarum} G/Virus/5(3) to the EPC cells was partially inhibited by peptidase treatment of the fish cells, low-temperature incubation, and addition of sugars that contained galactose (such as lactose and N-acetyl-D-galactosamine). \textit{De novo} synthesis of bacterial protein, viable bacteria and intact carbohydrate structure of vibrios were required for both super-adherence and galactose-linked adherence. These adherence characteristics were also found in ten other invasive vibrios, and galactose-linked adherence was found in nine invasive vibrios.

\textbf{Keywords:} \textit{Vibrio} spp., fish epithelial cells, adherence

\textbf{Abbreviations:} EPC, epithelioma papillosum of carp; EPEC, enteropathogenic \textit{E. coli}; FHM, fat-head minnow epithelial cells; GF, grunt-fin tissue cells.
are carbohydrate-binding proteins, akin to lectins (Ofek & Sharon, 1990). Afimbrial adhesins include outer-membrane proteins, lipoteichoic acids, lipopolysaccharide (LPS) and extracellular polysaccharides (Ofek & Doyle, 1994; Patrick & Larkin, 1995). Many bacterial strains are genotypically capable of producing more than one type of adhesin.

For the human bacterial pathogens enteropathogenic Escherichia coli (EPEC) (Puente et al., 1996; Ramer et al., 1996) and Vibrio cholerae (Herrington et al., 1988; Iredell & Manning, 1994), the bundle-forming pilus and the toxin-coregulated pilus, respectively, are used to adhere to the mammalian host cells. Group A streptococci bind to the fibronectin of host cells using the glycolipid end of lipoteichoic acid (Courtney et al., 1988), while Yersinia enterocolitica and Y. pseudotuberculosis encode a surface protein, invasin, which mediates adherence and uptake of these bacteria into epithelial cells (Isberg & Falkow, 1985; Isberg et al., 1987).

For fish bacterial pathogens, Aeromonas hydrophila and some Vibrio strains were found to attach to collagen, fibronectin (Ascencio et al., 1990), fish mucus (Krovacek et al., 1987) and fish epithelial cells (Chen & Hanna, 1992; Miliotis et al., 1995; Tan et al., 1998). The presence of flagella (Merino et al., 1997) and LPS (Merino et al., 1996b) in A. hydrophila was suggested to mediate adherence. The capsular polysaccharide of Aeromonas salmonicida was also reported to be an important factor in the adherence and invasion of fish cells (Merino et al., 1996a).

It has been postulated that the portals of entry for Vibrio species into fish are the gastrointestinal tract (Horne & Baxendale, 1983; Kanno et al., 1989; Olsson et al., 1996), gills (Baudin-Laurencin & German, 1987) and skin (Grimes et al., 1985; Kanno et al., 1989). Regardless of the route vibrios use to enter the fish, it is necessary for them to adhere to and penetrate through the epithelial cells to spread systemically. The adherence mechanisms of Vibrio species to fish cells have not been extensively investigated. Our previous study demonstrated that internalization and cytotoxicity are important virulence mechanisms in vibrio–fish cell interactions (Wang et al., 1998). Adherence and internalization are speculated to be interdependent events but regulated by different processes. Vibrios that have high adherence capability may not have a high invasion rate but poorly adherent strains had low invasion rates (Wang et al., 1998). In this study, the adherence abilities of Vibrio strains were investigated and characterized using various inhibitors and conditions. These studies may provide clues as to how fish pathogens adhere to and penetrate epithelial cells in order to initiate infections in fish.

**METHODS**

**Bacterial strains and media.** Fourteen Vibrio strains were chosen for this study; their characteristics were reported previously (Wang et al., 1998). These strains were tested using standard biochemical diagnostic kits (Microbact Identification Kit 24E, DP Diagnostics and BBL Crystal Enteric/Nonfermenter ID System, Becton Dickinson), and their identities were further confirmed according to the criteria of Breed (1996) and Hjelmes & Roberts (1993). Cultures were routinely grown at 25 °C in tryptic soy agar (TSA; Difco) or tryptic soy broth (TSB; Difco) supplemented with 0.5% NaCl. Stock cultures were maintained at −80 °C as a suspension in supplemented TSB containing 25% (v/v) glycerol.

