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Lectin, a Possible Basis for Symbiosis Between Bacteria and Sponges†

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From the marine sponge *Halichondria panicea* a lectin was isolated and characterized. The homogeneous lectin (composed of protein to 80.7% and of neutral carbohydrates to 14.1%) had a molecular weight of 78,000 (determined by gel filtration) and consisted of four subunits with a molecular weight of 21,000 each (determined by gel electrophoresis in the presence of sodium dodecyl sulfate). The hemagglutinating activity was only slightly dependent upon ionic strength and incubation temperature and did not require divalent cations, but it was inhibited by reagents for thiol groups. The *Halichondria* lectin was completely inhibited in hemagglutination competition experiments in the presence of fetuin, D-galacturonic acid, D-glucuronic acid, polygalacturonic acid, or L-fucose. The purified *Halichondria* lectin did not cause reaggregation of dissociated *H. panicea* cells. From the same sponge species bacteria were isolated and identified as *Pseudomonas insolita*. These bacteria were cultivated in marine broth 2216. Under these culture conditions the bacteria grew only in the presence of the homologous lectin; the lectin-caused effect was not abolished by D-glucuronic acid or D-galacturonic acid. However, after addition of a polysaccharide-containing fraction isolated from *P. insolita*, the lectin-caused, growth-promoting effect was abolished. Other lectins were found to exhibit no growth-promoting effect. On the basis of colony counts, *P. insolita* was the predominant bacterial species in the sponge extract; 1.9×10^6 *Pseudomonas* colonies were measured in extracts isolated from 1 g of sponge. The assumption of an interrelationship between the sponge and the bacterium is supported by the results indicating that the *Halichondria* lectin has no effect on the growth of such bacteria isolated from six other marine sponge species. Evidence is presented which indicates that the *Halichondria* lectin is not utilized during growth of the *Pseudomonas* species. Lectin activity was detected on the surface of mucoid cells from *H. panicea*. From the data obtained the possibility is discussed that the *Halichondria* lectin is a basis for a symbiotic relationship between the sponge and the bacterium.

Sponges live in symbiosis with algae (30), bacteria (2), fungi (28), and bryozoae (35). The physiological interrelationships between the sponges and the microorganisms are not known, although commensalism, mutualism, and inquilinism have been established in a few instances (19). Since the studies of Jakowska and Nigrelli (10), it is now accepted that specific antibiosis is one principle of symbiosis between sponges and bacteria. This biological system is based on the ability of some bacteria to adapt to the presence of antibiotics produced by the host.

In the present study a possible symbiotic principle in a sponge-bacterium system is described which is maintained by a profit-like mechanism for the bacteria living in a sponge. This system was species specific under the experimental conditions used. A lectin isolated from the marine sponge *Halichondria panicea* promoted the growth of the bacterium *Pseudomonas insolita*, which was isolated from the homologous sponge material.

† Manuscript no. XXVI in a series of publications from our laboratory dealing with aggregation of sponge cells.

MATERIALS AND METHODS

Sources of materials. The different culture media were obtained from Difco Laboratories, Detroit, Mich.; Labtrol and poly-L-lysine (molecular weight, 100,000) were from Serva, Heidelberg, West Germany; Sepharose 4B and dextran blue were from Deutsche Pharmacia, Freiburg, West Germany; Nonidet P-40 was from Deutsche Shell Chemie, Hamburg, West Germany; Bio-Gel P-300 was from Calbiochem, La Jolla, Calif.; all mono- and disaccharides, polygalacturonic

acid, and fetuin (cow; type IV) were from Sigma Chemical Co., St. Louis, Mo.; concanavalin A, *Ulex europaeus* agglutinin, and ricin (from *Ricinus communis*) were from Boehringer, Mannheim, West Germany.

Lectin from the siliceous sponge *Geodia cydonium* (Tetractinellida) was isolated and purified by the method of Vaith et al. (31); this lectin shows a high specificity for β-D-galactose-containing glyco substances (31).

The sponge *H. panicea* (Pallas; Halichondriidae) was collected in the vicinity of Rovinj, Yugoslavia, and Ghardaga, Egypt. The other sponges were collected exclusively near Rovinj: *Verongia aerophoba* (Schmidt; Spongiidae), *Tethya lyncurium* (Linnaeus; Tethyidae), *Tethya limski* (Müller; Tethyidae), *Hemimycale columella* (Bowerbank; Axinellidae), *Pellina semitubulosa* (Lieberkühn; Haplosclerina) and *Crambe crambe* (Schmidt; Axinellidae).

Solutions. Calcium- and magnesium-free artificial seawater (CMF) and calcium- and magnesium-free artificial seawater containing EDTA (CMFE) were prepared as described previously (17); the pH of the solutions was 8.2.

Hemagglutination assay. The lectin concentration (titer) was determined in roller tubes (23) containing 2.5 ml of a 2% suspension of paraformaldehyde-pretreated sheep erythrocytes (31) in CMF. A 0.5-ml amount of a lectin preparation serially diluted with equal volumes of CMF was added, and agglutination was allowed to proceed for 60 min at 20°C. The reciprocal of the greatest dilution at which agglutination (formation of cell clumps with diameters greater than 50 μm) occurred was defined as the titer of the undiluted lectin preparation. The size of the agglutinates was determined as described previously (25).

Adhesion assay. The method used for the adhesion assay was based on the formation of "rosettes." To perform the test, 2-ml portions of sponge cells (at a concentration of 2.5×10^7 cells per ml of CMF) were placed in plastic petri dishes (diameter, 3 cm) which were pretreated for 30 min with 2.5 ml of poly-L-lysine (50 μg/ml of distilled water) and subsequently washed twice with phosphate-buffered saline. After "fixation" of the cells on the plastic (incubation for 30 min at 25°C), the dishes were washed twice with a solution of bovine serum albumin (5 mg/ml of CMF). Then 1 ml of formalinized sheep erythrocytes (31) at a concentration of 2% (vol/vol) was added to the petri dishes. After incubation for 20 min at 25°C the cells were washed twice with phosphate-buffered saline and examined with a light microscope (magnification, ×800). A sponge cell with three or more erythrocytes on its surface was considered to be a rosette.

