Sulfide-oxidizing bacteria in the burrowing echinoid, *Echinocardium cordatum* (Echinodermata)

A. Temara 1, C. de Ridder 1, J. G. Kuenen 2, L. A. Robertson 2

1 Laboratoire de Biologie marine (C.P. 160), Université Libre de Bruxelles, 50 av. F.D. Roosevelt, B-1050 Bruxelles, Belgium
2 Kuyper Laboratory of Biotechnology, Delft University of Technology, P.O. Box 5057, Julianalaan 67, 2600 GB Delft, The Netherlands.

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**Abstract.** Symbiotic filamentous bacteria thrive in the intestinal caecum of the deposit-feeding echinoid *Echinocardium cordatum*. Specimens of *E. cordatum* were collected at Wimereux (Nord Pas-de-Calais, France) in 1991. Their symbiotic bacteria build nodules by forming multilayered mats around detrital particles that enter the caecum. The morphological features of the bacteria are those of *Thiobrix*, a sulfide-oxidizing genus. The filaments, which may form rosettes, are sheathed and made by a succession of hundreds of rod-shaped bacteria which store elemental sulfur in the presence of external sulfide. Live bacteria are restricted to the outer layers of the nodules. Their sulfide-oxidizing activity was investigated, using a Biological Oxygen Monitor, by measuring the O2 consumption when reduced sulfur compounds are provided. They oxidize thiosulfate and sulfide. Optimal sulfide oxidation occurs at intermediary pH (100 to 160 μM S02–3, 1–9). Spectrophotometry has confirmed that the sulfur content of the filamentous symbiotic sulfide-oxidizing bacteria depends on the presence of external sulfide. This is the first report of symbiotic intradigestive *Thiobrix* spp.-like bacteria; it lengthens the list of symbioses between sulfide-oxidizing bacteria and invertebrates from sulfide-rich habitats.

**Introduction**

Symbiotic sulfide-oxidizing bacteria often occur in marine invertebrates that live in sulfide-rich habitats. They have been reported in phyla from the hydrothermal vents (e.g. pogonophorans, Cavanaugh et al. 1981; molluscs, Cavanaugh 1983; and annelids, Gaill et al. 1987) and from sea grass beds and anoxic sediments (e.g. nematode, Ott and Novak 1989; molluscs, Vetter 1985; pogonophorans, Southward et al. 1986; annelids; Giere et al. 1988a; priapulida, Oeschger and Schmaljohann 1988; and protozoa, Fenchel and Finlay 1989). As a general rule, these symbiotic bacteria are assumed to, or have been demonstrated to, provide their hosts with chemosynthetically fixed CO2 (Rau and Hedges 1979, Felbeck 1981; Felbeck et al. 1981, Felbeck 1983, Felbeck 1985, Le Pennec and Fiala-Medioni 1988). In addition, a detoxifying role has also been suggested, as hydrogen sulfide is toxic to cytochrome c oxidase and thus to the aerobic metabolism of most animals (Vetter 1985, Gaill et al. 1987).

The echinoid *Echinocardium cordatum* lives in sediment and commonly burrows below or at the level of the oxidized-reduced interface. It ingests deep and surface sediments (De Ridder and Jangoux 1985). Surface sediment reaches the echinoid through a vertical tube that connects the burrow to the surface and provides the detritus on which it feeds. Although deep sediment are depleted in detritus, they are ingested in bulk by the echinoid and are suspected to play a mechanical role in the digestive process (De Ridder et al. 1985b). As a result, deep reduced sediment not only occurs in the habitat but also within the digestive tubule of the echinoid. Symbiotic sheathed filamentous bacteria have been observed in *E. cordatum* (De Ridder et al. 1985a). These bacteria live within the intestinal caecum (a pouch that opens at the intestine-rectum junction). By attaching to detrital particles that enter the caecum (e.g. wood or algal fragments, skeletal pieces of fish, small shells of bivalve or gastropod, etc.), the bacteria build typical multilayered nodules whose diameters range from 3 to 7 mm. The symbiotic bacteria thrive in the outer layers of the nodules, and empty bacterial sheaths accumulate in the inner layers. Preliminary observations based on morphological features indicate that these symbiotic bacteria could be sulfide-oxidizing, i.e., may be capable of oxidizing reduced inorganic sulfur compounds (De Ridder 1986, Temara 1990, Temara and De Ridder 1990).

