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Role of Chitin-Binding Proteins in the Specific Attachment of the Marine Bacterium *Vibrio harveyi* to Chitin

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We examined the mechanism of attachment of the marine bacterium *Vibrio harveyi* to chitin. Wheat germ agglutinin and chitinase bind to chitin and competitively inhibited the attachment of *V. harveyi* to chitin, but not to cellulose. Bovine serum albumin and cellulase do not bind to chitin and had no effect on bacterial attachment to chitin. These data suggest that this bacterium recognizes specific attachment sites on the chitin particle. The level of attachment of a chitinase-overproducing mutant of *V. harveyi* to chitin was about twice as much as that of the uninduced wild type. Detergent-extracted cell membranes inhibited attachment and contained a 53-kDa peptide that was overproduced by the chitinase-overproducing mutant. Three peptides (40, 53, and 150 kDa) were recovered from chitin which had been exposed to membrane extracts. Polyclonal antibodies raised against extracellular chitinase cross-reacted with the 53- and 150-kDa chitin-binding peptides and inhibited attachment, probably by sterically hindering interactions between the chitin-binding peptides and chitin. The 53- and 150-kDa chitin-binding peptides did not have chitinase activity. These results suggest that chitin-binding peptides, especially the 53-kDa chitin-binding peptide and chitinase and perhaps the 150-kDa peptide, mediate the specific attachment of *V. harveyi* to chitin.

In aquatic environments, bacteria probably need to attach in order to effectively utilize biogenic particles as nutrient sources. Attachment would optimize contact between cell surface enzymes and the substrate, allowing for efficient uptake of the soluble by-products of hydrolysis. This mechanism may describe degradation of chitin (poly-*N*-acetylglucosamine). This complex polysaccharide must be degraded to short oligomers, which are then transported across the cell membrane (7). In culture, many bacterial strains secrete enzymes such as chitinase (EC 3.2.1.14). In the natural environment, however, free-living bacteria that secrete chitinase may lose the nutritional benefit of producing these proteins. The enzyme itself may be degraded and assimilated by other bacteria, or the amino sugars released during chitin hydrolysis may be taken up by competing bacteria. A bacterial strain that attaches specifically to chitin particles may gain a nutritional advantage over those that attach to surfaces nonspecifically.

There are few examples of specific interactions between bacteria and common, nonliving surfaces. *Bacteroides thetaiotaomicron* appears to attach specifically to insoluble starch particles by using outer membrane proteins (2, 3). Attachment by some bacterial strains to cellulose also appears to be specific (16), one example being a marine bacterium that was isolated from a wood-boring shipworm (12). Imam et al. (12) found that a detergent-extracted fraction of this bacterium inhibited the attachment of whole cells. Also, cellulose oligomers and their analogs inhibited attachment (12). These short-chained oligomers probably inhibited attachment by occupying cell surface binding sites that mediate the specific attachment of the bacterium to the particle.

The specific attachment of pathogenic bacteria to epithelial tissue of mammalian hosts has been examined extensively (for a review, see reference 18). In many cases, this attachment is mediated by outer membrane proteins that belong to a general class of proteins called lectins. These lectins recognize specific sequences of sugars present on the glycoproteins of the host cells. Although many lectins from plants (24), some lectins from vertebrates, (14), and some lectins from eukaryotic microorganisms (10, 32) have an affinity for *N*-acetylglucosamine (GlcNAc), no bacterial lectin identified thus far has an affinity for this common amino sugar (33). There is some evidence, however, that the marine bacterium *Vibrio furnissi* produces a protein that binds specifically to the GlcNAc residues that make up chitin (33). This evidence is based on competitive inhibition by amino sugars of attachment to GlcNAc-containing beads and a requirement for protein synthesis during attachment (33).

We looked at whether the marine bacterium *V. harveyi* attaches specifically to chitin particles. We chose to work with *V. harveyi* because of the previous work on the genetics of its chitinase system (13, 25, 26). It has been suggested that in natural marine systems most bacteria attached to chitinaceous particles (e.g., copepod exoskeletons) are vibrios (20). We show here that the specific attachment of *V. harveyi* to chitin particles appears to be mediated by chitin-binding proteins associated with cell membranes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The mutant strain *V. harveyi* BB7-1 was produced by insertion of the transposon MiniMu (contained in P1::MiniMu) into the genome of the wild-type strain, BB7 (6, 34). Bacterial strains were maintained in LM broth (1% tryptone, 0.5% yeast extract, 2% NaCl) or on LM agar (LM broth, 1% agar) (Difco, Detroit, Mich.).

