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Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater

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ABSTRACT: The Caribbean sclerosponge *Ceratoporella nicholsoni* contains a morphologically diverse bacterial community. The bacteria are located in the sponge mesohyl, existing intercellularly. Bacterial isolates cultured from the sponge and ambient seawater were compared, to establish the specificity of the bacteria for their sponge habitat. Isolates from both habitats were analyzed by numerical taxonomy, employing 85 phenotypic traits for each isolate, permitting comparison of physiological and ecological attributes. Bacteria isolated from the sponge were found to be significantly different from bacteria present in seawater collected in the immediate vicinity of the sponge. The phenotypes of seawater bacteria were more diverse than the phenotypes of the sponge bacteria. Physiological profiles of the isolates generally reflected the nutrient profile of the habitat and were highly correlated with metabolic capabilities of bacteria isolated from each environment. Sponge-associated bacteria demonstrated greater metabolic capabilities, and were able to catabolize a large number of substrates, both oxidatively and fermentatively. In comparison, seawater isolates were potentially more restricted in their dissimilatory abilities, with respect to the substrates examined in this study, being capable only of oxidative metabolism.

INTRODUCTION

Survival and effective competition in nature often require specialized strategies, which may impart unique physiological, chemical, and ecological capabilities to the organism. Such strategies may include forming close associations with other species, as in symbiosis, or partitioning of resources by spatial separation. Unique properties resulting from symbiosis are observed among the chemosynthetic bacteria symbiotic in hydrothermal vent invertebrates, one of the most prominent examples (Childress et al. 1987). Marine bacteria have also demonstrated partitioning of resources by spatial separation, most frequently as stratification in the water column, reflecting nutrient profiles (Bianchi & Bianchi 1982, Simidu & Tsukamoto 1985, Muir 1986). Comparison of microorganisms from adjacent microenvironments can elucidate important properties and verify the specificity of associated bacteria in symbiosis. This is particularly important for bacteria found in marine sponges, because sponges filter planktonic bacteria from ambient seawater for food. Determining which bacteria are filtered from seawater and which bacteria are always associated with the sponge as symbionts is an important but difficult task.

The earliest published studies concluded that bacteria associated with sponges are the same bacteria as found in ambient seawater (Bertrand & Vacelet 1971, Madri et al. 1971). Bacteria isolated from the temperate, red beard sponge *Microcionia prolifera* were reported to be characteristic of the allochthonous and autochthonous bacterial flora of Long Island Sound (USA) (Madri et al. 1971). In studies examining the Mediterranean sponges *Verongia aerophoba* and *V. cavernicola*, bacteria of the genera *Pseudomonas* and *Alteromonas* were reported to have been isolated from the sponges, as well as from the surrounding seawater

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(Bertrand & Vacelet 1971). A decade later, Wilkinson et al. (1981) cultured a unique yellow-pigmented bacterium from *V. aerophoba* and concluded the bacterium was found only in the sponge, not in the surrounding seawater. In recent studies, a number of novel bacterial symbionts have been described from sponges (Wilkinson 1978a, b, Wilkinson et al. 1981, Santavy 1985, Santavy et al. 1990). Evidence has been provided which shows sponge-associated bacteria are true symbionts and are different from bacteria isolated from ambient seawater of the sponges studied (Wilkinson 1978a, 1984, Wilkinson et al. 1981).

Results of feeding studies employing radiolabeled substrates indicate that sponges differentiate between symbiotic bacteria and ambient seawater bacteria, the latter being consumed as food (Wilkinson et al. 1984). Sponges are prolific filter feeders and effectively remove 60 to 99% of the seawater bacteria which circulate through their aquiferous system, concentrating large quantities of seawater bacteria in their choanocyte chambers (Reiswig 1974, 1981), where the bacteria are presumably consumed by sponge archaeocytes. Most symbiotic bacteria are located in the intercellular matrix or mesohyl region of the sponge (Wilkinson 1978a), occupying up to 60% of the sponge volume and, at times, exceeding the total volume of sponge cells (Wilkinson 1978b, Santavy 1985, Santavy et al. 1990). Evidence from the studies reported here, employing numerical taxonomy analyses and immunological techniques (Santavy 1988), supports the idea put forward by Wilkinson (1978a) and Wilkinson et al. (1981) that only a small percentage of the bacterial flora isolated from sponges are related to bacteria isolated from seawater

A community of bacteria with diverse morphologies is associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* (Santavy et al. 1990). In an ultrastructure study of this sclerosponge, large numbers of symbiotic bacteria were found to comprise 57% of the intercellular mesohyl regions of the sponge (Willenz & Hartman 1985, 1989). The bacteria are rarely found associated with the dermal membrane or attached to the lining of the aquiferous system. Their size and morphology are highly variable. Most of the bacteria are rod or coccoid-shaped, possessing a typical Gram-negative wall (Forsberg et al. 1970), with an additional diffuse, loosely bound layer encapsulating most of the cells.

