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Phenotypic Study of Bacteria Associated with the Caribbean Sclerosponge, *Ceratoporella nicholsoni*

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Heterotrophic bacteria associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni* (Hickson), were found to occur extracellularly and were confined to the mesohyl regions of the sponge tissue. Physiological, metabolic, and morphological attributes of the culturable bacteria associated with the sponge were recorded by using numerical taxonomy methods for the analysis of 158 phenotypic attributes. Morphometric methods were used to determine the proportion of the total sponge-associated bacteria that were culturable by the methods employed, with the results ranging from 3 to 11% of the total bacteria inhabiting the sponge. Approximately 78% of the culturable bacteria clustered into four groups or phena, representing two previously undescribed *Vibrio* spp., an *Aeromonas* sp., and a coryneform- or actinomycete-like sp. Most of the bacteria were facultative anaerobes, fermenting sucrose and fucose but unusual in an inability to ferment glucose. This study was the first comprehensive study of heterotrophic bacteria associated with a sponge from the Caribbean basin, a region reputed to contain the most prolific sponge populations, with respect to biomass and diversity. The possible significance of these associations is discussed.

Microbial symbionts associated with marine sponges include bacteria, cyanobacteria, and unicellular algae. Most studies of the symbioses involving procaryotes and sponges are documented by electron microscopy (27, 35–37, 41). These ultrastructural studies are limited to morphological descriptions of the bacterial cells and approximations of their relative abundance within the sponge matrix. Few investigators have explored potential metabolic relationships and capabilities of the symbiont-host complex. A more informative approach is to isolate symbiotic bacteria and examine those physiological and biochemical processes that define their metabolic and taxonomic status.

Large numbers of heterotrophic bacteria of diverse morphology are reported from marine sponges (23, 36, 42, 48). In ultrastructural studies, it has been shown that often bacteria occupy more sponge volume than do the sponge cells—up to 60% of the mesohyl, the region between the thin external epithelium and internal flagellated epithelium (27, 37, 42). Published micrographs reveal that most symbiotic bacteria possess cell walls typical of gram-negative bacteria. Three classes of heterotrophic bacterial associations with sponges can be categorized as (i) small populations of cosmopolitan bacteria of a species composition similar to that in ambient seawater, most likely utilized as a food source by the host, (ii) large species-specific populations inhabiting the mesohyl region and not in the ambient seawater, most likely true symbionts, and (iii) very small bacteria located inside sponge cells (37).

A phenotypic analysis of heterotrophic bacterial symbionts of Great Barrier Reef and Mediterranean Sea sponges provides an understanding of functional relationships between the sponge host and its bacterial symbionts (42). Phenotypically similar bacterial symbionts are isolated from taxonomically diverse sponges collected from geographically distant localities (48). Most of the symbiotic bacteria differ from the bacteria isolated from ambient seawater, with the most prominent symbiont phenotype being an oxidase-negative, facultative anaerobe, able to metabolize a wide range of compounds and possessing characteristics most closely resembling those of the family *Enterobacteriaceae* (42).

A community of morphologically diverse bacteria is found associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni* (Hickson), and large numbers of sponge-associated bacteria compose up to 57% of the cellular composition of the mesohyl (49). The bacteria are located primarily extracellularly in the mesohyl regions and rarely are found associated with the dermal layers attached to the surface and lining of the aquiferous system. The sizes and morphologies of the bacteria are highly variable, with the majority being rod or coccoid shaped. Most of the bacteria possess cell walls typical of gram-negative bacteria. Culturable bacteria residing within the sclerosponge *C. nicholsoni* are very different from bacteria isolated from ambient seawater surrounding the sponge, a conclusion based on phenotypic and serological data (D. L. Santavy, Ph.D. dissertation, University of Maryland, College Park, 1988).

A numerical taxonomic study was done to determine the role of the bacteria associated with the Atlantic sclerosponge, *C. nicholsoni*. Phenotypic analysis was used to elucidate the ecological, physiological, and morphological attributes of bacteria associated with the sponge and their potential contribution to the physiology of the sponge. The objective of the study was to define the metabolic capabilities of the sponge-associated bacteria and to elucidate the interactions between individual bacterial species and the host, as well as other species of the microbial community, so that the sponge-bacteria association might be more precisely understood. Isolates examined in this study were aerobic bacteria readily cultured on the media employed. Relatively rare symbionts, or those more difficult to culture, are under-represented because of the limitations of the microbiological

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methods currently available. It is our intent to utilize the isolates characterized in this study for preparation of genetic probes so that, ultimately, molecular genetic methods may be used to define the bacteria-sponge relationship.

MATERIALS AND METHODS

Collection and culture of symbiotic bacteria. Bacteria associated with the sclerosponge *C. nicholsoni* were extracted from a single sponge species collected in a submarine cave at a depth of 30 m in Jamaica Bay at Acklins Island, Bahamas (74°17.1'W, 22°10.3'N), during August 1985. Collection was conducted as aseptically as possible underwater. All specimens were collected by using SCUBA and wearing sterile surgical gloves to reduce the contamination by allochthonous bacteria. Small sponge samples were collected by using a sterilized geologist's pick and cold chisel. Samples were cleaned of debris and sediment, placed in sterile Whirl pacs, and sealed underwater. Processing began within 1 h after collection.

All extraction and isolation procedures were performed aseptically. All instruments, glassware, and buffers were autoclaved or filter sterilized prior to use. Sponge surfaces were washed with jets of autoclaved, filtered seawater (0.22-μm-pore-size filter) until they were visibly free of debris. The sponge surface was sterilized in a rapid wash of 70% ethanol and immediately immersed in autoclaved, filtered seawater and aspirated. A measured area of sponge tissue was removed from the calcareous skeleton with a sterile scalpel. The tissue was immediately transferred to a sponge dissociation medium, CMFSW (2.7% NaCl, 0.008% KCl, 0.01% Na_2SO_4, pH 8.0 [13a]). The specimens were soaked for 20 min. The tissue and diluent were macerated with an Omni Mixer tissue homogenizer (Ivan Sorvall, Inc.). The homogenate was plated by using a dilution series to 10^{-5} and employing the spread plate technique. Each dilution was plated in quadruplicate on each of four media described below.

Three media were used to select for specific trophic modes, and a fourth medium was used to isolate gram-positive organisms. A modified marine agar (MMA; 1.8% marine M2216 medium [Difco Laboratories], 2% NaCl, 0.01% sodium glycerophosphate, 1.3% Difco agar, pH 7.4) was employed to isolate copiotrophic bacteria. A seawater-based medium (0.005% yeast extract [Difco], 0.05% tryptone [Oxoid Ltd.], 0.01% sodium glycerophosphate, 1.2% Noble agar, seawater from collection site) containing low nutrient concentrations was designed to promote growth of oligotrophic bacteria. Marine BG agar (Santavy, Ph.D. dissertation), a medium containing ions required for growth of marine cyanobacteria, consisting of trace elements, and supplemented with cyanocobalamin, was used to select for phototrophs. A selective medium for gram-positive bacteria was also used (4.3% phenylethanol agar [BBL Microbiology Systems, Cockeysville, Md.], 0.01% sodium glycerophosphate, 3.3% Instant Ocean).

All plates were incubated at 25°C. Colonies were selected by using a random grid pattern placed over each petri dish after incubation for 48 and 72 h and 1 and 2 weeks. Colonies were serially streaked until pure cultures were obtained. Plates were retained for an additional month to allow for the selection of slowly growing bacteria. Isolates were maintained on agar slants overlaid with mineral oil in vials and were also cryopreserved in liquid nitrogen (26% glycerol–logarithmic-phase liquid culture; 1:1).

Enumeration of culturable bacteria from the sponge. The average number of culturable bacteria isolated from the sclerosponge was estimated from plate count data, as described in the isolation procedure, and extrapolated to provide an estimate of the number of culturable bacteria per unit volume of sponge. Weight was concluded to be an inappropriate biomass measurement for bacterial enumeration because of the calcareous skeleton of the sponge. Instead, bacteria were isolated from a measured area of the sclerosponge surface. The total extracted sponge area was measured with millimeter calipers. Plate count data were obtained by using bacteria growing on MMA only, since this medium consistently yielded the largest number of colonies. The average number of culturable bacteria per sponge area was estimated from plate counts obtained from a total of 12 individual sclerosponges collected in the Bahamas in August 1985 and November 1986. Volumetric estimations for each sample were calculated by multiplying the scraped area by 200 μm, the average depth of the living tissue of *C. nicholsoni* covering the skeleton (49), times five to yield the number of culturable bacteria per cubic millimeter of sponge tissue. The mean and standard error of the mean (SE) are reported.