Attachment assay. Cells were grown overnight in 10 ml of yeast broth (0.2 g liter^{-1}) in artificial seawater (ASW) (23) at 30°C to a concentration of 5×10^7 cells ml^{-1}. To measure bacterial attachment, cells were radiolabelled by adding 0.1 mCi of [^{3}H]thymidine (NEN Research Products, Boston,

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Mass.) to 10 ml of yeast broth-ASW and 0.1 ml of exponentially growing cells (ca. 5×10^5 cells ml^{-1} [final concentration]). Cultures were radiolabelled overnight (30°C) to a final bacterial concentration of 5×10^7 cells ml^{-1}. Radiolabelled cultures were centrifuged (10,000 × *g*, 10 min, 25°C) and rinsed three times with 10-ml portions of filter-sterilized (0.2-μm-pore-size filters [Millipore Corp., Bedford, Mass.]) attachment medium (10 mM NH_3Cl in yeast broth-ASW). The specific activity of the cells (typically 35 to 50 cells dpm^{-1}) was calculated by filtering 0.1 ml of washed cells onto a 0.2-μm-pore-size filter (25-mm polycarbonate filter [Poretics Corp., Livermore, Calif.]), rinsing the filter three times with attachment medium (3 ml per rinse), and then radioassaying the filters. Bacterial abundance was measured with acridine orange staining and epifluorescence microscopy (11).

Radiolabelled cells (2.5×10^6 cells ml^{-1} [final concentration]) were added to filter-sterilized attachment medium with chitin purified from crab shells (2.5 mg ml^{-1}; Sigma Chemical Co., St. Louis, Mo.), and the mixture was incubated 0.5 to 30 min (25°C) with shaking. Before use in attachment assays, the chitin was rinsed three times with attachment medium. The presence of contaminating compounds (probably dissolved amino sugars) enhanced attachment to chitin (data not shown). After 30-min incubation, three to five replicates of each treatment were filtered onto 8-μm-pore-size filters (25-mm polycarbonate filter [Poretics]), which were then rinsed with attachment medium (3 ml). The filters were subsequently radioassayed. In addition, duplicate samples for each treatment were incubated without chitin and filtered to correct for unattached cells left on the filter. The radioactivity for these control filters (typically 100 to 300 dpm versus 10,000 dpm for samples with chitin) was subtracted from the sample values to measure the radioactivity of cells that had attached to the chitin particles. The total number of cells bound to chitin particles was calculated, using the specific activity of the cells. The specific activity of the cells did not change over the course of any experiment.

Chitinase activity. Methylumbelliferyl-chitobioside (MUF-DiGlcNAc; Sigma) was used to measure chitinase activity, because it has been shown to be a useful analog for chitin (21, 28). We added 10 μl of 5 mM MUF-DiGlcNAc dissolved in dimethylformamide to 1 ml (final volume) of sample in TBS (25 mM Tris buffer [pH 7.5] and 150 mM NaCl). The sample was incubated for 3 h at 30°C before fluorescence was measured (<365-nm-wavelength primary filter and >460-nm-wavelength secondary filter). Fluorochrome concentration was calculated by using a standard curve generated with methylumbelliferone (MUF; Sigma). One mole of MUF is released for every mole of substrate hydrolyzed.

Protein treatment of chitin and cellulose. Chitin or cellulose particles (Sigmacell type 20 microcrystalline; Sigma) were treated with wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, Calif.), chitinase (isolated from *Serratia marcescens*; Sigma), cellulase (isolated from *Trichoderma virdi*; Sigma), or bovine serum albumin (BSA; Sigma). Various concentrations (100 to 500 μg ml^{-1}) of protein were incubated with 5-mg portions of chitin particles in attachment medium for 30 min with shaking. More than 95% of the WGA bound to the chitin under these conditions (for a description of WGA, see reference 1). Treated particles were then used in the standard attachment assay (10-min incubation time) with radiolabelled *V. harveyi*.

Isolation of chitin-binding proteins. Cultures of *V. harveyi* BB7 and BB7-1 were grown overnight in LM broth (30°C) to a cell density of 5.0×10^9 cells ml^{-1}. Cells were centrifuged (10,000 × *g*, 20 min, 4°C) three times and resuspended in TBS. The following isolation procedure is from Soto-Gil and Zyskind (26) and has been shown to effectively remove chitobiase from the membranes of *V. harveyi* while retaining chitobiase activity. Concentrated cells were sonified (Branson Cell Disrupter 200; Branson Sonic Power Co., Danbury, Conn.) at setting six and 50% power for 30 s on ice. This sonification step was repeated nine times, with a 60-s cooling period between each sonification. Lysed cells were centrifuged (40,000 × *g*, 1 h, 4°C) to pellet the cell membranes. The supernatant (medium and cytoplasm) was removed, and the pellet was resuspended in TBS (1 ml). The membrane suspension was extracted (1 h, 37°C) with 0.1% (final concentration) *N*-dodecanoylsarcosinate (Sarkosyl) (Sigma). Undissolved material was removed by centrifugation (10,000 × *g* for 20 min at 25°C). Subsamples (0.1 ml) of the supernatant were incubated (30 min at 25°C) with 5-mg portions of chitin particles. The mixture was washed with TBS and centrifuged three times (10,000 × *g*, 10 min, 25°C) after incubation to remove proteins not binding to chitin.

