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Culture and isolation of phototrophic sulfur bacteria from the marine environment

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KURZFASSUNG: **Kultur und Isolierung phototropher Schwefelbakterien aus dem Meer.** Im Verlauf der letzten zehn Jahre sind von PFENNIG (1961, 1965, 1967) neue Kulturmedien und Techniken zur Isolierung und Reinzucht phototropher Schwefelbakterien (Thiorhodaceae und Chlorobacteriaceae) entwickelt worden. Seither sind viele Arten, die bislang nur von der mikroskopischen Beobachtung bekannt waren, in Reinkulturen untersucht worden. Phototrophe Bakterien treten in Massenentwicklungen in Küstenbereichen des Salzmarsch-Typs auf (z. B. US-Ostküste, nördliche Schwarzmeerküsten, dänische und schleswig-holsteinische Küsten). Weniger häufig findet man sie jedoch auch an anderen marinen Standorten. Unter Anwendung der PFENNIGschen Methoden wurde zum erstenmal eine große Zahl von Stämmen von marinen Standorten isoliert. Die meisten Proben wurden in Salzmarschen, von Küstenseen und flachen Stellen des Kontinentalschelfs um Cape Cod, Mass., USA, gesammelt. Vergleichsweise wurden auch Proben von den Galapagos-Inseln, Sizilien und verschiedenen salzhaltigen Inlandstandorten verwendet. Die Salinität des PFENNIGschen Mediums wurde der jeweiligen Probe angepaßt; in den meisten Fällen wurde 3 % NaCl zugesetzt. Die Schlamm- und Wasserproben wurden in flüssigem Medium bei Tageslicht (für die Selektion von Chlorobacteriaceae) oder gefiltertem Licht inkubiert (zur Anreicherung Bacteriochlorophyll-*a*-haltiger Thiorhodaceae wurde alles Licht unterhalb einer Wellenlänge von 750 nm ausgeschlossen; für Bacteriochlorophyll-*b*-haltige Bakterien wurde alles Licht unter 900 nm ausgeschlossen). Positive Anreicherungen wurden durch 3 oder 4 Passagen anaerober Agarschüttelkulturen, die mit normalen Glühbirnen beleuchtet wurden, gereinigt. Folgende neue bzw. verlorengegangene Arten wurden isoliert: *Chromatium buderi* TRÜPER und JANNASCH, *Ectothiorhodospira mobilis* PELSH, *Prosthecochloris aestuarii* GORLENKO; diese Arten erwiesen sich alle als unbedingt salzbedürftig. Ferner wurden Stämme von *Chromatium vinosum, C. violascens, C. gracile, Thiocystis violacea, Thiocapsa roseopersicina, T. pfennigii* (Bacteriochlorophyll-*b*), *Chlorobium limicola, C. vibrioforme* und *C. phaeobacteroides* isoliert. Versuche zum Salzbedürfnis an Reinkulturen ergaben, daß bei weitem nicht alle marinen Thiorhodaceae und Chlorobacteriaceae salzbedürftig sind. In der Verwertung anorganischer und organischer photosynthetischer Elektronendonatoren unterscheiden sich die marinen Stämme nicht wesentlich von vergleichbaren Süßwasserstämmen.

INTRODUCTION

Phototrophic sulfur bacteria (Thiorhodaceae and Chlorobacteriaceae) are regularly found where their ecological requirements are met; these are the presence of light and hydrogen sulfide and the absence of oxygen; the latter condition being due, in part, to the comsumption of oxygen during the decomposition of organic matter,

in part, to the presence of hydrogen sulfide. Basically, two types of suitable habitats exist which differ by the source of hydrogen sulfide: habitats with sulfur springs that carry H_2S and other reduced sulfur compounds, and habitats with H_2S production due to bacterial sulfate reduction. For the latter type which represents a bacterial sulfur cycle, BAAS BECKING (1925) introduced the term "sulfuretum".

The largest and most obvious developments of phototrophic bacteria are found in estuaries of the saltmarsh type which sometimes also have been called beach sulfureta (SUCKOW 1966). Since WARMING (1875) described these mass developments at the coast of Denmark they have been found at the coasts of Germany (Schleswig-Holstein), almost the whole eastern U.S. coast, the northern Black Sea coasts and others. In these environments, the phototrophic sulfur bacteria live at the expense of the H_2S produced by sulfate-reducing bacteria in the mud. Light is abundant. Since the phototrophic sulfur bacteria are able to photoassimilate quite a number of simple organic compounds such as fatty acids, alcohols and sugars, it can be assumed that part of their cell carbon is derived from such compounds which might in turn be produced by cellulose decomposing bacteria thriving on decaying plant material.

