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Attachment of *Vibrio alginolyticus* to chitin mediated by chitin-binding proteins

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Vibrio alginolyticus **is the only culturable vibrio associated with the chitinaceous carapace of the copepod *Tigriopus fulvus* (Fisher 1860) living in Ligurian coastal rock pools (Tyrrhenian Sea). The characteristics of the interaction between chitin particles and *V. alginolyticus* were studied by analysing strains isolated both from the copepod surface and from rock-pool water. The highest degree of attachment to chitin was observed at 20 °C, in the presence of 3% NaCl. Bacterial treatment with *N*-acetylglucosamine and pronase E caused a reduction in attachment of 52–62% and 77–94%, respectively. Chitin pretreatment with either wheat germ agglutinin or membrane proteins (MPs) from *V. alginolyticus* caused a reduction in attachment, of 50–57% and 53–70%, respectively. No inhibition was observed when bacteria were pretreated with D-glucose, D-fucose or D-fructose, or when chitin was pretreated with concanavalin A and *Escherichia coli* DH5α MPs. *V. alginolyticus* MPs able to bind chitin were isolated and analysed by SDS-PAGE. Four chitin-binding proteins were visualized in all tested strains (53, 35, 20 and 14 kDa); *in vivo* these peptides may efficiently mediate *V. alginolyticus* attachment to chitin-containing substrates.**

Keywords: plankton–bacteria interactions, *Vibrio alginolyticus*, attachment, chitin-binding proteins

INTRODUCTION

Chitin is one of the most abundant organic compounds in nature; it consists of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) units. Large reservoirs of chitin are derived from the cell walls of fungi and the cuticles of insects and crustacea (Jannatipur *et al.*, 1987). In natural marine environments, a significant percentage of total zooplankton biomass is generally represented by copepods (Carli *et al.*, 1994; Hirche & Mumm, 1992; Hopkins, 1985); these small crustacea produce several tons of the polysaccharide annually and significantly contribute to detrital chitin pool formation (Yu *et al.*, 1991).

In the aquatic environment chitin is heavily colonized by bacteria that are responsible for the mineralization of this insoluble polysaccharide by chitinase activity (Gooday *et al.*, 1991; Miyamoto *et al.*, 1991; Smith *et al.*, 1992). This process, which is essential for recycling carbon and nitrogen in marine ecosystems, can be separated into four steps: random collision or chemotaxis, attachment to the polymer, degradation to soluble oligosaccharides, and hydrolysis to GlcNAc (Yu *et al.*, 1991).

Despite the great importance of this process in several areas of microbial ecology, little is known about the specific attachment of bacteria to chitin-containing surfaces. It must be pointed out that this process is also important for bacterial colonization of living zooplankton organisms that are known to support a significant number of attached bacteria (e.g. *Vibrio* spp.) on their surface (Colwell, 1981; Huq *et al.*, 1983, 1984).

It has recently been suggested that marine microorganisms adhering to chitin surfaces have evolved specific systems to recognize and associate with this substrate (Yu *et al.*, 1987; Montgomery & Kirchman, 1993, 1994). A specific attachment to GlcNAc has been demonstrated in *V. furnissii* and *V. damsela*, and adhesion to immobilized GlcNAc has been shown for several *Vibrio* species (Yu *et al.*, 1987, 1991). Moreover, Montgomery & Kirchman (1993) demonstrated that *V. harveyi* can specifically attach to chitin particles through

Abbreviations: CBP, chitin-binding protein; MP, membrane proteins; GlcNAc, *N*-acetylglucosamine; WGA, wheat germ agglutinin; ConA, concanavalin A.

chitin-binding proteins (CBPs) associated with cell membranes.

We have previously shown that the copepod *Tigriopus fulvus* (Fisher 1860) living in Ligurian coastal rock pools is colonized during warmer months by *V. alginolyticus*, the only culturable vibrio found to be associated with the carapace (Carli *et al.*, 1993). The specific interaction occurring between *T. fulvus* and *V. alginolyticus* has prompted us to study the mechanism whereby these bacteria attach to chitin. Here we show the presence of CBPs in the envelope of strains colonizing the copepod carapace and those living freely in the rock-pool water. The role of these molecules in the interaction with both the copepod surface and detrital chitin is discussed.