Portions (50 μl) of loading buffer (24% sucrose, 120 mM dithiothreitol, 0.5% bromophenol blue, 2% lithium dodecyl sulfate) were added to the chitin particles in 50-μl quantities of TBS, and the samples were heated in boiling water (10 min) to remove chitin-binding proteins from the chitin particles (17). The samples were centrifuged (10,000 × *g*, 10 min, 25°C) and the supernatant, which now contained chitin-binding proteins, was removed and either assayed for total protein by the bicinchoninic acid assay (with BSA as the standard; Pierce, Rockford, Ill.) or analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15). The Sarkosyl-extracted membrane fractions typically contained 1.5 mg of protein ml^{-1}.

Detection of chitinase by SDS-PAGE. Proteins from the extracellular supernatant (secreted proteins), total extracts (proteins removed by SDS and heat treatment), and Sarkosyl-extracted fractions of both isolated membranes and whole cells were heated (5 min, 37°C) in loading buffer and then separated by SDS-PAGE. After electrophoretic separation, lanes were sliced horizontally into fragments (ca. 2 by 5 mm) and then placed into 1 ml of 50 μM MUF-DiGlcNAc for 3 h (30°C) in order to measure chitinase activity. Fluorescence was measured as described above.

Isolation of chitinase and preparation of antisera. Extracellularly secreted chitinase was isolated from the chitinase-overproducing strain of *V. harveyi* BB7-1 by the method of Zyskind (34). Cells were grown in LM broth for 4 days and then centrifuged (10,000 × *g*, 20 min, 4°C) to remove the cells. Colloidal chitin which was prepared by the method of Roberts and Cabib (22) was added to the cell supernatant (0.15 mg per unit of chitinase activity) and incubated on ice for 1 h. After centrifugation (10,000 × *g*, 20 min, 4°C), the pellet was washed three times with 10 mM Tris buffer (pH 7.3) and then resuspended in 1/10 the original volume in Tris buffer. It was incubated for 24 h at 30°C with periodic shaking by hand. After most of the chitin particles had been hydrolyzed by the chitinase, the remaining particles were removed by centrifugation and the supernatant was concentrated 20-fold with an Amicon-10 Centriprep concentrator (molecular mass cutoff of 10 kDa; Amicon Corp., Lexington, Mass.). The extracellular chitinase was then stored at 4°C in Tris buffer containing 0.2% sodium azide, 2 mM aminocaproic acid, and 0.4 mM benzamidine.

Extracellular chitinase from *V. harveyi* BB7-1 was further purified by preparative SDS-PAGE. Following electrophoresis, the gel slice with chitinase activity from the ca. 80- to

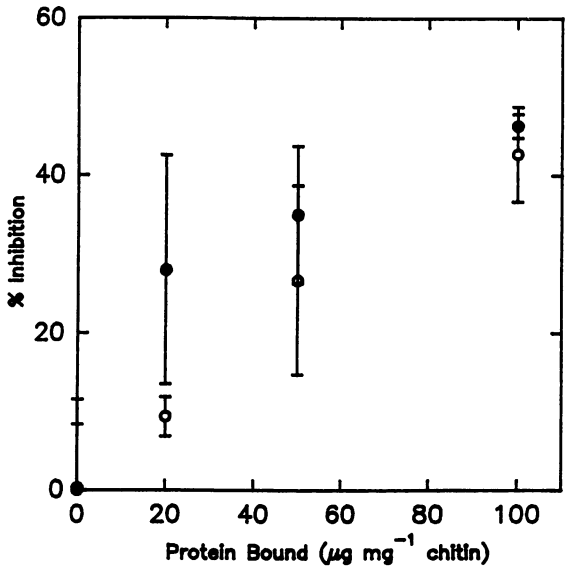

FIG. 1. Percent inhibition (relative to the number of cells attaching in the absence of added protein) by chitin-binding proteins of *V. harveyi* attachment to chitin. WGA (●) and chitinase (○) were bound to chitin (2.5 mg ml^{-1}) prior to incubation (10 min) with bacteria (2.5 × 10^6 cells ml^{-1}).

