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Studies on the *in situ* physiology of *Thiothrix* spp. present in activated sludge

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Summary

The *in situ* physiology of the filamentous sulphur bacterium *Thiothrix* spp. was investigated in an industrial wastewater treatment plant with severe bulking problems as a result of overgrowth of *Thiothrix.* Identification and enumeration using fluorescence *in situ* hybridization (FISH) with species-specific 16S and 23S rRNA probes revealed that 5–10% of the bacteria in the activated sludge were *Thiothrix* spp. By using a combination of FISH and microautoradiography it was possible to study the *in situ* physiology of probe-defined *Thiothrix* filaments under different environmental conditions. The *Thiothrix* filaments were very versatile and showed incorporation of radiolabelled acetate and/or bicarbonate under heterotrophic, mixotrophic and chemolithoautotrophic conditions. The *Thiothrix* filaments were active under anaerobic conditions (with or without nitrate) in which intracellular sulphur globules were formed from thiosulphate and acetate was taken up. *Thiothrix*-specific substrate uptake rates and growth rates in activated sludge samples were determined under different conditions. Doubling times of 6–9 h under mixotrophic conditions and 15–30 h under autotrophic conditions were estimated. The key properties that *Thiothrix* might be employing to outcompete other microorganisms in activated sludge were probably related to the mixotrophic growth potential with strong stimulation of acetate uptake by thiosulphate, as well as stimulation of bicarbonate incorporation by acetate in the presence of thiosulphate.

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Introduction

Thiothrix spp. are filamentous, colourless sulphur-oxidizing bacteria that may form rosettes and gonidia, and deposit sulphur when grown in the presence of sulphide or thiosulphate (Larkin, 1989). The growth habitat ranges from sulphide-containing natural waters and irrigation systems (Bland and Staley, 1978; Larkin and Strohl, 1983; Strohl and Schmidt, 1984) to aerated activated sludge wastewater treatment plants (Strom and Jenkins, 1984; Wanner, 1994; Nielsen *et al*., 1998). If *Thiothrix* filaments are present in large numbers in the treatment plants they often produce sludge with poor settling characteristics, known as filamentous sludge bulking. For this reason, several studies have been conducted to study the physiology and understand the presence of *Thiothrix* spp. in activated sludge in order to find suitable control measures.

The phylogenetic relationship of *Thiothrix* spp. is not completely resolved, although it is known that it is closely related to *Leucothrix* and the filamentous sulphur bacterium Eikelbooms Type 021N (Williams and Unz, 1985; Wagner *et al*., 1994). These organisms all belong to the gamma *Proteobacteria* (Polz *et al*., 1996; Howarth *et al*., 1999). The morphology is very similar for *Thiothrix* spp. and Type 021N, hence a proper identification can only be performed by using fluorescence *in situ* hybridization (FISH) by rRNA-targeted gene probes. This is important in activated sludge where both types can be present simultaneously causing bulking (Wagner *et al*., 1994; Nielsen *et al*., 1998).

Although Winogradsky suggested as early as 1888 that *Thiothrix* might be an aerobic chemolithoautotrophic organism, it has only recently been confirmed that some strains can grow autotrophically including *T. nivea* (McGlannan and Makemson, 1990), *T. ramosa* (Odintsova *et al*., 1993) and *Thiothrix* CT3 (Tandoi *et al*., 1994). *Thiothrix* had been considered obligately mixotrophic (Larkin and Shinabarger, 1983), but some heterotrophic strains have now been isolated from wastewater treatment plants (Williams and Unz, 1985; 1989).

The mixotrophic strains are supposed to benefit from the ability to oxidize reduced sulphur compounds simultaneously with the use of organic substrates as a carbon and energy source, thus increasing the growth yield compared with chemolithoautotrophic or heterotrophic growth (Odintsova *et al*., 1993). It is not known whether *Thiothrix* spp. in activated sludge treatment plants actually grow

mixotrophically or whether obligately heterotrophic strains predominate. Furthermore, some strains have been shown to be facultative with a capability of switching between organoheterotrophy, mixotrophy and chemolithoautotrophy, depending on the actual growth conditions (Odintsova *et al*., 1993; Tandoi *et al*., 1994). Such matters constitute an inherent problem in environmental microbiology because it is difficult or impossible to extrapolate knowledge obtained in pure culture with actual growth in complex microbial communities. However, the identification of microorganisms by using culture-independent methods such as FISH combined with microautoradiography (MAR) (Lee *et al*., 1999; Nielsen *et al*., 1999; Ouverney and Fuhrman, 1999) has now made it possible to study the *in situ* physiology of various probe-defined microorganisms.