Sclerosponges differ from common reef sponges in that they accrete a solid aragonite basal skeleton and the sponge tissue forms a thin veneer (0.1 to 0.3 mm) over the surface of the massive calcareous skeleton. A single individual may obtain a size of over 40 cm in diameter and living specimens have been aged to more than 400 yr (Willenz & Hartman 1985). Today, sclerosponges occupy submarine caves on reefs and areas deeper than 25 m, where low light intensity and sedimentation prevail.

In this study, bacteria isolated from the sclerosponge *Ceratoporella nicholsoni*, and from ambient seawater in immediate proximity to the sponge, were characterized, using physiological, morphological, and ecological characters, to discern similarities amongst the isolates. The strains selected for study were culturable, aerobic bacteria. It was hypothesized that the phenotypic profiles of the bacteria isolated from *C. nicholsoni* should be different from those bacteria isolated from the surrounding seawater in immediate proximity to the sponge. Association of specific traits with habitat was analyzed to discern ecological and physiological processes that might prove important in the symbiosis.

MATERIAL AND METHODS

Ceratoporella nicholsoni was collected in a submarine cave on the fore reef-slope at a depth of 30 m in Jamaica Bay at Acklins Island, Bahamas (74°17.1' W, 22°10.3' N), during August 1985. The collection was made while using SCUBA and aseptic procedures were employed, as far as possible underwater. Extraction of bacteria from the sponge tissue was performed using sterile techniques and the sponge dissociation method described by Santavy et al. (1990).

Two media were used to select for specific trophic modes. A modified marine agar (MMA) (1.8% Difco Marine M2216 Medium, 2% NaCl, 0.01% sodium glycerophosphate, pH = 7.4, 1.3% agar) was employed to isolate copiotrophic bacteria. A seawater-based medium [0.005% Bacto yeast extract (Difco), 0.05% tryptone (Oxoid), 0.01% sodium glycerophosphate, 1.2% Noble Agar, seawater from collection site], containing low nutrient concentrations, was designed to promote growth of oligotrophic bacteria. All plates were incubated at 25°C. Colonies were selected using a random grid pattern placed over each Petri dish, after incubation for 48 h, 72 h, 1 wk and 2 wk. Colonies were serially streaked until pure cultures were obtained. Plates were retained for 2 mo to allow for selection of slowly growing bacteria. Isolates were maintained on agar slants overlaid with mineral oil and cryopreserved in liquid nitrogen (26% glycerol: logarithmic phase liquid culture; 1:1).

Eight seawater samples were collected in the vicinity of the sponge sample using a sterile 2 l Niskin butterfly water sampler (General Oceanics, Miami), manually triggered by a SCUBA diver. The following quantities of seawater were filtered through a 0.22 µm membrane filter, in duplicate: 10, 25, 100, and 250 ml, for each

sample. The filters were incubated using the same media employed for the recovery of sponge-associated bacteria. Plates were incubated and colonies selected as described above. The isolates selected for the numerical taxonomy study maximized diversity, since significant diversity in colony morphology was observed.

Eighty strains isolated from *Ceratoporella nicholsoni* and 48 strains obtained from the surrounding seawater were included in a numerical taxonomy analysis, which was performed to determine whether the 2 groups of bacteria were phenotypically similar. Characters selected included physiological and ecological traits expected to yield useful information about the role of the bacteria within their respective habitats. Each strain was examined for 85 phenetic characters assessing cell morphology, physiological tolerances, and biochemical capabilities (Table 1). All strains were maintained on MMA, subcultured fortnightly with incubation at 25°C, and periodically examined for purity during the study.

General morphological features of the bacteria were observed using light and transmission electron microscopy (TEM). Negatively stained specimens viewed by TEM were used to determine cell shape, presence and type of flagella, mode of cell division, and unusual morphological features. The TEM fixation procedure has been described elsewhere (Santavy et al. 1990). Gram-reaction was determined employing Hucker's method (Doetsch 1981), and was confirmed using the KOH method (Buck 1982).

Physiological tolerances of the isolates to various concentrations of NaCl were evaluated, including 0, 3, 6, 8, and 10% in a basal medium containing 1% tryptone (Oxoid) and 0.01% sodium glycerophosphate. Oxidative versus fermentative pathways of carbohydrate metabolism were determined using a modified marine medium (Lemos et al. 1985). Oxidative respiration was determined using cultures grown aerobically, while fermentative respiration was determined by overlaying the medium with mineral oil. Acid production from the following carbohydrates was determined: arabinose, fructose, fucose, galactose, glucose, mannitol, sorbitol, and sucrose, employing 1% (v/w) carbohydrate concentration. All cultures were monitored daily and final results were recorded after 14 d.