Enumeration of total bacteria present in the sponge. Morphometric and stereological methods were employed to quantitate the total number of bacteria contained within the sponge tissue by using transmission electron micrographs (TEMs). Low-magnification TEMs of *C. nicholsoni* collected from Acklins Island were prepared as described elsewhere (49). The number of bacteria per unit volume (N_v) of sponge was estimated by using the formula of Weibel and Gomez (38): $N_v = (N_a^3/V_v)^{1/2}/\beta$, where N_a is the mean number of culturable bacteria per cubic millimeter of sponge tissue, V_v is the mean volume fraction of bacteria in sponge tissue, and β is the dimensionless shape coefficient.

Several assumptions were among the premises of the mathematical derivation which was required to use the morphometric technique of Weibel and Gomez (38) to estimate the number of particles in a three-dimensional volume from two-dimensional TEMs. The bacteria were assumed to be wholly convex, randomly distributed and orientated in the tissue, and nonspherical (1). It was assumed that no significant compression or shrinkage of the bacteria in the tissue occurred and that all sections were random and not serial or overlapping (3). Lastly, it was assumed that the smallest dimension of a bacterium was relatively large compared with the thickness of the TEM section and that there were no artifacts due to thick sections (12).

The progressions of errors for calculating N_v were obtained using the following expression (1, 12): $SE(N_v) = \{9/4\ [SE(N_a)/N_a]^2 + 1/4\ [SE(V_v)/V_v]^2\}^{1/2} \cdot N_v$, where $SE(N_v)$ is the standard error of the mean N_v, $SE(N_a)$ is the standard error of the mean N_a, and $SE(V_v)$ is the standard error of the mean Vv.

The mean number of bacteria per unit area (N_a) was estimated by counting the total number of bacteria in 10 TEMs of random sections of sponges, ranging in magnification from 2,800 to 7,100. A total area of 24,860 μm^2 was examined. Only bacteria which could be resolved unequivocally were counted, including partial cells. The use of low-magnification TEMs ensured minimal edge effects due to partial cells in the sections. All bacteria were counted, regardless of their orientation in the tissue. Dividing bacteria were counted as one bacterium, unless a septum could be resolved between the two cells.

The mean volume fraction of the bacteria in the sponge tissue (V_v) has been previously estimated for *C. nicholsoni*

TABLE 1. Reference strains used in the numerical taxonomy analysis

Identification no.	Species	ATCC no.
346	*Staphylococcus aureus*	27660
348	*Alteromonas macleodii*	27126
349	*Aeromonas caviae*	15468
350	*P. fluorescens*	13525
351	*Aeromonas hydrophila*	7966
352	*Escherichia coli*	11775
353	*Pseudomonas piscicida*	15251
354	*Vibrio campbellii*	25920
355	*V. alginolyticus*	17749
356	*Flectobacillus marinus*	25205
360	*Enterobacter cloacae*	13047
362	*Alteromonas haloplanktis*	14393
380	*M. luteus*	4698

by using planimetry. From earlier work, it is reported that bacteria occupy 18.53% ± 2.01% (standard deviation; n = 10) of the total sponge tissue, determined by examination of 13,078 μm^2 of sponge tissue (49). This estimate was used to determine the N_v and SE.

The shape coefficient (β) was determined by estimation of the mean axial ratios (R) of bacterial length to diameter and overall ellipsoidal shape (12). R was estimated from five random TEM sections ranging in magnification from 6,000 to 9,000 and encompassing a total area of 4,061 μm^2. Measurements of maximum length and minimum diameter were made on all complete profiles of bacteria contained within each TEM. Axial ratios were calculated by length and diameter measurements and the mean axial ratio. The SE was obtained for each TEM examined. A grand mean and variance were estimated by calculating a weighted average for both statistics for all the TEMs examined in this study (34).

The percentage of total bacteria cultured from the sponge volume (B_c) was estimated by calculating the average, maximum, and minimum percentages, by using 95% confidence limits (34), from the following equations: average $B_c = N_c/N_v$, maximum $B_c = \{N_c + [SE(N_c) \cdot 1.96]\}/\{N_v - [SE(N_v) \cdot 1.96]\}$, and minimum $B_c = \{N_c - [SE(N_c) \cdot 1.96]\}/\{N_v + [SE(N_v) \cdot 1.96]\}$, where N_c is the mean number of culturable bacteria per cubic millimeter of sponge tissue.

Numerical taxonomy analysis. Eighty strains isolated and purified from the collection of bacteria from the sponge were used for the numerical taxonomy analysis. Selected reference strains of marine bacteria were included in the study; thus, a total of 103 cultures were included in the analysis: 80 symbiont isolates, 15 reference strains, representing taxonomically diverse gram-negative and gram-positive strains of marine origin (Table 1), and 8 strains duplicated blindly to provide an estimate of test and operator error.

Each strain was characterized by using 158 characteristics (Tables 1 and 2). Duplicate strains were selected by using a random-number generator and treated as independent strains throughout the study. All test media were supplemented with appropriate ions reported by other investigators to be required by marine bacteria (16). The media used to test marine bacteria were supplemented with appropriate cations if the conventional test medium was designed for terrestrial or freshwater bacteria. The basal medium used for some of the characterization tests was MMA or modified marine broth (MMB), the latter being MMA without agar. Analytical-grade reagents and medium supplements were used in the preparation of media. Positive and negative controls were included in each test.

TABLE 2. Estimations of mean values and SE for parameters used in calculations for enumeration of bacteria per unit volume of sponge tissue (N_v)

Morphometric parameter	Mean	SE
N_a	6.93×10^{4}[a]	3.77×10^3
V_v	0.185	0.0636
R	1.55	0.0402
β	1.42	0.04

[a] Bacteria per square millimeter.

Morphological features were resolved employing light microscopy and TEM. Gram reactions were determined with heat-fixed smears of logarithmic-phase cultures grown in MMB and employing the Hucker modification of the Gram stain (11) and were confirmed by using the KOH method of Buck (8). Swarming was tested on MMA, and the luminescence test medium was examined after 24 and 48 h (40). Negatively stained specimens viewed by TEM were used to determine cell shape, presence and type of flagella, mode of cell division, and any unusual morphological features. Logarithmic-growth-phase cultures grown in MMB were fixed in a 2% glutaraldehyde–3% paraformaldehyde phosphate buffer solution (Millonig phosphate buffer [13]; osmolarity, 998 mM with sucrose) and placed on Formvar resin-coated copper grids. The cells were negatively stained with 1% phosphotungsten acid (pH 7.3) and viewed with a JOEL 100CX2 TEM (60 kV).

The biochemical traits of the strains were determined by employing conventional media modified by the addition of marine salts. The following characteristics were tested: oxidase reaction, reduction of nitrate and nitrite, denitrification, decarboxylation of lysine and ornithine, arginine hydrolase production (determined by the methods of both Moeller and Thornley [40]), gluconate oxidation after incubation for 7 days, indole production, and hydrogen sulfide production (10). The protocols of Smibert and Krieg (29) were utilized for tests of the following: Voges-Proskauer reaction, gas evolution from glucose, and catalase production (method two). The presence of β-galactosidase was determined by the detection of *o*-nitrophenyl-β-D-galactopyranoside by utilizing the fluorogenic substrate 4-methyl umbelliferyl-β-D-galactoside (20).

The physiological tolerances of the isolates to NaCl concentration, temperature, and pH were evaluated. The ability to grow at 4 and 42°C in MMB was tested and compared with growth at 25°C. Results were read after incubation for 2 and 5 days at 42°C and 14 days at 4°C. NaCl tolerances were tested by employing the following NaCl concentrations: 0, 3, 6, 8, and 10% in a medium consisting of 1% tryptone (Oxoid) and 0.01% sodium glycerophosphate. Growth at initial pHs of 4, 7, and 10 was observed when the same medium used to test 3% NaCl tolerance was employed (40).

