Search for unusual chemolithotrophic lifestyles: Oxidation of inorganic sulfur compounds coupled to manganese reduction

Growth cycle and "ixotrophy" of *Saprospira grandis*

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Abstract.

The enrichment of as yet unknown chemolithotrophic microbes that link the oxidation of inorganic sulfur compounds to the reduction of Mn(IV) was attempted. In a second project, the growth cycle of *Saprospira grandis*, a gliding bacterium that kills and lyses prey bacteria, was investigated using microscopical techniques.

1. Oxidation of inorganic sulfur compounds coupled to manganese reduction

Introduction.

Chemolithotrophy, the production of energy and reduction equivalents from inorganic compounds such as H_2, H_2S, NH^{4+}, and Fe^{2+}, is widespread in nature (for review, see Kuenen, 1999). Many chemolithotrophs use oxygen as terminal electron acceptor, but some facultatively anaerobic chemolithotrophs, for example *Thiobacillus denitrificans*, can also use nitrate or nitrite. Reduced inorganic sulfur compounds are abundant in the anoxic zone of microbial mats and marine sediments, mainly due to the activity of sulfate-reducing microorganisms, yet little is known about the oxidation of these compounds coupled to metal reduction (for review, see Jorgensen, 1987). In turn, manganese and iron reduction has been described for organisms that utilize hydrogen, organic acids, or aromatic compounds as electron donors (Lovley, 1991). There is preliminary evidence for the complete oxidation of S^0 or various sulfide forms in marine sediments by as-yet-unidentified microorganisms using Mn^{4+} as an electron acceptor (Aller and Rude, 1988). However, abiological reduction of Mn(IV) by sulfide does occur. In this study, it was attempted to enrich for microorganisms that combine both metabolic capabilities, i.e. the oxidation of reduced sulfur compounds coupled to the oxidation of manganese.

Materials and Methods.

Media and Chemicals. Saltwater medium containing 28 mg/l Na_2SO_4, 0.2 g/l KH_2PO_4, 0.25 g/l NH_4Cl, 20 g/l NaCl, 3.0 g/l $MgCl_2 \cdot 6\ H_2O$, 0.5 g/l KCl, and 0.15 g/l $CaCl_2 \cdot 2\ H_2O$ was prepared in a Widdel flask under anaerobic conditions. CO_2-saturated $NaHCO_3$ buffer was added at 30 ml/l final concentration, trace element solution, 10 vitamin solution, and Vitamin B12 solution were added at a final concentration of 1.0 ml/l. MnO_2 was synthesized as described (Kostka and Nealson, 1998). MnS was a gift from course member Rachel Whitaker. Sulfur flowers were prepared by shaking yellow sul (Sigma) in a Pfennig bottle containing glass beads.

Isolation and Enrichment. Samples were collected from the black, anoxic zone of microbial mats at two different locations (designated "1" and "2" in Tables 1, 3 and 5) at the Sippewisset Salt Marsh near Woods Hole, MA. All samples had a characteristic sulfide smell. For the enrichment of sulfide reducers, a gradient was established in hungate tubes by stacking different layers of agar (all in saltwater medium, Figure 1): The bottom layer contained 4 ml of 0.8 % agar and 20 mM Na_2S, the middle "buffer" layer contained 1 ml of 0.8 % agar, and the top layer contained 8 ml of approx. 1 g/l MgO_2 and 100 ul of inoculum taken from a slurry of Sippewisset mud. NaAcetate (Ac) and 2,6-anthraquinone disulfonate (AQDS) were added at 200 µM and 1 mM final concentration, respectively, where indicated (Table 1). The oxygen atmosphere above the agar was replaced with CO_2/N_2. For reinoculation of gradient tubes (Table 2), the concentrations of Na_2S and MnO_2 were 10 mM and approx. 2 g/l, respectively, and

AQDS was omitted. After five days, plugs of agar containing microorganisms were extracted using a pasteur pipet. The plugs were resuspended in 300 µl sterile seawater and 50 µl of suspension used for inoculation of new gradient tubes. For the enrichment of thiosulfate and sulfur oxidizers, Hungate tubes were used, filled with liquid medium identical in composition to that described above. Instead of Na_2S, NaS_2O_3 was added at a final concentration of 1.5 mM, or a few sulfur flowers were added. For reinoculation, 500 µl of culture were transferred to fresh medium. See Tables 1-6 for the various conditions.