Besides this predominant type of a marine habitat for the purple (Thiorhodaceae) and green (Chlorobacteriaceae) sulfur bacteria they inhabit a number of other ecological niches in the marine environment:

(1) Meromictic estuarine lakes, ponds and lagoons, which might not even be permanently connected with the open Sea, e.g. the ponds at the coast of Cape Cod, Massachusetts, or lake Faro near Messina (TRÜPER & GENOVESE 1968). Also anoxic fjords such as Lake Nitinat (RICHARDS et al. 1965) should contain phototrophic sulfur bacteria, since all environmental requirements are met to form a bacterial plate at the chemocline.

(2) Tide pools at rocky coasts of Japan have been found to contain mass developments during the summer months (TAGA 1967).

(3) Purple and green phototrophic sulfur bacteria were isolated from mud of shallow areas at the continental shelf of Cape Cod, Massachusetts (TRÜPER, unpublished).

(4) EIMHJELLEN (1967) isolated three species out of sponges, which obviously provide a suitable microenvironment for these bacteria as long as they are located in the photic zone.

(5) Finally, KRISS & RUKINA (1953) reported the enrichment of phototrophic sulfur bacteria from the anoxic deep waters of the Black Sea. Their theory that these organisms live there at the expense of radioactive radiation rather than of light, has been disproven by KUZNETSOV (1956). An attempt to find and isolate the organisms of KRISS & RUKINA (1953), using sterile sampling techniques and far more suitable growth media, was without success (TRÜPER, unpublished; R/V "Atlantis II" cruise No. 49, 1969).

Most of the earlier studies on the marine phototrophic bacteria (WARMING 1875, ISACHENKO 1914, BAVENDAMM 1924, GIETZEN 1931) are descriptive in character, due to the fact that no suitable culture media and techniques had been developed. The medium designed by VAN NIEL (1931) enabled scientists to cultivate at least a few species, nevertheless to the effect that knowledge about phototrophic bacteria and

photosynthesis in general could be greatly increased. A medium for green phototrophic bacteria was developed by LARSEN (1952), and finally PFENNIG (1961, 1965) designed a medium for both Thiorhodaceae and Chlorobacteriaceae, that so far has enabled us to isolate in pure culture most of the species of these families so far known from the descriptive literature (e.g., WINOGRADSKY 1888).

During this study, PFENNIG's medium was slightly modified and – for the first time – used in large scale isolations of phototrophic bacteria from the marine environment.

RESULTS

Preparation of PFENNIG's medium

The following recipe gives 3 liters of medium. Screw cap bottles of 125 ml (ca. 4 oz.) and 65 ml (ca. 2 oz.) volume were used. The medium was prepared as four different solutions:

Solution I:	distilled water:	2500 ml
	$CaCl_2 \cdot 2H_2O$	1.3 g
	NaCl	105.0 g (giving a final concentration of 3% NaCl)

Of solution I, 500 ml were autoclaved in an Erlenmeyer flask, and 2000 ml were distributed to the screw cap bottles, 80 ml for 125 ml-bottles, and 40 ml for 65 ml-bottles. Then the bottles were autoclaved with the caps not tightly closed. After autoclaving, the bottles were allowed to cool to room temperature slowly, then tightly closed.

Solution II:	distilled water:	67 ml
	trace element solution (PFENNIG & LIPPERT 1966):	30 ml
	Vitamin B_{12} (2 mg/100 ml) solution:	3 ml
	KH_2PO_4	1.0 g
	NH_4Cl	1.0 g
	$MgCl_2 \cdot 6H_2O$	1.0 g
	KCl	1.0 g
Solution III:	distilled water:	900 ml
	$NaHCO_3$	4.5 g

Solution III was enriched with CO_2 by bubbling until the pH was down to 6.2. Then solutions II and III were mixed and instantly sterilized by filtration through a sterile Seitz filter. (The sterilized filter was washed by filtration of 200 ml distilled water, before solutions II and III were sterilized.)