Acid production in carbohydrate media via fermentation was determined with a modified marine medium (17). Utilization of the following compounds was tested at a final concentration of 1% (vol/wt): glucose, sucrose, lactose, arbutin, trehalose, cellobiose, mannose, inositol, mannitol, sorbitol, salicin, arabinose, galactose, and fucose. All isolates were screened for the ability to utilize 70 different compounds as sole sources of organic carbon (Table 3). A modified basal medium containing electrolytes supporting growth of marine bacteria was used (40). Substrates were prepared with the sodium salt of the compound when available, filter sterilized with a 0.22-μm-pore-size membrane

TABLE 3. Percentage of strains of sponge-associated bacterial phena able to utilize a given carbon compound as its sole energy source

Compound	Value for phenon no.:			
	1	2	3	4
D-Arabitol	6	8	27	100
Dulcitol	100	100	100	100
Ethanol	76	88	70	22
D-Glycerol	59	96	55	11
D-Mannitol	94	100	50	11
1-Propanol	24	8	27	0
Sorbitol	0	0	0	11
D-Alanine	0	0	27	100
L-Alanine	59	68	55	0
L-Arginine	100	92	64	22
I-Asparagine	82	96	64	11
L-Aspartate	29	72	27	11
Gamma aminobutyrate	71	84	45	22
Citrulline	41	36	18	0
L-Glutamate	76	88	73	33
Glycine	59	72	18	0
L-Histidine	12	4	0	0
L-Proline	18	16	18	0
L-Hydroxyproline	41	92	27	0
L-Tyrosine	100	96	82	11
L-Threonine	18	8	9	0
Delta aminovalerate	88	84	55	22
D-Amygdalin	6	0	27	100
L-Arabinose	12	72	10	0
Arbutin	100	96	82	0
Cellobiose	0	0	0	11
Collagen	100	100	64	56
D-Galactose	100	100	73	33
D-Gluconate	0	0	0	100
D-Glucose	100	96	73	11
D-Glucosamine	59	84	100	11
N-Acetyl-D-glucosamine	100	100	100	100
Glycogen	100	100	100	0
Lactose	59	92	45	0
Maltose	100	100	64	22
D-Mannose	29	36	18	0
Melibiose	29	32	45	0
D-Ribose	100	100	64	33
Salicin	100	100	64	22
Sucrose	76	92	55	0
Acetate	100	96	50	11
Phenylacetate	100	96	100	50
p-Hydroxybenzoate	100	100	73	33
Butyrate	0	0	0	0
DL-3-Hydroxybutyrate	100	100	40	0
Citrate	100	96	64	11
Fumarate	47	48	36	0
Glutamine	94	100	73	11
Glutarate	94	100	36	33
2-Ketoglutarate	18	4	9	0
DL-Lactate	100	96	73	33
Malonate	100	100	55	33
Propionate	100	100	100	11
Succinate	88	6	64	22
Adenine	88	96	70	11
Ethanolamine	88	96	55	22
D-Galacturonate	35	28	18	0
D-Glucuronate	0	0	0	11
m-Inositol	29	44	27	0
Putrescine	88	92	82	0
Sarcosine	0	0	0	22
Taurine	12	8	0	0
Urea	71	92	55	0
Xanthine	94	96	82	0

filter, and added to a final concentration of 0.1% (wt/vol). Plates were multipoint inoculated and incubated for 14 days. A negative control plate containing only basal medium and a positive control plate supplemented with 0.1% (wt/vol) vitamin-free Casamino Acids (Difco) were also included.

Sensitivity to antibacterial compounds was determined with antibiotics and inhibitory substances as follows. Resistance to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl pteridine) was evaluated at concentrations of 10, 50, and 150 μg/ml on heart infusion agar (Difco) supplemented with marine salts (40). Antibiotic sensitivity was assessed by placing disks impregnated with the agent on confluent lawns growing on MMA. Sensi-Discs (BBL Microbiology Systems) containing the following antibiotic agents and quantities were used: ampicillin (10 μg), kanamycin (1 mg), nalidixic acid (30 μg), novobiocin (30 μg), penicillin (10 U), polymyxin B (300 U), streptomycin (10 μg), and tetracycline (30 μg). Zones of inhibition were noted after 2 and 7 days. Organisms were spot inoculated and tested for the ability to grow on the following inhibitory substances: brilliant green, crystal violet, and neutral red (0.002% each [wt/vol]), methyl violet (0.0002% [wt/vol]), and methylene blue (0.01% [wt/vol]). Filter-sterilized dye was added to MMA. The ability to grow on thiosulfate citrate bile salts sucrose (TCBS, Oxoid) agar supplemented with marine salts was also determined. Growth was monitored for 7 days and the plates were examined on days 2, 4, and 7 for detectable growth and the presence of yellow or green colonies.

Enzymatic and degradative properties of the isolates were assessed with a wide selection of substrates. MMA was used as the basal medium for tests of the following: tyrosine and xanthine degradation (39) and the presence of amylase, lecithinase, and chitinase (40); phosphatase and sulfatase (10); and elastase (28). Christensen urea agar supplemented with marine salts was used for the urease test (29), and the media employed to detect gelatinase, deoxyribonuclease, and lipase activity were similarly amended, the lipase activity being detected with a series of Tween compounds (Tween 20, Tween 40, Tween 60, and Tween 80) (40). The liquification of alginate was determined by the method of Billy (5). Testing for the presence of collagenase was done by overlaying a MMA plate with molten agar containing 1% purified collagen (Sigma Chemical Co.).

Statistical and cluster analyses. All characteristics were coded as binary data for statistical and cluster analyses. The value 1 was assigned if the characteristic was present or positive, and the value 0 was assigned if the characteristic was absent or negative. Multistate characteristics, such as range of tolerance to NaCl concentrations, were regarded as individual tests and recorded as individual two-state responses. Numerical analysis was done by calculating similarity matrices employing the Jaccard similarity coefficient (S_J) (30). Clustering was by unweighted average linkage (33) by using TAXAN (version 2.0, Sea Grant College, University of Maryland). The hypothetical median organism