Measurement of sulfate. Sulfate concentrations in thiosulfate enrichment cultures were measured using the barium sulfate turbidity method as described (Kelly and Wood, 1998).

Results and Discussion.

Rationale. Enrichment tubes for sulfide, thiosulfate, and elemental sulfur oxidizers were set up as shown in Tables 1-6. Initial enrichments were done in the presence of both electron donor and acceptor. A soluble electron shuttle, AQDS, was added to some tubes to facilitate the transfer of electrons to the insoluble manganese. Acetate was included as a carbon source to allow for the potential enrichment of chemolithoheterotrophs. To delineate the metabolic requirements of the organisms that emerged from the initial enrichments, successive enrichments were done in the absence of either electron donor or electron acceptor, and in the presence or absence of both donor and acceptor, respectively. AQDS and acetate were omitted, because microbial growth in the initial enrichments occurred in the absence of either compound. Acetate was only included to allow for the possible enrichment of thiosulfate reducers that may utilize acetate as electron donor and carbon source (Table 4, tubes 6 and 11, and Table 6, tubes 5 and 10).

Sulfide oxidizers. In the gradient tubes for sulfide-oxidizers (Tables 1 and 2), the color of MnO_2 changed from brown to clear, beginning at the bottom of the top agar layer, at an approx. rate of 1 cm/day. This is indicative of the establishment of a diffusion gradient of H_2S concomitant with the successive reduction of Mn^{4+}. In our hands, initial test-tube experiments with commercially available MnO_2 (Sigma) showed that it is relatively stable against reduction by Na_2S at neutral pH. Freshly synthesized MnO_2, which was used in our enrichments due to increased bioavailability, however, was immediately converted to MnS. Distinct bands of microbial growth formed at various distances within the top agar layer a few days following inoculation. Microscopic observation of these bands and of samples from liquid enrichments revealed cocci, diplococci, short and long rods. All morphotypes contained motile and apparently immotile species. A particular type of long rods was found to move in a spirochete-like fashion. The number of cells varied considerably among enrichment tubes, but was estimated to be between 10^4 and 10^5 cells/ml on average. In a few tubes, a narrow brown Mn layer (5-10 mm), remained and was apparently not reduced by sulfide over time. This may indicate the presence of O_2 in the top layer of the agar, which may readily reduce diffusing sulfide and therefore prevent the reduction of Mn^{4+}. The observed bands

of microbial growth, which in some enrichments formed exactly at the interface between reduced and oxidized manganese (Table 2, tubes 9, 14, and 18) may therefore contain microaerophilic organisms. The involvement of sulfide in the metabolism of these microbes is unlikely, because bands formed in the absence of S^{2-} as well (Table 2, e. g. tubes 6 and 8). We therefore propose that the enriched organisms represent agar-degrading microaerophiles rather that *Beggiatoa*-like H_2S oxizers. In any case, the desired chemolithotrophic lifestyle, oxidation of reduced sulfur compounds coupled to Mn reduction, was not obtained.

Thiosulfate and elemental sulfur oxidizers. Microbial growth occurred in all tubes, which did not allow us to determine particular metabolic requirements of the enriched organisms. Various morphological types were observed in a single enrichment culture without any obvious correlation between enrichment conditions and morphotype. For the elemental sulfur oxidizers, microbial growth was observed in uninoculated control tubes. This can probably be attributed to the source of sulfur, which was not sterile. For some enrichments, sulfate concentrations were measured. Sulfate production was particularly high in thiosulfate containing enrichments, and a characteristic sulfide smell was detectable in all enrichments but those containing manganese. Sulfide was probably also generated in these samples, but immediately precipitated by forming insoluble MnS. Microbial growth and sulfate or sulfide production in subsequent enrichments lacking thiosulfate or sulfur may stem from carry-over of these compounds during reinoculation. Evidence therefore points towards a disproportionation ("fermentation") of thiosulfate, i.e. the oxidation of thiosulfide to sulfate, and its reduction to sulfide. The same can be envisioned for the elemental sulfur enrichments, although sulfate concentrations were not measured.