The purpose of the present work is to characterize the bacteria associated with *Echinocardium cordatum* in order to discuss their possible symbiotic role. Their morphology, morphometry and sulfur metabolism are investigated.
Materials and methods

Specimens of the spantagoid echinoid *Echinocardium cordatum* were collected intertidally in March 1991 at Wimerex (Nord Pas-de-Calais, France). All echinoids were found buried at depths ranging from 15 to 20 cm, i.e., a few cm below the oxidized-reduced interface (Analogous to the RPD layer of Fenchel and Riedl 1970: ca. 10 to 13 cm, March 1991, Wimerex). They were maintained in a running seawater aquarium in the sediment in which they had been living and were dissected less than 24 h after having been collected. Once isolated from the intestinal caecum, the bacterial nodules were prepared for further observations.

For electron microscopy (transmission electron microscopy: TEM; scanning electron microscopy: SEM), the nodules were fixed in 3% glutaraldehyde in cacodylate buffer (0.1 M; pH 7.4), washed in buffer, post-fixed with 0.1% osmium tetroxide in 0.1 M cacodylate buffer, washed again in buffer, and dehydrated in graded ethanol. After this step, the nodules destined for TEM and SEM were treated separately. For TEM, the nodules were then embedded in Spur's medium. Ultra-thin sections (LKB V ultramicrotome) were stained with uranyl acetate and lead citrate before being observed with a Philips EM 300 transmission electron microscope. For SEM, the nodules were dried by the critical point method using CO₂ as transition fluid. They were then mounted on aluminum stubs, coated with gold in a sputter coater and observed with an ISIDS-130 scanning electron microscope.

Material destined for sulfur metabolism study was tested immediately after dissection, in order to restrict the spontaneous oxidation process. To test the "sulfide-oxidizing hypothesis", two series of observations were made on the symbiotic bacteria: (1) their oxygen consumption in the presence of external, reduced sulfur compounds was measured; (2) their storage and mobilization of intracellular elemental sulfur in the presence and in the absence of external sulfur was observed. The presence of sulfur in the nodules was estimated by measuring total acid volatile sulfide (titration by iodine) according to Charlot (1966).

The purpose of the respirometry was to determine whether the bacteria were able to use a reduced sulfur inorganic compound as an electron donor. Two types of samples were used: intact nodules, or suspensions of the outer layer containing the putative sulfide-oxidizing bacteria. The latter was peeled off the nodules under a stereomicroscope. Respirometry was measured in a closed chamber containing seawater (5% NaCl; T = 20°C; pH = 7.8). The initial pO₂ was controlled by bubbling air or N₂ into the chamber. Bacterial O₂ consumption was measured with a Biological Oxygen Monitor (Yellow Springs Instrument, Yellow Springs, Ohio, USA) after sulfide (Na₂S) or thiosulfate (Na₂S₂O₃) had been added. The oxidation reactions of the reduced sulfur compounds are illustrated in Table 1. Sulfide has a reduction state of −2. Thiosulfate contains a sulfone-sulfur at −6 state and a sulfane-sulfur at −2 state. Both substrates can donate eight electrons to oxygen when oxidized to sulfate (Reactions 3 and 6). Sulfide can partially be oxidized to elemental sulfur (1), releasing two electrons; thiosulfate can partially be converted to elemental sulfur according to the Reaction 4, followed by oxidation of sulfide to sulfate whereby again two electrons are donated to oxygen. An alternative route may be (6) followed by (1).

The final substrate concentration was assumed to be 20 μM. Na₂S and Na₂S₂O₃ stock solutions were prepared in O₂-free water to avoid the formation of oxidation products, i.e., sulfite. The negative control for measuring chemical oxidation was the O₂ consumption in seawater without bacteria after substrate addition. The protein content of the samples was measured according to the Micro-Biuret method described by Lowelur (1963).

