Thiomicrospira pelophila, gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium

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From marine mud flats a very thin, comma- or spiral-shaped bacterium was isolated. The new organism was an obligately chemolithotrophic sulfur bacterium. Its physiology was found to be essentially similar to that of *Thiobacillus thioparus*. Because of the spirillum-like appearance it was proposed to classify this bacterium into a new genus *Thiomicrospira*, with the species name *Tms. pelophila*. *Tms. pelophila* and a marine *T. thioparus*, which was isolated from the same mud, occupy different niches in this habitat. *Tms. pelophila* has a remarkable sulfide-tolerance as compared with *T. thioparus*. This property could be used for the specific enrichment of *Tms. pelophila*. The organism was also readily isolated in pure culture by filtering mud suspensions through a 0.22 μm membrane filter.

INTRODUCTION

In a study of the microbial flora of the intertidal mud flats of the shallow Dutch Waddenzee enrichments were made for colourless sulfur bacteria. Samples were plated on appropriate agar media. Among many typical colonies of thiobacilli we observed some very large colonies which appeared to contain small numbers of a bacterium with an unusual morphology. The spiral-shaped cells of this bacterium developed in enrichment cultures only at neutral pH in a mineral medium with a reduced sulfur compound as energy source, and a relatively high sulfide concentration. The isolation in pure culture and characterization of this organism will be described. The newly isolated organism has physiological properties similar to those of *Thiobacillus thioparus*, which appears to be very common in the same habitat. As it would be highly unlikely

that both organisms occupy the same ecological niche, a comparative study of the physiology and ecology of both organisms was made.

A short note on this organism has been published previously (Kuenen and Veldkamp, 1970).

MATERIALS AND METHODS

Isolation of the organisms. Synthetic seawater (see below) was supplemented with 0.8% (w/v) $Na_2S_2O_3 \cdot 5H_2O$ and occasionally with 0.01% (w/v) yeast extract. Conical flasks (100 ml) containing 40 ml of such media were inoculated with 20–30 g of a mixture of black and grey mud. The mud was collected from the mud flats occurring at low tide between the Frisian islands and the coastline of the Dutch province of Groningen. The enrichment cultures were incubated aerobically without shaking at 25C. Every two days samples were plated on the same medium supplemented with 1% agar (w/v) (Difco). Large colonies, sparsely populated with spirilla, developed after 2–5 days' incubation. The colonies were purified by preparing a dilution series in synthetic seawater–thiosulfate medium containing 0.8% (w/v) agar, which was subsequently poured into petridishes. Plates were incubated at 25C.

A marine strain of *T. thioparus* was isolated from the same mud and identified using Bergey's Manual of Determinative Bacteriology (Janke and Breed, 1957).

Maintenance of the organisms. Stock cultures were maintained in liquid medium consisting of $Na_2S_2O_3 \cdot 5H_2O$ (0.5% w/v) in synthetic seawater, on thiosulfate agar containing 0.8% (w/v) $Na_2S_2O_3 \cdot 5H_2O$, 1% agar in synthetic seawater, and in skimmed milk at —40 C. Liquid cultures were neutralized daily with sterile 0.4% (w/v) Na_2CO_3 solution. Bromothymol blue was used as an indicator.

Media. Synthetic seawater contained (% w/v): NaCl, 2.5; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.15; $CaCl_2$, 0.03; K_2HPO_4, 0.05 in deionised water. Of a trace element mixture described by Vishniac and Santer (1957), 0.2 ml was added per liter of medium. For cultivation of the marine *T. thioparus* the medium was supplemented with 0.25 mg $FeCl_3$/liter of medium and 0.03% K_2HPO_4 and 0.02% $KH_2PO_4 \cdot 3H_2O$ were used instead of 0.05% (w/v) K_2HPO_4. Vitamin B12 (Fluka) was included in the media as indicated. The medium was sterilized by autoclaving for 20 min at 120 C. Phosphate was sterilized separately. After autoclaving the pH was adjusted to 7.2 for the spirillum and 6.5 for *T. thioparus*.

Media for continuous culture. The medium used in the chemostat experiments

consisted of synthetic seawater supplemented with 0.8% $Na_2S_2O_3 \cdot 5H_2O$. The sodium thiosulfate was the growth-limiting factor. Increasing or lowering the thiosulfate concentration proportionally increased or lowered the cellular dry weight and protein. The medium was sterilized by filtration through a 0.15 micron pore size membrane filter (Sartorius, Göttingen, Germany).