In this study, we have used the MAR-FISH tool to investigate the *in situ* physiology of *Thiothrix* spp. in an industrial activated sludge treatment plant with severe bulking problems. We have focused on the ability of *Thiothrix* spp. to take up radiolabelled organic substrates and ^{14}C-labelled bicarbonate under distinct electron acceptor conditions and under different availabilities of reduced sulphur compounds. In addition, we have investigated the potential for anaerobic activity by *Thiothrix* spp. by studying the dynamics of sulphur globule formation and disappearance under various environmental conditions. The predominant *Thiothrix* spp. proved to be a very versatile facultative heterotrophic organism with mixotrophic and chemolithoautotrophic potential.

Results

Microscopic identification of Thiothrix *spp. and investigation of sulphur globule formation*

Investigations of activated sludge from Grindsted wastewater treatment plant (WTP) using microscopy revealed many filamentous microorganisms that were causing serious bulking problems (with a sludge volume index, SVI, typically in the range of 200–300 ml g^{-1}). Three predominating filamentous types could be detected based on morphology, Neisser and Gram staining. More than 80% of the filaments could be identified as *Thiothrix* spp. by using FISH with the species-specific probe (Fig. 1). The two other filamentous bacteria were not identified, but one belonged to the alpha *Proteobacteria and* one was a Gram-positive bacterium. Occasionally, a few filaments belonging to Type 021N were observed by FISH in the sludge. The *Thiothrix* filaments were 1–1.2 μm wide and the individual cells 1–2 μm long. The length of the filaments was typically 200–400 μm and they did not form rosettes. The longest filament observed was approximately 800 μm. Based on measurements of the total length of filaments in the sludge, and with an average cell length of 1.5 μm, the abundance of *Thiothrix* was estimated to be approximately 0.17×10^9 cells ml^{-1}. This represented 8.1% of the total number of bacteria in the sludge [2.1×10^9 cells ml^{-1} or 0.82×10^{12} g^{-1} volatile suspended solids (VSS)]. During the period of intensive investigation (April–May 1999), *Thiothrix* represented 5–10% of the total number of bacteria.

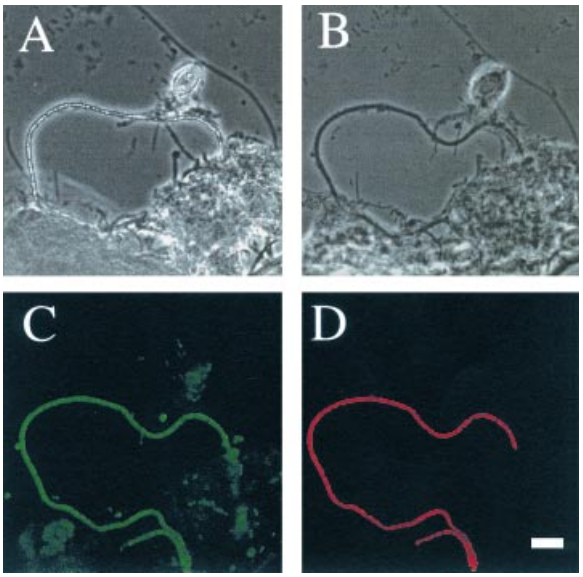

Fig. 1. Identification of *Thiothrix* by fluorescence *in situ* hybridization (FISH).
A. Phase-contrast micrograph showing a filament with sulphur (S) globules.
B. The same filament without S globules after ethanol treatment.
C. The same filament after hybridization with the probe for gamma *Proteobacteria* labelled with FLUOS.
D. The same filament after hybridization with the specific *Thiothrix* probe labelled with Cy3.
Bar = 5 μm.

Usually, *Thiothrix* did not have any intracellular sulphur (S) globules *in situ*, but was able to form them in the presence of thiosulphate under aerobic conditions. Many clear globules were present after a 3–4 h incubation under aerobic conditions. Figure 1 shows an example of a filamentous microorganism, identified with FISH as

Table 1. Formation of intracellular sulphur (S) globules from thiosulphate in *Thiothrix* filaments after 4 h incubation. The results after 24 h were similar.

Electron acceptor	Sulphur globules after 4 h	
	Globule size	Positive filaments
Oxygen	+ + +	> 90%
Nitrate	+ +	> 90%
Nitrite	+	80–90%
None added	+	80–90%

+ + + , Many large, bright S globules; + + , many S globules; + , few small S globules.