Acknowledgements.

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Table 1. Initial enrichment conditions for sulfide oxidizers.

Tube	Inoculum	pH	[H]-donor	[H]-acceptor	C-source	Observations		
						Band(s)	Cells	Remark
1	none	7.2	S^{2-}	Mn^{4+}	CO_2	-	-	
2	none	7.2	S^{2-}	Mn^{4+}	CO_2/Ac	+/-	-	
3	none	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2	+/-	-	
4	none	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2/Ac	+/-	-	
5	1	7.2	S^{2-}	Mn^{4+}	CO_2	+ (1,3)	+	
6	1	7.2	S^{2-}	Mn^{4+}	CO_2/Ac	+ (1)	+	
7	1	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2	+ (2)	+	
8	1	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2/Ac	+ (5-9)	+	Mn left
9*	2	7.2	S^{2-}	Mn^{4+}	CO_2	+ (1,3)	+	
10*	2	7.2	S^{2-}	Mn^{4+}	CO_2/Ac	+ (4-8)	+	Mn left
11	2	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2	+ (2,6)	n/d	
12	2	7.2	S^{2-}	Mn^{4+}/AQDS	CO_2/Ac	+ (1)	n/d	
13*	1	6.8	S^{2-}	Mn^{4+}	CO_2	+ (4,8)	+	Mn left
14	1	6.8	S^{2-}	Mn^{4+}	CO_2/Ac	+? (0)	n/d	
15	1	6.8	S^{2-}	Mn^{4+}/AQDS	CO_2	+? (0)	n/d	
16	1	6.8	S^{2-}	Mn^{4+}/AQDS	CO_2/Ac	1? (0)	n/d	

+/- indicates the presence/absence of bands or cells.
The number(s) in parentheses indicate the distance (in mm) of the respective band(s) below the agar/air interface.
n/d, not done.
(for all tables)

Table 2. Enrichment conditions for sulfide oxidizers, reinoculation.

Tube	Inoculum from tube	pH	[H]-donor	[H]-acceptor	C-source	Observations: Band(s)	Cells	Remark
1	-	7.2	S^{2-}	Mn^{4+}	CO_2	-	-	
2	9	7.2	-	-	CO_2	+ (0,2)	+	
3	9	7.2	S^{2-}	-	CO_2	+ (0)	+	
4	9	7.2	-	Mn^{4+}	CO_2	+ (1)	+	
5	9	7.2	S^{2-}	Mn^{4+}	CO_2	+ (1)	+	
6	10	6.8	-	-	CO_2	+ (2,3)	+	
7	10	6.8	S^{2-}	-	CO_2	+ (5)	+	
8	10	6.8	-	Mn^{4+}	CO_2	+ (8)	+	
9	10	6.8	S^{2-}	Mn^{4+}	CO_2	+ (10,15)	+	Mn (10)
10	10	7.2	-	-	CO_2	+ (1)	n/d	
11	10	7.2	-	-	CO_2/Ac	+ (0)	n/d	
12	10	7.2	S^{2-}	-	CO_2/Ac	+ (1)	n/d	
13	10	7.2		Mn^{4+}	CO_2/Ac	+ (1)	n/d	
14	10	7.2	S^{2-}	Mn^{4+}	CO_2/Ac	+ (1)	n/d	Mn (1)
15	13	7.2	-	-	CO_2	+ (2)	n/d	
16	13	7.2	S^{2-}	-	CO_2	+ (0)	n/d	
17	13	7.2	-	Mn^{4+}	CO_2	+ (5)	n/d	
18	13	7.2	S^{2-}	Mn^{4+}	CO_2	+ (5)	n/d	Mn (5)

Table 3. Initial enrichment conditions for thiosulfate oxidizers.