After sterile-filtration, the combined solutions II + III were distributed to the bottles which already contained sterile solution I. The portions were 40 ml and 20 ml, respectively.

Solution IV:	distilled water:	200 ml
	$Na_2S \cdot 9H_2O$	3.0 g

A teflon-covered stirring magnet was added, the solution autoclaved and set to cool. Then, under sterile conditions, about 1.5 ml of sterile (autoclaved) 2 M H_2SO_4 was added dropwise while the solution was stirred magnetically. This solution was distributed to the bottles in 6 ml and 3 ml amounts, respectively. Then the bottles were filled up with solution I; a pea-size air bubble was left to meet possible pressure changes. For the adjustment of the final pH, which should be 6.8 for the Chlorobacteriaceae and 7.2 for Thiorhodaceae, more or less H_2SO_4 was added to solution IV.

Enrichment techniques

With the medium described above, enrichment cultures in Winogradsky columns are no longer necessary. The natural water and mud samples were given into screw cap bottles with Pfennig's medium and incubated at 20°–25° C in the light. For selective enrichments, it was necessary to apply filtered light. Usually, daylight allows growth of Thiorhodaceae which, however, will be quickly outgrown by Chlorobacteriaceae. Repeatedly a rather high tolerance against H_2S was observed with the accompanying diatoms and blue-green algae of salt marsh samples. The growth of algae and Chlorobacteriaceae was completely inhibited when light filters were used, that allow to penetrate only light of wavelengths above 800 nm (filter No. IR 530, Göttinger Farbfilter GmbH). For the enrichment of Thiorhodaceae containing bacteriochlorophyll b it was necessary to use light filters that cut out all light of wavelenghts below 900 nm (filter No. IR 533, Göttinger Farbfilter GmbH). These filters were mounted on the sides of glass boxes which otherwise were painted black. The bottles were placed inside these boxes and cooled by rinsing with tap water. As light sources, incandescent lamps were installed outside the boxes in front of the filters.

Preparation of pure cultures by agar shake dilution

A solution of 0.1 % CaCl and 2.4 % agar (Difco or Oxoid 3) in distilled water was given in 3ml portions into long narrow test tubes which were then closed with cotton stoppers and autoclaved. The sterile tubes with the liquefied agar were kept in a 55° C water bath.

A 120 ml screw capped bottle containing PFENNIG's complete medium was supplied with filter-sterilized sodium ascorbate to an end concentration of 0.05 % and then kept with loosened screw cap in a 38° C water bath.

The agar tubes were then supplied with 6 ml of the prewarmed medium and immediately mixed by once turning them upside down and back (the wetting of the cotton plug does not disturb the further procedures). The mixed agar tubes were then kept in the 38° C water bath.

One of the tubes was inoculated with 1–3 drops of an enrichment culture and the contents immediately mixed by turning upside down and back.

Of this culture 0.5–1.0 ml were then given into a second tube containing the

agar medium, mixed immediately and so on. This dilution series was continued over eight to ten steps.

Each ready tube (after transfer to the next) was set into a water bath of 15°–20° C to harden the agar.

After hardening, the agar was immediately sealed with a sterile liquefied paraffin layer (1 part paraffin dissolved in 3 parts of paraffin oil) of about 2.5 cm thickness. The tubes were kept in the dark for one hour, then the upper part of the tubes was reheated, to achieve a better sealing effect.

The cultures were incubated at 20°–25° C at a light intensity of 50–100 foot-candles (about 500–1000 lux).

After growth, that tube of the series that contained less than 20 colonies was cut using a diamond, broken up at a proper point, and single colonies were removed by using a fine Pasteur pipette attached to a rubber tubing. The colony was suspended in a drop of sterile medium and the whole dilution series was repeated. In order to achieve pure cultures it was necessary to repeat the whole process three to four times.

Purity was checked by use of Difco AC medium and *Desulfovibrio*-Medium after ABD-EL-MALEK & RIZK (1960). As long as no growth in these media occurred and the culture was morphologically uniform, purity was established. (For the isolation of the large spirilla of the genus *Thiospirillum*, which will not grow in agar shakes, the capillary method [cf. JANNASCH 1965] may be used.)