Biochemical traits of the strains were determined employing conventional media modified by the addition of marine salts. The following tests were performed: oxidase activity, reduction of nitrate, decarboxylation of lysine and ornithine, and arginine hydrolase activity (West & Colwell 1984). The following attributes were examined: gluconate oxidation (7 d incubation), indole production, and hydrogen sulfide production (Cowan 1974). Smibert & Krieg's (1981) recommended protocols were utilized for the Voges-Proskauer reaction (VP) and detection of catalase (method 2). The presence of β-galactosidase was determined by detection of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), utilizing the fluorogenic substrate 4-methyl umbelliferyl-β-D-galactoside (Maddocks & Greenan 1975). Pigment production and luminescence were recorded for colonies growing on MMA.

Several selective and differential media were used to assign strains to taxonomic groups. Resistance to the antimicrobial agent Irgasan was tested by plating on *Pseudomonas* Isolation Agar (Difco), fortified with marine supplements (Leifson 1970). Growth was recorded after incubation for 14 d at 25°C. 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^{-1}, on MMA. The ability to grow on a medium containing thiosulfate, citrate, bile salts, and sucrose agar (TCBS, Oxoid), which is selective for vibrios, was also determined. Marine salts were supplemented to the TCBS medium. Growth was monitored for 7 d and plates examined for the presence of green or yellow colonies (the latter indicative of sucrose fermentation).

Enzymatic and degradative properties of the strains were assessed. MMA was used as the basal medium to test for the presence of: amylase, chitinase, collagenase, deoxyribonuclease, elastase, gelatinase, lecithinase, phosphatase, sulphatase, and urease. Swarming on MMA was also recorded. The strains were screened for ability to utilize 20 different compounds as sole sources of organic carbon: adenine, D-alanine, D-amygdalin, arabitol, cellobiose, citrulline, dulcitol, L-fructose, histidine, *m*-inositol, 2-ketoglutarate, L-leucine, maltose, melibiose, D-mannose, serine, sorbitol, succinate, trehalose, and xanthine (Table 2). The conditions under which these tests were conducted are described elsewhere (Santavy et al. 1990).

Experimental error was estimated for duplicate strains. Tests which yielded variance estimates greater than 0.10 (s_i^2) (Sneath & Johnson 1972) were considered of low reproducibility and were eliminated from subsequent analyses, as defined by criteria recommended by Bryant et al. (1986). Test attributes which were highly correlated, or characteristics which were positive or negative for all isolates, provided no additional information and were eliminated from the analysis.

All characters were coded as binary data, and multistate characters, such as percentage of NaCl tolerance, were regarded as individual tests and recorded as individual 2-state responses. Numerical analysis was done by calculating similarity matrices employing the Jaccard similarity coefficient (S_J), and clustering was by unweighted average linkage (Sneath & Sokal 1973), using TAXAN (Version 2.0, Sea Grant College, Univ. of

Table 1 Phenotypic traits listed as frequency of occurrence for 5 ambient seawater phena, unclustered strains not included

	Seawater				Sponge	
	1	2	3	4	1–3	4
Gram reaction						
Negative	77	100	100	0	100	0
Positive	23	0	0	100	0	63
Flagella	90	100	50	0	93	67
Rod	100	100	80	0	100	100
Coccoid	14	0	40	100	0	0
Filamentous	0	0	0	0	30	89
Polymorphic	0	0	0	0	9	89
Binary division	86	66	70	0	100	44
Growth at:						
0% NaCl	0	0	0	28	0	11
6% NaCl	100	100	100	100	100	89
8% NaCl	44	66	20	0	100	22
Acid production (fermentatively):						
L-arabinose	0	33	0	0	80	67
Fructose	0	33	0	0	65	18
D-fucose	0	33	0	0	86	100
D-galactose	0	66	0	0	60	100
D-glucose	9	100	0	0	0	100
D-mannitol	4	66	0	0	41	11
Sucrose	0	33	0	0	93	100
Acid production (oxidatively):						
L-arabinose	73	66	0	100	86	72
Fructose	33	33	0	100	71	18
D-fucose	24	66	30	100	100	100
D-galactose	33	100	0	60	83	100
D-glucose	24	100	20	100	9	100
D-mannitol	5	66	10	20	65	11
Sorbitol	5	0	0	0	0	9
Sucrose	64	66	0	0	100	100
Decarboxylation of:						
Arginine	5	0	0	0	0	0
Lysine	0	33	0	0	94	22
Ornithine	0	33	0	0	99	13
Catalase	73	100	50	75	69	100
Gluconate oxidation	0	33	0	0	0	0
Indole production	9	66	0	0	2	89
H_2S production	9	0	0	0	0	0
Nitrate reduction	23	40	30	35	60	45
ONPG	23	33	0	0	4	11
Oxidase	86	100	50	50	100	89
Pigmentation	64	33	10	100	0	0
VP	0	0	20	0	0	0
Resistance to:						
0/129 (50 μg ml^{-1})	100	100	100	100	97	100
0/129 (150 μg ml^{-1})	100	0	100	100	42	85
Growth on:						
TCBS	0	100	0	100	0	0
Pseudomonas agar	82	100	50	0	0	0
Production of:						
Amylase	91	100	80	80	35	0
Chitinase	9	66	0	0	78	0
Deoxyribonuclease	68	33	50	40	95	100
Gelatinase	77	100	30	20	100	100
Phosphatase	100	100	100	60	100	100
Urease	5	0	30	0	0	0
Total no. of strains	25	3	9	5	54	9
Percent of total strains	20	2	7	4	42	7
Average percent similarity	56.7	55.5	50.6	58.5	57.9	64.8