Isolation and purification of the lectin. All procedures were carried out at 0 to 4°C. A typical preparation (starting with 30 g of *Halichondria* material) is summarized in Table 1.

Step 1: initial extract. The sponge was cut into 2-mm^3 cubes and suspended 1:3 (wt/vol) in CMFE containing 10 mM 2-mercaptoethanol and homogenized with 10 strokes of a Dounce homogenizer. After 3 h of stirring the suspension was centrifuged at 20,000 × *g* for 30 min. The supernatant (78 ml) was collected and contained 5.9 mg of protein per ml (fraction 1).

Step 2: Sepharose 4B gel chromatography. Fraction 1 was passed through a Sepharose 4B column (Fig. 1). The lectin activity appeared within the K_{av} (6) range of 0 to 0.44 (fractions 16 to 31). The pooled fractions (120 ml; fraction 2) were analyzed with an electron microscope. After being mounted on a cytochrome *c* film, the preparation was transferred to carbon film which was then shadowed with platinum. After this procedure, round particles were detected (Fig. 2B) with a diameter of the central ring of 96.0 ± 7.5 nm and a circular contour length of 301.5 ± 23.5 nm. In contrast to similar particles first visualized in preparations from the sponge *Geodia cydonium* (19, 23), the *Halichondria* particles showed no radially arranged filaments.

Step 3: Nonidet P-40-urea treatment. From previous experiments (22, 24) it is known that the high-molecular-weight particles from *G. cydonium* can be disintegrated into smaller fragments by detergents. This procedure could also be applied successfully in the purification of the *Halichondria* lectin. Fraction 2 was dialyzed overnight against CMF containing 60 mM 2-mercaptoethanol, 5% (vol/vol) of the nonionic detergent Nonidet P-40, and 3 M urea. Subsequently, the lectin fraction was concentrated in dialysis tubing with polyethylene glycol to 7 ml (fraction 3). During this step the specific activity of the lectin decreased by 5% (Table 1).

Step 4: Bio-Gel P-300 gel chromatography. Fraction 3 was applied to a Bio-Gel P-300 column as described in the legend to Fig. 3. The lectin activity appeared after fractionation as a symmetric peak at

TABLE 1. *Purification of the Halichondria lectin*

Step and fraction	Total vol (ml)	UV-absorbing material (a) (A_{280}[a] × ml)	Total lectin (b) (titer × ml)	Specific activity (b/a)	Purification factor	Yield (%)
1. Initial extract	78	7.2	128	17.8	1.0	100
2. Sepharose 4B gel chromatography	128	1.8	64	35.0	2.0	82
3. Nonidet P-40-urea treatment	7	30.7	1,024	33.4	1.9	75
4. Bio-Gel P-300 gel chromatography	24	1.2	256	213.3	12.0	58
5. Hemadsorption	2.2	0.077	1,024	13,320.0	748.3	23

[a] A_{280}, absorbancy at 280 nm.

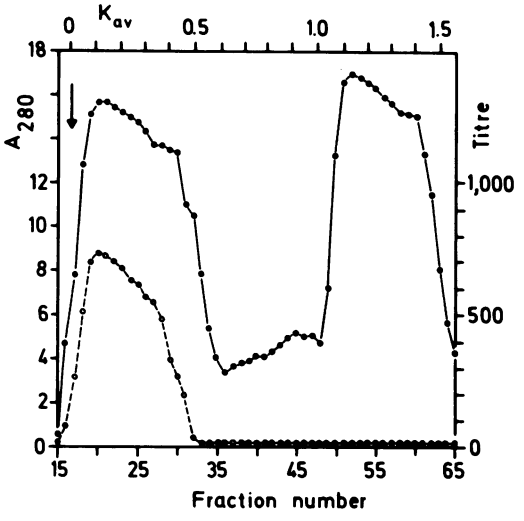

FIG. 1. *Purification of the lectin on Sepharose 4B. A column (4.5 by 42 cm) equilibrated with CMFE and containing 10 mM 2-mercaptoethanol was loaded with 78 ml of fraction 1. Fractions of 8 ml were collected, and 0.5 ml of each fraction was assayed for hemagglutinating activity* (○). (●), *absorbance at 280 nm* (A_{280}). *The arrow marks the position of dextran blue* (V_o). *Abscissa (upper scale),* K_{av} *values (6).*

the V_e/V_o value (6) of 1.7 ± 0.1. The active fractions 19 to 24 were pooled (24 ml); they contained a specific activity of 213.3 (Table 1) (fraction 4).

Step 5: hemadsorption. Final purification of the *Halichondria* lectin was achieved by taking advantage of the fact that the lectin-caused agglutination of sheep erythrocytes can be reversed by incubation in the presence of D-galacturonic acid (see below). Packed paraformaldehyde-pretreated sheep erythrocytes (4 ml) were fixed in 20 ml of 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) at 4°C for 2 h. The cells were then incubated in 0.2 M glycine at 4°C for 30 min and then washed three times in phosphate-buffered saline. The cell sediment after the last washing procedure was suspended in 24 ml of the lectin fraction 4. After 60 min of shaking, the cells were washed twice with CMF. The lectin was removed from the erythrocytes by incubation (60 min) with 3 ml of CMF containing 50 mM D-galacturonic acid. The cell suspension was then centrifuged (20,000 × *g* for 20 min), and the supernatant was collected and dialyzed overnight against CMF. The resulting lectin preparation (fraction 5), with a specific activity of 13,320 (Table 1), was used for the further studies. The protein concentration of this fraction was determined to be 115 μg/ml.