Pure cultures obtained

The strains isolated in pure cultures are listed in Tables 1 and 2. They were identified by morphological characteristics and physiological properties such as their in vivo absorption (TRÜPER & YENTSCH 1967) and their ability to utilize certain sulfur and carbon compounds as photosynthetic electron donors.

Ectothiorhodospira mobilis was originally described by PELSH (1937) but subsequently lost until its re-isolation in 1967 (TRÜPER 1968). This organism differs from the other Thiorhodaceae since it deposits the intermediarily formed elemental sulfur outside the cells instead of inside.

Chromatium buderi is a new species (TRÜPER & JANNASCH 1968) that appears to occur only in the marine habitat. Its cell shape is severely changed by lowering the salinity of the medium.

The species *Chromatium violascens* (TRÜPER & GENOVESE 1968) and *Chromatium gracile* have never been isolated in pure cultures since the time they were described and named from crude samples.

The species *Thiocapsa pfennigii* was described by EIMHJELLEN et al. (1967), under the name *Thiococcus* spec., nov. gen. nov. spec. This designation is taxonomically not validly published since no species name was given (International Code of Nomenclature of Bacteria 1966, rule 13a3). This organism will be included in the genus *Thiocapsa* as the new species *pfennigii* (EIMHJELLEN, pers. comm.). *Thiocapsa pfennigii* is the only species of the Thiorhodaceae so far known to contain bacteriochlorophyll-*b*.

Table 1

Isolated strains of Thiorhodaceae

Species	Strain	Source	Preferred salinity (% NaCl)
Ectothiorhodospira mobilis	8112	salt flat, Sta. Cruz, Galapagos	3
	8113	salt flat, Sta. Cruz, Galapagos	3
	8115	salt flat, Sta. Cruz, Galapagos	3
	8815	salt marsh, Barnstable, Mass.	3
Chromatium buderi	8111	salt flat, Sta. Cruz, Galapagos	3
	8812	salt marsh, Barnstable, Mass.	1
	8813	salt marsh, Barnstable, Mass.	1
Chromatium vinosum	8011	salt marsh, Milford, Conn.	1
	8114	salt flat, Sta. Cruz, Galapagos	1
	8211	brackish pond, Woods Hole, Mass.	1
	8212	brackish pond, Woods Hole, Mass.	1
	8214	brackish pond, Woods Hole, Mass.	2
	8411	estuarine pond, Falmouth, Mass.	1
	8412	estuarine pond, Falmouth, Mass.	1
	8417	estuarine pond, Falmouth, Mass.	2
	8511	salt marsh, Woods Hole, Mass.	1–3
	8711	30 m depth, contin. shelf, S. of Cape Cod	3
	8712	30 m depth, contin. shelf, S. of Cape Cod	3
	9011	salt flat, Etosha, S.W. Africa	1–3
	9511	salty water hole, Rietfontein, S.W. Africa	1–3
	9611	water hole, Ch. Marais Dam, S.W. Africa	1–3
Chromatium violascens	3311	marine lake Faro, Messina, Italy	1–3
	8313	salt marsh, Sippewissett, Mass.	2
	8314	salt marsh, Sippewissett, Mass.	2
Chromatium gracile	8611	Hadley Harbor, Naushon Island, Mass.	1
	8911	salt marsh, Martha's Vinyard Island, Mass.	1–3
Thiocystis violacea	8012	salt marsh, Milford, Conn.	1
	8213	brackish pond. Woods Hole, Mass.	1–3
	8311	salt marsh, Sippewissett, Mass.	1–3
	8314	salt marsh, Sippewissett, Mass.	3
	8316	salt marsh, West Falmouth, Mass.	1–3
	8512	salt marsh, Woods Hole, Mass.	2
Thiocapsa roseopersicina	8318	salt marsh, West Falmouth, Mass.	2
	8811	salt marsh, Barnstable, Mass.	1
Thiocapsa pfennigii	8013	salt marsh, Milford, Conn.	1
	8816	salt marsh, Barnstable, Mass.	1

Chlorobium vibrioforme was first described by PELSH (1937). Strains fitting PELSH's description were reported by PFENNIG (1967) and their properties justify the separation from *Chlorobium limicola*. *Chlorobium phaeobacteroides* is a brown colored species of the Chlorobacteriaceae which was first isolated in 1966 by PFENNIG (1968) from Norwegian meromictic lakes.

