Marine Sponges as Habitats of Anaerobic Phototrophic Bacteria

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Abstract. Enrichment cultures were prepared with different media for phototrophic bacteria from four species of marine sponges, collected from oxic coastal waters near Split (Yugoslavia). We obtained pure cultures of six strains of *Chromatiaceae* and two strains of *Rhodospirillaceae* by agar shake dilution. The *Rhodospirillaceae* were identified as *Rhodopseudomonas sulfidophila* and a marine form of *Rhodopseudomonas palustris*. The *Chromatiaceae* were identified as *Chromatium vinosum, Chromatium gracile, Chromatium minutissimum. Ectothiorhodospira mobilis*, and a Chromatium species, which in some respects resembles *Chromatium minus*. The occurrence of strictly anaerobic phototrophic bacteria in aerobic sponges is discussed with respect to nutrition and possible syntrophism.

Introduction

Without exception, marine sponges live in the oxic parts of the ocean [14]. On the contrary, phototrophic bacteria (except a few species which tolerate low oxygen tensions or are capable of aerobic, dark respirative metabolism) live in the anoxic and sulfide-enriched parts of the photic zone of the ocean.

According to Baas Becking and Wood [1], purple sulfur bacteria develop at Eh values between 0 and −200 mV and green sulfur bacteria between −100 and −300 mV. Since clean seawater has Eh values between +400 and +470 mV [5] and estuarine water has Eh values of +300 to +350 mV [2], phototrophic sulfur bacteria in the marine environment are restricted to a few ecological niches.

Phototrophic sulfur bacteria tend to develop in coastal areas of salt marshes [11, 25, 27, 29] or "Farbstreifensandwatt" [23, 24], where large amounts of sulfide are provided by sulfate-reducing bacteria.

As long as a sufficient production of sulfide in the sediment takes place, such as in coastal lagoons, fjords, and meromictic lakes that may not be in constant connection with the ocean, the formation of a zone containing phototrophic bacteria has been observed [6, 7, 12, 20, 28].

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In small splashwater ponds usually situated at rocky coasts, phototrophic bacteria may develop in upper sediment layers, or even in the supernatant water due to sulfide production. Taga [26] reported splashwater ponds with considerable enrichments of phototrophic bacteria at rocky coasts in Japan, and we have successfully studied similar environments on the island of Helgoland (North Sea) as well as at the Adriatic coast near Split, Yugoslavia.

A quite different habitat of phototrophic bacteria was first mentioned by Eimhjellen [10]. He set out to find phototrophic bacteria in the marine sponge *Halichondria panicea*. Startled by the occurrence of the carotenoid isorenieratene in *Chlorobiaceae*, which until then only had been reported from sponges, he studied a specimen of *Halichondria panicea* and succeeded in isolating *Chlorobium limicola, Thiocystis violacea*, and a nonmotile coccoid sulfur bacterium with spirilloxanthin as the main carotenoid—most probably *Thiocapsa roseopersicina*.

In a tentative experiment during a marine biology course at Naples (Italy), 1969, one of us (H.G.T.) carried out enrichment cultures with Pfennig's medium, inoculated with pieces from ten different sponges that had been collected from the waters around the island of Ischia. The series resulted in enrichments of *Chlorobium phaeovibrioides, Ectothiorhodospira mobilis*, and *Rhodopseudomonas spec.* which, however, were not purified and further studied.

The present work was carried out to substantiate the findings mentioned above using various enrichment media for the different groups of phototrophic bacteria.