All cultures were frequently checked for contaminants. Impurities in the cultures of the autotrophic spirillum could readily be detected by phase-contrast microscopy because of the unusual morphology of the spirillum. In addition cultures were streaked on thiosulfate agar and inoculated in yeast extract–acetate–glucose (YAG) medium. This medium contained (% w/v): sodium acetate or sodium lactate, 0.1; glucose, 0.2; yeast extract, 0.2; NaCl, 1.5; tap water; pH 7.0 Contamination was rare; when it occurred the cultures were discarded.

Growth conditions. Batch culture experiments were carried out in 500 ml erlenmeyer flasks containing 220 ml of medium and a few drops of a solution (1 mg/ml) of bromothymol blue indicator. The cultures were incubated at 28 C on a rotary shaker (Gyrotary shaker, New Brunswick, New Jersey, U.S.A.), and neutralized periodically with sterile 0.4 M Na_2CO_3 solution.

Continuous culture. In chemostat experiments the equipment used was similar to that described by Harder and Veldkamp (1967). The oxygen tension was monitored by an oxygen probe (New Brunswick, New Jersey, U.S.A.) and maintained between 40–60% air saturation.

The culture was automatically neutralized to pH 6.8 with 2 M Na_2CO_3 solution. At dilution rates below 0.25 hr^{-1}, the theoretical amount of sulfuric acid was produced from thiosulfate as measured by Na_2CO_3 needed for neutralization.

Respirometry. Respirometric studies were made using the conventional Warburg respirometer or a Gilson respirometer (Gilson Medical Electronics, Villiers-LeBel, France). Warburg flasks contained ca. 64 μg (dry weight) of cells from the chemostat ($D = 0.1$ hr^{-1}) in 2 ml synthetic seawater, and 0.2 ml containing 6 μM of sulfide, sulfur, thiosulfate or tetrathionate. All sulfur compounds were oxidized rapidly. Q_{O_2} values varied from 1500–3500 μl/hr/mg dry weight. It was found that centrifuging and washing the cells before use with synthetic seawater, reduced the Q_{O_2} values by 25%. Therefore samples to be used for respirometric studies were diluted with synthetic seawater directly after sampling, and used as soon as possible.

Estimation of maximum specific growth rate. Maximum specific growth rates (μ_{max}) were estimated from the critical dilution rate (D_c) in the chemostat. It was assumed, that when the dilution rate approaches D_c, the organism grows approximately at μ_{max} (Herbert, Elsworth and Telling, 1956). This ensured a

rapid estimation of μ_{max}, since under such conditions no sulfur is formed from thiosulfate, either by the spirillum or by *T. thioparus*.

Dry weight determination. Two hundred and fifty ml of a culture were centrifuged (12500 *g*, 45 min, 2 C) and the cells were washed twice with cold medium containing (% w/v): NaCl, 2.5; K_2HPO_4, 0.05 in deionized water; pH 7.5, for the spirillum and pH 6.8 for *T. thioparus*. The washed pellets were washed into weighing flasks with 10–12 ml deionized water, and dried overnight at 105 C. A correction for salt in the adhering water was made as described by Harder and Veldkamp (1967). Yields were expressed as grams dry weight per mole of thiosulfate consumed.

Protein estimation. Culture samples containing 0.3–1.5 mg of protein were centrifuged and washed twice to remove NH_4^+-ions. Protein was determined by a modified microbiuret procedure (Goa, 1953). In our modification the sample to which Benedicts' reagent had been added, was read against an identical sample to which an equivalent volume of water had been added, instead of the reagent. This procedure ensured reproducible results and was not interfered by sulfur. When growing chemolithotrophs on thiosulfate, very small amounts of sulfur nearly always precipitate. It appeared that the Lowry method (Lowry et al., 1951) could not be used since sulfur severely interfered.

DNA analysis. DNA of the spirillum and the marine *T. thioparus* were prepared as described by Marmur (1961). The T_m was determined in standard sodium chloride (SSC) (Marmur and Doty, 1962), using the following concentrations: 0.1, 0.5 and 1.0 SSC. The T_m increased linearly with the log of the SSC concentration. For the spirillum-DNA the T_m (1 SSC) was 89.0 C $\pm$ 0.1 C. The % G + C was calculated from this value using the equation of De Ley (1970), and found to be 48%. The same % was calculated from the density in a CsCl-gradient (Schildkraut, Marmur and Doty, 1962; De Ley, 1970), which was found to be $\rho = 1.708$. A DNA of $\rho = 1.725$ was used as a standard. The DNA of $\rho = 1.725$ was measured against DNA ($\rho = 1.703$) of *B. subtilis*. The T_m of the *T. thioparus*-DNA was 83.5 C $\pm$ 0.1 C which corresponds to a G + C% of 34%.