Table 2. Uptake of radiolabelled substrates by *Thiothrix* filaments under different conditions as visualized by microautoradiography (MAR) and fluorescence *in situ* hybridization (FISH). Labelling was assessed by silver grain density on the top of the filaments compared with the background.

Substrates tested	Electron acceptor		
	Oxygen	Nitrate	Anaerobic
Past. control ([^{3}H]-acetate)	–	–	–
[^{3}H]-acetate	+ +	–	–
[^{3}H]-acetate + thiosulphate	+ +	+ +	+
[^{3}H]-glucose	–	–	–
[^{3}H]-glucose + thiosulphate	–	–	–
[^{14}C]-bicarbonate	–	–	–
[^{14}C]-bicarbonate + thiosulphate	+ +	–	–
[^{14}C]-bicarbonate + thiosulphate + acetate	+ +	–	–
[^{14}C]-bicarbonate + S globules	+ +	ND	ND

+ +, Strong labelling; +, weaker labelling, but clearly positive; –, no labelling; ND, not determined.

Thiothrix spp., containing many S globules. It was possible to observe and localize a number of filaments with and without S globules on the microscopic slide, and then, following hybridization, to study the identity of the same filaments. Under aerobic conditions, most (> 90%) of the *Thiothrix* filaments were able to form many large, bright S globules (Table 1). All the observed filaments that contained S globules hybridized with the specific probe used for *Thiothrix*.

Thiothrix filaments were also able to store sulphur globules in the absence of oxygen (Table 1). With nitrate as an electron acceptor, most *Thiothrix* filaments produced S globules. In the absence of nitrate or nitrite, or with nitrite present, many filaments were able to produce S globules, but fewer S globules were produced than in the presence of nitrate. It was also noticed that the size and/or number of S globules within the cells was smaller under anaerobic conditions compared with aerobic conditions. When acetate (2 mM) was added to activated sludge during thiosulphate oxidation, some delaying effect on the accumulation of S globules was detected under all conditions tested (results not shown).

Uptake of labelled acetate, glucose and bicarbonate in Thiothrix *filaments*

A combination of MAR and FISH was used to study the uptake of organic substrates and bicarbonate by *Thiothrix* filaments and to investigate the potential for heterotrophic, mixotrophic or chemolithoautotrophic activity. [^{3}H]-acetate and [^{3}H]-glucose were used to study the uptake of organic substrate and the results are presented in Table 2 and Fig. 2. *Thiothrix* filaments were able to take up acetate as indicated by the MAR-positive result shown in Fig. 2. A dark layer of silver grains on the top of the filaments showed uptake of the tracer. Glucose was not taken up by *Thiothrix* although other types of filaments and some floc-forming bacteria in the sludge were able to consume glucose (Fig. 2D–F). The uptake was not dependent on