**Cell culture.** All tissue culture reagents were obtained from Gibco. epithelium papillosum of carp, Cyprinus carpio (EPC) (Wolf & Mann, 1980), fat-head minnow (Pimephales promelas) epithelial cells (FHM) (Gravell & Malsberger, 1965) and grunt-fin (Haemulon sciurus) tissue cells (GF) (Clem et al., 1961) were grown in minimal essential medium (MEM) with Hank’s salts (Sigma), 10 mM HEPES (pH 7.3), 2 mM glutamine, 0.23% (w/v) NaHCO₃ and 10% (or 20% for GF cells) (v/v) heat-inactivated fetal bovine serum at 25 °C in a 5% (v/v) CO₂ atmosphere. For the culturing of GF, MEM was supplemented with 58 mM NaCl for optimal growth. Cells were grown in 250 ml flasks and split at least once a week by trypsin/EDTA treatment and dilution at 1:10 in fresh media.

**Adherence assays using viable counts.** The adherence assays were performed as described before with minor modifications (Elsinghorst, 1994; Wang et al., 1998). Briefly, 5 ml stationary-phase cultures were prepared by inoculating supplemented TSB with vibrios from frozen glycerol stocks and incubating overnight at 25 °C. Three hours prior to infection of cells, mid-exponential-phase cultures were prepared by diluting the overnight culture 1:10 in fresh supplemented TSB and incubating at 25 °C. Bacterial cells were pelleted and washed three times in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 43 mM NaHPO₄ and 1.4 mM KH₂PO₄ at pH 7.2) before adding 5 µl to each tissue culture well (approx. 5 x 10⁸ bacteria). Monolayers of EPC cells were grown for 72 h in 24-well tissue-culture plates to about 95% confluence. After inoculation, the tissue culture plate was centrifuged (800 g, 5 min, 4 °C), then incubated at 25 °C. To measure the number of bacteria adhering to the monolayers, the plates were washed six times with Hank’s balanced salts solution, the EPC cells lysed with 1% (v/v) Triton X-100 in PBS, and then bacterial numbers estimated by plate counting. This assay quantifies the total number of bacteria bound to the outside of and internalized by the fish cells, as well as the bacteria non-specifically bound to the culture dish walls. The adherence rates were calculated from the mean of two wells in quadruple experiments.

For sugar inhibition studies, EPC cells were pre-incubated with the respective sugar (100 µg ml⁻¹, Sigma; see Table 3) in MEM for 30 min prior to the addition of bacteria. For trypsin (0·025%), proteinase K (1 mg ml⁻¹), periodic acid (10 mM) and sodium metaperiodate (1 mg ml⁻¹) treatments, chemicals in Hank’s solution or PBS were applied to EPC cells or to bacteria separately at 25 °C for 10 min as described previously (Giron et al., 1996; Boris et al., 1998). The host cells or bacteria were then washed twice with Hank’s solution or PBS before the addition of bacteria in MEM to the wells. The efficiency of bacterial adherence in the presence of inhibitors was expressed as the percentage of adhered bacteria in controls (absence of inhibitors).

**Microscopic count of adherent bacteria using Giemsa stain.** Glass coverslips were placed into each well of the 24-well tissue culture plate prior to seeding with EPC cells and grown...
for 72 h at 25 °C in a 5% (v/v) CO₂ atmosphere as described
above. After infection and incubation for 30 min, the mono-
layers were washed six times with Hank’s solution and fixed
with methanol for 30 min. The cells were washed twice with
PBS and stained with Giemsa stain (Merck) for 30 min. After
washing three times with PBS, the stained samples were
examined under an Axiosvert 25 CFL inverted microscope
(Carl-Zeiss) at 100x magnification. Photographs were taken
using Kodak colour ISO 100 film. The adherence rate was
expressed as the number of adhering bacteria per 100 EPC cells
after counting 300 EPC cells per cover slip. The adherence rate
was calculated from the mean of two coverslips in triplicate
experiments.

To examine the effect of heat treatment on bacterial adherence
to EPC cells, PBS-washed bacteria were heated at 56 °C for
5 min and added onto EPC monolayers. After incubation for
30 min, Giemsa staining was carried out as described above.
For adherence at 4 °C, the infected monolayer was incubated at
4 °C for 30 min, stained and examined under a microscope.
For the antibiotic-treated bacteria experiment, before bacteria
were inoculated onto the cell monolayer, they were treated
with various antibiotics at room temperature for 10 min as
described by Finlay et al. (1989). Treated bacteria were then
added into each well. In studies of the adherence ability of
vibrios on glass coverslips, washed bacteria were placed on
coverslips in a 24-well tissue culture plate and the plate was
centrifuged as described above. The plate was then incubated
in a 25 °C CO₂ incubator for 30 min. The coverslips were
washed six times, stained, and prepared for microscopic
examination as described above.

Statistical analysis. All data from adherence assays were
expressed as means ± standard errors (SEM). The data were
analysed using one-way and two-way ANOVA and Duncan
multiple range tests (SAS software, SAS Institute). Values of \( P < 0.05 \) were considered significant.