Sulfide gradients. Tall 500 ml glass cylinders were filled with 100 ml of synthetic seawater containing 1% (w/v) agar and 0.05%, 0.1%, 0.2%, 0.3%, 0.4% or 0.5% (w/v) $Na_2S{\cdot}9H_2O$ pH 7.2. The medium was heat-sterilized. Phosphate and sodium sulfide were sterilized separately. After solidification of the agar medium, 300 ml of sterile synthetic seawater, supplemented with 0.5% sodium thiosulfate, one drop of a 0.5% Na_2S solution, and bromothymol blue, was poured on top of the agar. The glass cylinders were incubated aerobically for one night before inoculation. Growth was estimated from direct counting in a counting chamber (Bürker-Türk, depth 0.02 mm). Samples for counting were

taken from three levels: just above the agar layer, in the middle of the liquid medium, and 0.5 cm below the surface.

RESULTS

Isolation. Synthetic seawater (pH 7.5) supplemented with 0.8% $Na_2S_2O_3$ and vitamin B12 (15 γ/liter) was added to conical flasks. These were inoculated with marine mud and incubated at 25C without agitation. The samples were collected from the mud flats on the North Sea coast. The enrichment cultures were inspected daily and showed the usual motile and non-motile rods. In addition a few very thin curved rods were sometimes encountered in cultures that were heavily inoculated with samples of rather black mud. In these cultures, especially when enriched with 0.01% yeast extract, sulfide producing *Desulfovibrio* developed in the mud layer. This appeared to stimulate the development of the thin spiral- or comma-like organism.

Enrichment cultures showing growth of this organism were plated out on a minerals–thiosulfate agar supplemented with vitamin B12. A few plates showed in addition to the usual *Thiobacillus* colonies, one or two larger colonies partly extending into the agar. These contained considerable amounts of sulfur and a few very thin, motile, comma- to spiral-shaped cells. However, when these colonies were transferred to agar plates of the same composition, no growth of the spirillum was obtained. Supplementing the agar medium with a wide variety of organic and inorganic compounds, as well as with mud extract failed to stimulate growth of the spirillum on plates.

Preparation of a dilution series in soft thiosulfate agar, poured into petridishes, appeared to be the only way to obtain the strikingly large colonies which again contained huge amounts of sulfur and a few very thin spirilla.

Initially, the isolation in pure culture was seemingly unsuccessful because the large colonies always were accompanied by small pinpoint-shaped sulfur-containing colonies of thin vibrio-like organisms. However, it soon became apparent that the high metabolic activity of this spirillum and its extreme acid intolerance caused a considerable variation in colony size and cell shape.

When the organism occurred in an enrichment in rather large numbers or was accompanied by large numbers of other thiosulfate-oxidizing bacteria such as thiobacilli, undiluted samples transferred to fresh solid medium resulted in rapid acidification of the agar. In such cases, no visible colonies were formed and diffuse growth in the agar was prevented.

In the dilution series in soft thiosulfate agar, those plates that received less than ca. 10 cells showed development of large colonies in the agar (Fig. 1).

Increasing numbers of cells in the inoculum reduced the colony size and eventually prevented colony formation. The development of the large colonies was made conspicuous by the extensive sulfur precipitation. Such colonies were very sparsely populated with spirilla, the diameter of which was so small that they could only be discerned by phase-contrast microscopy.

Once these particular characteristics of the organism were known, it appeared to be fairly easy to obtain a pure culture and reproducible enrichments. Also it appeared to be possible to trace very small colonies that occurred on plates that had been streaked from relatively densely populated enrichment cultures. However, in these plates the growth of the organism was inhibited by its own acid production or that of thiobacilli and the shape of the cells was not spirillum-like but rather that of a small vibrio. The pronounced tendency of producing involution forms under these conditions made the organisms difficult to recognize. Because of the extreme acid sensitivity, liquid cultures of the organism showed little or no turbidity. Even when neutralized manually at frequent intervals, cell yields were very low. In rapidly growing cultures, which were not neutralized frequently, growth was irreversibly inhibited and cells eventually lysed completely at low pH. The only way to obtain good and reproducible growth of the spirillum was to grow it in a chemostat with appropriate pH control.