Growth and identification of the bacteria. Freshly collected *H. panicea* specimens were cut into cubes of about 50 mm^3; these pieces were cleaned, rinsed to eliminate foreign material, and squeezed through a nylon net with a pore size of 10 μm. The filtrate obtained was passed through membrane filters (BSWP; 2 μm; Millipore Corp., Bedford, Mass.). A 2-ml amount of the resulting suspension was supplemented with 20 μg of the purified *Halichondria* lectin and transferred to a solid nutrient medium consisting of 4% (wt/vol) marine broth 2216 (Difco) and 1.3% (wt/vol) agar (Difco) in petri dishes (diameter, 8.2 cm). In the following procedures all growth media used were supplemented with 5 μg of *Halichondria* lectin per ml. The organisms were grown at the optimal incubation temperature of 20°C. After incubation for 48 h two species of bacteria were detected; first, gram-positive, rod-shaped organisms 0.8 to 1.2 by 9 to 12 μm (Fig. 2A) which formed filiform, violet-grey colonies and, second, straight rods, 0.2 to 0.5 by 1.5 to 3.0 μm (Fig. 2A), motile with polar monotrichous flagellation (14). Only the latter species was cultivated for characterization by five consecutive plating procedures starting from one colony each. The colonies were round (with a ridge along the edges) and characterized by a yellow-brown color. No resting stages were observed. The strain was gram negative as determined by the Hucker modification of the Gram method of staining (27). The bacteria were strict aerobes as tested by the "test tube assay" (27), were oxidase (13) and catalase (34) positive, and contained diffusible pigments (12, 34). Due to these characteristics the bacteria isolated from *H. panicea* were classified as the genus *Pseudomonas* (5, 29). For species identification the ability of the bacteria to utilize certain carbon compounds by oxidation or fermentation was determined by the method of Hugh and Leifson (8) with OF basal medium (Difco) dissolved in seawater. To promote growth of the bacteria, the medium had to be supplemented with 5 μg of *Halichondria* lectin per ml. The assays were performed in tubes containing carbohydrate media; one series was overlaid with mineral oil, and the other was left without overlay. The following carbon sources and final concentrations (wt/vol) were tested: glucose, 1%; lactose, 1%; sucrose, 1%; galactose, 1%; sorbitol, 1%; adonitol, 1%; mannitol, 1%; inositol, 1%, and ethanol, 0.5%. The results revealed that only glucose, lactose, galactose, and ethanol were oxidatively metabolized. For detection of the formation of indole and hydrogen sulfide, the bacteria were grown in TSU agar base (Difco) dissolved in seawater. These two decomposition metabolites could not be detected by using H_2S test strips (Difco) and indole test strips (Difco).

The summarized characteristics of the *Pseudomonas* species growing in *H. panicea* are identical to those of *Pseudomonas* (proche) *insolita,* described by F. Denis (Ph.D thesis, Université de Poitiers, Poiters, France, 1971).

Bacteria from other sponge species were isolated and cultivated by the methods described for *H. panicea;* the culture media used did not contain the *Halichondria* lectin. Primary bacterial cultures were used for the experiments determining the effect of the *Halichondria* lectin on bacterial growth.

Quantitation of bacteria. To quantitate the number of bacteria in *H. panicea,* 1 g of sponge tissue was cut into cubes of about 50 mm^3. Bacteria were isolated as described above, and 0.1 ml of the bacterial suspension was used for inoculation of solid medium (4% marine broth 2216 and 1.3% agar) in petri dishes. In one series the medium was supplemented with 5 μg of *Halichondria* lectin per ml. Inoculated medium was

kept at 20°C for 7 days, and then colonies were counted on a colony counter. For each experiment six petri dishes were inoculated. The optimal number of colonies was less than 200 per dish; if the concentration of colonies was higher, appropriate dilutions were made.

Sponge cells. The dissociation of sponge tissue into separate cells was performed in CMFE (23).

