Vibrio cholerae persistence in aquatic environments and colonization of intestinal cells: involvement of a common adhesion mechanism

Massimiliano Zampini a, Carla Pruzzo b,*, Vijay P. Bondre c, Renato Tarsi a, Mariangela Cosmo a, Alessandro Bacciaglia a, Arvind Chhabra c, Renjana Srivastava c, Brahm S. Srivastava c

a Istituto di Microbiologia e Scienze Biomediche, Università Politecnica delle Marche, Ancona 60131, Italy
b Dipartimento di Biologia, Università degli Studi di Genova, Genova 16132, Italy
c Division of Microbiology, Central Drug Research Institute, Lucknow 226001, India

Received 13 September 2004; received in revised form 28 December 2004; accepted 28 January 2005

First published online 8 February 2005

Edited by M. Mitsuyama

Abstract

Forty-one TnphoA mutants of Vibrio cholerae O1 classical strain CD81 were analyzed for their ability to interact with chitin particles, Tigriopus fulvus copepods and the Intestine 407 cell line compared to the parent strain. Thirteen mutants were less adhesive than CD81; in particular, T21, T33 and T87 were less adhesive towards all substrates and insensitive to inhibition by N-acetyl glucosamine (GlcNAc). By SDS–PAGE analysis of sarkosyl-insoluble membrane proteins (siMPs) isolated from mutants and parent, it was found that a 53 kDa siMP is missing in T21, T33 and T87 mutants. It is hypothesized that this protein might have the function to mediate adherence to GlcNAc-containing substrates both in the aquatic environment and in human intestine.

© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Vibrio cholerae; Adherence; Chitin; Zooplankton; Intestinal cells

1. Introduction

Vibrio cholerae is a marine microorganism that can infect humans through contaminated water and raw or partially cooked seafood. It survives in aquatic environments for long periods by adhering to different biotic and abiotic surfaces [1–10]; epibiotic bacteria are protected against environmental stresses and survive in sea water significantly longer than free forms [7,11].

Plankton organisms, mainly the chitin-containing plankton fraction (e.g., copepods), seem to be the most plausible aquatic reservoir of V. cholerae and other vibrios [1,2,6,8,12,13] in both active and dormant (e.g., viable but nonculturable) state [5,13–16].

The ability of V. cholerae to adhere to animal cells has long been studied [17,18] and different ligands involved in intestinal colonization have been investigated, including virulence-associated toxin co-regulated pilus (TCP) [19–22], outer membrane proteins [17,18,23,24], and lipopolysaccharide [25]. Its interactions with substrates present in the aquatic environment have been described more recently. Watnick et al. [26] have shown
that V. cholerae O1 El Tor does not use TCP to form biofilm on abiotic surfaces (borosilicate and cellulose) but rather the mannose-sensitive hemagglutinin (MSHA) pilus [19], which has no role in pathogenicity. Attachment to chitin has been shown to be independent of the MSHA pilus, suggesting divergent pathways for biofilm formation on nutritive and nonnutritive abiotic surfaces [26]. Chiavelli et al. [27] have found that MSHA is involved in V. cholerae O1 El Tor and O139 adhesion to the exoskeleton of the planktonic crustacean Daphnia pulex; other ligands are hypothesized to be utilized by V. cholerae O1 classical strains for zooplankton adhesion [27]. Finally, V. cholerae O1 classical strain membrane proteins have been shown to mediate N-acetyl glucosamine (GlcNAc)-sensitive attachment to chitin particles in vitro [28].

GlcNAc is a widespread compound that, besides being the constituent of chitin, is also present in complex carbohydrate structures of animal cell membranes. Datta-Roy et al. [29] and Sasmal et al. [30] showed that adhesion to rabbit intestine of most V. cholerae O1 and non-O1 isolates was inhibited to various extents by GlcNAc-sensitive attachment to chitin particles and non-O1 mutants exhibiting similar phenotypes towards chitin, suggesting that a 53 kDa protein may be one of the factors involved inGlcnAc-sensitive ligands in mediating adherence to intestinal cells.