A more rapid and specific enrichment method based on the tolerance of the spirillum to high sulfide concentrations will be described below.

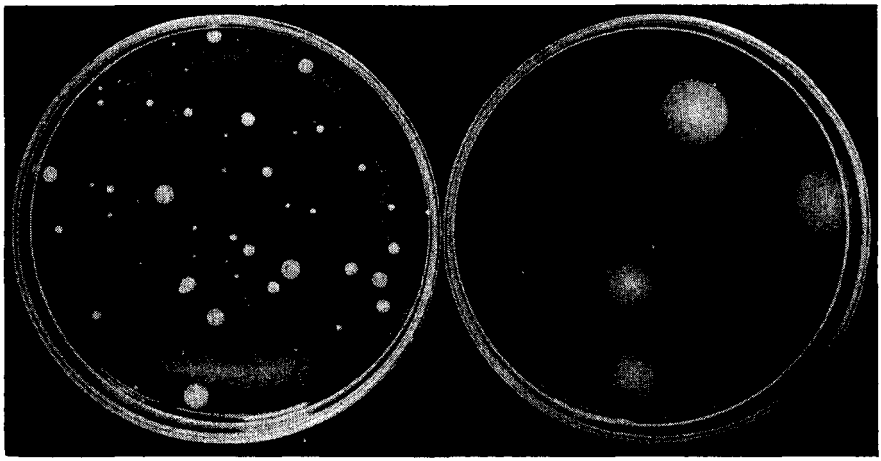

Fig. 1. 4-Day-old colonies of the isolated spirillum on two successive 10 cm plates of a dilution series in soft thiosulfate agar.

It appeared that the spirillum could be isolated very easily and rapidly by filtering a mud suspension through a 0.22 μm pore size membrane filter. In order to allow the organism to penetrate the filter, samples were taken after 30 min filtering with mild suction. A dilution series from this filtrate made in soft thiosulfate agar medium, showed the exclusive development of the spirillum.

Physiology. In contrast to its morphology, the physiology of the new isolate appeared to be similar to that of the obligately chemolithotrophic thiobacilli (Rittenberg, 1969).

Carbon dioxide could be used as the sole carbon source and was the main carbon source under all growth conditions. In one strain, growth appeared to be vitamin B12-dependent.

Energy was derived from the oxidation of reduced inorganic sulfur compounds only. Respirometric studies showed Q_{O_2} values of 1000–3500 μl/hr/mg dry weight for sulfide, sulfur, tetrathionate and thiosulfate. No growth was observed on organic media without this energy source. The maximum specific growth rate (μ_{max}) in autotrophic medium was 0.35 hr^{-1} at 28 C, and could not be increased by adding organic compounds.

Optimal growth of the organism occurred at neutral pH. In non-buffered cultures the pH dropped to 6.0–5.0, depending on the growth conditions. In cultures with high thiosulfate concentrations or a low oxygen tension a heavy precipitation of sulfur occurred.

When the spirillum was grown autotrophically in a thiosulfate-limited chemostat at pH 6.8, its cell yield was practically similar to that of a marine *T. thioparus* strain. The figures were 4.66 and 4.78 grams/mole thiosulfate respectively, at a dilution rate of 0.1 hr^{-1}.

The uptake of organic compounds in the spirillum showed the same pattern as was found in the obligately chemolithotrophic thiobacilli (Rittenberg, 1969). In addition, it was found that several organic compounds, such as acetate and succinate, caused a significant increase (10–30%) in dry weight and cell protein when the spirillum or *T. thioparus* were cultivated under energy limitation in a chemostat (Kuenen and Veldkamp, 1970, 1971). Further details on the uptake of organic compounds by the spirillum and the obligately chemolithotrophic thiobacilli will be published elsewhere.

Effect of sulfide on the growth of T. thioparus and the spirillum, and its use in enrichment procedures. The development of the spirillum in the initial enrichment cultures seemed to be promoted by the presence of sulfide produced by *Desulfovibrio*. To investigate the effect of sulfide on the growth of both the spirillum and the marine *T. thioparus* strain, the organisms were grown in a series of sulfide gradients in tall glass cylinders. These contained a bottom layer of agar supplemented with increasing amounts of sulfide. The agar layer was

covered by a column of liquid minerals–thiosulfate medium exposed to air (see also Materials and Methods).