The presence, the mobilization, and the storage of intracellular elemental sulfur were investigated using an interferential contrast phase microscope (ICPM) and a spectrophotometer (Philips PU 8700). All the observations were repeated on ten replicates of ten bacterial nodules of similar diameter (5 mm). For each nodule, the outer layer (i.e., the part of the nodule that consists of actively growing bacteria, see "Introduction") was peeled off and studied.

The observations and measurements were done on three sets of bacteria, namely: (1) freshly collected bacteria, which always contained large numbers of visible sulfur granules (Set 1); (2) the same bacterial samples suspended in sterile seawater and kept for 12 d in the absence of external sulfate (Set 2). These two samples were measured from Day 3 to 11 for sulfur content. (3) samples treated as (2) until visible sulfur had been reduced to a very low level (Day 9), and then incubated overnight, aerobically, in the same well aerated solution supplemented with 100 μM sulfate (Set 3). In order to minimize bacterial and algal contamination, samples of Set 2 and 3 were kept in sterile seawater at 4°C and in the dark. The sulfur content of each outer layer was extracted in 2 ml of acetone and compared to reference solutions (Na₂S: 0, 100, 200, 500, 1000 μM). Sulfur was measured spectrophotometrically according to Hazaa et al. (1988).

Results

Morphology and morphometry

Detrital particles that enter the intestinal caecum of *Echinocardium cordatum* are colonized by symbiotic filamentous sheathed bacteria. This results in nodule formation. Each "mature" nodule consists of a detrital central core wrapped in a three-layered coat of bacterial origin (see De Riddier et al. 1985b) (Fig. 1). Entangled sheathed filaments form the outer and intermediate layers, while stacked empty sheaths form the inner layer. Rosette structures may develop on the filaments from the outer layer (Fig. 2). Each filament consists of tens of rods placed end to end. The diameter of the rods ranges from 1.5 to 2 μm, and their length from 8 to 10 μm. A fibrillar sheath forms around each single filament and thickens with time. It reaches 30 nm in filaments from the outer layer (i.e., young filaments) and 200 nm in those from the intermediate layer (i.e., old filaments). The empty bacterial sheaths that accumulate in the inner layer show similar thickness and texture to those observed in the intermediate layer.

When observed in vivo under an ICPM, the filaments are flexible. Large refringent granules typically occur intracellularly and are centrally aligned in the rod (Fig. 3A). TEM observations indicated that each rod is limited by a three-layered wall. The cytoplasm is finely reticulated, not dense to the electrons and contains various-sized open spaces (0.1 to 0.8 μm in diameter). The larger ones (0.4 to 0.8 μm in diameter) are aligned centrally and their number varies from 0 to 6 rod⁻¹ (Fig. 4). These spaces appear empty (not dense to electrons); their contents could have been extracted by the solvents used.
Fig. 1. *Echinocardium cordatum*. Dissection of a bacterial nodule drawn from the intestinal caecum showing central core (bivalve shell) and outer layer of the filamentous bacteria. Scanning electron microscope (SEM) view. cc: central core; c: coat of bacterial origin

Fig. 2. *Thiothrix* spp.-like bacteria. Filaments from the outer layer of a nodule. SEM view. Arrow head: rosette

Fig. 3. *Thiothrix* spp.-like bacteria. (A) Bacteria containing intracellular refringent granules centrally aligned. Interferential contrast phase microscopic (ICPM) view. Arrow head: intracellular granule. (B) Bacteria having lost their refringent granules. ICPM view

Fig. 4. *Thiothrix* spp.-like bacteria. Transmission electron microscopic (TEM) view. cv: centrally aligned vacuole-like spaces; pv: peripheral granules

Fig. 5. *Thiothrix* spp.-like bacteria. TEM view. m: mesosome-like structures; s: sheath
during dehydration and embedding processes. Some rods contain numerous small peripheral granules (50 to 70 nm in diameter) that are dense to the electrons. Some of these are close to the cellular membrane (Fig. 4). Most rods also contain mesosome-like structures that extend into the cytoplasm (Fig. 5).