The above observations [28–30] suggested to us that V. cholerae may utilize the same ligand(s) to interact with GlcnAc-containing substrates present in the aquatic environment and in human intestine. To explore this possibility, TnphoA mutants of V. cholerae O1 classical strain CD81 [24] were investigated for their ability to attach to chitin-containing substrates (chitin particles and Tigriopus fulvus copepod exoskeleton [31]) and to cultured intestinal epithelial cells. It was reasoned that if the same V. cholerae ligand(s) is (are) involved in these interactions, it should be possible to isolate defective mutants exhibiting similar phenotypes towards chitin, copepods and epithelial cells. To our knowledge, this type of comparative study has not been performed before despite its importance to understanding the ecology of V. cholerae and its transmission from the aquatic environment to humans. The results, confirming the complexity of the adhesion process of V. cholerae, suggest that a 53 kDa protein may be one of the factors involved in GlcNAc-sensitive interactions with substrates present in both sea water and human intestine.

2. Materials and methods

2.1. Bacterial strains and culture conditions

V. cholerae O1 (classical biotype) strain CD81 [24] and previously generated CD81 TnphoA mutants (n = 41) containing an in frame insertion in genes encoding secreted or membrane proteins [24] were studied. Thiosulfate–citrate–bile salts–sucrose agar (Difco, Laboratories, Detroit, MI) and Brain-Heart Infusion Broth (BHB) and Brain Heart Infusion Agar (BHIA) (Difco) were used. To radiolabel bacteria, strains were grown in BHIB containing 10 μCi of [methyl-3H]thymidine (25 Ci/mmol) ml−1. After overnight growth, cells were harvested by centrifugation (3000g for 15 min at 4 °C), washed three times with phosphate-buffered 3% (w/v) NaCl solution (pH 8) and resuspended in the same buffer. Labeling efficiency (number of cells/count/min) was then evaluated in each suspension.

2.2. Attachment to chitin

Bacterial attachment to chitin particles was evaluated as described previously [28]. Briefly, an aliquot (1 ml) of labeled bacterial suspension (about 2 × 10^8 bacteria ml−1) was added to an aliquot (1 ml) of phosphate-buffered 3% (w/v) NaCl solution (pH 8) containing UV-sterilized chitin purified from crab shell (2.5 mg ml−1; Sigma Chemical Co., St. Louis, MO) and the mixture incubated for 2 h at 20 °C with shaking; a control sample without chitin was also prepared. Triplicate samples were prepared. After 2 h incubation, the mixtures were filtered through 8 μm pore size filters (25 mm polycarbonate membranes, Bio-Rad Laboratories srl, Milan, Italy) which were then rinsed with marine broth 2216 (10 ml) and radioassayed with a Beckman L5 1801 scintillation counter. The total number of cells attached to chitin particles was calculated based on efficiency of cell labeling. For background counts due to attachment of bacteria to the filtration membranes, duplicate samples for each treatment were incubated without chitin and filtered to correct for unattached cells left on the filter. The radioactivity of control filters (typically 50–250 cpm vs. 10,000 cpm in samples with chitin) was subtracted from the values of samples to calculate the radioactivity of cells attached to the chitin particles. Other tests were performed in the presence of GlcNAc at the final concentration of 10 mg ml−1.

2.3. Adherence to copepods

Adult Tigriopus fulvus copepods of the family Harpacticoida, commonly found in rock-pools around Genoa (Ligurian Sea, Italy) [31], were cultured in filter-sterilized artificial sea water (ASW) (salinity, 3.7%, pH7.5) and fed unicellular phytoplanktonic algae (Tetraselmis suecica). Before the association assay, they were washed 10 times with artificial sea water (ASW, Sigma) to remove adhering bacteria. One milliliter of radiolabeled bacterial suspension (about 2 × 10^8 bacteria ml−1) was then added to 1 ml ASW containing 10 washed copepods and incubated at 20 °C. Three replicates of each treatment were prepared. After 2 h incubation, suspensions were filtered through 12 μm pore size filters (25 mm polycarbonate membranes, Bio-Rad)
which were then rinsed with ASW and radioassayed with a Beckman L5 1801 scintillation counter. The total number of cells per copepod was calculated using efficiency of cell labeling. Some tests were performed in the presence of GlcNAc at a final concentration of 10 mg ml\(^{-1}\).