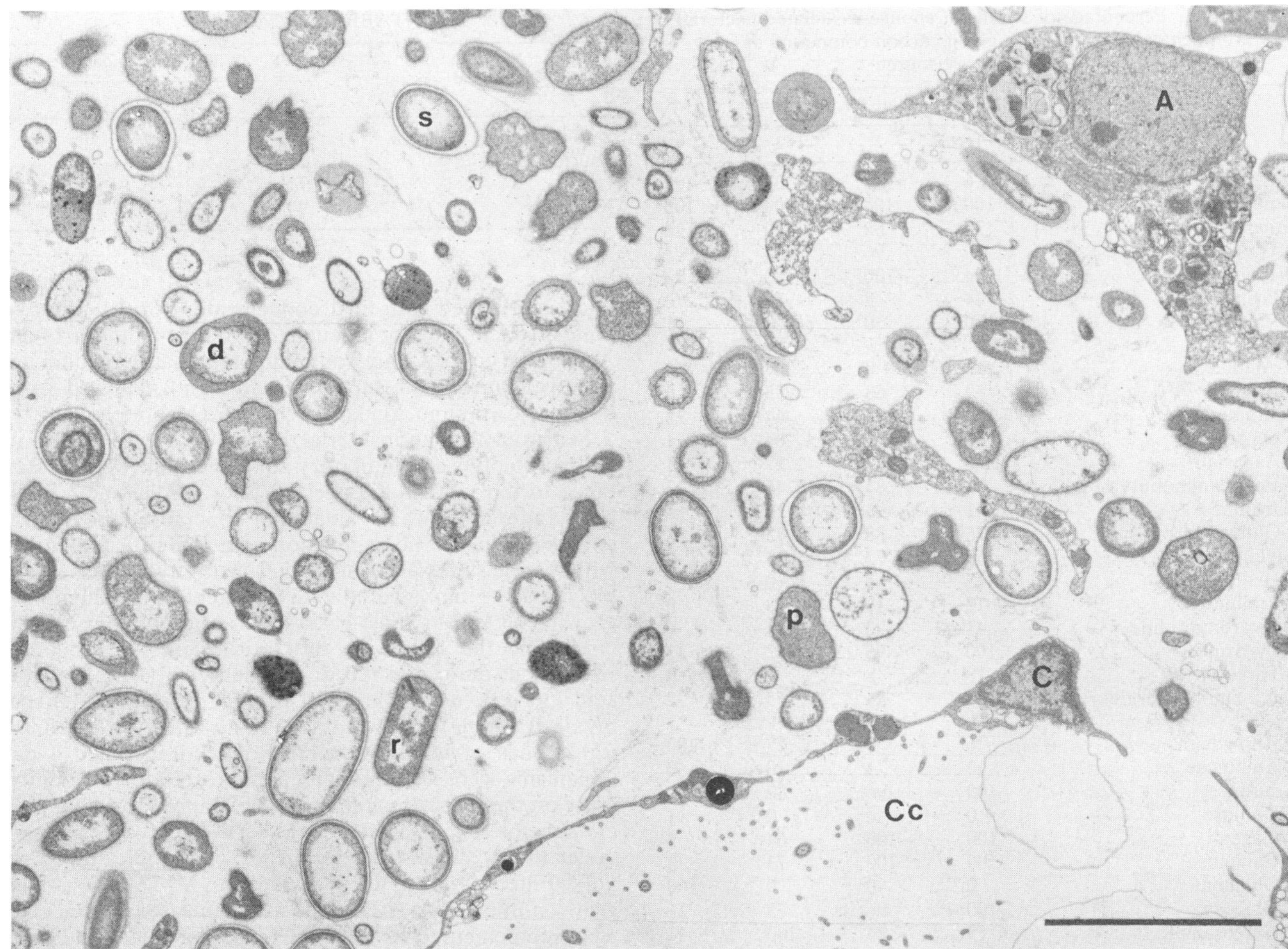

FIG. 1. TEM of *C. nicholsoni* tissue containing extracellular bacteria. Various bacterial morphologies, including rods (r), spherical to ovoid shapes with diffuse, loosely bound outer membranes (s), and pleomorphic forms (p), as well as bacteria with electron-dense outer membranes (d), were resolved and found among sponge archaeocytes (A) and choanocytes or flagellated cells (C) forming the choanocyte chamber (Cc). (Sponge cells and structure are denoted in upper case, while bacterial cells are denoted in lower case.) Bar, 5 μm.

(HMO) of each defined cluster was calculated (18), and the strain that most closely resembled it was selected to represent the cluster. The test results for all strains and their replicates were compared for all characteristics, and an estimate of test variance (s_i^2) was calculated. Individual tests which were all positive or negative for all the bacteria in the analysis or had variance estimates greater than 0.10 were eliminated from subsequent analyses (6, 7). These variances were used to determine the average pooled test variance and the average probability of an erroneous test result (31). Analyses were performed by using SAS version 5.16 and SAS/Graph (Statistical Analysis System, Cary, N.C.) on the University of Maryland IBM model 4381 computer.

RESULTS

Proportion of total bacteria represented by culturable bacteria. The estimated percentage of culturable bacteria associated with *C. nicholsoni* (B_c) ranged from 3.4 to 11% of the total number of bacteria contained with the sponge, as determined by enumeration and employing morphometry. Estimates of the mean values and respective SE for parameters used in the calculations ($N_v = [N_a^3/V_v]^{1/2}/\beta$) for the enumeration of bacteria per unit volume of sponge tissue (N_v) are given in Table 2, and a TEM of the tissue is provided in Fig. 1, an example from which the calculations were made. The range of values calculated for N_c and N_v, contained within the 95% confidence limits, used in the calculation of B_c are given in Table 4. The average number of bacteria cultured from each sponge, adjusted for the same volume, was not significantly different among all individuals or between the two sampling times (Santavy, Ph.D. dissertation), justifying the use of multiple samples collected at different times for calculation of the mean number of culturable bacteria per unit volume of sponge tissue.

Phenotypic analysis. All bacteria cultured from the sclero-

TABLE 4. Range estimates for the number of culturable bacteria (N_c) and total number of bacteria (N_v) per unit volume of sponge tissue

Morphometric parameter	Value (no. of bacteria/mm³ of sponge tissue)		
	Avg	Maximum	Minimum
N_v	9.44×10^7	11.0×10^7	7.88×10^7
N_c	6.20×10^6	8.64×10^6	3.76×10^6

TABLE 5. Characteristics of sponge symbiont phena as percentages of positive reactions

Characteristic	Value for phenon no.:				Value for others[a]
	1	2	3	4	
Gram reaction					
Negative	100	100	100	0	83
Positive	0	0	0	63	17
Variable	0	0	0	37	0
Flagellum	100	88	92	67	83
Rod shape	100	100	100	100	94
Filamentous shape	24	28	45	89	22
Polymorphism	6	16	0	89	17
Binary division	100	100	100	44	83
Growth in NaCl at:					
0%	0	0	0	11	28
6%	100	100	100	89	94
8%	94	100	100	22	39
Acid production:					
L-Arabinose	76	84	73	67	39
Cellobiose	0	100	18	0	28
Fructose	56	63	74	18	32
D-Fucose	88	80	91	100	61
D-Galactose	65	52	64	100	72
D-Glucose	0	0	0	100	28
D-Lactose	0	0	0	0	6
D-Mannitol	88	0	36	11	61
Salicin	0	0	0	0	22
Sorbitol	0	0	0	0	17
Sucrose	82	96	100	100	50
Trehalose	100	0	90	89	67
Decarboxylation of:					
Arginine	0	0	0	0	11
Lysine	94	88	100	22	61
Ornithine	100	96	100	13	83
Catalase	88	100	18	100	67
Gluconate oxidation	0	0	0	0	0
Indole production	0	4	0	89	22
H_2S production	0	0	0	0	17
ONPG[b]	12	0	0	11	0
Oxidase	100	100	100	89	83
Resistance to:					
0/129 (50 μg/ml)	100	100	91	100	89
0/129 (150 μg/ml)	12	96	18	85	44
Brilliant green (0.002%)	6	0	27	100	33
Crystal violet (0.002%)	0	0	27	100	28
Methylene blue (0.01%)	100	100	100	100	44
Methylene violet (0.0002%)	6	8	27	100	50
Neutral red (0.002%)	100	100	100	100	89
Sensitivity to:					
Ampicillin (10 μg)	0	0	0	11	0
Kanamycin (1 mg)	6	0	45	33	11
Nalidixic acid (30 μg)	0	0	0	11	0
Novobiocin (30 μg)	0	0	0	56	0
Penicillin (10 IU)	0	0	0	11	0
Polymyxin B (300 IU)	41	0	0	44	28
Streptomycin (10 μg)	0	0	0	78	6
Growth on TCBS	0	0	0	0	2
Yellow colonies on TCBS	0	0	0	0	11
Degradation of:					
Collagen	6	0	0	11	0
Tween 20	6	16	17	0	6
Tween 40	100	100	100	89	61
Tween 60	100	100	100	89	50
Tween 80	100	100	100	89	61
Tyrosine	100	100	100	89	56
Xanthine	14	10	0	0	39
Production of:					
Alginase	0	0	0	0	6
Amylase	6	0	100	0	44
Chitinase	88	58	89	0	22
Deoxyribonuclease	94	100	91	100	56
Gelatinase	100	100	100	100	44
Phosphatase	100	100	100	100	50
Sulfatase	0	0	0	11	0
Urease	0	0	0	0	3
No. of strains	17	25	12	9	17
% of total strains	21.5	31	15	11	21.5
% Similarity	79.3	79.4	77.9	67.0	

Continued

[a] All symbiotic isolates which did not form discrete clusters with the other four phena.
[b] OPNG, o-Nitrophenyl-β-D-galactopyranoside.

sponge were negative for seven characteristics: acid production from inositol, Voges-Proskauer test, swarming, luminescence, vibrioid morphology observed by TEM, growth on butyrate as the sole carbon source, and sensitivity to 30 μg of tetracycline per ml. All strains were positive for three characteristics: growth at 25°C and pH 7 and resistance to 10 μg of the vibriostatic agent 0/129 per ml.