At the highest sulfide concentration used (0.5% w/v) the spirillum formed a whitish veil of sulfur on the surface of the liquid and then developed homogeneously throughout the liquid medium. Excellent growth within 1–2 days, as measured by direct counting was observed. Concomitantly a large amount of sulfur was formed. *T. thioparus*, however, did not show any growth at this sulfide concentration (Fig. 2). After the sulfide concentration in the liquid medium had dropped (2–4 days incubation), due to gas exchange with the air, *T. thioparus* developed nearly exclusively at the surface of the liquid medium.

In contrast to the observations at high sulfide concentrations, we found excellent growth of *T. thioparus* in the same medium containing low concentrations of sulfide. At concentrations in the agar layer of 0.05% and 0.1% sulfide, *T. thioparus* grew well on the surface and throughout the liquid column at a faster rate than the spirillum.

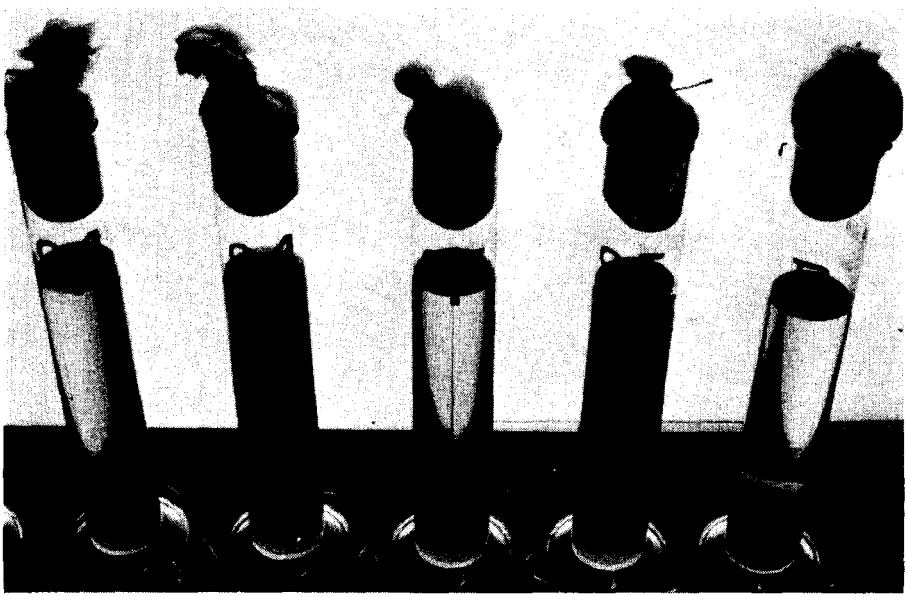

Fig. 2. Cultures of the spirillum (S) and the marine *T. thioparus* (T) in sulfide gradients. The gradients were prepared in 500 ml glass cylinders by pouring thiosulfate medium on top of an agar layer containing sulfide (see Materials and Methods). The figures 0.4 and 0.5 indicate the percentage sulfide in the agar layer.

The photograph was taken 2 days after inoculation and shows growth and sulfur formation in the spirillum cultures. Slight growth of *T. thioparus* could be observed at the surface of the liquid medium only. B is a blank gradient which was not inoculated.

From these experiments it was concluded that the spirillum developed better at higher sulfide concentrations than *T. thioparus*. On the basis of these observations a rapid enrichment procedure was developed for the spirillum. A sulfide gradient prepared as described above, using an agar layer containing 0.5–0.7% (w/v) of $Na_2S \cdot 9H_2O$ showed development of spirilla within 24 hr when inoculated with a small amount of black marine mud. This enrichment procedure is fairly specific for *Tms. pelophila*. When samples were taken from the gradient 24 hr after inoculation with mud and a dilution series was made in soft thiosulfate agar, generally 50–80% of the developing colonies were those formed by *Tms. pelophila*.

Some salient properties of the spirillum and *T. thioparus* are summarized in Table 1.

Table 1. Summary of some characteristics of the isolated spirillum and *T. thioparus*

	spirillum	*T. thioparus* (marine strain)
Morphology	spiral 0.2 × 1–4 μm	rod 0.4 × 2 μm
Temperature optimum	28–30 C	28–30 C
pH-Range	5.0–8.5	4.0–7.5
pH-Optimum	6.5–7.5	6.0–7.0
Maximum specific growth rate (28 C) in mineral thiosulfate medium	0.35±0.02 (t_d = 2 hr)	0.45±0.04 (t_d = 1.5 hr)
Reduced inorganic sulfur compound as energy source	obligate	obligate
Oxidation of thiosulfate	+	+
Oxidation of sulfide	+	+
Oxidation of sulfur	+	+
Oxidation of tetrathionate	+	—
Sulfur formation from excess thiosulfate	++	++
Main carbon source	CO_2	CO_2
Cell yield increase with 1 mM acetate (dry weight)[1]	15%	14%
Protein increase with 1 mM acetate[1]	12%	17%
Sulfide tolerance	high	low
NaCl requirement	obligate	obligate
G + C%	48%	34%

[1] Kuenen and Veldkamp, 1971.