90-kDa molecular mass range was diced into small pieces and brought to a total volume of 2.0 ml with TBS. The gel fragments and buffer were then mixed with an equal volume of Freund's complete adjuvant, emulsified, and injected subcutaneously and intramuscularly into two New Zealand White rabbits. Rabbits were immunized with 100 μg of chitinase initially and then with increasing doses at subsequent immunizations (200 μg at 3 weeks and 400 μg at 7 weeks) prior to bleeding after 10 weeks. Whole serum was used after the erythrocytes had been removed by standard methods.

SDS-PAGE and immunoblotting. Peptides separated by SDS-PAGE were transferred to Immobilon-P (Millipore) protein-binding membrane prior to immunoblotting (27). The blocking solution was 1% Carnation nonfat instant milk in TBS. Antichitinase serum was diluted 1:1,000 with blocking solution prior to incubation with the blots. The secondary antibody was anti-rabbit goat immunoglobulin G (IgG) conjugated to alkaline phosphatase diluted 1:1,000 in blocking solution. The substrates for detection of alkaline phosphatase were 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

RESULTS

Inhibition of attachment by WGA and chitinase. We hypothesized that marine bacteria use outer membrane-associated proteins to attach specifically to chitin particles. To examine this hypothesis, chitin particles were treated with proteins and then used in attachment assays with *V. harveyi*. About 40% fewer bacteria attached to chitin particles treated with the lectin WGA (100 μg of WGA mg of $chitin^{-1}$) (Fig. 1). Because WGA binds to a series of three consecutive GlcNAc monomers (1), it appears to sterically inhibit bacterial attachment that is dependent on the recognition of GlcNAc oligomers that make up the chitin particle. The inhibitory effect of WGA on bacterial attachment increased with increasing WGA concentration (Fig. 1), but the attachment to cellulose particles was not affected (Table 1).

TABLE 1. Effect on bacterial attachment of preincubating chitin and cellulose with various proteins

Preincubation treatment	Surface or particle	% Inhibition[a] (mean ± SD)
WGA	Chitin	46.3 ± 1.5
Chitinase	Chitin	42.7 ± 6.0
BSA	Chitin	13.7 ± 19
Cellulase	Chitin	−9.4 ± 11
WGA	Cellulose	−12.0 ± 16
Chitinase	Cellulose	−30.0 ± 23

[a] Percent inhibition was calculated as follows: [1 − (attachment with added protein/attachment with no added protein)] × 100. Negative values indicate stimulation of attachment.

Chitin particles were also treated with chitinase (purified from *S. marcescens*) and used in the attachment assay. Pretreatment with chitinase inhibited attachment as much as 42%, similar to inhibition by WGA (Fig. 1). Also, like WGA, chitinase did not inhibit bacterial attachment to cellulose particles (Table 1), suggesting that the inhibition of attachment to chitin by these two proteins was not due to some nonspecific effect on the cells.

Chitin particles were also treated with BSA or cellulase, and bacterial attachment was determined. BSA does not bind specifically to chitin and was a poor inhibitor of attachment (13.7% ± 19% at 100 μg mg of $chitin^{-1}$ [Table 1]). Cellulase, which neither binds nor degrades chitin, also had little effect on attachment to the chitin particles (−9% ± 11% at 100 μg mg of $chitin^{-1}$ [Table 1]).

Attachment of chitinase-overproducing mutant. The chitinase-overproducing mutant of *V. harveyi*, BB7-1, attached to chitin about twice as much as the wild-type strain (Fig. 2A). In the absence of chitin, the wild-type strain produced chitinase constitutively, but at a much lower level than the mutant. The BB7-1 mutant bound to cellulose particles to the same degree as that of the wild type, suggesting that the enhanced attachment to chitin by the mutant did not reflect an increased capability to attach to all surfaces (Fig. 2B). The two strains attached equally well to the polyethylene scintillation vials used in the attachment incubations; 3.3% ± 1.0% of added BB7-1 cells attached to the vials, similar to the level of attachment by BB7 (3.2% ± 1.3% of added cells). The absolute percentages of attachment are not comparable among surfaces and particles because of differences in the total surface area.

Isolation of chitin-binding proteins. Membrane-associated proteins were isolated from the wild type and chitinase-overproducing mutant of *V. harveyi* and separated by SDS-PAGE. The two strains differed primarily in the amount of a 53-kDa peptide, which was much more abundant in the chitinase overproducer than in the wild type (Fig. 3). We suspect that this 53-kDa peptide is associated with the outer membrane, although we do not have data directly supporting this hypothesis, because Osborn gradients do not work with this strain (13).