2.4. Adherence to the Intestine 407 cell line

Monolayers of Intestine 407 (ATCC CCL 6) cell line were prepared on 20 \(\times\) 22 mm glass coverslips placed in plastic culture dishes. Cells were grown in Eagle minimum essential medium with Earle salts (Flow Laboratories, McLean, VA) supplemented with 1% nonessential amino acids (Flow), 10% fetal calf serum (Gibco), 100 U ml\(^{-1}\) penicillin and 100 \(\mu\)g ml\(^{-1}\) streptomycin. Cells were incubated to confluence at 37°C in a 5% CO\(_2\) atmosphere. Bacterial ability to adhere to monolayers was tested by adding 1.5 ml of phosphate-buffered saline (PBS; 0.1 M Na\(_2\)HPO\(_4\), 0.1 M KH\(_2\)PO\(_4\), 0.15 M NaCl, pH 7.2–7.4) containing radiolabeled bacteria at a final concentration of about 5 \(\times\) 10\(^5\) bacteria ml\(^{-1}\); the dishes were incubated with gentle shaking at 37°C. Triplicate preparations were made for each sample. After 60 and 120 min incubation, the coverslips were rinsed three times in 3 ml cold PBS to remove nonadherent bacteria and transferred to PICO-FLUORTM15 scintillation fluid (Packard Instruments Company Inc., Meriden, CT). The radioactivity (cpm) of each monolayer was measured using a Beckman L5 1801 scintillation counter. The number of bacteria per monolayer was then calculated by multiplying the cpm values by efficiency of cell labeling. For background counts, control samples without cells were also prepared and their radioactivity was subtracted from that of the samples.

2.5. Isolation of N-dodecanoylsarcosinate (sarkosyl)- insoluble membrane proteins (siMPs)

Bacterial cultures were grown overnight in BHIB to an \(A_{650}\) of 1.5. Bacteria were centrifuged (10,000g, 20 min, 4°C) three times and resuspended in 125 mM Tris–HCl (pH 6.7). Concentrated cells were ultrasonicated (Ultrasonic Liquid Processor XL 2020, Heat System) at 20% power for 30 s on ice five times with 60 s cooling intervals between each sonication. The samples were centrifuged at 10,000g (20 min, 4°C) to pellet unbroken cells and then at 100,000g (60 min, 4°C) to pellet cell membranes. Pellets were suspended in 125 mM Tris–HCl (pH 6.7), treated for 30 min at 20°C with 0.5% (w/v) sarkosyl (Sigma), and then centrifuged at 100,000g (60 min, 20°C). This step was repeated twice and the last pellet was washed with 125 mM Tris–HCl (pH 6.7) and centrifuged as above. The undissolved material was removed by centrifugation at 10,000g (20 min, 4°C) and the supernatant, containing sarkosyl-insoluble membrane proteins (siMPs), was divided in aliquots that were used in further experiments. Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [32] using a 3% (w/v) acrylamide stacking gel and a 15% (w/v) separating gel; protein bands were visualized by silver staining (Bio-Rad). The determination of protein concentration was performed using a Bio-Rad protein assay.

2.6. Hydrophobicity assay

Bacterial surface hydrophobicity was evaluated as described by Rosenberg et al. [33]. Briefly, bacteria cultured overnight in BHIB were harvested, washed twice with PBS and resuspended in phosphate–urea–magnesium (PUM) buffer (K\(_2\)HPO\(_4\) : 3H\(_2\)O 22.2 g l\(^{-1}\), KH\(_2\)PO\(_4\) 7.26 g l\(^{-1}\), MgSO\(_4\) 0.2 g l\(^{-1}\), urea 1.8 g l\(^{-1}\), pH 7.1) to OD\(_{560}\) = 0.5; 1.2 ml samples were then placed in test tubes and 0.1 ml (or 0.5 ml) p-xylene was added. Following 10 min preincubation at 30°C, the tubes were vortexed for 1 min and allowed to stand at room temperature for 20 min; then the lower aqueous phase was removed and the OD\(_{560}\) of the suspension measured with a spectrophotometer (Kontron Instruments, UVICON 860). The results were expressed as percentage of the initial OD\(_{560}\).