Materials and Methods

Media and Isolation

As basic medium (containing mineral salts, vitamins, and trace elements) solutions I, II, and III of Pfennig's medium for phototrophic sulfur bacteria with the addition of 3% NaCl were used [16, 27]. On this basis, four different media were prepared adding the following supplements per liter:

Medium I	(a) 1.0 gm potassium acetate, 1.5 gm sodium succinate, 0.5 gm sodium lactate, 1.0 gm Difco yeast extract, 0.5 gm sodium ascorbate	pH = 6.9
	(b) as above but pH = 5.3	
Medium II	0.5 gm Difco yeast extract, 1.5 gm $Na_2S_2O_3 \cdot 5\ H_2O$	pH = 7.1
Medium III	(a) 0.5 gm Difco yeast extract, 0.5 gm $Na_2S \cdot 9\ H_2O$	pH = 7.0
	(b) as above, but pH = 8.3	
Medium IV	Pfennig's complete medium including his sulfide solution IV	pH = 7.2

For enrichment cultures these media were used in 50 ml screw-capped bottles. Pure cultures were obtained by repeated agar shake dilution series [17] using 0.5% oxoid agar. In all cases the incubation temperature was 28°C and the light intensity was about 400–800 Lux.

Absorption spectra of living cells were measured in 60% sucrose with a Perkin Elmer double beam spectral photometer, model 124. Growth on various organic substrates was tested in 50 ml screw-capped bottles. In the sterile bottles containing the organic substrates at a final concentration of 0.1%, the preinoculated basic medium with mineral salts and vitamins was added. For growth on S^{2-}, SO_3^{2-}, and $S_2O_3^{2-}$, 0.05% yeast extract was added; the blank contained only 0.05% yeast extract but no reduced sulfur compound. After incubation for 10 days at 28°C and about 800 Lux, growth was measured as absorption at 670 nm.

Results

Four different marine sponges were sampled from 2–4 m depth at the rocky coast 0.5 km southwest of the Institute of Oceanography and Fisheries, Split, Yugoslavia. The sponges grew in entirely clear, highly aerated moving water, attached to larger limestone rocks that had broken off the steep coast. All sponges were identified after Sara [22]:

sponge 1: *Ircinia spec.*, gray, clumpy, tough
sponge 2: *Verongia aerophoba*, which is known as containing cyanobacteria as symbionts [21] and, on the other hand, as producer of antibiotically active substances [4]
sponge 3: *Hippospongia communis*
sponge 4: *Euspongia officinalis*, containing a pinkish red fluid

The four specimens were taken alive from their natural environment and immediately brought to the laboratory. Pieces of about 1 cm^3 were cut from the cortical part of the sponges and used as inocula for liquid enrichment media. In addition, the pinkish red juice of *Euspongia officinalis* was directly inoculated into agar tubes. These tubes yielded a cell number of about 10^4 *Chromatium* cells per liter in the juice.

The enrichment cultures needed 10 to 20 days until growth could be detected by pigmentation. All enrichments with samples of the sponges 2 and 3 were negative. Cultures from samples of sponges 1 and 4 in the media Ia and IIIa contained predominantly small cell Chromatium species. Samples of sponge 1 in medium II resulted in an almost pure culture of *Chromatium spec.* strain BN 5500. Sample of sponge 4 in medium IIIb led to enrichments of mainly *Ectothiorhodospira mobilis* and *Rhodopseudomonas sulfidophila*. In all samples, sulfate-reducing bacteria of the genus *Desulfovibrio* developed as could be shown by microscopic observation and strong production of hydrogen sulfide as well as the occurrence of elemental sulfur in *Chromatiaceae* in enrichment media without any added reduced sulfur compounds. This group, however, was not further studied. Altogether the strains shown in Table I were isolated in pure culture.

Strains of *Chromatium gracile, Chromatium vinosum, Chromatium minutissimum*, and *Ectothiorhodospira mobilis* were identified after Bergey's Manual of Determinative Bacteriology [18]. Strain BN 191 was identified as a new isolate of *Rhodopseudomonas sulfidophila*. This species has been first

Table 1
Strains of Phototrophic Bacteria Isolated from Sponges by Different Enrichment Media