Table 2. Comparisons of characteristics of all bacterial strains isolated from ambient seawater and sponge host. Values are listed as percentage of reactions that were positive. Phenetic traits correlated with isolation habitat by Fisher's Exact Test recorded as probability value [(–) indicates $p > 0.05$]

Character	Sponge host	Ambient seawater	Prob. value
Gram reaction			
Negative	89	70	0.017
Positive	6.5	27.5	0.017
Variable	4.5	2.5	0.001
Flagella	89	60	0.004
Rod	99	77	0.004
Coccoid	1	23	0.001
Filamentous	35	7	–
Polymorphism	20	2.5	0.002
Binary division	90	78	–
Growth at:			
0% NaCl	8	10	0.018
6% NaCl	98	85	0.001
8% NaCl	64	55	–
Acid production (fermentatively):			
L-arabinose	69	2	0.001
Fructose	63	2	0.014
D-fuctose	81	2	0.001
D-galactose	67	4	0.001
D-glucose	18	6	–
D-mannitol	39	2	0.001
Sorbitol	4	0	0.001
Sucrose	84	2	0.001
(oxidatively):			
L-arabinose	84	68	–
Fructose	69	30	0.020
D-fucose	84	30	0.001
D-galactose	68	28	0.001
D-glucose	24	45	–
D-mannitol	41	8	0.001
Sorbitol	4	0	0.001
Sucrose	94	43	0.001
Decarboxylation of:			
Arginine	3	3	0.001
Lysine	78	0	0.001
Ornithine	85	0	0.001
Utilization of carbon sources:			
Dulcitol	13	5	0.001
Sorbitol	13	0	0.001
D-alanine	48	48	–
Citrulline	16	19	0.005
L-histidine	63	50	–
L-leucine	18	48	0.019
D-amygdalin	51	38	–
Arabitol	70	24	0.023
Cellobiose	30	48	–
Maltose	76	75	–
D-mannose	64	10	0.014
Melibiose	29	0	0.008
2-ketoglutarate	19	43	–
Succinate	65	81	0.010
Adenine	0	3	0.001
m-inositol	41	52	–
Xanthine	10	0	0.001
Catalase	79	60	–
Gluconate oxidation	0	3	0.001
Indole production	16	8	0.001
H_2S production	4	5	0.001
Nitrate reduction	51	25	0.029
ONPG	4	0	0.001
Oxidase	95	70	0.019
Pigmentation	1	53	0.003
VP	0	8	0.001
Resistance to:			
0/129 (50 μg ml^{-1})	93	100	0.002
0/129 (150 μg ml^{-1})	56	98	0.010
Growth on:			
TCBS	5	12	0.002
Pseudomonas agar	5	63	0.023
Production of:			
Amylase	25	70	0.005
Chitinase	48	5	0.009
Collagenase	3	0	0.001
Deoxyribonuclease	88	53	0.019
Gelatinase	88	60	0.016
Phosphatase	89	90	0.001
Sulfatase	1	0	0.001
Urease	8	5	0.001
No. of strains	80	48	
Av. % similarity	52	35	

Maryland). An average similarity value of 55% or greater was selected as the criterion for defining a phenon, a value discerning relationships at the genus or family level, depending on the current taxonomy defining individual levels of taxa (Liston et al. 1963). Variances were used to determine the average pooled test variance and average probability of an erroneous test result (Sneath & Johnson 1972). Provisional identification of clusters was made primarily to the genus level using Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1984, Sneath et al. 1986).