Seventeen tests were eliminated because they gave poor reproducibility when tested among the eight blind replicate strains. Individual tests which had variance estimates (s_i^2) greater than 0.10 (31) were eliminated from subsequent analyses by using the criteria recommended by Bryant and co-workers (6, 7). These tests measured growth at pHs 4 and 10, at 4 and 42°C or in 10% NaCl; acid production from arbutin and mannose; degradation of elastin and lecithin; the presence of arginine dihydrolase by the Thornley method; reduction of nitrate; and the ability to utilize serine, fructose, trehalose, malate, valerate, and glucuronate as sole carbon sources.

Experimental error was estimated for phenotypic analyses obtained for duplicate strains. High variance values were indicative of poor precision, and those tests were eliminated from the analysis. A total of 101 tests included in the analysis did not vary among all of the replicate strains. The pooled variance for all 148 tests in the study was 0.0302 (10 tests were eliminated which were the same for all strains). When tests which were poorly reproducible were omitted from the data matrix, the pooled variance estimate was 0.0161 (17 tests were omitted). The average probability of an erroneous test result (*P*) in this study was 1.7%, an acceptable level according to the criteria of Sneath and Johnson (31). In total, 27 characteristics showed poor or no differentiating value in the analysis and were eliminated from the data matrix.

The data analyzed to discern phenotypic relationships included all characteristics listed in Table 5 (data matrix 101 × 67). The sole carbon source data composed over half the tests in the study. Several studies have shown that sole carbon source data are often not reproducible and heavily influence clustering patterns (2, 6, 7, 21, 25). The ability to utilize a compound as a sole energy source can be lost in

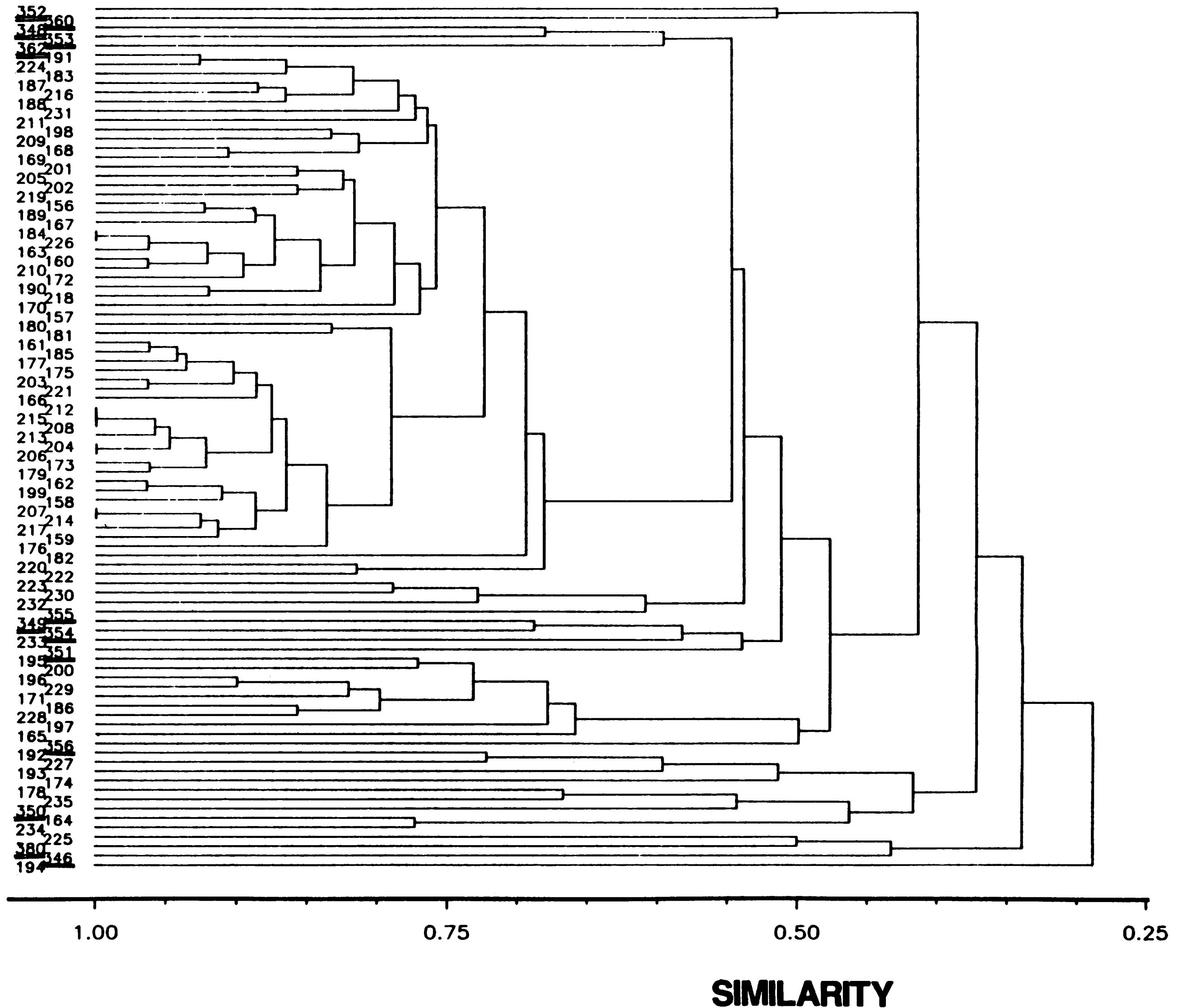

FIG. 2. Similarity dendrogram prepared from all taxonomic data coded for sponge isolates and reference strains. Clusters were obtained employing the UWAL and S_J similarity coefficient. The reference strains denoted by underlined identification numbers correspond to the strains indicated in Table 1.

cultures which are repeatedly subcultured and maintained in the laboratory (7). Since the reliability of sole carbon source data is controversial and the number of such tests could skew the analysis, the sole carbon source data were not included in the final matrix for cluster analysis but are reported for each of the clusters.

Description of major phena. Phena were defined as clusters of strains which, on average, shared ≥76% similarity values among all strains in that cluster, as determined by S_J and unweighted average linkage. Four discrete phena were identified among the 80 sponge-associated bacterial strains. The phena did not cluster significantly with any of the reference strains included in the study (Fig. 2). Twenty-five percent of the isolates did not cluster into phena but agglomerated into single- or double-member groups. Some of unclustered strains joined with reference strains at a higher similarity than with strains clustered into phena. The characteristics of the four phena and unclustered strains, the latter considered as one group for the purpose of this analysis and referred to as others, are given in Table 5.

Twenty-four traits were observed to occur in all HMOs. These traits included a rod shape; growth in 6% NaCl; fermentation of fucose and sucrose; oxidase; resistance to nalidixic acid, penicillin, methylene blue, and neutral red; no growth on TCBS agar; and degradation of Tween 40, Tween 60, Tween 80, tyrosine, DNA, gelatin, and phosphate. Traits which were absent from all HMOs were a presence of collagenase, alginase, sulfatase, and urease and utilization of Tween 20 and xanthine. Phena 1, 2, and 3 were most closely related to each other, but phenon 4 was very different from each of these.