2.7. Statistical analysis

Data, representing the means of three separate trials, were analyzed using the Mann–Whitney \(U\) test. Differences were considered significant at \(P \leq 0.05\).

3. Results and discussion

3.1. Attachment to chitin-containing surfaces and adherence to epithelial cells of \(V.\) cholerae CD81 TnphoA mutants

As a first approach to assessing whether \(V.\) cholerae CD81 presents GlcNAc-sensitive ligand(s) that bind to both chitin-containing substrates and intestinal cells, we analyzed the adhesive properties of 41 \(V.\) cholerae CD81 TnphoA mutants containing an in frame insertion in genes encoding secreted or membrane proteins [24], including mutants T69 and T92, which exhibit impaired ability to colonize freshly isolated rabbit intestinal tract cells, and T87, which is less toxigenic than the parent and lacks 53 and 38 kDa surface proteins [24]. As stated above, we tested the hypothesis that if at least one \(V.\) cholerae ligand bound to all substrates, we should find mutants exhibiting both impaired adherence to epithelial cells and a reduced association with chitin and copepods.
Thirteen mutants were shown to be less adhesive than the parent strain (Table 1). Mutants T3, T8, T10, T15, T23 and T31 interacted with chitin particles and copepods less efficiently than CD81, but their ability to adhere to intestinal cells was similar to that of the parent. In contrast, T7, T69 and T92 adhered less efficiently to Intestine 407 cells than CD81 and were as adhesive to chitin particles and copepods as the parental strain. Mutants T16, T21, T33 and T87 exhibited impaired interaction with both chitin-containing substrates and epithelial cells.

In the previous experiments we had noted that interaction of CD81 bacteria with the tested substrates was less efficient in the presence of GlcNAc than in control experiments. In fact, after 2 h incubation GlcNAc inhibited bacterial association with chitin, copepods and intestinal cells by 57%, 49%, and 43%, respectively (P < 0.05). To see whether in some mutants impaired adherence was associated with the loss of GlcNAc ligands, the experiments reported in Table 1 were also performed in the presence of GlcNAc. Most mutants showed reduced adhesion efficiency to the substrates compared with the respective untreated controls, with the exception of T21, T33 and T87 (Table 2). The mutants were thus grouped into four phenotypic groups: Groups 1 and 2 included mutants showing a reduced ability to interact with chitin-containing substrates and with intestinal cells, respectively (Table 1), but having GlcNAc-sensitive ligand(s) involved in interactions with all tested substrates, as shown by the fact that such interactions were affected by GlcNAc (Table 2). Accordingly, group 1 mutants might have lost GlcNAc-resistant ligand(s) towards chitin-containing surfaces, while group 2 mutants might have lost GlcNAc-resistant ligand(s) towards intestinal cell. The GlcNAc-sensitive ligand(s) were also present in the sole member of Group 3, T16 (Table 2), which exhibited impaired ability to interact with all tested substrates (Table 1). This mutant might have lost GlcNAc-resistant ligand(s) towards all tested substrates. Group 4 consisted of mutants T21, T33 and T87, with impaired interaction with both chitin-containing substrates and epithelial cells (Table 1) and defective in GlcNAc-sensitive ligand(s), as indicated by the fact that such interactions were not affected by GlcNAc (Table 2). Accordingly, distinct bacterial adherence factors for the tested substrates may be present on CD81; they include GlcNAc-resistant ligand(s) involved in interactions with chitin-containing substrates, GlcNAc-resistant ligand(s) involved in adherence to Intestine 407 cells, and GlcNAc-sensitive ligand(s) involved in interactions with both chitin-containing substrates and intestinal cells.