Sulfide-oxidizing metabolism

The black color and the fetid odor of the intradigestive nodules of *Echinocardium cordatum* suggest they are filled with reduced sulfur compounds. Total S\(^{2-}\) content in the nodules is in fact very important as revealed by titrimetry (1 mM). However, the sulfide concentration effectively available to the bacteria should be much lower because part of the sulfide may form not mobilizable compounds (e.g., ferrous sulfide which should be responsible for the black nodule coloration).

The bacterial oxygen consumption was measured with two substrates (i.e., Na\(_2\)S and Na\(_2\)S\(_2\)O\(_3\)) added to a closed, thermostatically controlled chamber. Respiratory assessments were made on tens of parallel measurements. Fig. 6 is a characteristic curve obtained in one of those measurements (redrawn from recorder tracing) and reflects the general pattern of the curves. Dealing with different initial pO\(_2\) and different bacteria suspensions, no error bars are applicable.

Intact nodules had a high endogenous respiration rate (0 to 2nd min, Fig. 6). With no external reduced sulfur compounds added, the respiration rate of an intact nodule was 1.3 \(\mu\text{M} \text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}\). The endogenous respiration is probably due to the sulfide present in the nodules or to intracellular S\(^0\) oxidation. As long as the nodules remained intact, they consumed oxygen when reduced sulfur compounds were provided (2nd min, Fig. 6; addition of thiosulfate). After thiosulfate addition, the respiration rate of the cell suspension increased 50%. However, as soon as the nodules desintegrated due to the stirring (4th min, Fig. 6), the solution turned black from the ferrous sulfide present in the inner layers. The \(\text{O}_2\) consumption rose dramatically because of chemical oxidation of internal hydrogen sulfide, and the bacterial suspension became insensitive to further substrate injection (5th min, Fig. 6; addition of thiosulfate).

The endogenous oxygen consumption rate of the inner layers was 6.5 \(\mu\text{M} \text{ O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ proteins}\). This value was modified neither by the addition of 20 \(\mu\text{M S}^{2-}\), nor by boiling the sample. This shows that added sulfur concentrations were too low compared to the inner ones and that a spontaneous chemical process occurred at these high substrate concentrations. It appears that the inner layers and the core of the nodules contained ferrous and hydrogen sulfide which was responsible for the apparent endogenous respiration and which buffered the response to additional reduced sulfur compound after the breaking of the nodules. To avoid this, filamentous bacterial mats were separated from the inner layers of the nodules and were studied singly.

Bacteria taken apart from the nodules maintained a slight endogenous respiration. Whether or not this respiration corresponded to oxidation of internal sulfur or of remaining sulfide was not determined. Table 2 shows that maximal sulfide oxidation was observed at oxygen concentrations ranging from 90 to 170 \(\mu\text{M O}_2 \text{ l}^{-1}\).

The presence of intracellular elemental sulfur was investigated by spectrophotometry on three prepared sets of bacteria (see "Materials and methods"). The results are presented in Fig. 7. Freshly collected bacteria (i.e., Set 1) had a high content of elemental sulfur. When maintained in the absence of external sulfide, the bacteria (i.e., Set 2) mobilized the sulfur. The time course of the mobi-
ization was illustrated by the daily decrease of the intracellular sulfur. The relatively high confidence interval calculated for the three last measurement series is due to the experimental conditions which are far from optimal (bacteria were isolated from their nodule and stored in a container at 4°C). Those suboptimal conditions most probably stop metabolism and growth or enhance mortality at the end of the experiment and explain the disparity in the last measurements. Bacteria that had lost their elemental sulfur content (Set 3) again stored high quantities of elemental sulfur when they were provided with Na₂S. When observed microscopically (ICPM), bacteria from Sets 1 and 3 contained intracellular refringent granules that were centrally aligned (Fig. 3 A) while these granules were occasional or absent in bacteria from Set 2 (Fig. 3 B). These observations could indicate that the refringent granules correspond to the elemental sulfur stored intracellularly, as the presence of granules is closely related to that of external sulfide.