Tube	Inoculum	pH	[H]-donor	[H]-acceptor	C-source	Observations		
						Tube	Cells	Remark
1	-	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	clear	-	unsol. material fine grains (tubes 1–4)
2	-	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2/Ac	clear	n/d	
3	-	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2	clear	n/d	
4	-	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2/Ac	clear	n/d	
5*	1	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	clear	+	unsol. material larger flakes (tubes 5–12)
6	1	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2/Ac	clear	++	
7	1	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2	clear	+	
8	1	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2/Ac	clear	+	
9*	2	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	clear	+	
10	2	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2/Ac	clear	n/d	
11	2	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2	clear	++	
12	2	7.2	$S_2O_3^{2-}$	Mn^{4+}/AQDS	CO_2/Ac	yellow	+	

Table 4. Enrichment conditions for thiosulfate oxidizers, reinoculation.

Tube	Inoculum from tube	pH	[H]-donor	[H]-acceptor	C-source	Observations		
						Cells	$[SO_4^{2-}]$	Remark
1	-	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	-	1 x	
2	5	7.2	$S_2O_3^{2-}$	-	CO_2	+	**6.3 x**	H_2S smell
3	5	7.2	-	Mn^{4+}	CO_2	+	3.8 x	
4	5	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	+	**6.9 x**	
5	5	7.2	-	-	CO_2	+	3.1 x	H_2S smell
6	5	7.2	(Ac)	$S_2O_3^{2-}$	CO_2/Ac	+	**10.3 x**	H_2S smell
7	9	7.2	$S_2O_3^{2-}$	-	CO_2	n/d	n/d	
8	9	7.2	-	Mn^{4+}	CO_2	n/d	n/d	
9	9	7.2	$S_2O_3^{2-}$	Mn^{4+}	CO_2	n/d	n/d	
10	9	7.2	-	-	CO_2	+	n/d	
11	9	7.2	(Ac)	$S_2O_3^{2-}$	CO_2/Ac	n/d	n/d	

Table 5. Initial enrichment conditions for elemental sulfur oxidizers.

Tube	Inoculum	pH	[H]-donor	[H]-acceptor	C-source	Observations		
						Tube	Cells	Remark
1	-	7.2	S^0	Mn^{4+}	CO_2		+	???
2	-	7.2	S^0	Mn^{4+}	CO_2/Ac		++	
3*	1	7.2	S^0	Mn^{4+}	CO_2		++	
4	1	7.2	S^0	Mn^{4+}	CO_2/Ac		++	
5*	1	6.8	S^0	Mn^{4+}	CO_2		+	
6	1	6.8	S^0	Mn^{4+}	CO_2/Ac		n/d	

Table 6. Enrichment conditions for elemental sulfur oxidizers, reinoculation.

Tube	Inoculum from tube	pH	[H]-donor	[H]-acceptor	C-source	Observations		
						Tube	Cells	Remark
1	-	7.2	S^0	Mn^{4+}	CO_2		+	
2	3	7.2	S^0	-	CO_2		+	H_2S smell
3	3	7.2	-	Mn^{4+}	CO_2		+	
4	3	7.2	S^0	Mn^{4+}	CO_2		+	
5	3	7.2	(Ac)	S^0	CO_2/Ac		+	H_2S smell
6	3	7.2	-	-	CO_2		+	H_2S smell
7	5	6.8	S^0	-	CO_2		n/d	
8	5	6.8	-	Mn^{4+}	CO_2		n/d	
9	5	6.8	S^0	Mn^{4+}	CO_2		n/d	
10	5	6.8	(Ac)	S^0	CO_2/Ac		n/d	
11	5	6.8	-	-	CO_2		n/d	