METHODS

Bacterial strains and culture conditions. *V. alginolyticus* strains were isolated from the *T. fulvus* carapace (T2, T3 and T4 strains) and rock-pool water (P1, P2 and P3 strains) as previously described (Carli *et al.*, 1993). Gram-negative, rod-shaped, oxidase-positive isolates grown on thiosulfate/citrate/bile salts/sucrose agar (Difco) were assigned to the various species by using the API20E identification system (API System) with 1·7% (v/v) saline as inoculum diluent, and by analysing their growth in nutrient broth supplemented with NaCl at concentrations ranging from 0 to 10% (Farmer *et al.*, 1991). A non-chitin-binding strain, *Escherichia coli* DH5α (Hanahan, 1983), was used as a control in experiments where both bacterial attachment to chitin and the effect of membrane proteins (MPs) on such an interaction were evaluated.

Marine broth 2216 (Difco) was used for all *Vibrio* strains; plates were poured adding Bacto-agar at a final concentration of 15 g l^{-1}. Luria–Bertani agar and broth (Sambrook *et al.*, 1989) were used for culturing *E. coli*. All cultures were grown at 20 °C for 18 h. To determine the effect of chitin, chitobiose or chitotriose on the expression of CBPs, bacteria were grown in presence of either colloidal chitin (Jeuniaux, 1966) or the other sugars at the final concentration of 0·2% (w/v). To radiolabel bacteria, strains were grown in marine broth containing 10 μCi (370 kBq) [*methyl*-^{3}H]thymidine [25 Ci (925 GBq) $mmol^{-1}$] ml^{-1}. After overnight growth, cells were harvested by centrifugation (3000 ***g*** for 15 min at 4 °C), washed three times with phosphate-buffered 3% (w/v) NaCl (pH 8) and resuspended in the same buffer to an OD_{650} of 0·1. The efficiency of cell labelling (number of cells per c.p.m.) varied from 700 to 2500 among strains.

Attachment assay. Bacterial attachment to chitin particles was evaluated as previously described (Montgomery & Kirchman, 1993). Briefly, 1 vol. radiolabelled bacterial suspension was added to 1 vol. phosphate-buffered 3% NaCl (pH 8) containing UV-sterilized chitin purified from crab shell (2·5 mg ml^{-1}; Sigma) and the mixture was incubated at 20 °C with shaking; a control sample without chitin was also prepared. At timed intervals, three replicates of each treatment were filtered onto 8 μm pore-size filters (25 mm polycarbonate membranes, Bio-Rad), 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 using the efficiency of cell labelling. To evaluate background counts due to attachment of bacteria to 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 these control filters (typically 50–250 c.p.m. versus 10000 c.p.m. for samples with chitin) was subtracted from the sample values to give the radioactivity of cells that had attached to the chitin particles. To evaluate the efficiency of the filtration process, a non-chitin-binding strain, *E. coli* DH5α, was incubated with chitin and filtered as above. No more than 5% of bacteria present in the inoculum (versus 80–90% for *V. alginolyticus* strains) bound to the filters.

To evaluate the effect of NaCl on adsorption, experiments were performed using NaCl solutions varying from 0·5 to 5·5% (w/v). When the effect of pH on the adsorption onto chitin particles was studied, experiments were performed in 3% NaCl solutions at a pH ranging from 6 to 10. To study the effects of $MgSO_4$, $MgCl_2$ and $CaCl_2$, experiments were run using the same NaCl solution (3%, pH 8) with 0, 0·01, 0·1 and 1% (w/v) of each ion added to the solution.

Some experiments were performed by incubating chitin with MPs [5–15 μg (mg chitin)$^{-1}$], wheat germ agglutinin (WGA) or concanavalin A (ConA) [100 μg (mg chitin)$^{-1}$]; in other tests bacteria were treated with GlcNAc, D-glucose, D-fructose or D-fucose (final concn 100 mg ml^{-1}). All incubations were performed for 30 min at 20 °C; thereafter, either chitin or bacteria were harvested by centrifugation and used in standard adsorption experiments. To analyse the effect of bacterial treatment with pronase E (Sigma), the enzyme was added to bacterial suspensions at a final concentration of 100 μg ml^{-1}. The suspensions were then incubated for 1 h at 37 °C in a shaking waterbath and centrifuged. The pellets were resuspended in phosphate-buffered 3% NaCl (pH 8) to the original volume. Sodium *m*-periodate pretreatment of bacteria was performed in PBS containing sodium *m*-periodate at a final concentration of 10 mM. The suspensions were incubated at room temperature for 10 min, washed twice and resuspended in phosphate-buffered 3% NaCl.