**Discussion**

The morphological characteristics of the symbiotic filamentous sheathed bacteria that occur in *Echinocardium cordatum* are those of the Beggioatoaceae. As pointed out by Strohl (1974): “members of this family are among the few bacteria to be recognized and differentiated mostly on the basis of their morphology”. They are “giant” rod-shaped bacteria, their width and length ranging, respectively, from 1 to 122 μm (Nelson et al. 1989) and from 2 to 10 μm. Cells are attached end-to-end to form long flexible filaments that are generally motile (“gliding bacteria”); this motility allows filaments to migrate towards adequate environments. The peripheral distribution of the bacteria and the subsequent layered structure of the nodules could result from a continuous centrifugal migration of the bacteria. The cells lack flagella and do not contain photosynthetic pigments (“colorless bacteria”). They accumulate elemental sulfur when external reduced sulfur compounds are present in their environment. Four genera of Beggioatoaceae are known (Strohl 1974) namely, *Beggioa, Thiotrix, Thioplaca* and *Thiospirilopsis*. The symbiotic bacteria that occur in *E. cordatum* have the very distinctive characteristics of the genus *Thiotrix* such as their size, the presence of a sheath, the number of filament per sheath (i.e., one), and the formation of rosettes. According to Harold and Stanier (1955), rosette formation is one of the most discriminatory features of the *Thiotrix* genus.

The distinctive morphological features of the symbiotic bacteria of *Echinocardium cordatum* allowed direct observations without obtaining pure cultures, yet with the assurance that the same type of bacteria were always involved. Investigations on the sulfur metabolism were therefore done directly on bacterial mats from the outer layers of the nodules. The spectrophotometry measurements demonstrate that the bacteria from *E. cordatum* are able to store and mobilize sulfur in the presence and absence of external reduced sulfur compounds. Their oxygen consumption also increases when reduced sulfur compounds are available. ICPM observations indicate that sulfur may be stored in the form of refringent granules aligned in the rods. In *Beggioata* spp. sulfur globules occur in the periplasmic space created by the invaginated cytoplasmic membrane (Lawrey et al. 1981, Strohl et al. 1981). A similar sulfur location was observed in unidentified sulfide-oxidizing bacterial symbionts of bivalves (Vetter 1985). When observed under a TEM, the symbionts of *E. cordatum* contained large vacuole-like spaces aligned in the rods, but no continuity with the periplasmic space was seen. There is no evidence that these “vacuoles” correspond to the refringent granules.

Previously reported symbioses between sulfide-oxidizing procaryotes and marine invertebrates generally involved rod- or sphere-shaped bacteria: bivalves harbor their symbionts within their gills (Vetter 1985, Reid and Brand 1986), oligochaetes harbour their bacteria in a subcuticular space (Giere and Langhein 1987), while pogonophorans house them within their trophosome (Cavanaugh et al. 1981). In polychaetes, various morphological types of bacteria simultaneously occur externally on the body wall (Gaill et al. 1984, 1987). The *Thiotrix* spp.-like bacteria associated with *Echinocardium cordatum* are extracellular and strictly confined to a particular diverticulum of the digestive tube, the intestinal caecum. *Thiotrix* spp.-like bacteria and the other Beggioatoaceae have not before been reported to be intradigestive. However *Thiotrix* spp. attached to the exoskeleton of mayfly larvae (Larkin et al. 1990), *Thiotrix* spp.-like epibacteria associated with the priapulid worm *Halicyrpus spinulosus* (Oeschger and Schmaljohann 1988), unidentified filamentous sulfur-containing bacteria attached to the parapodia of *Alvinella pompejana* (polychaete, Laubier et al. 1983) or to the periostracum of vents mussels (Jannasch and Wirsén 1981) have been described.