### 3.2. Analysis of siMPs and surface hydrophobicity

The results obtained suggested that mutants T21, T33 and T87 might have lost (or undergone a modification of) the ligand performing the function to bind to GlcNAc-containing substrates present in the aquatic environment and in human intestinal cells. Since T87 lacks 53 kDa and 38 kDa surface proteins [24], siMPs of the wild-type strain CD81 and of its TnphoA mutants were extracted and analyzed by SDS-PAGE. As shown in Fig. 1, mutants T21, T33 and T87 lack a 53 kDa protein; as expected, T87 also lacks a 38 kDa protein. Interestingly, a 42 kDa band, not found in the parent strain, is observed in T21. None of the other CD81 derivatives tested showed any difference from the parent (Fig. 1 illustrates the results obtained with two representative mutants, T31 and T16). These results, indicating that a

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean number of bacteria(^a) per 2.5 mg chitin ± SD (x10^6)</th>
<th>Copepod ± SD (x10^3)</th>
<th>Intestine 407 monolayer ± SD (x10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control CD81</td>
<td>92 ± 6</td>
<td>45 ± 3</td>
<td>142 ± 8</td>
</tr>
<tr>
<td>T3</td>
<td>49 ± 7 (47%)</td>
<td>22 ± 4 (51%)</td>
<td>136 ± 15 (4%)(^b)</td>
</tr>
<tr>
<td>T8</td>
<td>45 ± 4 (51%)</td>
<td>21 ± 2 (53%)</td>
<td>140 ± 12 (1%)(^b)</td>
</tr>
<tr>
<td>T10</td>
<td>47 ± 5 (49%)</td>
<td>22 ± 2 (51%)</td>
<td>130 ± 9 (8%)(^b)</td>
</tr>
<tr>
<td>T15</td>
<td>40 ± 5 (57%)</td>
<td>20 ± 1 (56%)</td>
<td>133 ± 11 (6%)(^b)</td>
</tr>
<tr>
<td>T23</td>
<td>49 ± 6 (47%)</td>
<td>21 ± 2 (53%)</td>
<td>132 ± 12 (7%)(^b)</td>
</tr>
<tr>
<td>T31</td>
<td>48 ± 7 (48%)</td>
<td>22 ± 1 (51%)</td>
<td>144 ± 11 (^b)</td>
</tr>
<tr>
<td>T7</td>
<td>102 ± 11(^b)</td>
<td>48 ± .6(^b)</td>
<td>71 ± 6 (50%)</td>
</tr>
<tr>
<td>T69</td>
<td>107 ± 14(^b)</td>
<td>49 ± 4(^b)</td>
<td>74 ± 8 (47%)</td>
</tr>
<tr>
<td>T92</td>
<td>101 ± 16(^b)</td>
<td>48 ± 6(^b)</td>
<td>89 ± 8 (44%)</td>
</tr>
<tr>
<td>T16</td>
<td>40 ± 5 (57%)</td>
<td>21 ± 2 (53%)</td>
<td>59 ± 6 (58%)</td>
</tr>
<tr>
<td>T21</td>
<td>40 ± 3 (57%)</td>
<td>30 ± 4 (33%)</td>
<td>76 ± 18 (47%)</td>
</tr>
<tr>
<td>T33</td>
<td>51 ± 6 (45%)</td>
<td>27 ± 5 (40%)</td>
<td>83 ± 9 (42%)</td>
</tr>
<tr>
<td>T87</td>
<td>52 ± 4 (44%)</td>
<td>29 ± 3 (36%)</td>
<td>66 ± 8 (54%)</td>
</tr>
</tbody>
</table>

\(^a\) Values in parentheses indicate percentage of adherence inhibition compared with parental strain CD81. Results were obtained after 2 h incubation. The differences with the respective controls are significant (P < 0.05) except for \(^b\).
53 kDa protein is absent in all group 4 mutants and in these mutants only, suggest that this protein might be involved in GlcNAc-sensitive interactions with the tested substrates. The fact that mutant T16, though exhibiting defective interaction with all tested substrates, is sensitive to inhibition by GlcNAc and shows the same siMP electrophoretic pattern as the parent, suggest that *V. cholerae* may carry other unidentified adhesin(s) capable of binding to a broad range of substrates.