RESULTS

Adherence of vibrios to different fish cell lines

The adherence assay was performed on EPC, FHM and
GF cells with two invasive strains, \( V. \text{anguillarum} \)
811218-5W and G/Virus/5(3), which can adhere to
EPC cells before internalization (Wang et al., 1998), and
two non-invasive strains, \( V. \text{damselae} \) ATCC 33539 and
\( V. \text{anguillarum} \) S2/5/93(2). The results (Table 1)
showed that for each strain the adherence capability to
the three different types of fish cells was similar (\( P > 0.05 \)). EPC cells were therefore used for the rest of this
work. The invasive strains had higher adherence abilities
than the non-invasive strains. \( V. \text{anguillarum} \) 811218-
5W exhibited the highest adherence capability, followed
by G/Virus/5(3) and S2/5/93(2); the cytotoxic strain \( V. \text{damselae} \) ATCC 33539 exhibited the lowest adherence
to fish epithelial cells.

Adherence at different temperatures

When the adherence assay was performed at low
temperature (4 °C), significant reductions in adherence
(\( P < 0.05 \)) were observed for \( V. \text{anguillarum} \) G/
Virus/5(3) (about 69%) and S2/5/93(2) (about 63%)
(Table 2, column 5) when compared to the control
(Table 2, column 3). On the other hand, low-tem-
perature incubation did not significantly affect (\( P >
0.05 \)) the adherence ability of \( V. \text{anguillarum} \) 811218-5W
and \( V. \text{damselae} \) ATCC 33539. Heat treatment (56 °C
for 5 min) killed and drastically decreased the adherence
of all \( V \text{ibrio} \) strains (Table 2). After heat treatment,
the viability of vibrios was less than 0.0026% (\( n = 3 \)) for all
four strains. Nonviable \( V. \text{anguillarum} \) 811218-5W
demonstrated about 91% reduction in adherence, while
\( V. \text{anguillarum} \) G/Virus/5(3) showed about 59% re-
duction of adherence to EPC cells.

Microscopic examination of adherent vibrios using
Giemsa stain

The adherence pattern of each strain on the EPC
monolayers was then examined. \( V. \text{anguillarum} \) 811218-5W had an aggregate adherence pattern (Fig.
1a): clustering of bacterial cells was found on the EPC
cells before internalization (Wang et al., 1998), and
two non-invasive strains, \( V. \text{damselae} \) ATCC 33539 and
\( V. \text{anguillarum} \) S2/5/93(2). The results (Table 1)
showed that for each strain the adherence to the three
different types of fish cells was similar (\( P > 0.05 \)). EPC cells were therefore used for the rest of this
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to fish epithelial cells.

Table 1. Proportion of adherent \( V \text{ibrio} \) species in different fish cell lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>10^5 x Inoculum</th>
<th>Adherence*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EPC</td>
</tr>
<tr>
<td>( V. \text{anguillarum} ) 811218-5W</td>
<td>5.4 ± 0.5</td>
<td>101 ± 5 ± 5*a</td>
</tr>
<tr>
<td>( V. \text{anguillarum} ) G/Virus/5(3)</td>
<td>6.0 ± 0.7</td>
<td>24 ± 4 ± 0*b</td>
</tr>
<tr>
<td>( V. \text{damselae} ) ATCC 33539</td>
<td>4.6 ± 0.9</td>
<td>21 ± 0 ± 6*c</td>
</tr>
<tr>
<td>( V. \text{anguillarum} ) S2/5/93(2)</td>
<td>6.2 ± 1.0</td>
<td>12.5 ± 2 ± 8*e</td>
</tr>
</tbody>
</table>

*a Three-day-old cells and exponential-phase vibrios were used to perform adherence assays at 25 °C
for 30 min. The viable count method was used to estimate adherence, which is expressed as percentage
of bacteria still adherent after washing six times with PBS (\( n = 4 \)). Values were recorded as mean ± SEM
for four trials, each in triplicate. Within each column, values followed by different superscript letters are
significantly different (\( P < 0.05 \)).
Table 2. Proportion of adherent Vibrio species in EPC cells at different temperatures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inoculum</th>
<th>Adherence†</th>
<th>Heat treatment (56 °C, 5 min)‡</th>
<th>Low temp. (4 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^3 x</td>
<td>Direct count control (25 °C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. anguillarum 811218-5W</td>
<td>7.7±1.4</td>
<td>93.0±2.4a</td>
<td>9.6±0.6as</td>
<td>90.8±8.2a</td>
</tr>
<tr>
<td>V. anguillarum G/Virus/5(3)</td>
<td>5.3±1.5</td>
<td>39.9±3.1b</td>
<td>17.4±1.4as</td>
<td>12.4±1.6bs</td>
</tr>
<tr>
<td>V. damsela ATCC 33539</td>
<td>5.5±0.5</td>
<td>6.8±1.2c</td>
<td>3.2±0.5s</td>
<td>7.9±0.6b</td>
</tr>
<tr>
<td>V. anguillarum S2/5/93(2)</td>
<td>4.3±0.9</td>
<td>28.6±1.5d</td>
<td>2.3±0.2es</td>
<td>10.7±1.7es</td>
</tr>
</tbody>
</table>