Prosthecochloris aestuarii was recently described by GORLENKO (1968) as a new green sulfur bacterium; the cells are covered with prosthecae which contain the

Table 2

Isolated strains of Chlorobacteriaceae

Species	Strain	Source	Preferred salinity (% NaCl)
Chlorobium limicola f. *thiosulfatophilum*	3331	marine lake Faro, Messina, Italy	1
Chlorobium limicola	8432	estuarine pond, Falmouth, Mass.	1–3
	8433	estuarine pond, Falmouth, Mass.	1–3
Chlorobium vibrioforme	8230	brackish pond, Woods Hole, Mass.	1
	8430	estuarine pond, Falmouth, Mass.	1–3
	8431	estuarine pond, Falmouth, Mass.	1–3
	8730	30 m depth, contin. shelf, S. of Cape Cod	3
	8731	30 m depth, contin. shelf, S. of Cape Cod	3
Chlorobium phaeobacteroides	3330	marine lake Faro, Messina, Italy	3
	L.T.	exit of underwater saltspring, Lake Tiberias	1–3
Prosthecochloris aestuarii	8530	salt marsh, Woods Hole, Mass.	1

typical *Chlorobium* vesicles carrying the photopigments. A description and designation by GORLENKO is in press (pers. comm.).

Most of the isolated strains have been included in the culture collection of the Institut für Mikrobiologie, Göttingen, and are presently under further investigation with respect to ultrastructural, physiological and pigment characteristics.

General observations

Besides the isolated forms, also a number of other species were repeatedly observed during microscopical examinations of natural specimens.

In samples from estuarine lakes near Falmouth, Massachusetts, *Thiospirillum sanguineum* EHRENBERG was found in between mass developments of *Thiocystis violacea*. *T. sanguineum* differs clearly from *T. jenense*, since its single cells appear purple-red under the microscope, a property typical for okenone-containing Thiorhodaceae *(Chromatium okenii, C. weissei)*, while cells of *T. jenense* appear yellowish under the microscope. In salt marsh samples, also *Chromatium warmingii*, easily discernible by its polar arrangement of intracellular sulfur globules, was found a few times. These large forms, however, could not be isolated, since in the natural samples they represented less than 1 % of the photosynthetic bacteria and were rapidly outgrown by the species with smaller cells during enrichments. One sample from the Barnstable salt marshes contained a natural enrichment of the platelet-forming, gas vacuole containing *Thiopedia rosea*. Cell masses of this species appeared distinctly purple-violet like the species containing rhodopinal (warmingone) as the main carotenoid (e. g. *Chromatium warmingii, C. buderi, Thiocystis violacea*).

Attempts to isolate this species failed. Appearently the gas vacuole containing species will not easily grow in agar shake cultures.

The natural samples of the salt marshes usually represented clusters of purple-violet colored immotile phototrophic bacteria, often encapsuled in more or less conspicuous slime masses. Usually these clusters, resembling the descriptions of WINOGRADSKY (1888) for *Lamprocystis, Thiocystis, Thiopolycoccus* and *Thiocapsa,* started to disintegrate into smaller clusters and single cells in the enrichment bottles. The enrichments and isolations showed that usually a non-motile *Thiocapsa* and a motile *Thiocystis* or *Chromatium* together were responsible for these natural "colonial" clusters. A *Thiopolycoccus* was never seen in pure culture; it appears doubtful whether this species exists at all.

Of the species isolated, only *Ectothiorhodospira mobilis, Chromatium buderi, Chlorobium vibrioforme, Chlorobium phaeobacteroides,* and *Prosthecochloris aestuarii* are absolutely salt requiring. It is, however, not clear whether this is a requirement for a certain osmolality of the medium or for the Na^+ or the Cl^- ion. PFENNIG (1968) found that all strains of *Chlorobium phaeovibrioides* so far isolated required NaCl in the medium. A definite requirement for the sodium ion was found in the non-sulfur photoheterotroph, *Rhodopseudomonas spheroides* (SISTROM 1960). So far as tested, most of the strains isolated during this study (e. g. all strains of the two *Thiocapsa* species) are able to grow in non-saline medium. Optimal growth of fresh isolates, however, was found at the salinities indicated in Tables 1 and 2.