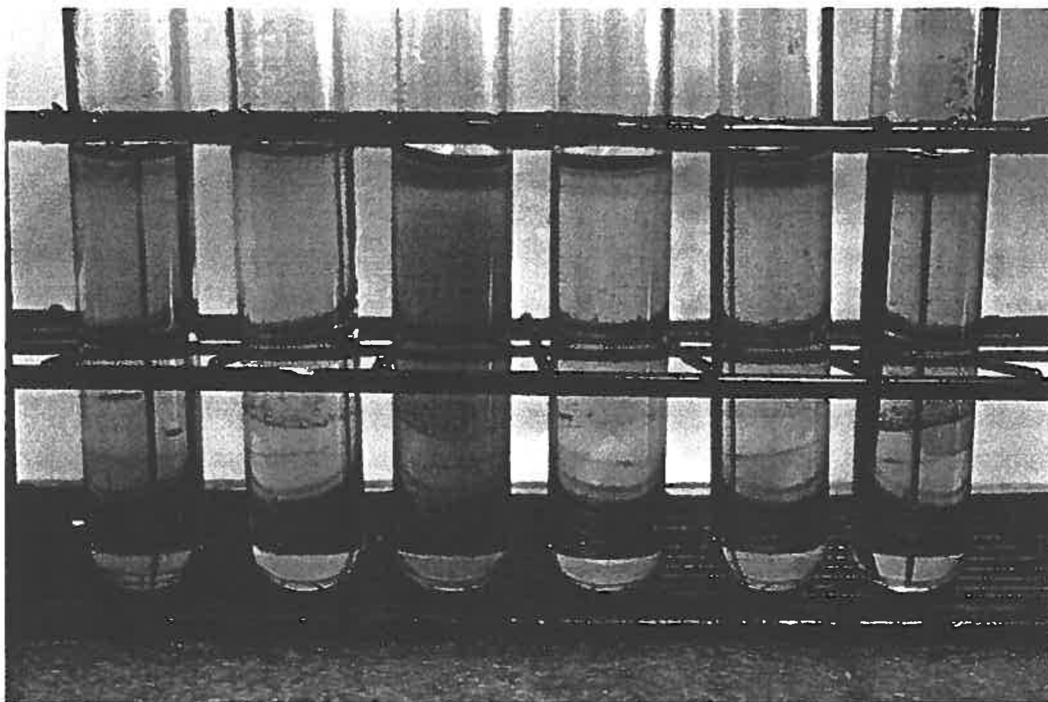

Figure 1. Gradient tubes for initial enrichments of sulfide oxidizers. Shown are tubes 1, 5, 8, 9, 10, and 13 (from left; see Table 1).

2. Growth cycle and "ixotrophy" of *Saprospira grandis*.

Introduction.

Saprospira grandis is a gram-negative gliding bacterium, that forms helical, multicellular filaments of up to 500 µm in length (for review, see Reichenbach, 1992). Phylogenetically, the genus *Saprospira* it is most closely related to the cytophagas and flexibacteria. The organism, commonly found in marine littoral sand and other coastal habitats, is a strictly aerobic organotroph and seems to prefer amino acids as carbon, nitrogen, and energy source. It can exist as a predator, killing and lysing other microorganisms over which it glides. More recently, it was discovered that *S. grandis* can also catch motile bacteria by ixotrophy, which has been defined as feeding on prey caught on a sticky substance (Lewin, 1997). The initial bacterial attachment is thought to be flagella-mediated. Filaments multiply by breaking in two, but reproduction can apparently also occur by simultaneous fragmentation into many short, one- or few-celled units. Short pieces move only slowly if at all, and movement speeds up with growing length. This study attempts to investigate the growth cycle of the organism, and more specifically, a relationship between ixotrophy and fragmentation pattern.

Materials and Methods.

Isolation and Enrichment. *S. grandis* was abundant in a biofilm covering the surface of two-week old seawater samples, including small rocks and seaweeds, from Stoney Beach, Woods Hole, MA. Subsequent isolation of the organism was based on published methods (Reichenbach, 1992, and Lewin, 1997). Samples of the film were serial diluted and plated, or directly struck, on 1.5% natural seawater agar with or without yeast extract and tryptone (0.5 g/l or 5.0 g/l each). Isolated colonies were examined microscopically for the presence of *S. grandis*, and restreaked on the same medium as before, and later in the purification process, on a richer medium. In addition, samples of the film were placed in the center of an agar plate. Spreading edges of swarm colonies were transferre to a new plate by cutting out a piece of the agar and streaking for isolates.

Time-lapse microscopy. Seven individual *S. grandis* fragments (short and long, with and without attached bacteria) were monitored over a period of seven hours by phase contrast microscopy at 400 x magnification. Cartoons of the original images were drawn at one hour intervals. Cells were taken directly from plates and embedded in 0.4 % seawater agar supplemented with yeast extract and tryptone (1.2 % each; Fig. 1).