† Adherence is expressed as number of adherent bacteria per 100 EPC cells. Values were recorded as mean ± SEM for three trials on duplicate coverslips for a total of 600 EPC cells observed each time. Within each column, values followed by different superscript letters are significantly different (P < 0.05). Values followed by an asterisk are significantly different (P < 0.005) from the direct count control for the same Vibrio strain.

‡ For heat treatment, vibrios were heated at 56 °C for 5 min before being inoculated onto the EPC monolayers.

Vibrios based on direct microscopic count (Table 2, column 3) were comparable with the viable count result (Table 1, column 3), and in a similar order. V. anguillarum 811218-5W exhibited the highest adherence, followed by G/Virus/5(3) and S2/5/93(2), whereas V. damsela ATCC 33539 showed the lowest adherence. Furthermore, V. anguillarum 811218-5W could adhere on non-biological surface such as glass coverslips (Fig. 1d), whereas V. anguillarum G/Virus/5(3) (Fig. 1e) and the non-invasive strains could not (data not shown).

Inhibition of adherence using sugars and other inhibitors

The abilities of various sugars to inhibit adherence of V. anguillarum 811218-5W and G/Virus/5(3) to EPC cells were examined (Table 3). Adherence of V. anguillarum G/Virus/5(3) to the EPC cells was significantly inhibited (P < 0.005) in the presence of lactose, galactose and N-acetyl-b-galactosamine, while no inhibition was observed for V. anguillarum 811218-5W.

Vibrio strains and EPC cells were treated with trypsin, proteinase K, periodic acid and sodium metaperiodate separately. The adherence of V. anguillarum G/Virus/5(3) to EPC cells was significantly inhibited (P < 0.005) by treating EPC cells with trypsin and proteinase K, while adherence of V. anguillarum 811218-5W was not affected by any of the treatments (Table 3). Treatment of vibrios with periodic acid or sodium metaperiodate inhibited adherence in both strains. The inhibition of adherence by treating the vibrios with chloramphenicol, nalidixic acid and rifampin (Table 3) showed that these both strains require de novo protein synthesis for adherence.

The results of the inhibitor studies, together with the effects of temperature and microscopic examination, suggested that the adherence of V. anguillarum 811218-5W and G/Virus/5(3) to host cells was different.

Adherence characteristics of 12 invasive Vibrio strains

Twelve invasive Vibrio strains were tested for their adherence characteristics as established from V. anguillarum 811218-5W and G/Virus/5(3) (Table 4). Using the viable count method to estimate the number of adherent bacteria, V. anguillarum 811218-5W and V. alginolyticus 240/89 showed significantly greater adherence (102–117%) (P < 0.005) to EPC cells than the other strains, followed by V. damsela E311 and V. parahaemolyticus W368-1p (41–58%). In contrast, all the strains of V. vulnificus (ATCC 33147, ATCC 33148, ATCC 33149, S1/4/93(4) and S1/7/93(1)) showed significantly lower adherence to EPC cells than the other strains (0.5–5%), followed by V. harveyi W618, V. anguillarum G/Virus/5(3) and 01/10/93(2) (19–24%).

By phase-contrast microscopy, V. anguillarum 811218-5W (Fig. 1a) and V. damsela E311 (Fig. 1c) exhibited formation of aggregated patterns on EPC cells while the rest did not. Among the 12 strains we studied, the auto-agglutination phenotype was observed only in V. damsela E311 when incubated in PBS and in MEM. V. anguillarum 811218-5W (Fig. 1d) and V. alginolyticus 240/89 (Fig. 1f) showed significantly greater adherence to cell-free glass coverslips than the other strains tested; most of the other Vibrio strains showed little or no adherence (Table 4). V. damsela E311 demonstrated moderate adherence to glass coverslips, probably due to its auto-agglutination characteristics. The adherence abilities of V. anguillarum 811218-5W and V. alginolyticus 240/89 and the five V. vulnificus strains were not significantly affected by low-temperature incubation, while other vibrios such as V. damsela E311 (47% reduction) and V. parahaemolyticus W368-1p (73% reduction) were drastically affected. This further
Fig. 1. Giemsa-stained bright-field micrographs of EPC cells (a to c) and glass coverslips (d to f) inoculated with V. anguillarum 811218-5W (a, d), G/Virus/5/3 (b, e), V. damselae E311 (c) and V. alginolyticus 240/89 (f). Bar, 20 µm.
Table 3. Adherence of Vibrio anguillarum 811218-W and G/Virus/5(3) in the presence of various putative inhibitors