Sample	Medium of enrichment/ isolation	Species	Strain no.
Sponge 1	II	*Chromatium spec.*	BN 5500
	Ia/IIIa	*Chromatium gracile*	BN 5410
Sponge 2	All media	None	—
Sponge 3	All media	None	—
Sponge 4	Ia	*Chromatium vinosum*	BN 5102
	IA	*Rhodopseudomonas palustris*	BN 126
	IIIa	*Chromatium minutissimum*	BN 5700
	IIIb	*Ectothiorhodospira mobilis*	BN 9901
	IIIb	*Rhodopseudomonas sulfidophila*	BN 191
Sponge 4 juice	IIIa, IV, I	*Chromatium vinosum*	BN 5101

described by Hansen and Veldkamp [13]. This organism is rather similar to *Rhodopseudomonas capsulata*. The new isolate is 0.8–1.0 μm in width and 2.0–2.5 μm in length. Anaerobically grown cultures have a brown color. If cultures become aerobic the color changes to red, therefore the cells most probably contain spheroidene as the main carotenoid, which is aerobically oxidized to spheroidenone. Absorption spectra of living cells are identical with those obtained from strains of *Rps. sulfidophila* [13] and *Rps. capsulata* [3]. Comparison of growth on some selected substrates of strain BN 191 and *Rps. sulfidophila* strains W_4 and W_{12} is shown in Table 2. Strain BN 191 prefers 3% NaCl. Strain BN 126 is a new isolate of *Rhodopseudomonas palustris*, differing in some respects from typical strains of *Rps. palustris*.

Absorption spectra of living cells show maxima at 490, 520, and 550 nm, thus very similar to that of *Rhodospirillum rubrum*, which until now was the only species of the *Rhodospirillaceae* known to contain predominantly spirilloxanthin as carotenoid. A comparison with spectra of typical *Rps. palustris* strains (e.g., BN 124) shows, that in strain BN 126 the maximum at 460 nm, which is due to rhodopin content, is lacking. Analysis of the carotenoid contents kindly carried out by Dr. Karin Schmidt, Göttingen, (Table 3) makes it clear that rhodopin is completely lacking. Contrary to known *Rps. palustris* strains, strain BN 126 grows very well on sugars and sugar alcohols and more slowly on citrate. It tolerates sulfide without any growth inhibition up to 1 mM in the presence of 0.05% yeast extract.

Strain BN 5500 is a small *Chromatium* apparently not identical with any of the *Chromatium* species described thus far. The cell suspension is of an intense blue-violet color. Autotrophically grown cells are motile, 1.2–1.5 μm in width, and about 3.0–4.0 μm in length and somewhat smaller than described for *Chromatium minus* [18]. Cells grow on thiosulfate as well as on sulfide, as

Table 2
Comparison of Substrate Utilization between the New Isolate Rps. sulfidophila *Strain BN 191 and the Originally Described Types*

Substrate	Strain BN 191	Strains W_4/W_{12}[a]
Propionate	++	++
Formate	+	++
Citrate	−	−
Glycerol	−	++
Gluconate	++	n.d.
Fructose	++	−/+
Mannitol	−	−
Glutamate	++	++
Aspartate	+	−/+
Ethanol	++	−/++
Benzoate	−	−
Yeast extract	+	++
S^{2-}	+	++
$S_2O_3^{2-}$	++	++
SO_3^{2-}	−	−

[a]Data from Hansen and Veldkamp [13]. Optical density at 670 nm within 1 week: ++, >0.25; +, <0.25; −, no growth; n.d., not determined.

does *Chromatium minus*. As seen from absorption spectra of living cells (Fig. 1), the carotenoid contents differ from that of *C. minus*. Our new isolate possesses, as preliminary results of Dr. K. Schmidt indicate, a new type of carotenoid composition.

Discussion

From the work of Eimhjellen [10] and the results reported above it becomes evident that at least part of the marine sponges regularly contain anaerobic phototrophic bacteria. This is rather surprising, because marine sponges live in the oxic parts of the oceans and are known to be sensitive to oxygen deficiencies [14].