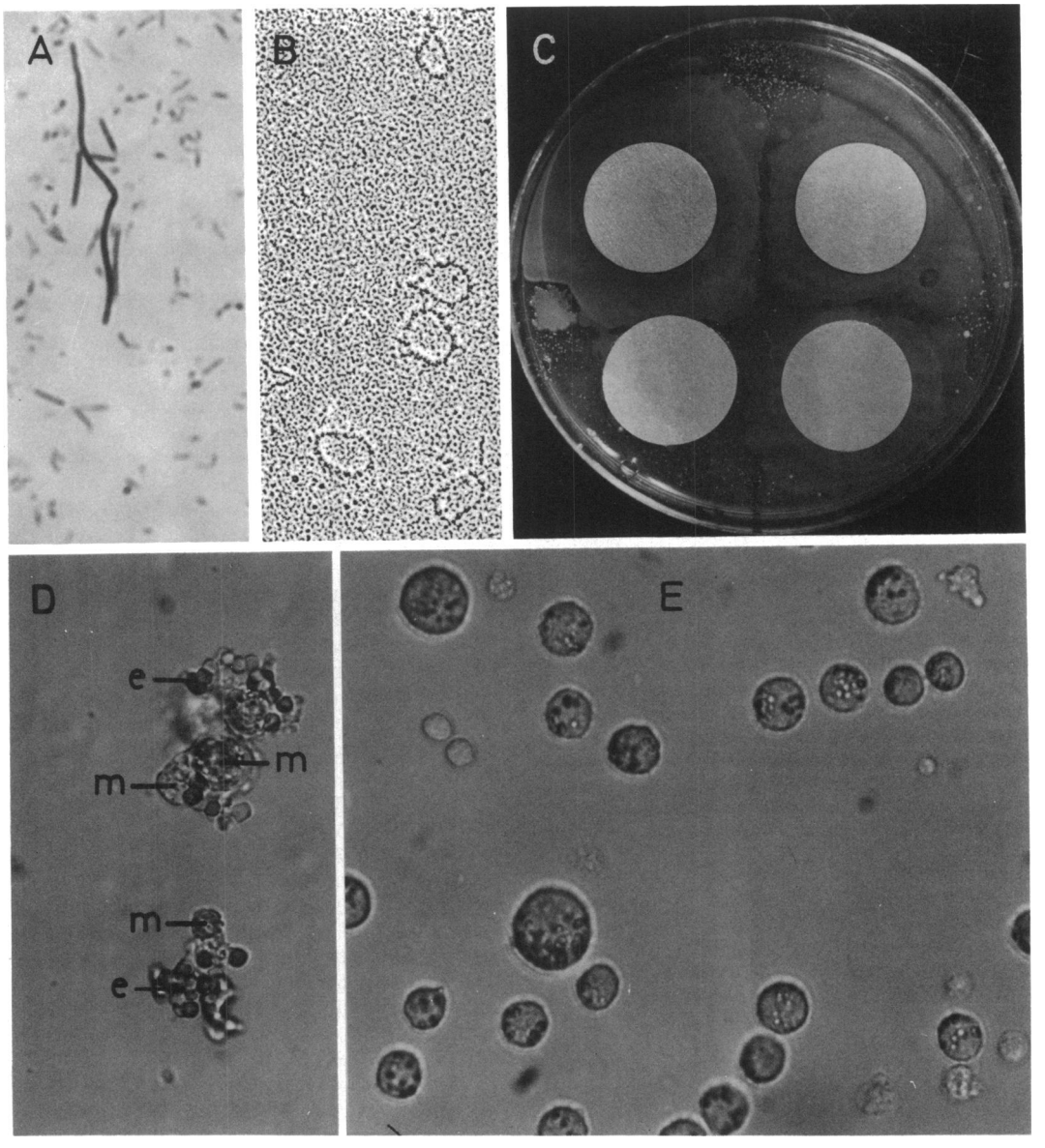

FIG. 2. *(A) Photomicrograph of P. insolita cells isolated from H. panicea (small rods) and an undetermined bacterial strain (large rods); magnification, ×1,000. (B) Electron micrograph of the particles detectable in the lectin fractions obtained after the purification on Sepharose 4B; magnification, ×60,000. (C) Influence of the Halichondria lectin on the growth of P. insolita. A 100-μl amount of the lectin preparation (0.5 μg) was applied onto a filter paper disk (diameter, 2.3 cm) placed on the surface of the seeded plates, and the cultures were incubated for 48 h at 20°C. Above left, 0.5 μg of lectin; above right, 0.5 μg of lectin plus 1 mg of D-galacturonic acid; below right, 0.5 μg of lectin plus 1 mg of D-glucuronic acid; and below left, control (without lectin). (D) Rosette formation of mucoid cells (m) from H. panicea with erythrocytes (e); magnification, ×550. (E) Inhibition of rosette formation by addition of 10 μM D-galacturonic acid during the incubation of sponge cells with erythrocytes; magnification, ×850.*

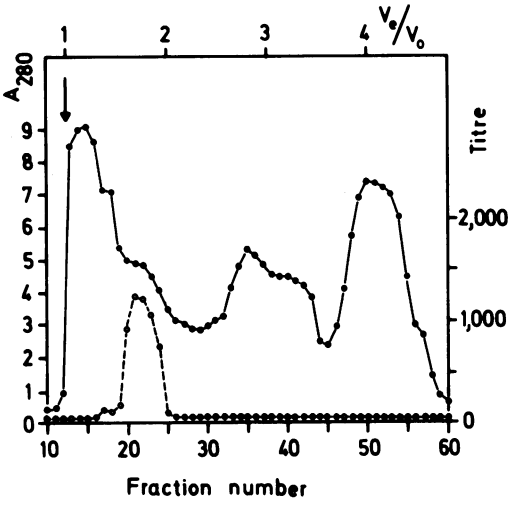

FIG. 3. *Purification of the lectin on Bio-Gel P-300. A column (2.0 by 50.0 cm) previously equilibrated with CMF containing 10 mM 2-mercaptoethanol and 3 M urea was loaded with 7 ml of fraction 3 and eluted with the equilibration buffer; 4-ml fractions were collected and 0.5 ml of each fraction was assayed for hemagglutinating activity* (○). (●), *absorbance at 280 nm* (A_{280}). *The arrow marks the position of dextran blue* (V_o). *Abscissa (upper scale),* V_e/V_o *value (6).*

Counting of cells was performed as described previously (23). For the identification of the different cell types, the procedure introduced by John et al. (11) was applied (21). With this method mucoid cells (diameter, 9 ± 1.5 μm) are stained blue-green by alcian blue; archaeocytes (6 ± 2 μm), characterized by prominent nucleoli, are stained purple by hematoxylin, and the small choanocytes (0.7 ± 0.2 μm) show pronounced basophila and are not stained by alcian blue. The cells obtained after dissociation of sponge tissue were centrifuged in CMFE at 12,000 × *g* for 5 min (20°C). The supernatant was collected and found to contain choanocytes (purity, >95%) and bacteria. The cell sediment was suspended in CMFE and fractionated further by Ficoll discontinuous density gradient centrifugation (11, 21); the mucoid cells were recovered on top of the 20% Ficoll (purity, 85%), and the archaeocytes were recovered on top of the 25% Ficoll (purity, >90%). The cells were washed three times with CMF; the last supernatant did not contain dissolved lectin, as determined by the hemagglutination assay. The viability of the sponge cells was 85 to 90% as determined by the trypan blue dye exclusion test (23).