Description of the new chemolithotrophic sulfur bacterium

The cells are spiral-shaped when cultured at neutral pH (Fig. 3). The width of the cells is 0.2 micron; the length 3–4 micron. Vibrio-shaped cells are formed in non-buffered media; the dimensions then are 0.2 × 1–2 micron. Long spirilla

(10–20 micron) are sometimes encountered in media with suboptimal concentrations of different constituents.

The cells are non-sporeforming.

Motile strains have a polar flagellum (Fig. 4). Non-motile strains are easily selected in thiosulfate-limited continuous cultures.

On sparsely seeded minerals–thiosulfate agar, colonies develop after 2–4 days'

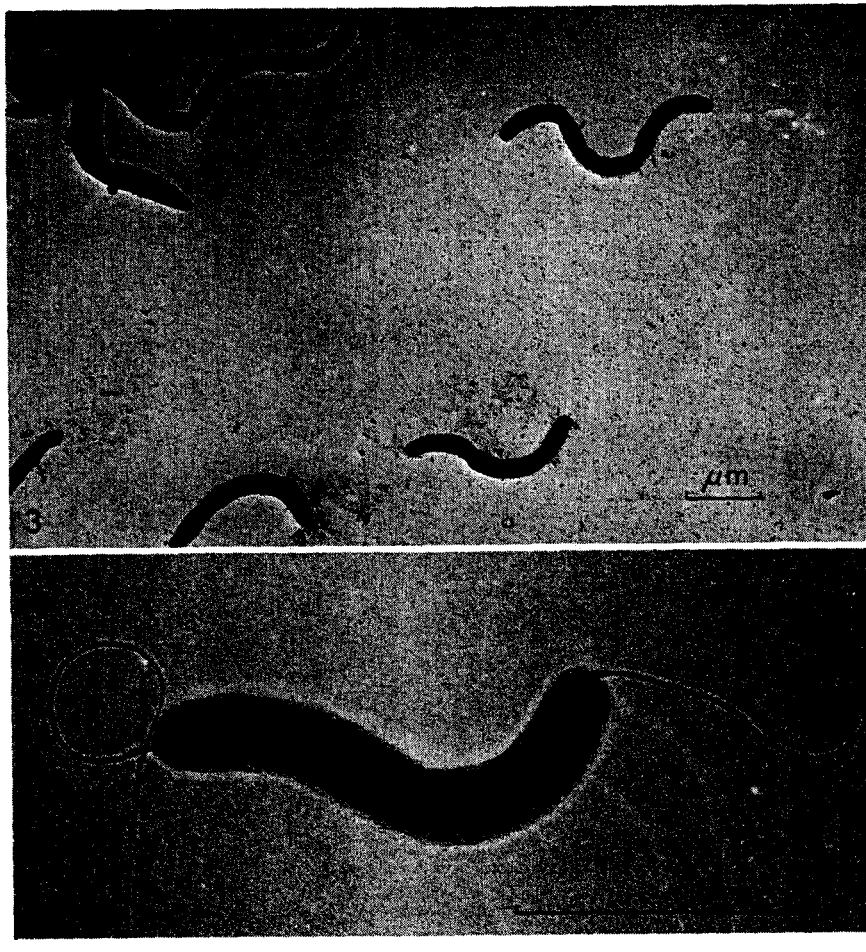

Fig. 3. Electron micrograph of a culture of the spirillum showing vibrio- and spirillum-shaped cells. Cells were fixed in osmium tetroxide and shadowed with carbon.
Fig. 4. Electron micrograph of a flagellated cell of the spirillum. The cell was fixed in aldehyde and shadowed with platinum.

incubation at 28 C as small white pinpoints, partly growing in the agar, and surrounded by a small halo of precipitated sulfur. If the organism is suspended in soft agar (0.8% w/v), large diffuse sulfur-containing colonies, up to 1 cm in diameter, are formed.

Marked sulfide tolerance as compared to *Thiobacillus* species.

The optimum temperature for growth is 28–30 C.