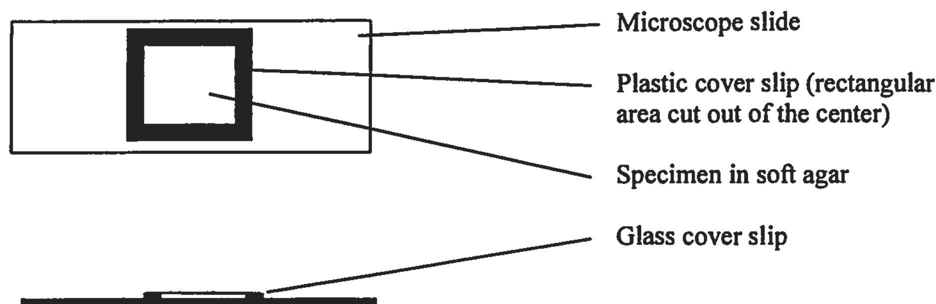

Fig. 1. Set-up for time-lapse microscopy of *S. grandis*. (Top and side view).

Results and Discussion.

Identification of *S. grandis*. Identification of the organism was based on morphological characteristics. Observed filament size (approx. 5 to 100 μm), helical shape, the presence of intracellular granules and cross walls, movement by gliding, as well as the tendency to spread on low nutrient agar are consistent with characterictics previously reported for *S. grandis* (Reichenbach, 1992).

Enrichment. The isolation of *S. grandis* was attempted by serial streaking and plating on low-nutrient agar plates (see Material and Methods). Colonies were greatly enriched for *S. grandis* filaments, but never appeared pure. They always contained a fraction of small, rod-shaped cells that grew as pigmented, agar degrading colonies. These cells appeared to be prey for *S. grandis*, because some of them were stuck to the *S. grandis* helical filaments. Moreover, the colony morphology of this faster growing, putative prey species gradually changed from discrete and firm to diffuse and almost liquid, probably due to the invasion and growth of *S. grandis*, and concomitant lysis of prey cells. In our hands, this association with prey bacteria appears to be essential for growth, but previous reports note the isolation of pure cultures without difficulties (Reichenbach 1992, Lewin, 1997). Therefore, it cannot be excluded that the described *Saprospira* species is not *S. grandis*, despite the striking morphological resemblance. 16s rRNA analysis of a clone library from our enrichment culture would resolve this uncertainty.

Growth cycle. It was observed that prey cells adhered to *S. grandis* filaments predominantly when the cells were fragmenting, i.e. breaking into many small pieces, whereas intact, longer, motile filaments were rarely found in association with prey (Fig. 2). This result suggested that attachment of prey bacteria might trigger a fragmentation response. The growth cycle of *S. grandis* might thus be separable into a motile phase where grown filaments hunt for prey and a subsequent growth phase where individual cells of a fragmented filament grow in a nutrient-rich environment feeding off their prey. This hypothesis could ideally be tested with the controlled addition of various prey bacteria to a pure culture of *S. grandis*. However, since a pure culture was not obtainable, it was attempted to monitor the growth cycle of a mixed culture (multiple filaments with or without adherent prey bacteria) over several hours using time-lapse

microscopy (data not shown). Filaments of *S. grandis* were immobilized by embedding them in soft agar. Significant growth of small filaments (up to 100% increase in length) was observed, whether they were associated with prey or not. Large filaments did not increase in size, but they also did not fragment. Therefore, no conclusions can be drawn with respect to a possible relationship between ixotrophy and fragmentation of *S. grandis*. From our microscopic observations, the minimum doubling time can be estimated to be about seven hours, compared to only two to three hours in comparable medium at 30° C (Reichenbach, 1992). This result, together with the relatively small number of filaments monitored, may explain why fragmentations were not observed. Growth may have been slowed by experimental conditions; agar entrapment may have stressed the cells and limited their oxygen supply. The filaments appeared to be relatively active throughout the experiment, however, as they continued to move within the constraints imposed by the agar, and particles or bacteria were constantly translocated across the filament surface. It has also been reported that environmental stress induces rather than prevents fragmentation (Reichenbach, 1992). This, in turn, might explain our initial observation that prey bacteria were only associated with fragmented *S. grandis*. The leakage of cytoplasmic components due to stress-induced fragmentation and lysis might have attracted prey bacteria, which then adhered to the dead, but still sticky, fragments.