Preparation of Sarkosyl-resistant membrane proteins (MPs). Cultures of *V. alginolyticus* were grown overnight in marine broth 2216 to an OD_{650} of 1·5. Bacteria were centrifuged (10000 ***g***, 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. This sonication step was repeated five times, with a 60 s cooling period between each sonication. The samples were centrifuged at 10000 ***g*** (20 min, 4 °C) to pellet unbroken cells and then at 100000 ***g*** (40 min, 4 °C) to pellet cell membranes. The sediment was resuspended in Tris, treated for 30 min at 20 °C with 0·5% (w/v) Sarkosyl (Sigma) and then centrifuged at 100000 ***g*** (40 min, 20 °C). This step was repeated three times and the last pellet washed with Tris and centrifuged as above. Undissolved material was removed by centrifugation at 10000 ***g*** (20 min, 4 °C) and the supernatant, containing Sarkosyl-resistant MPs, was subdivided into aliquots that were used in further experiments. Proteins were analysed by SDS-PAGE (Laemmli, 1970), using a 3·85% (w/v) acrylamide stacking gel and a 12·5% (w/v) separating gel. Protein concentration was measured using a Bio-Rad protein assay.

Isolation of chitin-binding proteins (CBPs). CBPs were isolated according to the procedure described by Montgomery & Kirchman (1993). Briefly, aliquots (0·1 ml) of the Sarkosyl-extracted membrane fraction were incubated (30 min at 25 °C) with 5 mg portions of chitin particles. The mixture was then washed with TBS (25 mM Tris buffer pH 7·5 and 150 mM NaCl) and centrifuged (10000 ***g***) to remove proteins not binding to chitin; this step was repeated five times. Aliquots (40 μl) of the pellet resuspended in TBS were then added to 40 μl loading buffer [100 mM Tris/HCl pH 6·8, 100 mM dithiothreitol, 2%

(w/v) SDS, 0·2% (w/v) bromophenol blue, 20% (v/v) glycerol], heated in boiling water (10 min) to remove CBPs from the chitin particles, and centrifuged (10000 ***g***, 10 min, 25 °C). The supernatant was assayed for total proteins and analysed by SDS-PAGE. The Sarkosyl-extracted membrane fraction and the CBP samples typically contained 2 and 0·1 mg protein ml^{-1}, respectively.

Statistical calculation. Differences in adsorption efficiencies between control and inhibitor-treated samples were analysed for significance using the Student's *t* test. Differences were considered significant at $P \leqslant 0{\cdot}01$.

RESULTS

V. alginolyticus attachment to chitin particles

Conditions for optimal attachment to chitin particles were studied by varying temperature, pH, and NaCl and other salt concentrations. Analysed values were selected with respect to the range of conditions found in natural environments (sea and rock-pool water) (Carli *et al.*, 1993). Two strains were used, one isolated from the copepod carapace (T3) and one from the rock-pool water (P2). The efficiency of attachment was found to be dependent on NaCl concentration and temperature. The percentage of bacteria attached to chitin increased by up to 80% in presence of NaCl concentrations varying from 0·5 to 3% and then decreased. Attachment to chitin progressively increased from about 65% to about 80% as temperature increased from 5 to 20 °C; a progressive reduction in attachment was observed at higher temperatures. The other tested salts ($MgSO_4$, $MgCl_2$ and $CaCl_2$) and pH did not have any relevant effect on *V. alginolyticus* interactions with chitin beads.

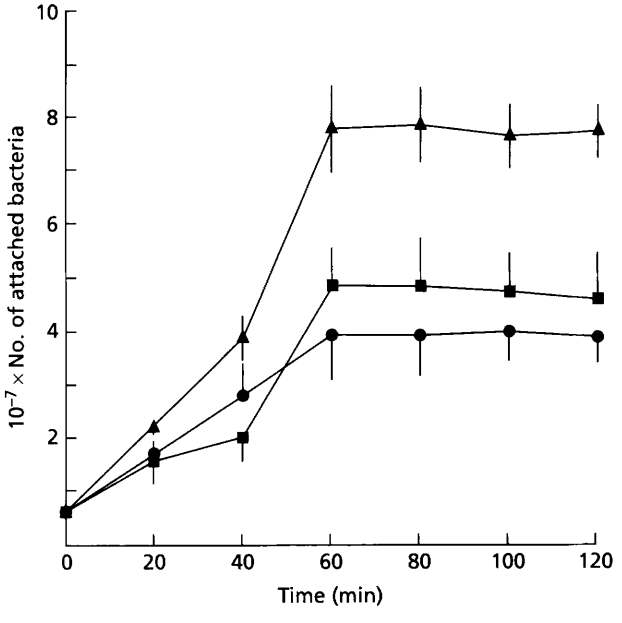

Fig. 1. Time course of *V. alginolyticus* T3 attachment to chitin particles. Results are means of three experiments performed with three cultures on different days; bars represent SD. Similar results were obtained with strain P2. The original bacterial inoculum was 5×10^7 (●), 1×10^8 (■) and 3×10^8 (▲).