A series of 2 × 2 contingency tables were constructed, using Fisher's Exact Test (Sokal & Rohlf 1981), to determine correlations of specific phenotypic traits with the 2 environments from which the isolates were obtained. Characters exhibiting probability values of $p < 0.05$, derived from the 2 × 2 contingency table analysis, were considered to be dependent on habitat. All analyses were performed using SAS Version 5.16 & SAS/Graph (Statistical Analysis System, Cary, N. Carolina 1985).

RESULTS

Bacteria isolated from seawater were phenotypically different from bacteria isolated from the sponge

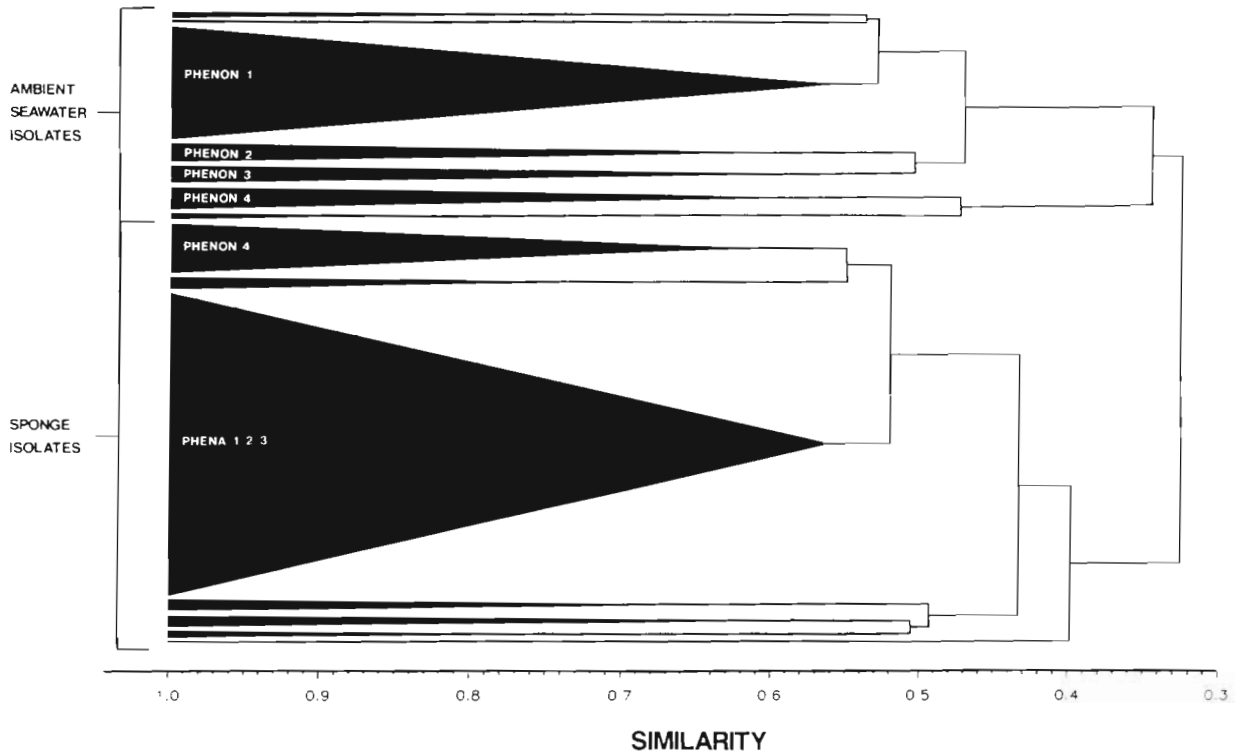

Fig. 1. Dendrogram showing clustering patterns for phena of ambient seawater and sponge isolates. Phena are described as groups of strains possessing average S_J values of 55% or greater

Ceratoporella nicholsoni (Fig. 1). In the numerical taxonomic analysis, all bacteria isolated from the same habitat clustered most closely with other bacteria from the same habitat. The 2 clusters of bacteria isolated from the 2 habitats were linked at an average similarity value of 32.7%.

Overall, bacterial strains associated with the sponge were much more similar to each other than seawater bacterial strains were to each other. A total of 18% of the isolates in the analysis did not cluster into discrete groups. These isolates included 6 seawater strains and 17 sponge strains.

Tests which yielded variance estimates greater than 0.10 and were eliminated from the analysis included: elastinase, lecithinase, growth in 10% NaCl, and ability to utilize serine, fructose, and trehalose as a sole energy source. None of the bacteria demonstrated swarming, luminescence, or vibrioid morphology, the latter observed by TEM. All strains were positive for growth at 25°C and at pH 7 in MMA, and were resistant to 10 μg ml^{-1} of the vibriostatic agent 0/129. The pooled variance estimate for traits included in the analysis was 0.0192 and the average probability of an erroneous results test result (p) was equivalent to 2.1%, an acceptable level according to the criteria of Sneath & Johnson (1972).