Two major classes described the overall clustering patterns of the phena (Fig. 3). The first class contained phena 1 through 3, which were most similar to one another. The second class contained phenon 4 and was distantly removed from the first class. The average similarities among all strains

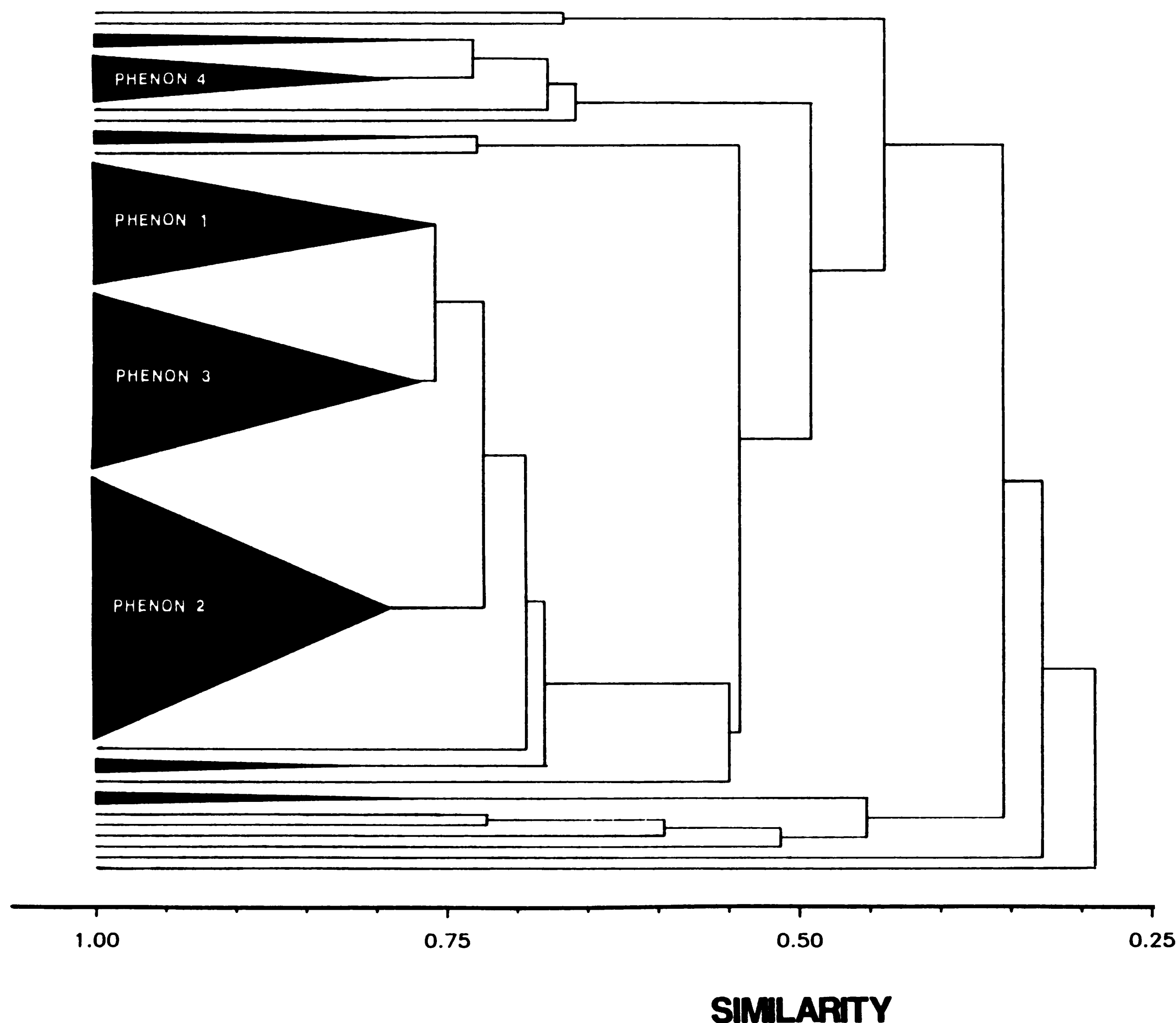

FIG. 3. Dendrogram showing clustering patterns for four phena of sponge isolates. Phena are described as groups of strains possessing average S_J values of 76% or greater.

within phena 1, 2, and 3 were 79.3, 79.4, and 77.9%, respectively. Traits differentiating each phenon are listed in Table 6. All strains contained within the first major class were gram-negative rods with a polar flagellum and oxidase positive and demonstrated an obligate requirement for 3% NaCl for growth. All strains grew well in 6% NaCl, and most grew in 8% NaCl. The typical strain from this class was able to ferment sucrose and fucose but was unusual in lacking the ability to ferment glucose. Decarboxylation of lysine and ornithine was positive for these strains, but they could not hydrolyze arginine. The strains were resistant to 50 μg of 0/129 per ml, methylene blue, neutral red, ampicillin, nalidixic acid, novobiocin, penicillin, and streptomycin. Most strains were able to degrade Tween 40, Tween 60, and Tween 80, tyrosine, DNA, gelatin, and phosphate compounds. The second major class, including phenon 4, was composed primarily of polymorphic gram-positive or gram-variable bacteria.

Phenon 1 was distinguished from the other phena by a unique combination of attributes (Fig. 3 and Table 6). These isolates were able to ferment mannitol and trehalose, and over half of the strains could ferment arabinose and galactose. The median strain produced catalase but was sensitive to 150 μg of the vibriostatic agent 0/129 per ml and produced a chitinase but not an amylase. Of the strains in this group, 76% clustered at an average similarity value of 82%.

Phenon 2 clustered most tightly compared with the other groups, with 92% of its members displaying an average similarity of 85% with other strains in the group. This group was distinguished by the ability to ferment arabinose and cellobiose, with about half of the strains able to ferment galactose. Most strains were catalase positive and resistant to all concentrations of 0/129 used in this study. They did not possess amylase, but over half of the strains were chitin degraders. This was the largest single phenon and comprised approximately 30% of the total symbiont isolates.

Phenon 3 closely resembled phenon 1, with several differences observed (Table 6). The strains were characterized by the ability to ferment trehalose, with most strains able to ferment arabinose and galactose and degrade chitin, but only

TABLE 6. Distinguishing characteristics of sponge-associated bacterial phena 1 to 4

Characteristic	Results[a] for no.:			
	1	2	3	4
Gram reaction				
Negative	+	+	+	−
Positive	−	−	−	d
Variable	−	−	−	d
Filamentous shape	d	d	d	+
Polymorphism	−	−	−	+
Binary Division	+	+	+	d
Growth in 8% NaCl	+	+	+	d
Acid production:				
L-Arabinose	d	+	d	d
Cellobiose	−	+	−	−
Fructose	d	d	d	−
D-Galactose	d	d	d	+
D-Glucose	−	−	−	+
D-Mannitol	+	−	d	−
Trehalose	+	−	+	+
Decarboxylation of:				
Lysine	+	+	+	−
Ornithine	+	+	+	−
Resistance to:				
0/129 (50 μg/ml)	+	+	+	+
0/129 (150 μg/ml)	−	+	−	+
Kanamycin (1 mg)	−	−	d	d
Novobiocin (30 μg)	−	−	−	d
Polymyxin B (300 IU)	d	−	−	d
Streptomycin (10 μg)	−	−	−	d
Brilliant green (0.002%)	−	−	d	+
Crystal violet (0.002%)	−	−	d	+
Methyl violet (0.0002%)	−	−	d	+
Production of:				
Amylase	−	−	+	−
Chitinase	+	d	+	−
Catalase	+	+	−	+
Indole	−	-	−	+
Sole C source				
D-Arabitol	−	−	d	+
Ethanol	d	+	d	d
D-Glycerol	d	+	d	−
D-Mannitol	+	+	d	−
1-Propanol	d	−	d	−
D-Alanine	−	−	d	+
L-Alanine	d	d	d	−
L-Arginine	+	+	d	d
L-Asparagine	+	+	d	−
L-Aspartate	d	d	d	−
Gamma aminobutyrate	d	+	d	d
Citrulline	d	d	−	−
L-Glutamate	d	+	d	d
Glycine	d	d	−	−
L-Hydroxyproline	d	+	d	−
L-Tyrosine	+	+	+	−
δ-Aminovalerate	+	+	d	d
D-Amygdalin	−	−	d	+
L-Arabinose	−	d	−	−
Arbutin	+	+	+	−
Collagen	+	+	d	d
D-Galactose	+	+	d	d
D-Gluconate	−	−	−	+

Continued

TABLE 6—*Continued*

Characteristic	Results[a] for no.:			
	1	2	3	4
D-Glucose	+	+	d	−
D-Glucosamine	d	+	+	−
Glycogen	+	+	+	−
Lactose	d	+	d	−
Maltose	+	+	d	d
D-Mannose	d	d	+	−
Melibiose	d	d	d	−
D-Ribose	+	+	d	d
Salicin	+	+	d	d
Sucrose	d	+	d	−
Acetate	+	+	d	−
Phenylacetate	+	+	+	d
p-Hydroxybenzoate	+	+	d	d
DL-3-Hydroxybutyrate	+	+	d	−
Citrate	+	+	d	−
Fumarate	d	d	d	−
Glutamine	+	+	d	−
Glutarate	+	+	d	d
DL-Lactate	+	+	d	d
Malonate	+	+	d	d
Propionate	+	+	+	−
Succinate	+	+	d	d
Adenine	+	+	d	−
Ethanolamine	+	+	d	d
D-Galacturonate	d	d	−	−
m-Inositol	d	d	d	−
Putrescine	+	+	+	−
Sarcosine	−	−	−	d
Urea	d	+	d	−
Xanthine	+	+	+	−

[a] +, At least 80% of the isolates positive for the characteristic; −, at least 80% of the isolates negative for the characteristic; d, character present in 21 to 79% of the isolates.