Cells can grow autotrophically in inorganic media, showing an obligate need for carbon dioxide as C-source, and reduced inorganic sulfur compound as energy source. In addition to thiosulfate, sulfide, sulfur and tetrathionate can be used as energy source.

In neutralized cultures the theoretical amount of sulfuric acid is produced from the oxidation of thiosulfate. At high thiosulfate concentrations, low pH or at oxygen tensions below 20% air saturation, sulfur is precipitated in thiosulfate-containing cultures.

Growth occurs in the pH range 5.0–8.5. The pH optimum is 6.5–7.5. Cells may lyse at pH < 5.0.

The maximum specific growth rate in autotrophic growth medium is about 0.35 hr^{-1} at 28 C. Although carbon dioxide is the main carbon source under all growth conditions, various organic carbon compounds can be incorporated to a limited extent. Enrichment of the autotrophic medium with acetate (1 mM) does not increase the maximum specific growth rate. It does, however, increase the total yield of structural cell material in continuous culture if the energy source (thiosulfate) is growth-limiting (Kuenen and Veldkamp, 1970, 1971).

The relative guanine + cytosine content (G + C) of the DNA is 48%. (See also Materials and Methods).

The marine organism needs 1.5–3% NaCl.

Habitat: marine mud flats in which sulfide is produced.

DISCUSSION

Isolation and properties of the spirillum

Growth in enrichment cultures of the obligately chemolithotrophic spiral-shaped organism described above, was observed only when the medium was heavily inoculated with black marine mud.

Sulfide production by *Desulfovibrio* which appeared to promote the development of the spirillum, was stimulated by adding yeast extract and vitamin B12 to the medium. Using this enrichment method, it has been possible to reisolate the spirillum several times at different intervals during three years.

At present the enrichments for the spirillum are made in the 'sulfide gradients'

described above, because it was found that growth of the spirillum was invariably excellent under these conditions. This property might enable the spirillum to survive purification procedures for *Desulfovibrio*. For instance, the method described by Postgate (1965) which prescribes the use of 5 ml of sulfide agar in tubes exposed to air would not exclude growth of our aerobic spirillum. In fact, one of Baars' cultures of *Desulfovibrio desulfuricans* might have been contaminated by an organism similar to our organism. One of his photomicrographs (Baars, 1930, Plate 1, Fig. 5) shows very thin spirilla which are strikingly similar to our organism which are at least four times smaller than the *Desulfovibrio* cells present in the same picture. It seems recommendable to check *Desulfovibrio* cultures for autotrophic contaminants, in addition to the usual controls performed by streaking samples on organic agar media.

The cell yield of the spirillum cultures which are not neutralized frequently is very low as compared to *T. thioparus*. However, satisfactory yields were obtained in automatically neutralized cultures, as is shown by the results of continuous cultivation.

No growth was observed on any purely organic growth medium. Organic compounds added to the autotrophic medium do not enhance the maximum specific growth rate as is found in mixotrophic thiobacilli such as *T. intermedius* (London, 1963).

Since an inorganic reduced sulfur compound is the obligate energy source and autotrophic growth is possible in inorganic media, our organism belongs to the obligately chemolithotrophic colourless sulfur bacteria as defined by Rittenberg (1969).

Growth of one strain is vitamin B12-dependent. The B12-requirement of this strain is not surprising since vitamin B12 is nearly always present in marine environments (Duursma, 1965). The same dependency has also been demonstrated for *Chlorobium* species (Pfennig and Lippert, 1966) and many other bacteria living in mud and natural waters.

Taxonomy

In some aspects the properties of our bacterium are reminiscent of *Thiobacillus coproliticus* (Lipman and McLees, 1940). *T. coproliticus* has the same varying cell shape and also shows no turbidity when grown in a non-neutralized thiosulfate medium. The "regenerative bodies", as described by Lipman and McLees probably represent lysing cells. Similar involution forms occur in cultures of our spirillum and are characterized by spherical extrusions of the curved cells. A major difference, however, is the failure of our organism to grow mixotrophically, i.e. on peptone–soil extract, as was observed in *T. coproliticus*.

In the review of Vishniac and Santer (1957) it was proposed to exclude *T. coproliticus* from the genus *Thiobacillus* because of the poor growth in non-neutralized thiosulfate media. However, in view of our results, it seems likely that the genus *Thiobacillus* may contain organisms which are extremely sensitive to acid.