<table>
<thead>
<tr>
<th>Substance</th>
<th>Target</th>
<th>V. anguillarum 811218-W</th>
<th>V. anguillarum G/Virus/5(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sugars (100 µg ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Fucose</td>
<td>EPC surface protein</td>
<td>116.2 ± 15.0</td>
<td>116.3 ± 12.0</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>EPC surface protein</td>
<td>123.2 ± 5.2</td>
<td>123.0 ± 13.1</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>Carbohydrate structure on EPC</td>
<td>108.6 ± 4.2</td>
<td>68.3 ± 9.3*</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Carbohydrate structure on EPC</td>
<td>119.8 ± 5.2</td>
<td>97.0 ± 3.8</td>
</tr>
<tr>
<td>α-Lactose</td>
<td></td>
<td>94.1 ± 5.8</td>
<td>56.5 ± 5.0*</td>
</tr>
<tr>
<td>N-Acetylmannosamine</td>
<td></td>
<td>100.2 ± 7.7</td>
<td>90.3 ± 7.3</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td></td>
<td>105.9 ± 9.8</td>
<td>92.8 ± 6.8</td>
</tr>
<tr>
<td>N-Acetylnearcinic acid</td>
<td></td>
<td>97.4 ± 5.3</td>
<td>68.9 ± 5.0*</td>
</tr>
<tr>
<td>Trypsin (0.025%)</td>
<td>Bacterial surface protein</td>
<td>115.2 ± 18.4</td>
<td>62.0 ± 7.6*</td>
</tr>
<tr>
<td>Proteinase K (1 mg ml⁻¹)</td>
<td>Bacterial surface protein</td>
<td>120.0 ± 6.6*</td>
<td>62.0 ± 16.0*</td>
</tr>
<tr>
<td>Periodic acid (10 mM)</td>
<td>Carbohydrate structure on EPC</td>
<td>108.4 ± 5.6</td>
<td>133.7 ± 9.6*</td>
</tr>
<tr>
<td>Sodium metaperiodate (1 mg ml⁻¹)</td>
<td>Carbohydrate structure on EPC</td>
<td>113.3 ± 7.9</td>
<td>95.5 ± 9.7</td>
</tr>
<tr>
<td>Trypsin (0.025%)</td>
<td>Bacterial surface protein</td>
<td>106.1 ± 5.1</td>
<td>104.2 ± 4.6</td>
</tr>
<tr>
<td>Proteinase K (1 mg ml⁻¹)</td>
<td>Bacterial surface protein</td>
<td>101.6 ± 14.7</td>
<td>105.7 ± 20.4</td>
</tr>
<tr>
<td>Periodic acid (10 mM)</td>
<td>Carbohydrate structure on bacteria</td>
<td>11.3 ± 3.9*</td>
<td>6.7 ± 1.8*</td>
</tr>
<tr>
<td>Sodium metaperiodate (1 mg ml⁻¹)</td>
<td>Carbohydrate structure on bacteria</td>
<td>35.7 ± 1.8*</td>
<td>16.5 ± 24*</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg ml⁻¹)</td>
<td>Bacterial protein synthesis</td>
<td>19.2 ± 2.4*</td>
<td>10.8 ± 0.2*</td>
</tr>
<tr>
<td>Rifampin (10 µg ml⁻¹)</td>
<td>Bacterial RNA synthesis</td>
<td>15.9 ± 4.8*</td>
<td>20.3 ± 7.8*</td>
</tr>
<tr>
<td>Nalidixic acid (20 µg ml⁻¹)</td>
<td>Bacterial DNA synthesis</td>
<td>33.0 ± 9.2*</td>
<td>36.0 ± 5.7*</td>
</tr>
</tbody>
</table>

suggested that V. anguillarum 811218-W and V. alginolyticus 240/89 were in the same group due to their high adherence on EPC cells and coverslips.

Adherence of bacteria to the host cell depends on the interaction between the bacterial surface structure and the host-cell surface receptors. The effectiveness of galactose as an inhibitor of vibrio adherence to EPC cells was examined. Galactose significantly inhibited (P < 0.05) the adherence of nine of the 12 Vibrio strains tested; adherence of V. anguillarum 811218-W, V. damsela E311 and V. vulnificus S1/4/93(4) was not inhibited (Table 4).