Bacteria from sponges

Bacteria isolated from *Ceratoporella nicholsoni* comprised 2 major clusters, or phena, when the average similarity of 55% or greater was used to define an individual cluster or phenon. When the criterion for phenon formation was a higher similarity value, most of the sponge isolates were contained within 4 discrete clusters and demonstrated average similarity values ranging from 73 to 82%. In contrast, the seawater isolates formed only one additional group when the more strict criterion for S_J was employed. In general, the seawater bacteria displayed a greater heterogeneity among the strains, whereas 70% of the sponge isolates comprised a large cluster, comprising Phena 1, 2, and 3 (Fig. 1).

A total of 42% of the sponge strains were contained within the large cluster of sponge bacteria. Phena 1 to 3 comprised Gram-negative, polarly flagellated, rod-shaped bacteria that were oxidase positive and facultative anaerobes. All of the strains were members of the genera *Vibrio* and *Aeromonas* (Table 1). This cluster of strains demonstrated an obligate requirement for 3% NaCl for growth and grew well in both 6 and 8% NaCl. Most strains were able to ferment sucrose and fucose, but lacked the ability to ferment glucose. Most were

able to decarboxylate lysine and ornithine, but could not hydrolyze arginine. All were unable to grow on TCBS. The presence of chitinase was a common characteristic, whereas the presence of amylase was not.

A second cluster labelled Phenon 4 comprised only 7% of the total isolates. The bacteria in this cluster were mostly Gram-positive and Gram-variable cells characterized by polymorphism. Cell morphology varied from rods that often included club-, Y- and T-shaped cells, to long filaments. Most were oxidase-positive, facultatively anaerobic, and fermented a wide variety of carbohydrates, including glucose. The strains did not decarboxylate lysine or ornithine. All strains were resistant to all concentrations of the vibriostatic agent 0/129 tested. The presence of amylase or chitinase was not detected. This phenon appeared very different from bacterial species described to date, but was concluded to be distantly related to the Coryneform bacteria (Krieg & Holt 1984).

Bacteria from seawater

Bacteria isolated from seawater comprised less homogeneous clusters, compared with the sponge isolates, with 95% of the seawater isolates agglomerating into 4 phena (Fig. 1). Of all the seawater isolates, none expressed the following traits: Gram-variable, polymorphism, fermentation of arabinose and sorbitol, collagenase, and sulfatase. Very few of the phenotypic traits were common to all seawater bacteria. All bacteria isolated from the water column grew in 6% NaCl and were resistant to 50 $\mu g\ ml^{-1}$ vibriostatic agent 0/129. Most of the bacteria isolated from seawater demonstrated an obligate requirement for sodium chloride.

Phenon 1 contained 56% of the seawater isolates, with an average similarity value of 56.7%. The cluster comprised Gram-negative rods that were polar or laterally flagellated. Most strains were oxidase-positive, obligately aerobic bacteria, catalase-positive, resistant to all concentrations of 0/129 tested, and able to produce acid oxidatively from a variety of carbohydrates (Table 1). Approximately half of the strains in Phenon 1, comprising seawater isolates, were pigmented, ranging from yellow to purple, pink, and an intense metallic red color. Most were able to utilize a wide range of carbon sources for energy and degrade starch, glycogen, DNA, and gelatin. Most of the strains within this cluster were presumptive members of the family Pseudomonadaceae, based on the following characters: Gram-negative, polar flagella, oxidase-positive, and aerobic metabolism of carbohydrates (Krieg & Holt 1984).

Phenon 2 contained strains which most closely resembled members of the genus *Vibrio* (Krieg & Holt 1984). A total of 8% of the seawater isolates were Gram-negative rods with a polar flagellum, oxidase-positive, and facultatively anaerobic. These non-pigmented bacteria were characterized by glucose fermentation, sensitivity to 150 $\mu g\ ml^{-1}$ of 0/129, and ability to grow on TCBS. This cluster demonstrated an average similarity coefficient value of 55.5%.

Phenon 3 isolates comprised 21% of the seawater isolates and were negative for most of the traits tested. All were characterized as Gram-negative rods or coccoid cells, oxidase variable, and obligate aerobes. All strains were resistant to all concentrations of 0/129 tested and did not grow on TCBS. Some of the strains possessed a polar flagellum. Most were not pigmented, although one strain produced a bright yellow, diffusable pigment. Carbohydrates rarely produced acid oxidatively and did not ferment substrates. The strains most closely resembled members of the genera *Acinetobacter* and *Moraxella*, possessing the diagnostic characteristics of being Gram-negative, coccoid-rod shaped, and capable of oxidative metabolism (Sneath et al. 1986).