The attachment of *V. alginolyticus* to chitin particles, as a function of time, is shown in Fig. 1. The total number of attached bacteria increased with time and levelled off after 60 min, when equilibrium was achieved; the number of bacteria attached to chitin was approximately 4×10^7, 5×10^7 and 8×10^7 when 5×10^7, 1×10^8 and 3×10^8 bacteria, respectively, were added to the assay, suggesting that saturation of the chitin surface occurred as more cells were added to the system.

No difference in binding efficiency was observed between strains isolated from the copepod surface and those from the water.

Inhibition of *V. alginolyticus* attachment to chitin

The effect of treatment of bacterial cells with pronase E and sodium *m*-periodate on the attachment was studied as a preliminary molecular characterization of the system involved in the interactions between *V. alginolyticus* and chitin. As shown in Table 1, while pronase E treatment caused a 77–94% reduction of attachment, sodium *m*-periodate, which oxidizes polysaccharides, did not affect the interaction, suggesting that *V. alginolyticus* attaches to chitin particles through a protein system. To confirm this hypothesis, we isolated Sarkosyl-resistant MPs from the tested strains and evaluated their effect on *V. alginolyticus* attachment to chitin (Table 2). A 53–70% reduction was observed when bacterial strains were treated with homologous MPs; in contrast, MPs isolated from the non-chitin-binding strain *E. coli* DH5α had no effect.

To analyse the sugar specificity of the protein(s) involved in binding to chitin, bacterial attachment was evaluated after either cell treatment with GlcNAc, the sugar that makes up chitin, or chitin treatment with WGA, a lectin that binds GlcNAc. As a control, bacteria were also treated with D-glucose, D-fructose and D-fucose, and chitin was treated with ConA, a lectin that binds polymers containing D-mannose and D-glucose. As shown in Table 1, bacterial treatment with GlcNAc inhibited *V. alginolyticus* attachment to chitin by 52–62%; in contrast, D-glucose, D-fucose and D-fructose had no effect. Chitin treatment with WGA (Table 2) reduced *V. alginolyticus* attachment to chitin by 50–57%, while ConA had no effect. These results clearly show that *V. alginolyticus* uses surface proteins to attach specifically to GlcNAc residues of chitin particles.

No difference was observed between strains deriving from the copepod (T2, T3, T4) and those isolated from the rock-pool water (P1, P2, P3).

Isolation of chitin-binding proteins from *V. alginolyticus* strains

Sarkosyl-resistant MPs isolated from the tested strains were incubated with chitin. SDS-PAGE analysis of peptide(s) bound to chitin revealed four peptides of 53,

Table 1. Effect of treatment of *V. alginolyticus* with pronase E, sodium *m*-periodate and sugar on attachment to chitin

Attachment is expressed as 10^{-6} × mean no. of bacteria (2·5 mg chitin)$^{-1}$ ± SD. Results are means of three independent experiments performed with three cultures on different days. Only the differences between samples treated with GlcNAc or pronase E and their untreated controls were statistically significant ($P \leqslant 0{\cdot}001$).

Strain*	Treatment								
	Control	Pronase E		Sodium *m*-periodate		GlcNAc		D-Glucose†	
		Attachment	% Inhibition‡	Attachment	% Inhibition‡	Attachment	% Inhibition‡	Attachment	% Inhibition‡
T2	79±3	15±1	81	80±2	–	34±3	57	83±3	–
T3	83±9	17±2	80	86±12	–	38±7	54	86±11	–
T4	81±5	19±2	77	82±7	–	30±4	62	81±8	–
P1	80±5	13±2	94	83±2	–	33±2	59	78±5	3
P2	82±3	12±5	85	81±2	1	31±2	62	86±4	–
P3	81±9	17±3	79	78±4	4	39±3	52	78±4	–

* Strains T2, T3 and T4 were isolated from copepod carapace; strains P1, P2 and P3 were isolated from rock-pool water.

† Results similar to those obtained with D-glucose were obtained with D-fucose and D-fructose.

‡ Percentages of attachment inhibition were calculated by comparing the treated samples with their untreated controls. –, No inhibition.