Beggioatoaceae characteristically occur in aquatic sulfide-rich environments (Bland and Staley 1978, Larkin 1980, Kuenen and Bos 1988). They have gained recent attention as microorganisms from the vicinity of deep-sea hydrothermal vents. In these areas, thick mats of predominate *Beggioata* spp.-like and *Thiotrix* spp.-like filaments represent sites of substantial chemosynthetic production and are actively grazed by a variety of invertebrates (Jannasch and Wirsén 1981, Jannasch and Motil 1985). In *Echinocardium cordatum, Thiotrix* spp.-like bacteria occur in the intestinal caecum where they form mats of entangled filaments around detrital particles. Both sulfide and oxygen are continuously supplied to the symbionts. Hydrogen sulfide (easily detectable by its pronounced smell) regularly forms in the ingested sediment that stagnates in the intestine (De Ridder et al. 1985 b, De Ridder 1986); another source of sulfide is the inner part of the nodule itself where bacterial sulfate-reduction occurs (Temara 1990). Oxygen is provided by the respiratory tube feet whose ampullae overlap the caecum in the general coelomic cavity. Oxygen presumably reaches the bacteria by diffusing through the caecal wall. The fact that symbiotic *Thiotrix* spp.-like bacteria only grow at the very periphery of the nodule indicates that their preferred environment is strictly localised. Such behaviour
has been described in *Beggiaota spp.* that live in the sedi-
ment at the oxidised-reduced interface and that are sub-
sequently called "gradient organisms" (Jørgensen and
Revsbech 1983).

The intradigestive ectosymbionts of *Echinocardium
cordatum* should protect host tissues against diffusion of
sulfide from the sediment stagnating in the hindgut. This
process is similar in effect to the one observed in marine
sediments by Jørgensen and Des Marais (1986) who de-
scribe the creation of a steep chemical gradient by *Beggia-
toa* spp. in a bacterial mat. This exclusion of *H₂S* has
been proposed to be important for animals harbouring
ectosymbiotic sulfur bacteria on their body surface; e.g.
a hydrothermal vent limpet (Burgh and Singla 1984), the
polychaete *Alvinella pompejana* (Prieur and Jeannelth
1987), the priapulid *Halicyryptus spinulosus* (Oeschger and
Schmaljohann 1988), the oligochaete *Tubificides benediti*
(Giere et al. 1988 b), and the nematode *Stibnometae*
(Ott and Novak 1989). Although Powell et al. (1979)
deny the occurrence of sulfide exclusion at the body wall
of meiofauna, their results confirm an uptake of labelled
sulfide by the prokaryotic epiphytes of the nematode
*Eubostrichus* spp.

Marine animals of sulfide-rich environment have de-
veloped various defense mechanisms permitting sulfide
tolerance (Somero et al. 1989). Those mechanisms have
been grouped into a two-“level” hierarchy of sulfide de-
fenses (Vismann 1991): peripheral mechanisms that pre-
vent *H₂S* to diffuse into the tissues, and internal mecha-
nisms that correspond to *H₂S* detoxification. *Echi-
ocardium cordatum* meets hydrogen sulfide either in its
surroundings, when burrowed below the oxidised-reduced
interface, or in its hindgut where the sediments are stag-
nating. According to Vismann’s classification, three pe-
ripheral defense mechanisms against hydrogen sulfide
might be recognized in *E. cordatum*. These are the re-
newed flow of well-oxygenated water circulating through
its burrow, the mucus layer that covers around the body
surface (it has already been suggested that mucus traps
*H₂S* externally in an oligochaete; Giere et al. 1988 b), and
the intradigestive sulfide-oxidizing ectosymbionts. The
mechanisms involving sulfide tolerance in *E. cordatum*
could be of ecological importance by extending the echi-
noid’s ecological niche, as has been demonstrated for the
polychaete *Nereis diversicolor* (Vismann 1990).

Whether or not the bacteria provide their host with
chemosynthetic food has not been investigated. A nutri-
tive role would, however, not be surprising as the intesti-
nal caecum is a strongly absorbent organ (De Ridder
1986, Temara 1990), and the symbiotic bacteria contain
an essential polyunsaturated fatty acid that the host must
have in its diet (Temara et al. 1991).

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