Phenon 4 comprised 10.5% of all the seawater isolates. All members of this cluster were Gram-positive, coccoid-shaped cells, most of which were pink pigmented. These bacteria were obligate aerobes, able to acidify oxidatively arabinose, fructose, galactose, fucose, and glucose. Flagella were absent and the oxidase reaction was variable. They were able to grow on TCBS, the medium selective for many *Vibrio* spp., but were resistant to all concentrations of 0/129 that were tested, a perplexing combination of traits. The majority of these isolates also were positive for amylase, catalase and phosphatase. This phenon most closely resembles the description of *Micrococcus*, although many of the diagnostic characters were variable (Krieg & Holt 1984).

Attributes correlated with habitat

A total of 52 attributes were correlated with bacterial groups segregated by habitat (Table 2), with 19 traits showing no differentiating value for habitat specificity, even though they were all dependent with respect to frequency of occurrence with habitat. Characters found to be highly correlated with a specific environment did not necessarily require inclusion or exclusion of that trait from another environment. Often, a specific trait was possessed by bacteria from both habitats, but expression was much more common in one habitat, compared to the other.

Fermentative abilities and amino acid decarboxyla-

tion by the sponge isolates were the most prominent metabolic attributes which distinguished the presumptive sponge symbionts from seawater bacteria. Few seawater isolates were capable of fermentative metabolism. Bacteria from sponges were most readily able to catabolize arabinose, fucose, galactose, and sucrose anaerobically. Oxidative catabolism of fucose and galactose was unique to the sponge isolates, though seawater isolates did possess limited abilities for oxidation of selected carbohydrates. Most sponge isolates utilized sucrose both fermentatively and oxidatively. Presence in a sponge habitat was correlated with the ability to decarboxylate lysine and ornithine, as well as utilization of arabitol as a sole carbon source. Most of the sponge isolates demonstrated the ability to degrade large organic molecules, such as DNA, gelatin, and chitin. Pigmentation was not observed to occur among the sponge-associated bacteria, and most could not utilize leucine as a sole energy source. Most of the sponge isolates did not demonstrate amylase activity.

Absence of attributes revealed the seawater bacteria to be metabolically less opportunistic, compared to the sponge-associated bacteria. Traits not found associated with seawater bacteria were the ability to produce acid from mannitol oxidatively, reduce nitrate, utilize mannose as a sole carbon source, and degrade chitin. Most of the seawater isolates were pigmented and resistant to Irgasan, a trait common to *Pseudomonas* spp. Resistance to the vibriostatic agent 0/129 was positively associated with bacteria isolated from seawater. Other traits also found to be positively correlated with seawater bacteria were degradation of starch and glycogen by amylase.

Most of the bacteria isolated from both environments were Gram-negative rods exhibiting little polymorphism, and were rarely coccoid in shape. They were unable to grow in 0% NaCl, and tolerated up to 6% NaCl. Succinate was utilized by most of these bacteria as a sole energy source, whereas, dulcitol, sorbitol, citrulline, melibiose, adenine, and xanthine were not utilized as sole energy sources.

DISCUSSION

Bacteria isolated from the sponge were found to be associated only with the sponge and there was no evidence to support the hypothesis that they were simply bacteria filtered from the seawater by *Ceratoporella nicholsoni*. None of the sponge-associated isolates were detected among isolates from the surrounding seawater. These conclusions are supported by phenotypic evidence presented in this study and serological data from a companion study reported elsewhere (Santavy 1988). Species-specific antibodies prepared to each of the 4 species of sponge symbionts were used to probe all the isolates included in this numerical taxonomic study, as well as other isolates obtained from additional seawater samples (Santavy 1988). Recent studies of sponge symbionts conducted by other investigators have led to similar conclusions when numerical taxonomy (Wilkinson 1978a, Wilkinson et al. 1981) and immunological analyses were employed (Wilkinson 1984).