Sponges are typical filter feeders. The main contents of their food consists of bacteria that are actively swirled in and digested intracellularly [19]. Because anaerobic bacterial photosynthesis in the oxic part of the ocean is impossible, phototrophic bacteria may occur only at very low concentrations. Therefore they cannot form the major food component of sponges and cannot be enriched within sponges to a greater extent only through filtering.

We found that sponges collected from the same environment, i.e., with identical nutritive seawater, were partly negative and partly positive with respect to the occurrence of viable phototrophic bacteria in their bodies. This difference may be explained by the fact that a number of sponges produce antibiotically active substances. Burkholder and Rützler [4] showed that *Verongia aerophoba, Crambe crambe, Aplysilla sulfurea*, and other sponges from the Mediterranean Sea contain substances that inhibit the growth of gram-

Table 3
Carotenoid Composition of Rps. palustris *Strain BN 126*

Carotenoid	Percentage of content
Lycopene	1
Rhodopin	0
Anhydrorhodovibrin	8
Rhodovibrin	24
Monodemethylated spirilloxanthin	2
Spirilloxanthin	65

positive and gram-negative bacteria, *Verongia aerophoba* exhibiting the strongest activity. Of 31 tested species, 18 were found to produce antibiotically active materials.

Halichrondria panicea, however, the species from which Eimhjellen [10] isolated phototrophic bacteria, produces antibiotics [14]. These authors were able to prove that the antibiotic substances did not act upon those microorganisms that live in a specific commensal association with the respective sponge.

There are numerous reports in the literature that bacteria do live freely in the mesogloea of healthy sponges [8, 9]. This was confirmed by the fine structure studies of Levi and Porte [15]. Whether this holds for phototrophic bacteria, too, is not yet certain. We assume, however, that phototrophic bacteria do not just serve as a diet for sponges but that between some species of sponges and phototrophic bacteria as well as sulfate-reducing bacteria a more close association may exist. The relatively high number (as compared with seawater)

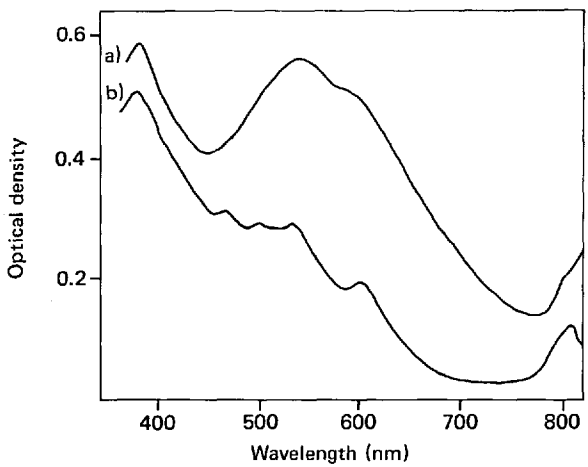

Fig. 1. Absorption spectra of (a) *Chromatium* species strains BN 5500 and (b) *Chromatium minus* strains 1211 both grown autotrophically on sulfide.

of 10^4 viable *Chromatium* cells per liter in the juice of Euspongia officinalis would support this assumption.

As generally more than one species of phototrophic bacteria was isolated from the positive sponge specimens, it appears that a special microenvironment is formed allowing suitable growth conditions for anaerobic phototrophic bacteria. Since we always detected sulfate-reducing bacteria in the sponges, the hydrogen sulfide produced by this group could serve to provide the anoxic conditions as well as the photosynthetic electron donor for the phototrophic sulfur bacteria.

In symbiosis between algae and sponges the algae are transmitted from one sponge generation to the next either by sexual larvae or by the gemmules. In some cases, if algae-free gemmules are elaborated, infection occurs anew when sponges are given free algae, which are ingested through the same pathway as food particles [19].

We are of the opinion that phototrophic bacteria growing in anaerobic sulfide-enriched environments in the mud of the estuarine zone are carried off by the streaming water into oxic parts of the sea, where they are ingested by sponges. Thus marine sponges may assume the role of active enrichments for phototrophic bacteria which are present in oxic estuarine waters only in extremely low numbers.

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