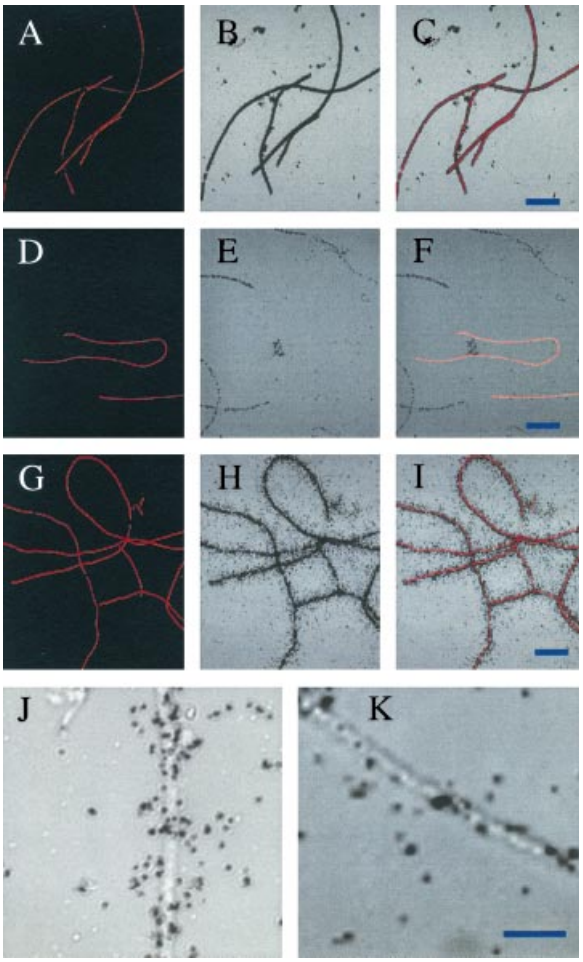

Fig. 2. Uptake of [^{3}H]-acetate, [^{3}H]-glucose and [^{14}C]-bicarbonate by *Thiothrix* filaments detected by microautoradiography (MAR) and fluorescence *in situ* hybridization (FISH) under aerobic conditions.
A–C. Uptake of [^{3}H]-acetate.
D–F. No uptake of [^{3}H]-glucose.
G–I. Uptake of [^{14}C]-bicarbonate in the presence of thiosulphate.
J and K. An example of silver grains on the top of the filaments from uptake of [^{14}C]-acetate and [^{14}C]-bicarbonate respectively.
FISH images are presented on the left-hand side (A, D, G) and MAR images in the middle (B, E, H), while the images are overlaid on the right-hand side (C, F, I). Bar = 20 μm in images A–I, and 5 μm in images J and K.

Table 3. Quantitative microautoradiography (MAR) determination of the aerobic uptake of [^{14}C]-bicarbonate and [^{14}C]-acetate by *Thiothrix* filaments by silver grain density on the top of the filaments.

	Grains μm^{-1} filament	Grains μm^{-1} filament
	Background	(Background subtracted)
Pasteurized control ([^{14}C]-acetate)	0	0
[^{14}C]-acetate	0.8	9.4
[^{14}C]-acetate + thiosulphate	0.8	15.0
[^{14}C]-bicarbonate	0.3	0
[^{14}C]-bicarbonate + thiosulphate	0.9	1.7
[^{14}C]-bicarbonate + thiosulphate + acetate	0.2	2.8

the presence of thiosulphate. In the absence of oxygen, *Thiothrix* filaments did not take up [^{3}H]-acetate unless thiosulphate was present (Table 2).

[^{14}C]-bicarbonate was incorporated into *Thiothrix* filaments only when thiosulphate was present, indicating a capability for chemolithoautotrophic growth (Table 2). [^{14}C]-bicarbonate was also incorporated when acetate was added and concomitantly consumed. No incorporation was observed under anaerobic conditions with or without nitrate (Table 2). All *Thiothrix* filaments were able to take up [^{14}C]-bicarbonate indicating that all filaments had similar autotrophic capabilitites. Intracellular S globules could also be used as an energy source for [^{14}C]-bicarbonate fixation, as shown by experiments using *Thiothrix* filaments with intracellular S globules in the absence of external thiosulphate (Table 2). All cells within the *Thiothrix* filaments were usually MAR-positive showing that all cells were viable in each filament and able to fix [^{14}C]-bicarbonate (Fig. 2G–J).

The uptake of [^{14}C]-bicarbonate in *Thiothrix* filaments was estimated to be approximately 20% of the uptake of [^{14}C]-acetate. This could be estimated from the silver grain density on top of the filaments (Table 3 and Fig. 2J and K) when they were incubated, exposed and processed under identical conditions. Furthermore, it was important to notice that addition of thiosulphate stimulated the incorporation of [^{14}C]-acetate into the filaments with about 60%, suggesting mixotrophic activity. It was also found that acetate stimulated the incorportion of [^{14}C]-bicarbonate into the filaments with 60–70%.

Removal of S globules under anaerobic conditions

When *Thiothrix* filaments with intracellular S globules (produced under aerobic conditions from thiosulphate) were exposed to anaerobic conditions with or without nitrite or nitrate present, the S globules in the filaments disappeared. After 2 h, a reduction in globule size and some cells with a loss of globules were observed, and after 5 h a significant reduction in globule size was visible in most filaments, and many cells lost their S globules. After 24 h almost all S globules had disappeared. It was also observed that the removal occurred faster in the presence of oxygen, where all S globules in the filaments were completely removed after only 5 h. The removal rates were not clearly affected by acetate addition.

Consumption of thiosulphate, acetate and bicarbonate in activated sludge

The activated sludge exhibited a high thiosulphate removal rate in the presence of oxygen (Fig. 3). The removal rates measured during a 6 month period ranged from 0.35 to 0.50 mmol g^{-1} VSS h^{-1}. These rates were significantly higher than the rate observed in a normal domestic wastewater treatment plant (Aalborg East WTP, Fig. 3), where reduced sulphur compounds are uncommon in the wastewater. No (or only very limited) removal of thiosulphate was found in the absence of oxygen (Fig. 3). If nitrate or nitrite was added as an electron acceptor for thiosulphate oxidation, the thiosulphate removal rates did