To determine the binding properties of these proteins, detergent-extracted membranes from both strains were incubated with chitin or cellulose particles. The particles and associated proteins were washed to remove nonspecifically bound proteins, and then the specifically bound proteins were eluted by using SDS and heat. None of the peptides in the extract bound to cellulose particles, as visualized by

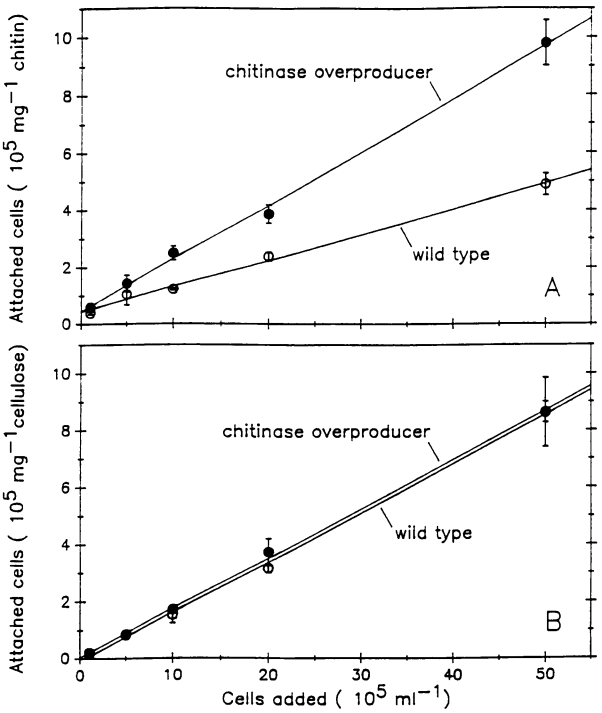

FIG. 2. Attachment of *V. harveyi* wild-type and chitinase-overproducing mutant to chitin (A) and cellulose (B).

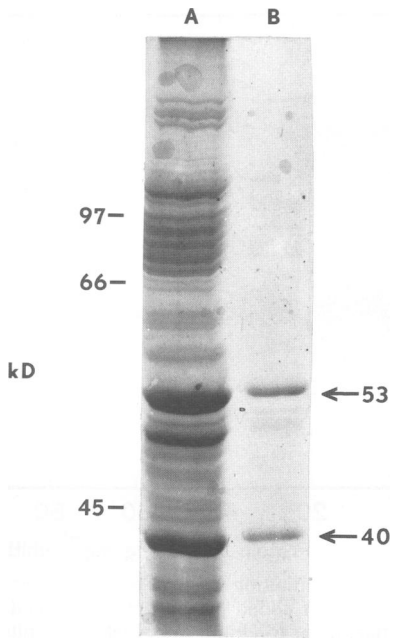

FIG. 4. SDS-PAGE (9% acrylamide) separation of membrane extracts of the *V. harveyi* mutant (30 μg of protein loaded) (lane A) and those membrane proteins that bound to chitin particles and were eluted by SDS and heat (1 μg of protein loaded) (lane B).

Coomassie blue staining (data not shown), but the 53-kDa peptide and a 40-kDa peptide bound to chitin (Fig. 4). The 53-kDa peptide appeared to be the overproduced peptide (on the basis of apparent molecular mass), and thus much higher amounts were recovered in extracts from the mutant than from the wild type. In contrast, the wild type and the chitinase-overproducing mutant produced similar amounts of the 40-kDa peptide on the basis of Coomassie blue-stained gels of the proteins removed from chitin after exposure to membrane extracts (data not shown). This observation was confirmed when we reexamined Coomassie blue-stained gels of membrane extracts (Fig. 3).

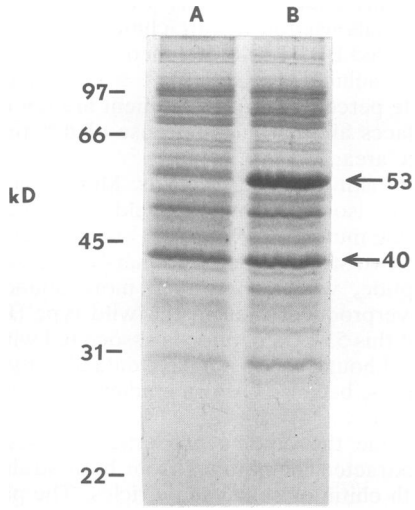

FIG. 3. SDS-PAGE (10% acrylamide) separation of detergent-extracted bacterial membranes of *V. harveyi* wild type (lane A) and of chitinase-overproducing mutant (lane B). Fifteen micrograms of protein was loaded in each lane.

Inhibition of attachment by membrane-associated proteins. Chitin particles were exposed to Sarkosyl-extracted *V. harveyi* membranes to determine whether the chitin-binding proteins would inhibit attachment of *V. harveyi*. The chitin-binding proteins from the wild-type membrane extract inhibited attachment by 22% at 12.9 μg of bound protein per mg of chitin (Fig. 5A). The inhibition by chitin-binding proteins from the mutant membranes was much greater, 40% at 9 μg of bound protein per mg of chitin (Fig. 5B). The maximum levels of inhibition by chitin-binding proteins from the mutant membrane extract, WGA, and *S. marcescens* chitinase were nearly equal at 40% (Fig. 1). To achieve this same level of inhibition, however, 100 μg of WGA or the chitinase was necessary, about 10-fold more protein than was needed from mutant membrane extracts to inhibit attachment.