Extraction of polysaccharides from *P. insolita*. *P. insolita* colonies were transferred from a pure culture growing on solid medium into a liquid medium containing 10 ml of 4% marine broth 2216 and 5 μg of *Halichondria* lectin per ml and aerated at 20°C (overnight culture). A 1,500-ml bottle containing 800 ml of the liquid medium was inoculated with 5 ml of an overnight culture and incubated at 20°C under aeration. After 12 h the culture reached an optical density at 596 nm of 0.44 (corresponding to 7×10^8 cells per ml); then, the culture was centrifuged at 20,000 × *g* for 30 min to obtain the cells. The bacteria were washed 3 times with CMF, and the resulting 220 mg of fresh cells was extracted with 45% aqueous phenol (33). The extract was freeze-dried and then dissolved in 1 ml of water and dialyzed for 24 h against distilled water. The dialysis residue (polysaccharide fraction) contained 0.8 mg of neutral carbohydrate.

Analytical methods. Crude preparations were assayed by their absorbance at 280 nm. For protein determination the method of Lowry et al. (16) was used (standard, Labtrol). Neutral carbohydrates (standard, starch) were determined by the method of Dubois et al. (7), and hexuronic acid (standard, D-glucuronic acid) was determined by the method of Avigad (1).

The determination of molecular weight was performed by gel filtration on a calibrated Bio-Gel P-300 column (6, 31). Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis of protein was done by modification of the procedure of Weber and Osborn (32) under conditions described previously (18); the gels were stained with Coomassie brilliant blue.

Details of the electron micrographic method used were described previously (19).

RESULTS

Properties of the lectin. (i) Physical characterization. The molecular weight of the lectin was estimated on a calibrated Bio-Gel P-300 column. From the elution behavior (Fig. 3; $V_e/V_o = 1.7 \pm 0.1$) a molecular weight of about 78,000 was calculated. With sodium dodecyl sulfate gel electrophoresis, the lectin fraction showed only one band with a molecular weight of 21,000. Thus, the native lectin appeared to be a tetramer which had been purified to homogeneity.

The lectin was not heat stable; the hemagglutination activity was completely destroyed after heating for 1 min at 60°C. The activity was sensitive to reagents for thiol groups; when the lectin was assayed in the presence of 5 mM *N*-ethylmaleimide or 7 mM *o*-iodobenzoate, the reaction was inhibited by 75 or 81%, respectively. The UV adsorption spectrum showed a maximum at 268 nm and a minimum at 244 nm; the ratio at 268 nm/244 nm was 1.8, and the ratio at 268 nm/280 nm was 1.6.

(ii) Chemical characterization. The chemical analysis revealed that the *Halichondria* lectin consisted mainly of protein (80.7%, wt/wt); neutral carbohydrates were present only in the low concentration of 14.1% (wt/wt). Hexuronic acid was not detected in the preparation. The contents of the remaining 5.2% of the material have not been determined.

(iii) Hemagglutination. The parameters controlling the hemagglutinating activity were determined in the standard assay described above. Time course experiments (Fig. 4) showed

that at high lectin concentrations (40 μg/ml) the process of hemagglutination started after a slight lag phase. This lag increased in assays containing lower lectin concentrations. The reaction reached a plateau after an incubation period of 60 min. The lower limit of sensitivity of the assay was 0.025 μg of lectin protein per ml; the upper limit was 1 μg/ml, due to the cell concentration chosen for the assay. The agglutination occurred at 6°C with kinetics and rate similar to those at 20 and 30°C; at higher incubation temperatures the agglutination was reduced. The agglutination showed a broad pH optimum; within the pH range of 7.2 to 9.5 (the pH value was adjusted with the Tris-hydrochloride buffer system present in CMF) no significant change in the extent of agglutination was observed. At pH 5.0 (50 mM citrate-phosphate system) the size of the agglutinates was 21%, and at pH 12 (Tris-NaOH system) it was 65% (controls at pH 8.2, 100%). The agglutination process was affected at an osmolarity higher than $\mu = 1.6$ (with respect to NaCl) and $\mu = 1.85$ (urea), respectively. The lectin-caused agglutination was independent of the presence of divalent cations and occurred even in the presence of 20 mM EDTA.

The lectin-caused hemagglutination could be inhibited by the monosaccharides L-fucose, D-glucuronic acid, D-galacturonic acid, the polygalacturonic acid as well as by the glycoprotein fetuin (26) and the polysaccharide-containing fraction isolated from *P. insolita*. The 50% inhibition doses (Fig. 5) were estimated to be as follows: fetuin (molecular weight, 48,330 [26]), 19.4 nM; D-galacturonic acid, 1.7 μM; D-glucuronic acid, 9.3 μM; and L-fucose, 11.0 μM. Under the conditions described in the legend to Fig. 5, polygalacturonic acid and the polysaccharide-containing fraction from the bacteria were found to inhibit hemagglutination by 50% at concentrations of 17.4 and 1.3 μg/ml, respectively (data not shown). The slopes of the dose-response curves were almost identical and varied between 3.27 and 3.73. This finding may indicate that these compounds interfere with the identical or chemically related binding site of the lectin to the erythrocytes. The inhibitory potencies of the compounds differed considerably. The highest affinity (ratio between one lectin molecule and the number of molecules of the respective inhibitory compound at 50% inhibition) was exerted by fetuin with 1:3.9; lower were the affinities of D-galacturonic acid (1:340), D-glucuronic acid (1:1,860), and L-fucose (1:2,210). The molar ratio of inhibition between polygalacturonic acid or the polysaccharide-containing fraction and the *Halichondria* lectin could not be calculated because of the unknown molecular weight of the two inhibitors. The following glyco substances (at concentrations of 50 mM) were found not to interfere with the lectin-caused agglutination: L-arabinose, D-deoxyribose, D-ribose, D-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, D-galactosamine, 1-*O*-methyl-D-mannoside, *N*-acetyl-D-galactosamine, *N*-acetyl-D-neuraminic acid, *N*-acetyl-D-glucosamine, sucrose, lactose, cellobiose, and glycogen. The effect of amino acids was not tested.