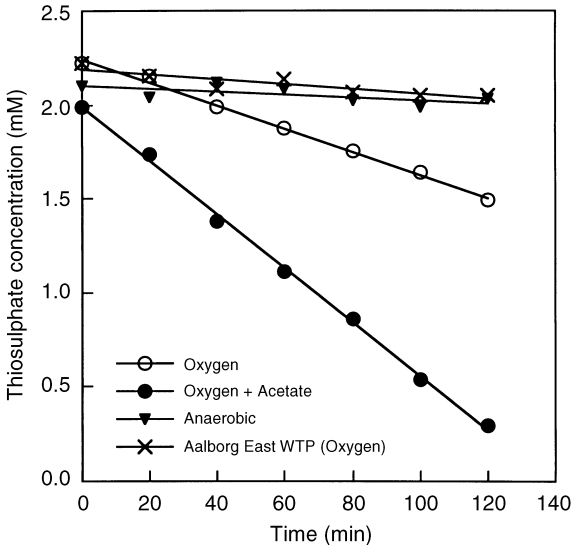

Fig. 3. Removal of thiosulphate in activated sludge from Grindsted wastewater treatment plant (WTP) under aerobic and anaerobic conditions. Stimulation of thiosulphate removal by acetate is shown. Thiosulphate removal in a municipal plant (Aalborg East WTP) under aerobic conditions is also shown. The volatile suspended solids (VSS) content was 1.6 g l^{-1} and 2.7 g l^{-1} in the two plants respectively.

Table 4. Removal rates of thiosulphate, acetate and bicarbonate in activated sludge from Grindsted wastewater treatment plant under aerobic or anaerobic conditions. Average rates are presented for duplicate or triplicate experiments. Standard errors of the mean were less than 5%.

Substrates	Incubation conditions	Substrate removed	Removal rate (mmol g^{-1} VSS h^{-1})
Thiosulphate	Aerobic	Thiosulphate	0.254
Thiosulphate + acetate	Aerobic	Thiosulphate	0.574
Acetate	Aerobic	Acetate	2.71
[^{14}C]-bicarbonate	Aerobic	Bicarbonate	0.039
[^{14}C]-bicarbonate	Anaerobic, nitrate	Bicarbonate	0.020
[^{14}C]-bicarbonate + thiosulphate	Aerobic	Bicarbonate	0.099
[^{14}C]-bicarbonate + thiosulphate + acetate	Aerobic	Bicarbonate	0.197
[^{14}C]-bicarbonate + thiosulphate	Anaerobic, nitrate	Bicarbonate	0.009
[^{14}C]-bicarbonate + thiosulphate	Anaerobic	Bicarbonate	0.007
[^{14}C]-bicarbonate + thiosulphate	Pasteurized	Bicarbonate	0.001

not significantly increase compared with anaerobic conditions (results not shown).

Acetate strongly stimulated the thiosulphate removal rate (Table 4 and Fig. 3). The removal rate of acetate varied between 2.5 and 3 mmol g^{-1} VSS h^{-1} during the 6 month investigation period. The removal rates of acetate under anaerobic conditions with and without nitrate were 15–23% and 0–5% of the aerobic rate respectively.

[^{14}C]-bicarbonate was incorporated into the activated sludge under mainly aerobic conditions. The incorporation was stimulated by thiosulphate (Table 4). A small amount was also incorporated in the absence of thiosulphate under aerobic conditions, most probably the result of nitrifying activity. The incorporation of [^{14}C]-bicarbonate was strongly stimulated by addition of acetate (a two- to threefold increase in the rate of incorporation). The maximum thiosulphate-dependent [^{14}C]-bicarbonate fixation rate was around 6–7% of the observed acetate removal rate. No [^{14}C]-bicarbonate incorporation was observed under anaerobic conditions.

Discussion

The microbial system studied was highly loaded with organic matter and the regular presence of reduced sulphur compounds. The microbial community exhibited a large capacity for the aerobic removal of acetate, the oxidation of thiosulphate and a capacity for fixation of bicarbonate. This system proved highly suitable for the study of the *in situ* physiology of facultatively heterotrophic, mixotrophic and chemolithoautotrophic probe-defined *Thiothrix* filaments using the recently developed MAR-FISH approach (Lee *et al.*, 1999).

All *Thiothrix* filaments in the activated sludge samples formed S globules from thiosulphate and they were closely phylogenetically related, hybridizing with the *Thiothrix* probe used. Only occasionally a few filaments hybridized with the probe for Type 021N, but no other filaments belonging to the gamma *Proteobacteria* were observed. In the various experiments relating to the ecophysiology of *Thiothrix*, the same metabolic features were observed, suggesting that the same strain was the predominant strain in the sludge. However, regarding the formation and disappearance of sulphur globules under anaerobic conditions, some variability among the filaments was observed. Whether this reflects different growth stages for the same strain or several different *Thiothrix* strains detected by the same oligonucleotide probe is not known.