Chitinase activity in membrane extracts. Chitinase activities of peptides separated by SDS-PAGE were measured by using MUF-DiGlcNAc. Chitinase activity (ca. 80 to 90 kDa) was detected in the Sarkosyl-extracted membranes from the mutant (231 μmol/mg of protein per h) but not from the wild type (less than 1 μmol degraded mg^{-1} h^{-1}). Chitinase was not detected in the wild type, because it was not induced and the amount that is produced constitutively was below the detection limit. The apparent molecular mass of chitinase-positive peptides roughly corresponds to that reported for the major chitinase of this strain, the 85-kDa peptide (34). These samples were heated at only 37°C for 10 min (not boiling water as in standard methods), so the apparent molecular mass likely differs from that of proteins heated at 100°C. The enzymatic activity of the chitinase decreased, and the enzyme appears to be degraded by proteases after heating to 100°C. Notably, there was no chitinase activity

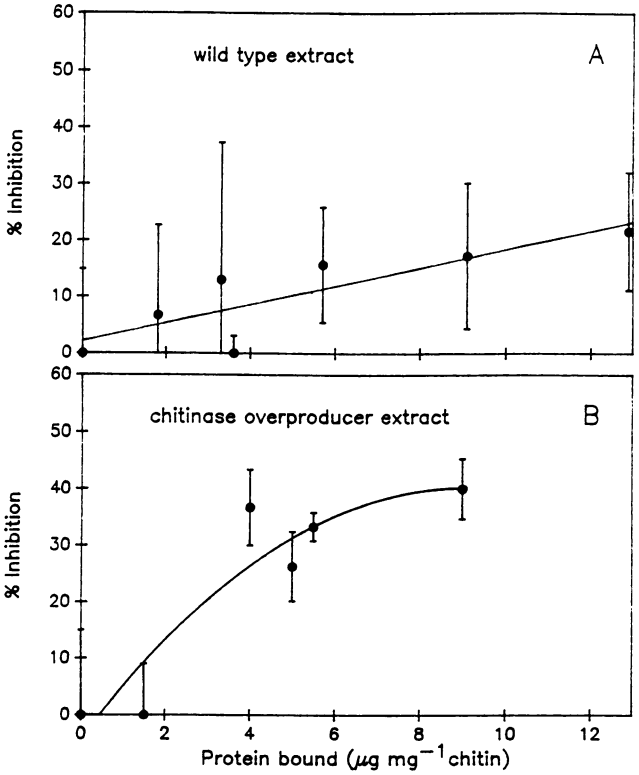

FIG. 5. Percent inhibition of attachment of *V. harveyi* to chitin by extracts of wild-type (A) and mutant (B) membranes.

associated with the 53-kDa peptide overproduced in the chitinase-overproducing mutant.

Immunoblot of SDS-extracted membranes and chitin-binding proteins. Primarily two peptides associated with membranes of *V. harveyi* BB7-1 reacted with the antichitinase serum (Fig. 6, lane A). The molecular mass of one cross-reacting peptide corresponded to the 53-kDa chitin-binding peptide overproduced by the mutant (Fig. 6, lane C). The reactivity of the antiserum with this peptide was weak relative to the 85-kDa extracellular chitinase that was used to derive the antiserum. The other peptide that cross-reacted with the antiserum had an apparent molecular mass of 150 kDa, which is much greater than the 85-kDa chitinase.

Using the antichitinase serum, we probed a Western blot (immunoblot) of the membrane-associated peptides that bound to chitin, i.e., the chitin-binding peptides. Two peptides cross-reacted with the antiserum, the overproduced 53-kDa peptide (albeit weakly) and the 150-kDa peptide (Fig. 6, lane B), although the 150-kDa peptide was not visible in the Coomassie blue-stained gel of the chitin-binding peptides (Fig. 6, lane D). The 40-kDa peptide, which was observed with the 53-kDa peptide in Coomassie blue-stained gels (Fig. 6, lane D), did not cross-react with the antiserum (Fig. 6, lane B).

Inhibition of attachment with the antichitinase serum. Treatment of the chitinase-overproducing *V. harveyi* with antichitinase serum inhibited attachment to chitin particles (Fig. 7). Preincubation of the cells with preimmune serum IgG (Fig. 7) or with goat IgG (data not shown) did not inhibit attachment (attachment actually increased 11% ± 4% at 175 μg ml^{-1}), whereas IgG from the antichitinase serum inhibited attachment as much as 40% (Fig. 7).