The water column overlying coral reefs is classified as somewhat oligotrophic, with available nutrients quickly assimilated, especially nitrogenous compounds. Increased metabolic capability and physiological diversity of symbiotic bacteria isolated from *Ceratoporella nicholsoni* were observed to be directly correlated with increased levels of potentially available nutrients in the sponge. Seawater isolates were more limited in their ability to utilize different nutritional sources, and appeared to be metabolically restricted by the reduced concentration of nutrients in the water column. This phenomenon has also been observed in microbiological studies of other oceanic habitats, where physiological attributes correlated well with the nutrient status of the environment (Simidu & Tsukamoto 1985, Muir 1986). Bacteria isolated from seawater were generally characterized as Gram-negative rods, but included coccoid forms and strains that were oxidative in respiration, and limited in metabolism of organic and high molecular weight compounds, pigmentation, and the ability to reduce nitrate (although starch and glycogen were degraded). In comparison, the following attributes were positively correlated with a relatively eutrophic environment: Gram-negative rods, motility, fermentative respiration, nitrate reduction, metabolism of organic and high molecular weight compounds, and oxidase-positive reactions.

These correlations were also reflected in the classification of species comprising the bacterial communities. Bacteria found to be most numerous in oligotrophic waters were related to the Pseudomonadaceae, with fewer numbers of *Acinetobacter* and *Moraxella*-like organisms, and even fewer numbers of Vibrionaceae observed. In contrast, bacteria associated with *Ceratoporella nicholsoni* were composed primarily of facultative anaerobes of the Vibrionaceae family. Taxonomic similarities correlated with the nutrient status of the environment of this study were comparable with the results of another study of bacterial communities of the Benguela Upwelling off South Africa (Muir 1986).

Sponges can provide a relatively nutrient-rich environment for a symbiotic bacterial community by concentrating particulate organic matter filtered from the water column and by supplying nitrogenous

excretory products accumulated by the organism. During periods of low pumping rates, anaerobic pockets may develop inside the sponges, which allow fermentative bacteria to function as a important nutritional source (Reiswig 1974). This view is supported by observation of the presence of facultatively anaerobic bacteria in *Ceratoporella nicholsoni,* recorded in this study, and observed in other sclerosponges (Wilkinson 1984), as well as in other species of sponges (Wilkinson 1978a, Wilkinson et al. 1981). Massive sponges containing bacteria have been postulated to have a significant carbon deficit if the only source of dissolved organic particulates was from the water column. Bacterial symbionts are believed to provide up to 70% of the required dissolved organic carbon, especially during periods of low filtering by the sponge (Reiswig 1981).

The 4 species of sponge symbionts overlapped greatly in their nutritional capabilities, with the ability to utilize relatively few substrates separating different groups. On the other hand, bacteria from seawater possessed relatively little overlap in nutritional capabilities. The mesohyl of the sclerosponge, which contains most of the sponge bacteria, represented a much smaller spatial scale, compared with the surrounding seawater. Thus, specialization of metabolic capabilities by the sponge symbionts may have developed as a result of increased competition in a confined area, eventually leading to differentiation of bacterial species, an interesting hypothesis verifiable by nucleic acid sequence analysis.

The seawater isolates employed in the numerical taxonomy study were selected from samples including many more than the 48 isolates employed in this study, to obtain maximum diversity in the analysis. It was obvious from the morphological and biochemical traits that the seawater community includes a greater diversity of bacterial species. To ensure maximum confidence in the comparison of bacteria from both habitats, seawater isolates which were obviously different morphologically from other seawater isolates were selected for the numerical taxonomy study. It is understood that morphology is not an adequate criterion to distinguish amongst bacteria, but may be useful for selecting strains for analysis of biological diversity.

Numerical taxonomy studies can reveal significant phenotypic information about bacteria, but they are also very labor-intensive. Often, compromises in the number of strains and the choice of tests employed must be made. A companion study, employing species-specific antibody probes, yielded results showing that the species composition and relative abundance of the bacterial community residing in different individuals of *Ceratoporella nicholsoni* in the Bahamian population did not significantly differ over time (Santavy 1988). Therefore, isolates from the sponge are concluded to be representative of the host bacterial flora.

In conclusion, bacteria isolated from the sclerosponge *Ceratoporella nicholsoni* were taxonomically different from bacteria isolated from ambient seawater, supporting the hypothesis that they represent true symbionts. The sponge symbionts were taxonomically much less diverse compared with isolates from the surrounding water column. Over 65% of the sponge isolates demonstrated 72% or greater similarity, which was clearly not the case for clusters of the seawater isolates. Sponge symbionts related to the Vibrionaceae and coryneforms, observed in this study, and to the Enterobacteriaceae, concluded from other studies (Wilkinson 1978a, Wilkinson et al. 1981), appear more adept in exploiting the nutrient-rich environment of the sponges. Fermentative bacteria may be able to function as an important nutritional source for sponges, especially during anaerobiosis developing during periods of low pumping. In contrast, representatives of the families Pseudomonadaceae and Micrococcaceae and *Acinetobacter* and *Moraxella*-like organisms, isolated from seawater, appear to be adapted to increased survival in relatively low nutrient waters, such as those found overlying coral reefs.

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