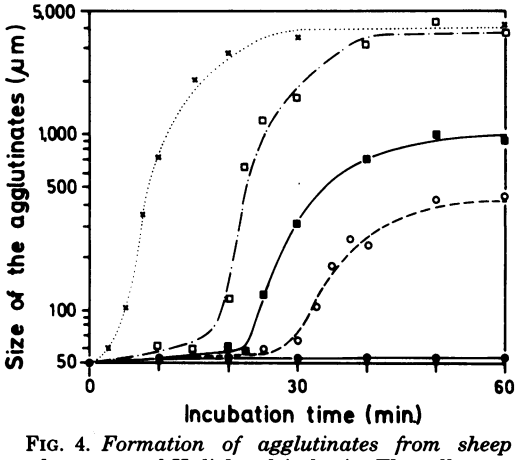

FIG. 4. *Formation of agglutinates from sheep erythrocytes and Halichondria lectin. The cells were incubated in the presence of the following lectin concentrations: 0 μg/ml (●), 0.2 μg/ml (○), 0.4 μg/ml (■), 2 μg/ml (□), and 40 μg/ml (×). The results come from five parallel determinations; the mean values presented have a maximum standard deviation of 20%.*

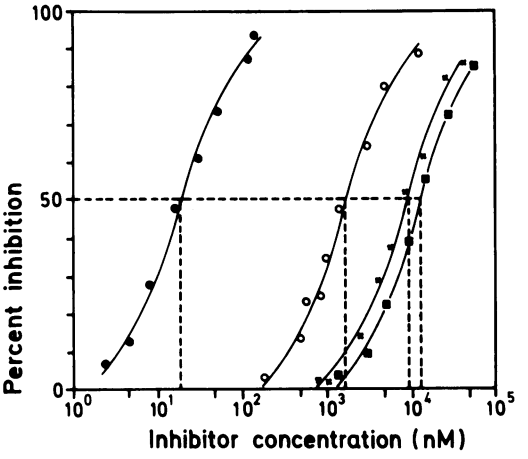

FIG. 5. *Inhibition by glyco substances of sheep erythrocyte agglutination with Halichondria lectin. The lectin concentration in the standard assay was 0.4 μg/ml. Symbols: ●, fetuin; ○, D-galacturonic acid; ×, D-glucuronic acid; ■, D-fucose; and - - -, 50% inhibition dose. Each value comes from 10 parallel assays; standard deviation is less than 10%.*

Influence of the lectin on bacterial growth. The influence of *Halichondria* lectin on the growth of *P. insolita* isolated from the homologous material was determined with pure cultures of the bacteria in 4% marine broth 2216 in the absence or presence of 1.3% (wt/wt) agar. In the liquid medium (incubation temperature, 20°C) the bacteria do not grow during an incubation period of 12 h. Addition of *Halichondria* lectin to the cultures immediately after inoculation resulted in a rapid proliferation of the bacteria. At a concentration of 5 μg of lectin per ml the optical density at 596 nm of the culture increased to 0.44 (100%); at lower lectin concentrations (3, 1, and 0.3 μg/ml) the growth rate was reduced (85, 53, and 15%, respectively). For the determination of the lectin effect on solid medium, filter paper disks soaked with the lectin preparation were layered on the surface of the seeded plates (Fig. 2C). In the absence of lectin (Fig. 2C, lower left) only few isolated colonies developed around the disk, whereas in the assays using disks soaked with lectin, the bacteria grew to confluence. In some assays, especially if high lectin concentrations were used, central and peripheral zones of growth were observed. Both zones were found to consist of the same colony types, developed from *P. insolita*. The width of the peripheral zone is a semiquantitative measure for the growth rate in dependence on the lectin concentration (Fig. 6). In view of the findings that D-galacturonic acid, polygalacturonic acid, and D-glucuronic acid inhibited hemagglutination, growth experiments with these mono- and polysaccharides were performed. The glyco substances alone exhibited no growth-stimulating activity (up to concentrations of 10 mg/ml); given in combination with the lectin, they showed no influence on the activity of the lectin to promote bacteria growth (Fig. 2C). Heat-denatured lectin (5 min at 60°C) did not exhibit any growth-stimulating activity (data not shown).

To rule out the possibility that the *Halichondria* lectin differs in its growth-promoting activity depending on the habitat from which the sponge specimen was collected, the lectin was isolated from sponges grown in the Mediterranean Sea (near Rovinj) as well as from specimens collected in the Red Sea (near Ghardaga). Both preparations caused almost the same potency of stimulation of the growth of *P. insolita*.

In control experiments the following lectins (in concentrations between 0.1 μg and 100 μg) were tested for their influence on the growth of *P. insolita* under the assay conditions described in the legend to Fig. 2C: concanavalin A (α-D-mannose > α-D-glucose > α-D-*N*-acetylglucosamine specificity), *Ulex europaeus* agglutinin (α-L-fucose), ricin (β-galactose), and *Geodia* lectin (β-D-galactose). None of these lectins caused a stimulation of bacterial growth at the concentrations tested.