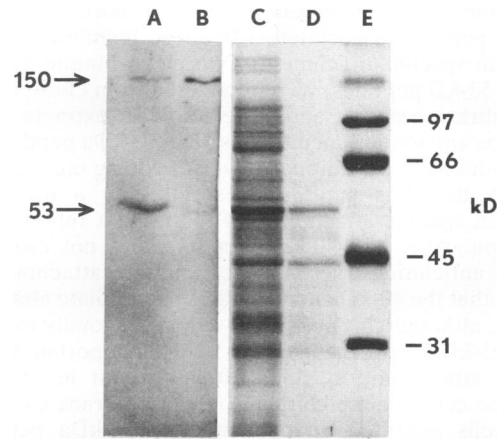

FIG. 6. Immunological analysis of chitin-binding peptides. Lanes A and B are a Western immunoblot probed with antichitinase serum. Lane A was loaded with detergent-extracted (1% SDS, 100°C, 10 min) membranes of the *V. harveyi* mutant (15 μg of protein loaded), and lane B was loaded with the proteins from the extract that bound to chitin and were eluted off the particles (1 μg of protein loaded). Lanes C and D are these same proteins separated by SDS-PAGE and stained with Coomassie blue. The membrane extract is in lane C, and the chitin-binding proteins are in lane D. Lane E contains the molecular mass standards.

DISCUSSION

There have been few studies demonstrating the specific attachment of bacteria to nonliving surfaces. Here we present evidence that the marine bacterium *V. harveyi* uses chitin-binding peptides to mediate attachment to chitin. The chitinase-overproducing mutant attached to chitin much more than the wild type. Treating chitin particles with proteins that bind to chitin, such as chitinase (from *S. marcescens*) or WGA, inhibited attachment of *V. harveyi*. In addition, detergent-extracted membranes from the wild type and a chitinase-overproducing mutant of *V. harveyi* inhibited attachment. Finally, attachment to chitin is inhibited by incubating the cells with antiserum generated against chitinase.

Inhibition of attachment by preincubating chitin with

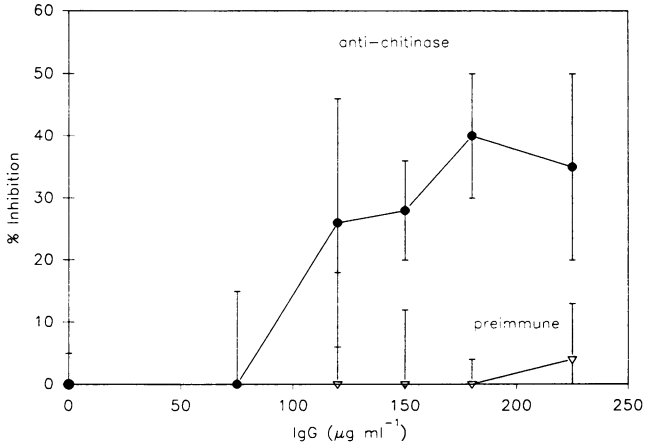

FIG. 7. Effect of preincubation of chitin particles with preimmune antiserum or antichitinase serum on the attachment of *V. harveyi* chitinase-overproducing mutant to the particles.

membrane extracts suggests that one or more of the chitin-binding peptides associated with these membranes are involved in specific attachment. Two chitin-binding peptides, 40- and 53-kD peptides, were recovered from chitin exposed to membrane extracts and were found in extracts of both wild-type and mutant membranes. The 40-kDa peptide is not overproduced in the chitinase-overproducing mutant, which suggests that this peptide does not play a role in the enhanced specific attachment of the mutant relative to the wild type. Also, the 40-kDa peptide does not cross-react with the antichitinase serum which inhibited attachment. We suspect that the 40-kDa peptide does not mediate attachment directly, although this possibility cannot be totally excluded.

The 53-kDa peptide appears to be most important in initial specific attachment to chitin. It is present in uninduced wild-type cells, unlike chitinase, and membrane extracts of these cells inhibited attachment. The 53-kDa peptide is overproduced in the mutant that attaches better than the wild type and cross-reacts with the antiserum that inhibits attachment. There may be still another chitin-binding peptide that is also overproduced in the mutant but which binds to chitin so tightly that boiling the chitin in detergent is ineffective at removing the peptide. However, we were able to recover over 98% of the membrane-associated protein added to the chitin particles, which suggests that this is an unlikely possibility.