Heterotrophic, mixotrophic and chemolithoautotrophic activity

All the *Thiothrix* filaments were capable of heterotrophic activity under aerobic conditions without the presence of thiosulphate or intracellular S globules. The uptake of [^{3}H]-acetate into the filaments could be visualized by MAR-FISH (Fig. 2, Tables 2 and 3). Heterotrophic *Thiothrix* strains are known (Williams and Unz, 1985) and the preincubation step of adding non-labelled acetate for 1 h before the labelled acetate was added and taken up (during the following 2–3 h incubation) strongly suggests that it was not a transient uptake and storage, but actual growth. *Thiothrix* filaments were not able to take up glucose which agrees with both the pure culture studies and our earlier studies of the *in situ* physiology of *Thiothrix* in different water treatment plants (Andreasen and Nielsen, 1997; Nielsen *et al.*, 1998).

The *Thiothrix* filaments also exhibited a mixotrophic activity. Under aerobic conditions, the presence of thiosulphate strongly stimulated the uptake of acetate, as indicated by the MAR results as well as the general uptake measurements in the sludge (Tables 3 and 4). This is in accordance with several studies where strains are obligately mixotrophic (Larkin and Shinabarger, 1983) or facultatively mixotrophic (Odintsova *et al.*, 1993; Tandoi *et al.*, 1994).

The results suggest that the *Thiothrix* filaments carried

out chemolithoautotrophic activity. [^{14}C]-bicarbonate was incorporated when thiosulphate or intracellular S globules were present and did not require the presence of acetate (Tables 2 and 3). Furthermore, the amount of [^{14}C]-bicarbonate incorporated into the biomass was similar to that found in pure culture (see below) for *T. ramosa* (Odintsova *et al*., 1993). The amount of [^{14}C]-bicarbonate incorporated into cell carbon was, however, small compared with the acetate uptake, as indicated by MAR (about 20%, see below), hence the reported effect of thiosulphate was probably the result of the production of energy used for acetate uptake rather than for incorporation of bicarbonate. The results suggest that growth was stimulated by the mixotrophic activity rather than the heterotrophic activity, as is also described for *T. ramosa* (Odintsova *et al*., 1993). However, such a stimulation was not found for another facultatively mixotrophic strain, *Thiothrix* CT3 (Tandoi *et al*., 1994).

Apart from thiosulphate, other reduced sulphur compounds such as sulphide may stimulate the mixotrophic or autotrophic activity of *Thiothrix*. Otte *et al*. (1999) found that *Thioploca* was stimulated more by sulphide than by thiosulphate. In this study, the effect of sulphide was not investigated because any sulphide added formed black FeS precipitates with the ferric iron present in the sludge, indicating that soluble sulphide was not commonly present in the treatment plant.

An important observation was that the [^{14}C]-bicarbonate incorporation rate was significantly stimulated by the presence of acetate, as indicated by the MAR results as well as the general uptake measurements in the sludge (Tables 3 and 4). This has not been reported before among the known chemolithoautotrophic *Thiothrix* strains (Odintsova *et al*., 1993; Tandoi *et al*., 1994), but has been observed for freshwater *Beggiatoa* strains (Strohl *et al*., 1981; Nelson, 1989). Only in one field study of natural *Thiothrix* tufts has a similar effect been observed in an artesian well (McGlannan and Makemson, 1990). In that study, different organic compounds were tested for potential stimulation of the uptake of [^{14}C]-bicarbonate and some stimulation was found by adding acetate and lactate. Thus, this physiological capability has not yet been described in pure cultures of *Thiothrix*, but it could be of great significance for competition and survival in complex dynamic systems.

Quantification of substrate consumption and growth rate of Thiothrix

The use of acetate, thiosulphate and bicarbonate by *Thiothrix* was generally reflected in the overall consumption rates in the activated sludge. This was probably because a large part of the biomass (5–10%) was *Thiothrix.* This also resulted in very similar substrate uptake patterns, as measured by overall consumption rates and by MAR (Tables 3 and 4). MAR is usually considered as qualitative or semiquantitative and [^{14}C], in particular, which is a relatively strong beta-emitter, is considered difficult to use in quantitative MAR studies (Rogers, 1977). It is also clear from our experiments that the resolution is far better for [^{3}H]-acetate than for ^{14}C-labelled acetate or bicarbonate (Fig. 2). However, as we carefully incubated, exposed and developed all samples in batches under identical conditions, we believe that the number of silver grains on the top of the filaments corresponds, at least in relative terms, to the real uptake.