Table 2. Effect of treatment of chitin with MPs and lectins on *V. alginolyticus* attachment

Attachment is expressed as 10^{-6} × mean no. of bacteria (2·5 mg chitin)$^{-1}$ ± SD. Results are means of three independent experiments performed with three cultures on different days. Only the differences between WGA and MP treated samples and their controls were statistically significant ($P \leqslant 0{\cdot}01$).

Strain*	Treatment								
	Control	MPs†		EMPs‡		WGA		ConA	
		Attachment	% Inhibition§	Attachment	% Inhibition§	Attachment	% Inhibition§	Attachment	% Inhibition§
T2	89±6	42±4	53	94±10	–	36±4	56	89±7	–
T3	83±2	25±3	70	83±2	–	41±4	51	84±4	–
T4	80±6	30±9	62	79±4	1	40±9	50	80±6	–
P1	81±4	33±3	59	80±10	1	38±2	53	85±7	–
P2	85±6	28±3	67	85±4	–	40±2	53	86±6	–
P3	83±3	29±3	65	80±3	4	36±3	57	79±5	–

* See Table 1.

† For each strain, its own Sarkosyl-extracted MPs were utilized. Results refer to a protein concentration of 5 μg (mg chitin)$^{-1}$.

‡ Sarkosyl-extracted MPs isolated from *E. coli* DH5α were used; 15 μg MPs (mg chitin)$^{-1}$ were used.

§ Percentages of attachment inhibition were calculated by comparing the treated samples with their untreated controls. –, No inhibition.

35, 20 and 14 kDa (Fig. 2); in most strains smaller quantities of the last three proteins were found. All peptides were observed in extracts derived from both adhering and non-adhering bacteria. Bacterial growth in the presence of either chitin or chitobiose or chitotriose did not modify either the MP electrophoretic profiles or the type and the amount of visualized chitin-binding proteins (data not shown).

DISCUSSION

The results presented in this paper indicate that *V. alginolyticus* carries a protein system on its surface able to recognize chitin-containing substrates. Four membrane peptides able to bind chitin were revealed: a 53 kDa protein that seems to correspond to one of the major outer-membrane proteins, and three other peptides (35, 20 and 14 kDa) that are recovered at very low levels in

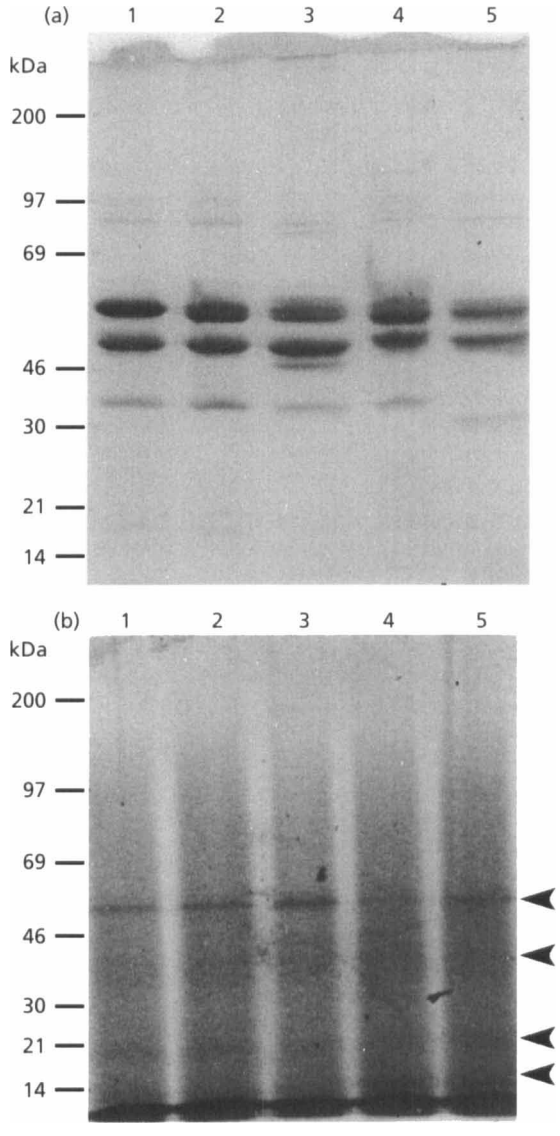

Fig. 2. SDS-PAGE separation of *V. alginolyticus* Sarkosyl-resistant MPs (a) and of the fraction able to bind chitin (b). Fifteen micrograms of protein were loaded in each lane. Positions of molecular mass standards are shown on the left. The positions of CBPs are indicated by arrowheads. Lanes: 1, strain T2; 2, strain T3; 3, strain T4; 4, strain P1; 5, strain P2.

most strains. These proteins are present both in bacteria isolated from copepod carapace and in those deriving from rock-pool water, and their expression is not dependent on the presence of chitin, chitobiose or chitotriose in the culture medium.