Another Sarkosyl-extracted peptide that binds to chitin has a molecular mass of 150 kDa. We could not detect it with Coomassie blue staining, but it could be detected in immunoblots of the peptides removed from the chitin. This 150-kDa peptide may also be involved in attachment, because attachment to chitin was inhibited when the cells were preincubated with antichitinase serum but not with the preimmune serum. Neither the 150-kDa peptide nor the 53-kDa chitin-binding peptide exhibited chitinase activity after separation by SDS-PAGE. Perhaps the 150-kDa peptide is a preprocessed form of the 85-kDa chitinase that binds but does not hydrolyze chitin.

The 85-kDa chitinase may be another peptide that is involved in attachment. Chitinase activity is found in membrane extracts which inhibit attachment of whole cells. Also, the antiserum raised against chitinase inhibits attachment. However, the 85-kDa chitinase was not observed in immunoblots of membrane extracts or among the peptides (i.e., 150-, 53-, and 40-kDa peptides) recovered from chitin exposed to membrane extracts. Apparently, measuring chitinase activity is more sensitive than Coomassie blue staining and immunological detection. We hypothesize that the 85-kDa chitinase is less important than the 53-kDa peptide in initial attachment because of the low amount of the 85-kDa chitinase in uninduced wild-type cells. Also, induction studies show that the amount of the 85-kDa chitinase increases over time after exposure to chitin, whereas the amount of the 53-kDa peptide remains constant (19).

Bacteria may use an outer membrane-associated chitinase to mediate attachment to chitin particles, but this mechanism also has a complicating factor. Chitinase would degrade the particle upon contact and thus be a poor anchor for attaching the cell to the surface. One plausible mechanism is that the protein (or a portion of it) could bind chitin as a lectin while associated with the outer membrane and then lose binding capacity and gain enzymatic activity after processing and secretion.

Watanabe et al. (28) describes this sort of processing for a bacterial chitinase. They found that *Bacillus circulans* produces two major chitinases, one being the proteolytic degradation product of the other. The proteolytically processed chitinase maintains its ability to hydrolyze chitin but can no longer bind to chitin. Watanabe et al. (28, 29) suggest that the C terminus of the original chitinase is responsible for strong binding of the enzyme to chitin. Gilboa-Garber and Garber (9) suggest that lectins can be lytic enzyme-positioning sites (LEPS) that act synergistically with enzymes. These LEPS are important for efficient hydrolysis of biopolymers (9).

The need for both catalytic enzymes and LEPS suggests that bacteria use a multimolecular complex to bind and degrade chitin, which is analogous to that hypothesized in cellulolytic systems (5). Cellulolytic bacteria have cellulosomes, which are complexes that contain cellulases and other peptides that mediate the degradation of cellulose particles (4, 5, 8, 30). There is some evidence that these cellulosomes mediate bacterial attachment to cellulose. Lamed et al. (16) found a correlation between cellulase activity and attachment to cellulose among various bacterial strains. Mutant strains of *Clostridium thermocellum* lacking cellulase activity exhibit lower levels of bacterial attachment to cellulose than those strains with wild-type cellulase activity (16). More work is needed in identifying the specific components of the cellulosome that mediate cellular attachment to cellulose.

It is curious that the antiserum raised against a secreted chitinase cross-reacts with the membrane-associated 53-kDa peptide. There are three possible explanations. First, the 53-kDa peptide could have been a contaminant in the gel slice harboring the 85-kDa chitinase, although this seems unlikely, given the resolution of SDS-PAGE separation. Second, the 53- and 40-kDa chitin-binding peptides could be proteolytic degradation products of the 85-kDa chitinase, although protease inhibitors were present in all extractions. Also, the 40-kDa peptide fails to react with the antiserum, and the regulation of both the 53- and 40-kDa peptides appears to differ from that of the 85-kDa chitinase (19). The most likely reason that the 53-kDa peptide reacts with the antichitinase serum is that this peptide and the 85-kDa chitinase share common epitopes, perhaps in chitin-binding domains.

Many strains of marine vibrios are both chitinolytic and pathogenic for higher organisms (31) and may use a specific attachment mechanism to colonize both chitin particles and vertebrate hosts. Strains that attach specifically to chitin particles, which are common in seawater, may use these same attachment proteins to bind GlcNAc residues of glycoproteins of host epithelial tissue. Although much of the work on specific attachment of bacteria has focused on pathogenic strains, other aquatic bacteria probably use such attachment and substratum recognition systems to attach to surfaces in aquatic ecosystems. In aquatic environments, the likelihood of an individual bacterium encountering a vertebrate host is extremely small compared with the likelihood of encountering a chitin particle or other naturally occurring biopolymers. From this study, it appears that the 53-kDa chitin-binding peptide, the 85-kDa chitinase and possibly other antigenically related peptides (e.g., 150-kDa peptide) contribute to specific attachment. Information about cellular processing and metabolic control of chitin-binding proteins may be important in understanding particle degradation and attachment to surfaces by bacteria.

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