**DISCUSSION**

Bacterial adherence to host cells is often an essential step to initiate infection because it localizes pathogens to the appropriate target tissues (Finlay & Cossart, 1997; Finlay & Falkow, 1997). The host cell may then be triggered to internalize the bacteria, by either phagocytosis or endocytosis (invasion), via the host’s signal transduction mechanisms. The adherent bacteria also colonize, and produce toxins that cause tissue damage to facilitate the infection (Ofek & Sharon, 1990). Therefore, it is important to understand the mechanisms involved in the adherence of bacteria onto host cells so that we can devise preventive measures to reduce infection.

**Adherence of vibrios in various conditions**

Adherence mechanisms of vibrios have not been examined in detail in fish models. It was proposed that vibrios penetrated the mucosal surfaces (i.e. gastrointestinal tract), adhered to and invaded the fish epithelial cells, and then spread to other tissues and organs (Krovacek et al., 1987; Chen & Hanna, 1992; Olsson et al., 1996). Tissue culture cells, especially epithelial cells, have played a crucial role in investigating bacteria–host interactions because cultured cells are easy
Table 4. Adherence characteristics of 12 Vibrio strains

The data for the prototype strains showing super-adherence [811218-5W] and galactose-linked adherence [G/Virus/5(3)] are shown in bold.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$10^{-5} \times$ Inoculum</th>
<th>Adherence (%)†</th>
<th>Adherence at 4°C (%)†</th>
<th>Galactose inhibition (%)‡</th>
<th>Adherence pattern§</th>
<th>Bacteria on glass coverslip††</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. alginolyticus 240/89</td>
<td>7.2 ± 0.2</td>
<td>117.1 ± 6.5‡</td>
<td>82.7 ± 16.6‡</td>
<td>74.6 ± 4.3‡</td>
<td>—</td>
<td>123.8 ± 2.2‡</td>
</tr>
<tr>
<td>V. anguillarum 811218-5W</td>
<td>6.4 ± 0.5</td>
<td>101.7 ± 5.9‡</td>
<td>91.9 ± 2.5‡</td>
<td>105.7 ± 9.4‡</td>
<td>+</td>
<td>119.1 ± 4.2‡</td>
</tr>
<tr>
<td>V. anguillarum 01/10/93(2)</td>
<td>5.1 ± 0.4</td>
<td>18.6 ± 1.2‡</td>
<td>68.1 ± 2.2‡</td>
<td>41.2 ± 7.1‡</td>
<td>—</td>
<td>1.9 ± 1.4†</td>
</tr>
<tr>
<td>V. anguillarum G/Virus/5(3)</td>
<td>5.0 ± 0.8</td>
<td>24.4 ± 4.0‡</td>
<td>5.1 ± 0.4‡</td>
<td>68.3 ± 9.3‡</td>
<td>+</td>
<td>13.0 ± 2.8‡</td>
</tr>
<tr>
<td>V. damselae E311</td>
<td>6.1 ± 0.9</td>
<td>41.0 ± 7.1†</td>
<td>21.8 ± 2.3†</td>
<td>99.7 ± 5.6†</td>
<td>+</td>
<td>44.9 ± 6.2b</td>
</tr>
<tr>
<td>V. harveyi W618</td>
<td>8.2 ± 0.4</td>
<td>19.0 ± 1.4‡</td>
<td>4.0 ± 1.1‡</td>
<td>64.9 ± 7.0‡</td>
<td>—</td>
<td>2.1 ± 1.4†</td>
</tr>
<tr>
<td>V. parahaemolyticus W368-1p</td>
<td>6.6 ± 0.6</td>
<td>57.9 ± 4.4‡</td>
<td>15.9 ± 3.4‡</td>
<td>66.4 ± 3.0‡</td>
<td>—</td>
<td>21.7 ± 5.6e</td>
</tr>
<tr>
<td>V. vulnificus ATCC 33147</td>
<td>3.6 ± 0.4</td>
<td>2.3 ± 1.0‡</td>
<td>4.7 ± 2.3†</td>
<td>48.2 ± 7.4†</td>
<td>—</td>
<td>2.1 ± 0.7f</td>
</tr>
<tr>
<td>V. vulnificus ATCC 33148</td>
<td>3.2 ± 0.4</td>
<td>0.5 ± 0.06‡</td>
<td>0.0 ± 0.2‡</td>
<td>63.7 ± 6.9‡</td>
<td>+</td>
<td>2.5 ± 0.9f</td>
</tr>
<tr>
<td>V. vulnificus ATCC 33149</td>
<td>5.1 ± 1.3</td>
<td>0.9 ± 0.3‡</td>
<td>1.0 ± 0.4‡</td>
<td>79.5 ± 6.1†</td>
<td>—</td>
<td>0.9 ± 0.3c</td>
</tr>
<tr>
<td>V. vulnificus S1/4/93(4)</td>
<td>5.5 ± 0.4</td>
<td>1.6 ± 0.4‡</td>
<td>0.8 ± 0.2‡</td>
<td>111.8 ± 15.5</td>
<td>—</td>
<td>1.2 ± 1.5d</td>
</tr>
<tr>
<td>V. vulnificus S1/7/93(1)</td>
<td>7.7 ± 1.4</td>
<td>5.0 ± 1.5‡</td>
<td>9.1 ± 2.3‡</td>
<td>73.1 ± 6.1‡</td>
<td>—</td>
<td>1.4 ± 0.8d</td>
</tr>
</tbody>
</table>