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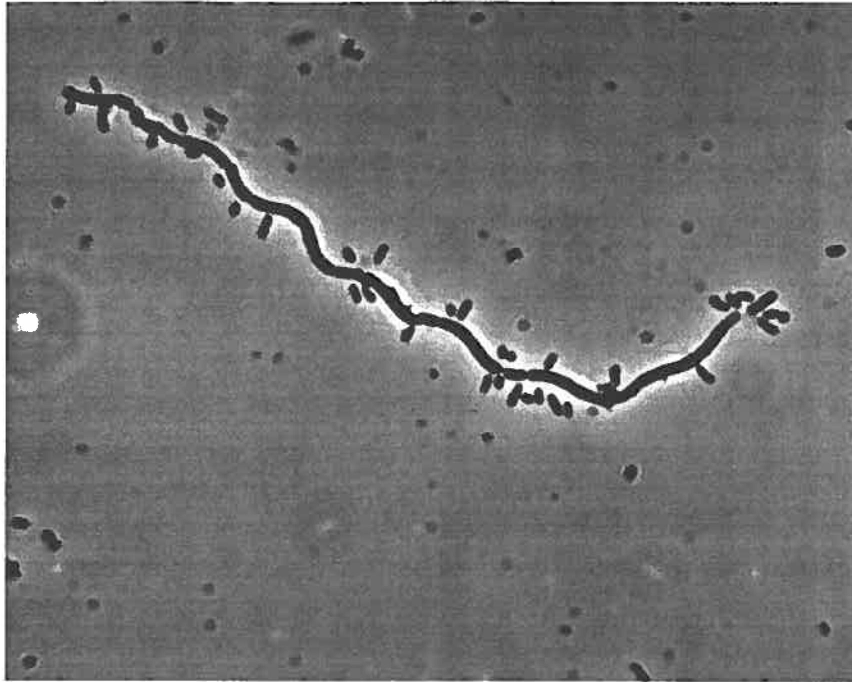

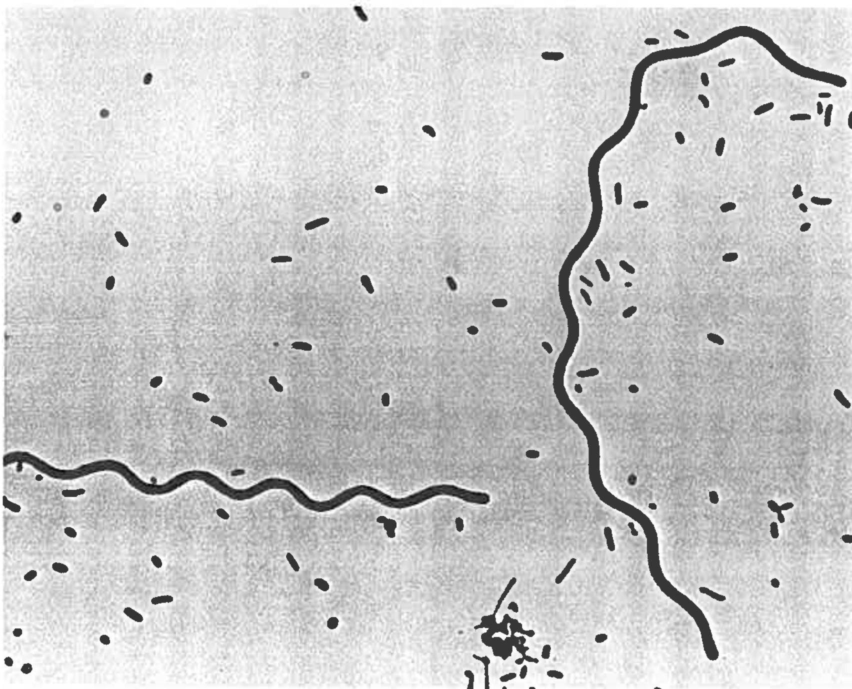

Figure 2. Fragmenting (top) and intact (bottom) filaments of *S. grandis*.