Some quantitative information about the growth of *Thiothrix* in activated sludge can be obtained if it is assumed that the uptake of [^{14}C]-bicarbonate was mainly due to *Thiothrix* filaments, and that the MAR results on a relative basis are valid. By observing the MAR signals it appeared that some floc-forming bacteria were able to incorporate [^{14}C]-bicarbonate, but they were much less numerous than *Thiothrix*. Thus, assuming that all thiosulphate was used for fixation of bicarbonate into *Thiothrix*, 2.8 g of carbon was assimilated per mol thiosulphate consumed. In the presence of acetate this yield increased to 3.3 g of carbon per mol thiosulphate. These yields are very similar to those reported for *T. ramosa* growing chemolithoautotrophically in a chemostat (Odintsova *et al*., 1993) of 3.6 g of cell protein per mol thiosulphate. Assuming the protein content of a cell is 50% of the dry weight and the carbon is 50% of the dry weight, this yield corresponds to 3.6 g of carbon per mol thiosulphate.

The amount of acetate consumed by *Thiothrix* is more difficult to estimate as many other bacteria present were also able to consume this substrate. If, however, it is assumed that the uptake was approximately five times larger than the [^{14}C]-bicarbonate incorporation (as could be estimated from the MAR experiment (Table 3), the uptake rate corresponded to 0.3–0.4 mmol acetate g^{-1} VSS h^{-1}. This is 11–15% of the overall acetate consumption rate, which is a reasonable estimate with a biomass proportion of *Thiothrix* of 8.1%. Thus, using the MAR results quantitatively seems to give a fair and reasonable estimate of the actual *in situ* consumption of substrates by *Thiothrix* in activated sludge.

The apparent growth rates of *Thiothrix* under different conditions can also be calculated from the results. The amount of *Thiothrix* biomass in the sludge can be estimated to 18 mg of carbon per g of VSS from cell counts, cell size and a conversion factor of 0.25 pg of carbon per μm^3 (Nagata and Wanatabe, 1990). The incorporation of bicarbonate was 0.7 and 1.5 mg of carbon g^{-1} of VSS h^{-1} without and with acetate present respectively. This corresponds to a doubling time of 12–25 h, which is slightly longer than the maximum autotrophical growth rate reported for *Thiothrix* CT3 of 1.8 d^{-1} or a doubling

time of 9.2 h (Tandoi *et al.*, 1994). The growth on acetate was faster, as estimated from the MAR experiment (Table 3), giving an incorporation of 3.5–5 mg of carbon g^{-1} of VSS h^{-1} or a doubling time of 4–6 h. This is very similar to the mixotrophic and heterotrophic maximum growth rate found for *Thiothrix* CT3 of 2.5 d^{-1} or a doubling time of 6.6 h (Tandoi *et al.*, 1994).

Anaerobic activity

Isolated strains of *Thiothrix* (Larkin and Shinabarger, 1983; Williams and Unz, 1985, 1989; Howarth *et al.*, 1999) are all reported as being obligately aerobic. Nitrate cannot be used as an electron acceptor for growth, although other filamentous sulphur bacteria have this potential, such as *Beggiatoa* (Sweerts *et al.*, 1990; McHatton *et al.*, 1996) and *Thioploca* (Jørgensen and Gallado, 1999; Otte *et al.*, 1999). Some *Thiothrix* strains are able to form nitrite from nitrate in the absence of oxygen, but not able to grow (Williams and Unz, 1985, 1989). Interestingly, our *in situ* investigation revealed that *Thiothrix* was physiologically very active with nitrate as an electron acceptor as it could form S globules from thiosulphate and take up acetate in the presence of thiosulphate or intracellular S globules (Tables 1 and 2).

Such anaerobic activity has not previously been reported for *Thiothrix* and there may be a possibility for a real anaerobic metabolism where elemental sulphur is oxidized to sulphate and nitrate is reduced to nitrogen, as described for *Beggiatoa* (Sweerts *et al.*, 1990; McHatton *et al.*, 1996), or to ammonium as described for *Thioploca* (Otte *et al.*, 1999). This process seems to be mainly important for marine species (McHatton *et al.*, 1996) and, if this is also true for *Thiothrix*, this could explain why we observed it in this particular treatment plant as it has a salinity comparable to brakish water. However, although *Thiothrix* was physiologically active and took up external substrates under anaerobic conditions, it is still uncertain whether it could grow. For instance, the overall removal rates of thiosulphate and acetate in the sludge were low with nitrate as an electron acceptor compared with aerobic conditions. This could indicate a transient activity with formation of S globules, as previously described, under aerobic conditions (Odintsova *et al.*, 1993) and with storage of some organic products, but without a significant further oxidation.