† Adherence is expressed as percentage of inoculum bacteria still adherent after washing six times ($n = 4$). Values were recorded as mean ± SEM for four trials. Within each column, values followed by different superscript letters (a, b, c, d) are significantly different ($P < 0.05$). When comparing adherence at 25 °C versus 4 °C (columns 3 and 4), values followed by different superscript symbols ($x$ and $y$) are significantly different ($P < 0.05$).

‡ Values represent the percentages of adherent bacteria in the presence of galactose to adherent bacteria without galactose treatment ($n = 4$). Values followed by an asterisk are significantly different from the untreated control ($P < 0.05$).

§ +, Vibrios adhered to EPC cells in an aggregated pattern; -, absence of an aggregated pattern.

†† Microscopic count was used for measuring bacterial adherence on glass coverslips. Adherence is expressed as number of adherent bacteria per field. The adherence rate was calculated from the mean of two coverslips in triplicate experiments. Within this column, values followed by different superscript letters (a, b, c, d) are significantly different ($P < 0.05$).

to work with, can be maintained under controlled conditions, and may be relevant to the diseases under study (Quinn et al., 1997). In this work, EPC and FHM were used as a freshwater fish tissue culture model and GF as a marine fish tissue culture model for adhesion studies.

V. anguillarum 811218-5W induced morphological changes to 1-d-old EPC cells in 35.0 ± 30 min ($n = 3$) and this might have compromised the integrity of the monolayers during the 30 min adherence assay. For 3-d-old EPC cells, 43.3 ± 1.5 min ($n = 3$) was required. The use of 3-d-old cells delayed the cytopathic process and therefore provided a more accurate model for comparative adhesion studies than 1-d-old cells. Observations on adhesion of group B streptococci (Kubin & Ryc, 1988; Tamura et al., 1994) had indicated that the growth phase of the bacteria had minimal effects on adherence. Similar observations were found in our Vibrio strains when comparing adherence of exponential- and stationary-phase vibrios to 3-d-old EPC cells (data not shown). Hence, 3-d-old EPC cells and exponential-phase bacterial cultures were used throughout this work.

Continuous bacterial protein synthesis was required for stable bacterial adherence. Incubation at low temperature (4 °C) was used to inhibit continuous bacterial protein synthesis and produce an inhibition of adherence for Haemophilus influenzae (St Geme & Falkow, 1990) and Salmonella species (Finlay et al., 1989). On the other hand, heat treatment (56 °C) will affect bacterial surface structures that are heat-sensitive and decrease the viability of vibrios. We wanted to find out whether nonviable vibrios could still adhere to fish cells. The invasive strains V. anguillarum 811218-5W and G/Virus/5(3) were found to adhere to EPC cells before internalization. Heat treatment inhibited their adherence. The non-invasive strain V. damselae ATCC 33539 was found to be non-adherent; low-temperature incubation and heat treatment did not significantly affect ($P > 0.05$) its adherence (Tables 1 and 2). On the other hand, the non-invasive strain V. anguillarum S2/5/93(2) was able to adhere and its adherence ability was sensitive to the low temperature and heat treatments. This further suggests that internalization and adherence in these vibrios were regulated by different processes and proteins.

Molecules involved in adherence of two invasive strains

When EPC cells were treated with trypsin and proteinase K, adherence of V. anguillarum G/Virus/5(3) was partially inhibited (Table 3). This suggested that these
peptidases might modify EPC cell-surface proteins involved in bacterial adherence. Bacteria–host-cell interactions may depend on non-specific hydrophobic interactions or specific peptides such as the RGD sequence from the host cells for adherence (Ofek & Doyle, 1994). When the adherence assay was carried out in the presence of galactose, lactose and N-acetylgalactosamine, adherence of *V. anguillarum* G/Virus/5(3) to EPC cells was partially inhibited (Table 3). This suggests that vibrio structures involved in interactions with host-cell receptors contained galactose. Similar observations were reported for the binding of *Campylobacter jejuni* to human epithelial cells (McSweenan & Walker, 1986; Russell & Blake, 1994). The treatments with various mono- and disaccharides failed to completely inhibit adherence of *C. jejuni* (about 20–50% reduction in adherence). The partial inhibition may indicate that the process of adherence is very complex and affected by a number of factors.