36% were able to ferment mannitol. The HMO did not possess catalase and was sensitive to 0/129 (150 μg/ml). Slightly less than half of the strains displayed sensitivity to kanamycin. This phenon was unique in that the strains demonstrated amylase activity.

Phenon 4 comprised organisms very different in phenotype compared with the other three phena (Table 6), including gram-positive or gram-variable organisms displaying extreme polymorphism, ranging from rod shapes (often forming club-, Y-, or T-shaped cells) to long filaments. These cells appeared to replicate by fragmentation of existing cells and not by binary division, as was characteristic of cells for isolates from the other phena described in this study. Most strains within this phenon were not able to grow in 8% NaCl. All fermented glucose, sucrose, fucose, and galactose. This was the only phenon of which any strain was able to ferment glucose. Most strains fermented arabinose and trehalose and produced indole and catalase but did not decarboxylate lysine or ornithine. The HMO was characterized by resistance to all concentrations of the vibriostatic agent 0/129 tested in this study, kanamycin, and polymyxin but was sensitive to novobiocin, streptomycin, brilliant green, crystal violet, and methyl violet. The presence of amylase or chitinase was not detected.

Phenon 4 was the most diverse and loosely formed cluster compared with the other phena. If the average similarity value of 76% or greater among all strains within the cluster were employed to define the cluster, only four strains would

be included (Fig. 3). The fact that these isolates were very different from the other three phena resulted in a larger number of shared negative traits. In fact, strains included in phenon 4 joined at average S_J of 67%, thereby including a total of nine strains.

Twenty-three percent of the isolates (classified as others in Table 5) did not form closely linked, discrete clusters. Three strains clustered at 60.8% similarity with *Vibrio alginolyticus* (Fig. 2), the highest value at which an isolate from the sponge linked with a reference strain. One strain clustered at 58.2% with *Aeromonas caviae*. Two strains clustered with *Pseudomonas fluorescens* at 54.3% similarity. *Micrococcus luteus* was linked at 50% to a strain which did not require NaCl for growth. The majority of the isolates were gram-negative rods that were oxidase positive, possessed a polar flagellum, and required 3% NaCl for growth (Table 6). Most of these strains were able to ferment at least four of the carbohydrates tested, but only a small fraction was able to ferment glucose. The majority of strains decarboxylated ornithine and were resistant to 50 μg of 0/129 per ml.

Utilization of sole carbon sources. The symbionts were able to utilize a broad spectrum of organic compounds as sole carbon and energy sources, including carbohydrates, organic acids, and amino acids. Dulcitol and *N*-acetyl-D-glucosamine were utilized by most of the isolates of the four phena. The following substrates were not utilized by most of isolates: sorbitol, L-histidine, L-proline, L-threonine, cellobiose, butyrate, 2-ketoglutarate, D-glucuronate, and taurine. Strains of phenon 4 were less frequently able to utilize substrates as sole carbon and energy sources, whereas strains of phenon 2 were the most metabolically versatile. In some cases, most of the isolates comprising phena 1, 2, and 3 were able to utilize a specific carbon source, while strains of phenon 4 were not, as in the cases of L-tyrosine, arbutin, D-galactose, glycogen, propionate, putrescine, and xanthine. The converse was true with D-arabitol, D-alanine, D-gluconate, and D-amygdalin.

DISCUSSION

This study represents the first comprehensive analysis of heterotrophic bacteria associated with a single sponge species found in the Caribbean basin, a region reputed to contain the most prolific sponge populations, with respect to biomass and diversity (43). Similar or related studies have been done in the Pacific Ocean and Mediterranean Sea (42, 48). The significant abundance of bacteria found to occur within the sponge alludes to their overall importance in this consortium. In a sponge, where the average proportion of the bacterial biomass exceeds the proportion of sponge cells, the potential implications of this association are obvious. In this study, a foundation for future work to explore the molecular genetic interactions between bacterial symbionts and the host sponge was laid, in that culturable bacteria were isolated and characterized. Future work will include the preparation of gene probes for determination of the location of these cells in the sponge and, ultimately, the nature of the association.

The percentage of culturable bacteria associated with the sclerosponge was observed to be significant and correlated with similar data reported elsewhere (49). Bacteria associated with the sponge were not detected among the culturable bacteria isolated from the ambient water column surrounding the sponge, according to the results of phenotypic and serological analyses (Santavy, Ph.D. dissertation). In earlier studies of marine bacteria isolated from seawater, it was estimated that less than 1% of the total bacteria are culturable. Historically, knowledge of marine microorganisms is based on data gathered for the relatively few species and strains which could be cultured. The recovery and culture of symbiotic bacteria are a first step in gaining an understanding of the complex microbial community comprising symbiosis among the marine invertebrates. Molecular and immunological approaches will be applied to analyze the fundamental basis of symbiosis but will not negate the need to understand the metabolism and physiology of both the symbiont and the host.

The estimation of the percentage of culturable symbionts was achieved by an indirect method, by extrapolating the number of particles in a given volume from two-dimensional measurements to three-dimensional space. Obviously, there is some inherent error, but at the present time, morphometry offers a good approach for obtaining volumetric estimations with TEM (12). The composite shape coefficient (β) corrects for multiple bacterial morphologies within the sponge but assumes an overall ellipsoidal shape (1). Bacterial shapes observed within the sponge were, in general, ellipsoidal, but multiple populations of several cell shapes were observed.

Volumetric calculations employed an average sponge tissue thickness (49), whereas in reality, the thickness and region containing the bacteria may be highly variable. The pinacoderm and aquiferous channels are not distributed uniformly throughout the sponge tissue, and the calcareous skeleton varies in the degree of penetration into the tissue layer (49). Direct counts by epifluorescent microscopy were made, but the results were variable because eucaryotic cellular material from the homogenized sponge could not be discriminated from bacterial cells. Therefore, fluorochome dyes or direct count methods could not be used effectively to enumerate bacteria within the sponge. The ratio of the number of bacteria cultured from the sponge to the total number of bacteria within the sponge was very similar to the ratio reported in studies comparing the enumeration of bacteria by plate count and direct count methods (14).

Approximately 78% of the culturable bacteria associated with the sponge demonstrated phenotypic characteristics most closely related to species in the families *Vibrionaceae* and *Aeromonadaceae* (9), with most of the strains examined in this study demonstrating a requirement for 3% NaCl for growth. All strains of phena 1, 2, and 3 possessed features diagnostic for these families. All members were gram negative and oxidase positive and possessed fermentative metabolism without the ability to ferment glucose (15, 26, 39). A distinctive but unusual attribute was the ability to ferment sucrose but not glucose. These organisms were classified as marine, sucrose-fermenting species of the genera in the families *Vibrionaceae* and *Aeromonadaceae*. The phena did not closely resemble any species previously described in the literature and are concluded to represent new species.

Phena 1 and 3 most closely resembled the genus *Vibrio*, a conclusion supported by the observation that the strains were sensitive to the vibriostatic agent 0/129. None of the isolates were able to grow on TCBS, hydrolyze arginine, or ferment lactose, characteristics diagnostic for many species of the genus *Vibrio* (40). *V. alginolyticus* shared the most attributes with these groups, but the isolates displayed little similarity to the type strains included in the analysis. The strains shared most attributes with unidentified marine vibrios included in the extensive numerical taxonomy study by West and co-workers (39), again corroborating their uniqueness and representing new species.