With a view to establishing preliminarily whether the different adhesive properties of CD81 and its mutants could also depend on modifications in surface hydrophobicity, which strongly affects bacterial interaction with different substrates, we evaluated the degree of adherence of the bacteria to xylene [33] using a decrease in absorbance of the aqueous bacterial suspension after xylene addition as a measure of cell surface hydrophobicity. The results did not show significant differences between the wild-type parent and its Tn*pho*A mutants, as all tested strains showed a similar decrease in absorbance of the aqueous phase (20–30% and 35–45% of the initial optical density, with the addition of 0.1 and 0.5 ml xylene, respectively). These data make it unlikely that mutant strain defects in adherence efficiency are related to differences in surface hydrophobicity.

### Table 2

<table>
<thead>
<tr>
<th>Phenotypic group</th>
<th>Strain</th>
<th>Mean number of bacteria* per 2.5 mg chitin ± SD (×10^6)</th>
<th>Copepod ± SD (×10^3)</th>
<th>Intestine 407 monolayer ± SD (×10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control + GlcNAc % inhibition</td>
<td>Control + GlcNAc % inhibition</td>
<td>Control + GlcNAc % inhibition</td>
</tr>
<tr>
<td>1</td>
<td>T3</td>
<td>45 ± 6</td>
<td>35 ± 3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>T8</td>
<td>40 ± 4</td>
<td>282 ± 3</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>T10</td>
<td>49 ± 5</td>
<td>253 ± 5</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>T15</td>
<td>45 ± 5</td>
<td>222 ± 4</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>T23</td>
<td>46 ± 4</td>
<td>283 ± 3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>T31</td>
<td>44 ± 3</td>
<td>313 ± 2</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>T7</td>
<td>99 ± 8</td>
<td>56 ± 7</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>T69</td>
<td>102 ± 9</td>
<td>617 ± 40</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>T92</td>
<td>110 ± 10</td>
<td>62 ± 6</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>T16</td>
<td>38 ± 3</td>
<td>17 ± 2</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>T21</td>
<td>44 ± 5</td>
<td>45 ± 4</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>T33</td>
<td>49 ± 4</td>
<td>48 ± 5</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>T87</td>
<td>55 ± 4</td>
<td>57 ± 6</td>
<td>–</td>
</tr>
</tbody>
</table>

*: no inhibition by GlcNAc.

Results were obtained after 2 h incubation. The differences between GlcNAc-treated samples and the respective controls are significant (*P* ± 0.05) except for b.

Fig. 1. SDS–PAGE separation of siMPs isolated from parental strain CD81 and its Tn*pho*A mutants T16, T21, T31, T33 and T87. Fifteen micrograms of proteins were loaded in each lane. Molecular sizes of markers (kDa) are shown on the right. The position of the 53 kDa protein is indicated by an arrow.