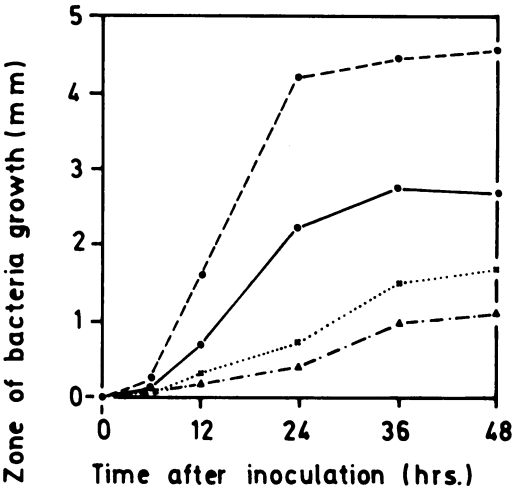

FIG. 6. *Growth-stimulating activity of the Halichondria lectin in the P. insolita system. As described in the legend to Fig. 2, disks containing 0.5 μg (○), 0.3 μg (●), or 0.1 μg (×) of lectin were layered on the seeded plates. In one series, 5 μg of the polysaccharide-containing fraction isolated from P. insolita was added to 0.5 μg of lectin (▲). After different incubation periods the width of the zone around the application (growth of the bacteria to confluence) was determined; (standard deviation does not exceed 20%).*

A series of experiments was designed to determine whether the agar in the solid culture contained substances which were growth inhibitory for *P. insolita* and which might be neutralized by the *Halichondria* lectin. The bacteria were grown in agar-free liquid medium (30-ml culture volume) containing 0 or 0.5 μg of *Halichondria* lectin per ml. After cultivation in spinner cultures for 12 h (20°C) the bacterial growth, as measured by determination of the optical density at 596 nm, was low (increase in optical density of 0.01) in the lectin-free culture, whereas in the lectin-containing culture an increase in optical density of 0.15 was determined.

From a series of other sponge species, bacteria were isolated and cultivated to determine a possible species-specific influence of the *Halichondria* lectin on other bacterial strains. Under the cultivation conditions described above, bacteria could be isolated and cultivated from the following sponge species: *V. aerophoba, T. lyncurium, T. limski, H. columella, P. semitubulosa,* and *C. crambe*. In no case could an inhibitory or stimulating effect of the *Halichondria* lectin (at

concentrations between 0.05 and 50 μg/ml) on the different bacterial cultures from heterologous sponge species be detected.

Possible ecological significance of the lectin. The following three approaches were performed to elucidate the possible role of the *Halichondria* lectin in intact sponges.

(i) Quantitation of *P. insolita* in sponges. To quantitate the number of bacteria in the sponge extract, 0.1-ml samples were inoculated on plates of solid marine broth 2216. In the absence of *Halichondria* lectin in the medium, $1.2 \times 10^4 \pm 0.5 \times 10^4$ colonies were determined in 1 ml of sponge extract (derived from 1.8 g of sponge tissue). Eighty percent of the colonies had a filiform appearance and contained gram-positive, rod-shaped organisms (Fig. 2A). No *P. insolita* colonies were detected. Cultivation of bacteria from the sponge extract on solid medium containing *Halichondria* lectin resulted in the formation of $3.4 \times 10^6 \pm 0.8 \times 10^6$ colonies per ml of extract; 87% of the colonies had an appearance typical of *P. insolita*. This means that this *Pseudomonas* species is predominant in the extract from *H. panicea* under the culture conditions used; 1.9×10^6 *P. insolita* colonies have been isolated from extracts of 1 g of sponge.

(ii) Interaction of the lectin with the bacteria. As summarized above, none of the synthetic glyco substances showed an influence on the lectin-caused growth stimulation of *P. insolita*. However, addition of the polysaccharide-containing fraction isolated from this bacterium reduced the lectin effect. As shown in Fig. 6, 5 μg of the polysaccharide-containing fraction lowered the growth-stimulated effect of 0.5 μg of lectin by 25%. From this competition experiment we can draw the conclusion that the bacteria contain a polysaccharide which binds to the *Halichondria* lectin.

The next step was to determine whether *Halichondria* lectin is utilized by the bacteria. Bacteria were allowed to grow in 100 ml of liquid medium in the presence of 1 μg of lectin per ml (titer, 8.9), a concentration which was found to be growth limiting. After incubation, the bacteria-free culture medium was recovered by centrifugation (20,000 × *g* for 30 min); the lectin was isolated from the medium by the hemadsorption procedure described above. After removal of the lectin from the erythrocytes with D-galacturonic acid and a subsequent dialysis, a lectin titer of 512 (equivalent to 57 μg) was determined. This means that 57% of the amount of lectin added at the beginning of the incubation could be recovered from the medium after growth of the bacteria. Taking into consideration that only 40% of the lectin activity was recovered after the hemadsorption procedure (Table 1; standard purification), we conclude that either no lectin or only a small percentage of active lectin was removed from the broth culture during growth of the bacteria.

(iii) Localization of the lectin in sponge cells. As a consequence of the above-mentioned result demonstrating that *Halichondria* lectin agglutinates formalinized erythrocytes, erythrocytes might be used as assay cells to identify *H. panicea* cells that are provided with lectin on the cell surface. The rosette formation experiments were performed under conditions (see above) in which the sponge cells (test cells) were attached to plastic dishes. The highest percentage of rosette formation (70 ± 15%; mean ± standard deviation of 10 parallel assays) was determined in the assays with mucoid cells (Fig. 2D); only 20 ± 5% of the archaeocytes had the potency of rosette formation. The rosette formation was inhibited by 80% in the presence of 10 μM D-galacturonic acid (Fig. 2E). The last finding is taken as an indication that lectin molecules on the cell surface of *H. panicea* cells cause rosetting with erythrocytes.

DISCUSSION

From a previous study (20) it is known that a lectin in the *Geodia* sponge system controls the aggregation potency of the sponge cells. In the present work it is shown that the biological role of the sponge lectins might not be restricted to reaggregation processes, but might have an additional biological function, the maintenance of a symbiosis between a marine sponge (*H. panicea*) and a bacterium (*P. insolita*).