Initial experiments about the utilization of carbon and sulfur compounds as photosynthetic electron donors, showed that the usual pattern as demonstrated by THIELE (1968) for freshwater Thiorhodaceae is also present with the salt water strains (TRÜPER & GENOVESE 1968, TRÜPER & JANNASCH 1968, TRÜPER 1968). In general, in the marine strains there appears to be a tendency for the utilization of fructose, propionate and butyrate, which is not as common in the freshwater forms.

DISCUSSION

The medium of PFENNIG was originally developed to grow the large freshwater species *Chromatium okenii* and *Thiospirillum jenense* (PFENNIG 1961). This was done by stepwise replacing sewage and mud extracts of the natural environments (SCHLEGEL & PFENNIG 1961).

The medium is based on a delicate bicarbonate buffer system, a relatively low content of the usual mineral salts, a new trace element formula (PFENNIG & LIPPERT 1966), including EDTA as a chelating agent, and the addition of vitamin B_{12} (cyanocobalamine). This vitamin is required by many species of the phototrophic sulfur bacteria (PFENNIG 1961, 1965, PFENNIG & LIPPERT 1966, TRÜPER & GENOVESE 1968, TRÜPER & JANNASCH 1968, TRÜPER 1968).

The medium has meanwhile been used for all types of phototrophic sulfur bacteria to the effect that it is far better, gives more stability in the cultures, and higher cell yields than any other medium developed so far for this physiological group.

As the above experiments have shown, 3 % NaCl may be added to the basic formula without any negative effect upon the buffer capacity, which partly depends

on the dissolved carbon dioxide gas. The extremely halophilic phototrophic sulfur bacterium, *Ectothiorhodospira halophila* (RAYMOND & SISTROM 1967, and pers. comm.), requiring an optimal salinity of 20 % NaCl, however, does not grow in PFENNIG's medium (TRÜPER, unpublished results); the buffering capacity as well as the total bicarbonate/CO_2 content is severely disturbed by this extreme salinity.

In a rather recent computation, BRISOU (1955) considered the following *Thiorhodaceae* species as marine: *Thiocystis violacea, Thiocystis rufa, Thiocapsa roseopersicina, Thiopedia rosea, Amoebobacter granula, Thiodyction elegans, Thiothece gelatinosa, Thiopolycoccus ruber, Chromatium gobii, Chromatium okenii, C. weissei, Rhabdomonas rosea, Thiospirillum jenense, Thiospirillum violaceum, Thiospirillum rosenbergii, Rhodothece pendens.* Besides the facts that most of these species have been originally described from freshwater, and strains of nine of them have been isolated from freshwater, BRISOU's list was based entirely on observations of crude natural samples. Several of these organisms were never reported by any other investigator but the original author, and since they were never even accumulated in enrichment cultures or isolated in pure cultures their actual existence appears doubtful. Some of these species have to be considered as synonyms since their morphology is changed by specific influences of their natural habitats (PFENNIG & TRÜPER, in: Bergey's Manual of Determinative Bacteriology, 8th ed., in press). Such cases have been described by PFENNIG & LIPPERT (1966) during a pure culture study on the vitamin B_{12} requirement by the large cell Thiorhodaceae: Vitamin B_{12} deficient cells of *Chromatium okenii* fit exactly into the purely morphological description of SKUJA's (1948) *Chromatium densegranulatum.* When *Chromatium buderi* is grown at lower salinities (TRÜPER & JANNASCH 1968) the cells start to elongate and assume shapes rather typical for members of the genus *Rhabdomonas* (synonym: *Rhabdochromatium*), a genus that not too frequently was observed, but never brought into pure culture.

These examples may demonstrate that mere morphological descriptions of new species are worthless for modern microbiology. Pure culture studies – for phototrophic sulfur bacteria facilitated by PFENNIG's medium – are absolutely necessary before a new species should be even recognized as such. The knowledge of the photosynthetic pigments, utilization of photosynthetic electron donors (H_2, sulfur compounds, simple organic compounds) and relationships to salinity of the medium is as important as the microscopic observation. Standard methods of modern microbiology are now applicable and commendable for the phototrophic bacteria, too.

SUMMARY

1. A new growth medium for Thiorhodaceae and Chlorobacteriaceae was adopted for the isolation of members of these bacterial families from marine habitats.
2. 47 strains comprising 13 species were enriched and isolated in pure cultures.
3. The strains were classified by physiological and morphological characteristics.
4. Many of the strains represent species in pure culture for the first time.

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