3.3. Inhibition of *V. cholerae* adherence by siMPs

To partly confirm these results other experiments were performed, challenging chitin, copepods and intestinal monolayers with strain CD81 in the presence of siMPs extracted from CD81 and phenotypic group 4 mutants, T21, T33 and T87. As shown in Table 3, CD81 interactions with chitin, copepods and monolayers decreased by 60%, 53% and 64%, respectively, in the presence of CD81 siMPs. In contrast, CD81 interactions with chitin, copepods and intestinal monolayers decreased by 43%, 20% and 43%, respectively, in the presence of CD81 siMPs. In all cases (not shown). These data suggest that the lesser inhibition of CD81 adherence induced by T87 (or T21 or T33) siMPs compared with CD81 siMPs may depend on the fact that the former fractions do not contain the 53 kDa protein.
Overall, our data suggest that the 53 kDa protein can be one of the candidates of the factor responsible for GlcNAc-sensitive interactions of V. cholerae CD81 with chitin particles, copepods, and Intestine 407 cells. In fact, the protein is not found only in Group 4 mutants (T21, T33 and T87), which exhibit a severely impaired ability to adhere to the substrates and are not sensitive to inhibition by GlcNAc. Moreover, T87 has previously been shown to be very poor at colonizing freshly isolated rabbit intestinal tract cells and to be less toxigenic in vitro than the parent [24]. The nature of the TnphoA mutation in T87 mutant has been analyzed (Chhabra and Srivastava, unpublished). The transposon insertion appears to have occurred in a regulatory gene which controlled coordinate expression of a number of virulence determinants [24]. The nucleotide sequence of regulatory gene (1296 bp) revealed the presence of two open reading frames constituting an operon (EMBL Accession # AJ277893). The 1296 bp sequence covering ORFI and ORFII shows 100% homology to a contig of V. cholerae genome chromosome I representing genes VC0973 and VC0974 [34]. Analysis with various proteomic tools suggests that ORFI codes for a sensor protein whereas ORFII appears to be a transcriptional regulatory protein. Therefore the defect in T87 class of mutants lies in a regulatory gene which down regulates a number of virulence determinants including adhesins such as 53 kDa protein.

Since a 53 kDa chitin-binding protein was observed in V. cholerae O1 classical biotype [28], Vibrio alginolyticus [35], V. cholerae O1 El Tor biotype, other V. cholerae serotypes and other Vibrio species (unpublished results), it is our endeavour to purify it in order to reveal gene identity. Moreover, experiments are in progress in our laboratory to evaluate the expression of the 53 kDa protein in different environmental conditions.

Overall the data reported herein, confirming that the process of V. cholerae adherence to biotic and abiotic surface is very complex, indicate that this microorganism carries a repertoire of adhesins that include ligand(s) (e.g., the 53 kDa protein) with the function to bind GlcNAc-containing receptors present in both sea water and human hosts. It may be hypothesized that V. cholerae, evolving as a marine bacterium able to interact with chitin-containing substrates present in sea water, has eventually adapted its adhesive properties to the human host. The ability to use the same structure to interact with different substrates may be a common property of pathogenic bacteria that have environmental reservoirs, and may represent a discriminating feature between harmless and potentially pathogenic environmental bacteria.

Acknowledgments

This work was supported by Ministero dell’Università e della Ricerca Scientifica e Tecnologica (COFIN2000 and COFIN2003) and Ministero Affari Esteri, Italy–India Research Program.

References


Table 3
Role of siMPs in attachment of V. cholerae CD81 to chitin particles, copepods and intestinal cell monolayers

<table>
<thead>
<tr>
<th>siMPs from</th>
<th>Mean number of CD81 bacteria$^*$ per 2.5 mg chitin ± SD (×10$^6$)</th>
<th>Copepod ± SD (×10$^3$)</th>
<th>Intestine 407 monolayer ± SD (×10$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD81</td>
<td>36 ± 6 (60%)</td>
<td>21 ± 3 (53%)</td>
<td>48 ± 7 (64%)</td>
</tr>
<tr>
<td>T21</td>
<td>50 ± 5 (44%)</td>
<td>37 ± 4 (18%)</td>
<td>81 ± 6 (39%)</td>
</tr>
<tr>
<td>T33</td>
<td>49 ± 6 (46%)</td>
<td>38 ± 4 (16%)</td>
<td>80 ± 7 (40%)</td>
</tr>
<tr>
<td>T87</td>
<td>51 ± 7 (43%)</td>
<td>36 ± 2 (20%)</td>
<td>76 ± 11 (43%)</td>
</tr>
<tr>
<td>Control without siMPs</td>
<td>90 ± 9</td>
<td>45 ± 2</td>
<td>133 ± 15</td>
</tr>
</tbody>
</table>

$^*$ Results were obtained after 2 h incubation using 5 μg siMPs mg $^{-1}$ chitin. Values in parentheses indicate percentage of inhibition compared with untreated controls. The differences between siMP treated samples and the respective controls are statistically significant ($P < 0.05$).