The new lectin from *H. panicea* which was purified to homogeneity is obviously not specific for *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, or D-galactose as described for the sponge lectins from *Aaptos papillata* (4), *Axinella polypoides* (3), and *G. cydonium* (31). Hemagglutination inhibition experiments revealed that D-galacturonic acid, D-glucuronic acid, and L-fucose are inhibitors of the *Halichondria* lectin. The strongest reactivity of the lectin was determined with D-galacturonic acid; 340 molecules of this sugar reduced the activity of one lectin molecule by 50%. Under comparable conditions the *Axinella* agglutinin I is 8.5-fold more strongly inhibited by the monosaccharide D-galactose (3). The lectin in its native state has a molecular weight of about 78,000. Taking this figure and the amount of extractable lectin, 1.1 mg of protein, (Table 1; 2.2 ml of purified lectin with a protein concentration of 115 μg/ml was obtained at a yield of 23%) obtained from 30 g of

H. panicea tissue, the concentration of lectin molecules per mg of *H. panicea* tissue has been estimated to be 3×10^{11} molecules per mg of tissue. In the average, 5×10^7 cells have a weight of 1 mg (unpublished data), resulting in an estimate of 6×10^3 lectin molecules per cell. In the *Geodia* system the lectin concentration was found to be 100-fold higher (W. E. G. Müller, R. K. Zahn, B. Kurelec, I. Müller, G. Uhlenbruck, and P. Vaith, submitted for publication). Like all of the other lectins isolated from sponges (3, 4, 31), the *Halichondria* lectin consists also of subunits corresponding in this system to a molecular weight of 21,000. The characteristic feature of the *Halichondria* lectin is its association with high-molecular-weight, round-shaped particles. These particles were found in the *Geodia* sponge system to carry the aggregation factor (22, 23). *Halichondria* lectin is not involved in the aggregation process of dissociated *H. panicea* single cells (unpublished data). In view of the fact that a soluble aggregation factor has been detected in *H. panicea* (15), it is evident that the lectin and the aggregation factor are two distinct molecules, as described previously for the *Geodia* system (31).

Of interest is the finding that under the culture conditions used in the present study, the *Halichondria* lectin was required for the growth of a distinct bacterial species (isolated from *H. panicea*) which was identified as *P. insolita*. The bacteria can only grow in the presence of the *Halichondria* lectin (isolated from specimens collected in the Mediterranean Sea or in the Red Sea). The biological action of the *Halichondria* lectin on the growth of *P. insolita* is species specific under the culture conditions used in the present study. This conclusion might be drawn from the finding demonstrating that (i) other lectins were not found to stimulate the growth of *P. insolita* and (ii) the *Halichondria* lectin exhibited no effect on the growth of bacteria isolated from heterologous sponge extracts.

A second bacterial species was detected after the first plating procedure. This organism, which has not been identified, was not influenced in its growth behavior by the *Halichondria* lectin. Compared with *P. insolita*, this species was present in the crude extract from *H. panicea* in a low concentration. Based on colony counts, *P. insolita* was detected at a concentration of 1.9×10^6 cells per extract from 1 g of sponge, and the unidentified species was found only in a concentration of 6.7×10^3/g of sponge. Future experiments will show whether this bacterium belongs to the physiological microbiota of the sponge or is present as an accidental contamination. Concerning *P. insolita*, it seems likely that this bacterium represents a significant component of the flora in vivo.

At the present state of knowledge the biochemical basis for the interrelationship between the sponge and *P. insolita* is not understood. From the hemagglutination inhibition tests a specificity of the *Halichondria* lectin especially for D-glucuronic acid and D-galacturonic acid was determined. However, in the biological growth assay no competition between these monosaccharides and the lectin was observed. Therefore, different binding sites for the lectin on the sponge cells and on the bacteria might exist. The high activity of fetuin in the hemagglutination assay is hard to explain due to the complexity of the macromolecule; however, because it is known that fetuin contains a high percentage of fucose (which is also active), we postulate that this carbohydrate moiety is the underlying molecular mechanism causing inhibition of hemagglutination. The influence of fetuin on the growth of *P. insolita* was not checked because of its complex chemical composition; therefore, the results would not contribute further information which could help to elucidate the mode of action of the *Halichondria* lectin on the biological level.

In a series of experiments we attempted to elucidate the role of the *Halichondria* lectin in the homologous biological system. The results revealed that *H. panicea* cells are capable of binding erythrocytes. From the competition experiments with D-galacturonic acid we can conclude that this binding is mediated by the lectin isolated in the present study. The observation that mucoid cells of *H. panicea* are predominantly charged with lectin is confirmed by earlier findings performed with the sponge *G. cydonium* (Müller et al, submitted for publication). This in vitro observation suggests that on the surface of *H. panicea* mucoid cells a factor, presumably the described lectin, is present that binds to the surface of erythrocytes. This factor (lectin) obviously binds not only to erythrocytes but also to *P. insolita* cells. The latter suggestion resulted from the finding that the lectin-caused growth stimulation was prevented by the addition of a polysaccharide-containing fraction from this bacterium to the culture medium.

Preliminary experiments described in this paper seem to indicate that the *Halichondria* lectin does not act as a nutrient source for the bacteria, because during the process of heat denaturation the lectin lost its growth-stimulating activity. Because of the high molecular weight of the *Halichondria* lectin it is very likely that the lectin protein is irreversibly denatured during heating, resulting in a loss of its secondary

structure. Therefore, we assume at the present stage of knowledge that the secondary or even tertiary structure of the lectin is required for its growth-stimulating activity. The suggestion that the *Halichondria* lectin is not utilized by the bacteria as a growth factor or carbon source is supported by an additional observation. Determinations of the hemagglutination titers in the uninoculated broth medium and in the medium after growth of the bacteria revealed comparable levels. Taking these results together, we assume that the *Halichondria* lectin acts as an extracellular factor to *P. insolita* which triggers bacterial nucleic acid synthesis after binding to the cell wall. This concept would partially explain the symbiotic relationship between marine sponges and their bacterial flora (10).

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