Phenon 2 most closely resembled species of the genus

Aeromonas but was not significantly similar to the type strains of this genus included in the analysis. Phenon 2 was similar to phena 1 and 3, with the important distinction of being resistant to 0/129. Some marine vibrios may be resistant to 0/129, but serological data supported a significant relationship of this phenon with the genus *Aeromonas* (Santavy, Ph.D. dissertation). They did not ferment glucose and trehalose or hydrolyze arginine, characteristics diagnostic for most aeromonads (15, 22, 26, 39). Only half of the members were chitinolytic. Fermentative abilities differed in the strains composing phena 1 and 2 in that strains in phenon 2 were able to reduce cellobiose but not trehalose.

Phenon 4 represented the most diverse cluster of strains, consisting of both gram-positive and gram-variable isolates. The expression of extreme polymorphism in this group ranged from filaments to the unusual shapes of club-, Y-, and T-shaped cells, and most of the isolates possessed a polar flagellum. These asporogenous bacteria were facultative anaerobes, able to metabolize a wide range of substrates. Unlike other clusters, most members of this phenon were sensitive to streptomycin. The bacterial strains clustering within phenon 4 were most similar in cell morphology and biochemical properties to the coryneform or actinomycete microorganisms. However, the colony morphology of the isolates did not include formation of mycelium, true hyphae, or aerial spores, and the cells were not acid fast. Generic relationships could not be determined from descriptions given in *Bergey's Manual of Determinative Bacteriology* (15, 32). The nonpathogenic coryneform bacteria in general are relatively poorly described, with most diagnostic features being based on cell morphology. Distinction between these two groups is difficult at best, with diagnostic characteristics at the genus level based predominantly upon the carbohydrate composition of the cell wall. Cell wall carbohydrate composition was not determined in this study.

Cluster relationships in numerical taxonomy are products of the data analyzed and are influenced by the choice of strains, characteristic selection, and the statistical methods used. Often compounds tested for their ability to be utilized as sole carbon sources are anabolized via the same metabolic pathways (25), thus being highly correlated with one another (4). The results of several studies show that carbon utilization test results can be inaccurate, produce highly biased clustering patterns (6, 7), and give results which conflict with those obtained by other techniques to evaluate taxonomic relationships (21), especially if they compose up to 50% of the attributes being analyzed. The sole carbon source utilization data in this study purposely were not incorporated into the clustering algorithm in order to eliminate these ambiguities. Nevertheless, carbon utilization data can be informative, especially when metabolic potential is of interest, if the limitations of the data are considered when the analyses are done.

The phenotypic characteristics of bacterial symbionts isolated from *C. nicholsoni* differed from those previously reported in studies of bacterial sponge symbionts. The dissimilarities can be explained as being related to the different geographical locations, phylogenetic hosts, and dissimilar ecological and environmental parameters examined in other investigations. Bacteria associated with sponges from the Great Barrier Reef and Mediterranean Sea were examined in previous studies (42, 48). Sponges of the Caribbean are classified as heterotrophic (43) and are believed to rely greatly on their heterotrophic bacterial populations for obtaining nutrients (24) rather than on the phototrophic mode which characterizes sponges from the Great Barrier Reef (44).

Phenotypically similar bacteria were isolated from sponges collected from the Mediterranean Sea and the Great Barrier Reef in earlier numerical taxonomy studies (41, 48). One bacterial cluster described in the earlier studies resembles bacteria isolated from *C. nicholsoni* in this study. Common attributes include a requirement for marine cations, being facultatively anaerobic and gram negative, cell flagellation in culture, and the ability to metabolize a wide range of carbohydrates and amino acids. The largest cluster of strains, most closely resembling the family *Enterobacteriaceae*, was found in 9 of the 10 sponges surveyed from the Mediterranean Sea and the Great Barrier Reef (48). Other clusters of bacteria observed at a lower frequency of occurrence in those sponges were also isolated from the water column samples and included gram-positive coccoid forms not requiring seawater for growth and pseudomonad-like bacteria (48). Bacteria resembling these two major groups were not isolated from *C. nicholsoni*.

Valid comparisons between this study and the phenotypic analyses of past studies (42, 48) can be made after considering the different methods employed in the numerical taxonomic analyses. Over 50% of the traits included in earlier studies were based on colony and cell morphology and the compounds utilized as sole carbon energy sources, which are now recognized as being of limited value for determining taxonomic status. In general, cell and colony morphologies are highly variable and depend upon many environmental factors. Thus, potentially ambiguous results may be obtained from tests assaying compounds utilized as sole carbon energy sources, as discussed earlier (see above). Fermentative metabolism is usually assayed employing only glucose (42, 48). Since most of the bacteria isolated from *C. nicholsoni* could not ferment glucose but did ferment sucrose, the fermentative abilities, an important taxonomic criteria, may be overlooked if only glucose is used to test for the presence of this trait. Other metabolic activities which were assayed in this survey were not assayed in the earlier surveys. In addition, preselection for certain groups of bacteria may have occurred in earlier studies, since strains were isolated by the pour plate method, which eliminates bacteria unable to tolerate temperatures of ≥45°C. In this study, the less-selective spread plate method, which does not expose the bacteria to temperatures above their maximum viable growth temperature, was used, since many of the bacteria in this study were incapable of growth at 42°C or above.

Other studies provide evidence that sponges and their symbiotic bacteria may cooperate in a nutritive exchange of substrates that one may make available to the other (24); such exchanges may involve dissolved amino acid uptake (46) and bacterial collagen degradation (45). Results of radiolabeling studies indicate that sponges can differentiate between ambient seawater bacteria consumed as food and symbiotic bacteria (47). Most sponges are characterized by the production of slimes and mucins, which are found to be rich in polysaccharides. The results of analyses of polysaccharides from different sponges show significant variations in carbohydrate composition, often correlated with species specificity, although most of the sponge species which have been examined contained large amounts of galactose and fucose (19), both of which were utilized by most of the bacteria associated with *C. nicholsoni*. Investigations of the partial carbon and energy budgets of bacteriosponges from the Caribbean apparently show a gross deficit compared with nonbacteriosponges, permitting the prediction that the dis-

solved organic matter produced by the bacteria must be a primary nutrient source (24). It is hypothesized that bacteria play an important role in the acquisition and remineralization of dissolved organic matter. If so, then heterotrophic bacterial symbionts may be very important for the physiology of Caribbean sponges, given that the overall biomass of Caribbean oceanic reef sponges is eight times greater than that of similar Pacific oceanic reef sponges; furthermore, the average size of a sponge is also greater on Caribbean reefs (43).

It is useful to speculate which substrates a sponge may contribute to the catabolic processes of its associated bacterial symbionts. Products made available to bacteria by the sponge may be acquired by concentrating nutrients in the filtrate from ambient seawater or in sponge excretory or secretory products, such as sponge mucus. Most of the bacteria associated with *C. nicholsoni* were able to ferment fucose and sucrose, decarboxylate lysine and ornithine, and utilize chitin, DNA, gelatine, tyrosine, and a range of fatty acids (Tween 40 to Tween 80). Fermentative metabolism of the symbionts may provide a mechanism for coping with periods of greatly decreased pumping rates in the sponge, when anaerobiosis may exist (23), thereby maintaining the viability of the symbionts and generating nutrition for the sponge. In the sclerosponge, anaerobic zones may enhance calcification of its aragonite skeleton, providing an acidic environment and facilitating calcium carbonate precipitation.

In summary, the association between the *C. nicholsoni* and its associated bacterial flora is a very complex relationship in which different symbiont species may be important in generating and utilizing selected nutrients. Examination of the phenotypic traits of the culturable bacteria allows a better understanding of the role of the associated bacteria and the sponge host. The results of this study did not elucidate the roles of obligate anaerobic bacteria which may reside in the sponge, of rare species of bacteria, or of those bacteria poorly able or unable to be cultured. Preliminary evidence suggests a role of the bacteria in antifouling or prevention of overgrowth by encroaching organisms on the surface of the sponge. Antimicrobial activity displayed against marine bacteria and antineoplastic activity was observed among the four phena of bacteria isolated from the sponge (R. R. Colwell, D. L. Santavy, F. Singleton, T. Breschel, and T. Davidson, *Proceedings of the First International Marine Biotechnology Conference, September 1989*, in press). Future studies will employ molecular and immunological techniques to explore other aspects of this relationship, in order to verify the conclusions reported here as well as reveal any molecular genetic relationships between bacterial symbionts and their sponge host.

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