The G + C content of the genus *Thiobacillus* covers a wide range. Jackson, Moriarty and Nicholas (1968) reported values from 51% to 67%. The following percentages were found for *T. thioparus*: 68–70% (Marmur, Falkow and Mandel, 1963), 63–66% (cf. Jackson et al., 1968), 52–53% (Matin, 1969). Our marine *T. thioparus* strain has a G + C content as low as 34% (see Materials and Methods). This means that the genus contains strains which are physiologically similar but are genetically not really closely related. Our spirillum is physiologically similar to *T. thioparus* and has a G + C content of 48%, and for these reasons might be included provisionally in the genus *Thiobacillus*. However, when the organism is cultivated in the chemostat under optimum growth conditions with controlled pH, its morphology is so strikingly different from *T. thioparus* grown under similar optimal conditions, that the genus *Thiobacillus* would have to be redefined if our organism were included in this genus. We prefer to create a new genus to classify our organism, named *Thiomicrospira*, with the type species *Tms. pelophila*. The generic definition is as follows.

Thi.o.mi.cro.spi'ra. Gr. noun *thium* sulfur; Gr. adj. *micros* small, little; Gr. *spira* a spiral. *Thiomicrospira* generic name: a small sulfur spiral.

Small spiral-shaped cells forming long screws or portions of a turn. Dimensions: 0.2–0.3 micron wide and at least 1–2 micron long. Involution forms may be 20–30 micron long. Motile by means of a polar flagellum. Non-motile mutants are easily formed in the laboratory. No sulfur granules formed within the cells. Energy derived from the oxidation of reduced inorganic sulfur compounds, principally from sulfide, sulfur and thiosulfate. The principal product of oxidation is sulfate, but sulfur is formed in growth media containing more than 0.2–0.3 mM thiosulfate. Carbon dioxide can be used as the sole carbon source and is under any growth condition the major C-source. Organic compounds can be metabolized to a limited extent.

The type species of the genus *Thiomicrospira* is the obligately chemolithotrophic organism described above, which has a G + C content of 48%, and for which we propose the specific name *Thiomicrospira pelophila*. Gr. noun *pelos* mud; Gr. adj. *phila* loving; *pelophila*: mud loving.

Because of its type of metabolism we propose to assign the genus *Thiomicrospira* to the family of the *Thiobacteriaceae*.

Ecology

The isolation from marine muds of an organism having characteristics similar to *T. thioparus* was very interesting from an ecological point of view.

As stated in the introduction it was unlikely that both organism would have the same ecological niche. Table 1 shows that *T. thioparus* and *Tms. pelophila* have physiological characteristics that are practically similar.

A conspicuous difference between *T. thioparus* and *Tms. pelophila* is the cell shape. One could easily imagine that the curved shape and extreme thinness of the motile *Thiomicrospira* would facilitate the penetration by this organism into very small capillaries between mud particles as compared with *T. thioparus*. This is further substantiated by the finding that *Tms. pelophila* could be isolated directly from mud in pure culture by filtering a mud suspension through a 0.22 μm filter. However, from an ecological point of view, probably the most important difference between both species is the higher sulfide-tolerance of *Tms. pelophila*. In the marine mud, a continuous production of sulfide occurs due to the activity of sulfate-reducing bacteria. Since the surface of the mud is aerobic a sulfide- and oxygen-gradient tends to establish in the upper layer of the mud. In the interface where sulfide, or sulfur, and oxygen are both present, colourless sulfur bacteria thrive. In this area the differences in ability to grow at relatively high sulfide concentrations or low oxygen tensions will be of a decisive importance in competition. The difference in the behaviour towards sulfide of *Tms. pelophila* and *T. thioparus* provides an explanation for the coexistence of both organisms in the same habitat in which these organisms occupy different niches.

In this context it should be emphasized that in this habitat both the obligately chemolithotrophic thiobacilli and *Tms. pelophila* are not completely restricted to the use of carbon dioxide as carbon source. As mentioned before, organic acids like acetate and succinate have an energy-sparing effect when growth of these organisms is limited by the energy source. Under natural conditions the uptake of such compounds might also be important. In the anaerobic mud layer the breakdown of carbohydrates will lead to fermentation products such as acetate and succinate. In addition acetate is one of the main end products of the anaerobic respiration of *Desulfovibrio*. The obligately chemolithotrophic sulfur bacteria living in the interface of anaerobic and aerobic conditions may locally be limited in their growth by lack of either oxygen or energy source. In such cases the ability to utilize organic acids certainly may have survival value for the obligate chemolithotrophs.

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