Components involved in the adherence process of *V. anguillarum* G/Virus/5(3) may be LPS or glycoproteins. Treatment of bacteria with chemicals that modified carbohydrates inhibited adherence (Table 3). Furthermore, nonviable *V. anguillarum* G/Virus/5(3) obtained by heat treatment still showed some adherence capability (Table 2). This further supports the idea that a carbohydrate structure of the *V. anguillarum* G/Virus/5(3) is responsible for adherence (carbohydrates are relatively heat resistant). LPS molecules have been suggested to function as adhesins that mediate the binding of *C. jejuni* (McSweenan & Walker, 1986) and *A. hydrophila* (Merino et al., 1996b) to epithelial cells. The LPS has also been shown to mediate the interaction of bacteria with phagocytic cells (Perry & Ofek, 1984; Wright et al., 1989). Our ongoing project is to examine whether this adhesin molecule of *V. anguillarum* G/Virus/5(3) is LPS in nature.

In contrast to strain G/Virus/5(3), the adherence ability of *V. anguillarum* 811218-5W was not affected by most of the conditions and treatments except heat, periodic acid and antibiotic treatments of the bacteria (Tables 2 and 3). Furthermore, it was found to adhere to non-biological surface such as glass coverslips (Fig. 1d). Giemsa stain showed that it adhered to EPC cells in an aggregated pattern (Fig. 1a) and this strain did not exhibit any auto-aggregation phenotype in TSB, PBS or MEM. All of these results indicated that this strain is a super-adherent or non-specifically adherent strain. EPEC can preferentially attach to jejunal epithelial surface as discrete microcolonies in a pattern called localized adherence (LA) (Puente et al., 1996; Ramer et al., 1996). Expression of the bundle-forming pilus was correlated with the ability of EPEC strains to exhibit LA or auto-aggregation phenotypes. However, no pilus structure was found in *V. anguillarum* 811218-5W and G/Virus/5(3) (data not shown). This suggests that the molecules responsible for adherence in these strains may be afimbrial adhesins. De novo bacterial protein synthesis and intact carbohydrate structure were required for both strains to have effective adherence.

**Adherence characteristics of vibrios**

The adherence characteristics of super-adherence [represented by *V. anguillarum* 811218-5W] and galactose-linked adherence [represented by *V. anguillarum* G/Virus/5(3)] were found in other invasive vibrios (Table 4). Among the 12 *Vibrio* strains studied, *V. anguillarum* 811218-5W and *V. alginolyticus* 240/897 were found to have the super-adherence characteristics of high adherence index on EPC monolayers and glass coverslips as well as adherence at low temperature. However, aggregated patterns on EPC monolayers were only observed in *V. anguillarum* 811218-5W, *V. damselaE* E311 and *V. parahaemolyticus* W368-1p were not placed in this group due to their low adherence on glass coverslips and at low temperature. The aggregated adherence pattern of *V. damsela* E311 on EPC monolayers may be due to its auto-aggregation property rather than non-specific or super-adherence. Galactose inhibited the adherence of 9 out of 12 of the invasive vibrios we tested, the exceptions being *V. anguillarum* 811218-5, *V. damsela* E311 and *V. vulnificus* S1/4/93(4). The super-adherence and galactose-linked adherence characteristics could be present in the same strain, as evident in *V. alginolyticus* 240/89. On the other hand, the adherence characteristics of *V. damsela* E311 and *V. vulnificus* S1/4/93(4) do not belong to either of these two types.

Thus, at least two possible adherence characteristics were observed in our *Vibrio* isolates. It is possible that vibrios have evolved to possess many types of adhesins to maintain their niche in interacting with the host, as seen in *V. cholerae* (Sperandio et al., 1995; Manning, 1997) and other bacteria such as *E. coli* (DeGraaf, 1990; Hacker, 1990) and group *B* streptococci (Teti et al., 1987; Wibawan et al., 1991, 1992). Expression of different adhesins may depend on the different environmental conditions as well as the host-cell receptors. The understanding of these adherence processes will yield useful information on the initial steps of vibrio pathogenesis. This knowledge will be vital for the design and planning of fish health management programmes against vibriosis.

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**REFERENCES**


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