Montgomery & Kirchman (1993) have recently shown that in *V. harveyi* a 53 kDa outer-membrane protein is the most important CBP. These results suggest that *V. alginolyticus* and *V. harveyi*, although not closely related (Bauman & Schubert, 1984), share the same chitin-binding system. Considering that bacteria colonizing copepods and detrital chitin belong almost entirely to the genus *Vibrio*, it would be interesting to evaluate the presence of similar CBPs in other marine *Vibrio* species. Vibrios could efficiently compete with other micro-organisms for this ecological niche by reason of their CBP(s). Moreover, since many marine vibrios can be pathogenic for humans and animals, the possibility that CBPs are involved in bacterial attachment to host epithelia should also be investigated. It is interesting in this context to recall that in *V. cholerae*, a micro-organism known to survive in the marine environment due to its ability to associate with crustacean exoskeleton (Nalin *et al.*, 1979; Roszak & Colwell, 1987; Tamplin *et al.*, 1990), a 53 kDa protein is involved in colonization of the intestine of infant mice (Singh *et al.*, 1994). Of course, this does not imply that this protein is the same as the one described here for *V. alginolyticus*; nevertheless, it would be interesting to evaluate the peptide's ability to mediate attachment to chitin-containing surfaces.

Our results allow us to approach the general question of bacterial attachment to non-living surfaces. Through the CBP system, bacteria can firmly attach to detrital chitin that, being very common in sea water, is the substrate they most frequently encounter. This specific attachment gives a nutritional advantage to adhering bacteria, which can more efficiently degrade chitin to soluble products in comparison to free micro-organisms. Our preliminary results have shown that no chitinolytic activity is associated with the CBPs studied; a protein of about 80 kDa, not recovered from chitin exposed to membrane extracts, shows this activity. Therefore, as previously suggested (Montgomery & Kirchman, 1993), CBPs could position bacteria so that chitinolytic enzymes can efficiently hydrolyse the substrate. Very few other examples of specific interactions between bacteria and chitin-containing surfaces are known. In addition to *V. harveyi* (Montgomery & Kirchman, 1994) and *V. alginolyticus* (this paper), lectins with sugar specificity for GlcNAc were found in *V. damsela* and *V. furnissii* (Yu *et al.*, 1987, 1991); in this last species, the lectin exhibits a broad sugar specificity with the highest affinity toward GlcNAc followed by D-glucose and D-mannose.

This paper also reports data on environmental factors that modify *V. alginolyticus* association with chitin and, consequently, affect the ability of this micro-organism to decompose this polysaccharide in natural sea water. As in the case of other vibrios (Belas & Colwell, 1982; Huq *et al.*, 1984; Koneko & Colwell, 1975), the efficiency of attachment was found to be dependent on NaCl concentration and temperature; the highest levels of adsorption were observed at 20 °C, in the presence of 3% NaCl, corresponding, approximately, to the salinity of sea water. These results confirm our *in vivo* findings (Carli *et al.*, 1993) showing that the number of *V. alginolyticus* bacteria adhering to copepod exoskeleton reaches maximum values when rock-pool water temperature and salinity are around 20 °C and 3–4%, respectively.

Bacterial attachment to copepods is a very complex phenomenon and entails several types of interactions between lipids, carbohydrates and proteins (Koneko & Colwell, 1975; Tamplin *et al.*, 1990). Moreover, the crustacean outer surface is covered with an epicuticle of

waxes (Koneko & Colwell, 1975) that could, at least partially, hide the chitin layer. Nevertheless, since the ability of *Vibrio* to interact directly with the chitin exoskeleton of crustacea and to induce lesions is well known (Alderman, 1986; El-Gamal *et al.*, 1986), we can imagine that the enzymic activities produced by these bacteria (e.g. lipase) may facilitate the bacterial approach to the chitin layer. Therefore the specific chitin-binding system reported here may provide marine bacteria with an efficient attachment system to living substrates as well. These bacteria, after having overcome repulsion forces and come close to the chitin surface, could permanently and irreversibly adhere to the substrate through the use of CBPs.

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