Under anaerobic conditions in the absence of nitrate, small S globules were also formed from thiosulphate and some acetate was taken up (Tables 1 and 2). However, the overall removal rate of thiosulphate and acetate was very low, so it seemed not to be an important process. Instead, there were strong indications of an ability of the stored elemental sulphur to act as an electron acceptor, being reduced to sulphide when the external thiosulphate was not present. This is supported by the observation that sulphide was observed to be formed (as black FeS) in the activated sludge when *Thiothrix* filaments with intracellular S globules were kept in anaerobic conditions overnight. The sulphide production was not as a result of sulphate reduction because addition of 5 mM molybdate (that inhibits sulphate reduction) did not prevent the sulphide formation, and controls carried out on *Thiothrix* filaments without intracellular S globules did not produce sulphide (data not shown). This ability is comparable with that described for a freshwater strain of *Beggiatoa* (Nelson and Castenholz, 1981). This interesting anaerobic versatility ought to be investigated further in *Thiothrix* spp.

Ecological implications

An activated sludge wastewater treatment plant is a dynamic system with strong variations in the main substrates (organic matter and reduced sulphur compounds), as well as oxygen and nitrate. Variations typically take place in time scales of seconds, minutes or a few hours, so the great physiological versatility observed by *Thiothrix* makes these bacteria well suited for competition for substrates in these systems. In particular, the strong stimulation of acetate uptake by thiosulphate, and the stimulation of thiosulphate oxidation and bicarbonate incorporation by acetate, are probably key competitive properties. The significance of the capability to be active under anaerobic conditions still remains to be better understood, but is likely to be of importance in systems where anaerobic phases (e.g. in clarifiers) are present. The study presented here shows that *Thiothrix* spp. is a physiologically versatile group of filamentous sulphur-oxidizing bacteria that can exist in a variety of habitats.

Experimental procedures

Activated sludge samples

The experiments were carried out with activated sludge from an industrial wastewater treatment plant (WTP) in Grindsted, Denmark. The plant receives easily degradable wastewater with a high content of low molecular weight alcohols, organic acids and other compounds from food additives and medical manufacturing. It usually contains some sulphides, but these have never been quantified. Owing to a low content of ammonium in the wastewater, some ammonium is continuously added to secure a good removal of organic matter. The salinity is rather high with a conductivity between 15 and 23 mS cm^{-1} and a sodium concentration around 5–7 g l^{-1}. The mean cell residence time (sludge age) is 8–10 d. The plant is operated with an oxygen concentration of 0.5–2 mg l^{-1} in the process tank and at an annual average temperature of 15–25°C. The plant has been affected by bulking problems for several years.

The activated sludge was harvested the day before the experiments were performed and kept at 4°C. The sludge had a dry matter content (suspended solids, SS) of 3–4 g SS l^{-1}. The organic fraction (volatile suspended solids, VSS) of the dry solids was 75 ± 2% of SS. Before use, the sludge was diluted to appropriate concentrations (0.1–3 gSS l^{-1}) with supernatant from the activated sludge.

Consumption rates of thiosulphate, acetate and [^{14}C]bicarbonate

The consumption rate of acetate and thiosulphate in the sludge was determined under different electron acceptor conditions. Diluted sludge samples (25–200 ml with 1–3 g SS l^{-1}) were placed in glass bottles, the air was evacuated and the gas phase replaced by ultra pure nitrogen gas. Substrates were added from anaerobic stock solutions to a final concentration of 2 mM for acetate and 5 mM for thiosulphate. The samples were shaken at 22 ± 2°C at 200 r.p.m. and removal rates were measured over 2–3 h by continuous sampling. Incubations were conducted in duplicate or triplicate. After sampling, the samples were immediately chilled to 0–1°C, centrifuged, filtered (0.2 μm Millipore, Millipore, Bedford, MA, USA) and frozen for later high-performance liquid chromatography (HPLC) analysis.

Uptake of [^{14}C]-bicarbonate in the sludge was investigated under different electron acceptor conditions. Five millilitres of activated sludge (1 g SS l^{-1}) was transferred to 7 ml serum vials for anaerobic incubations and 25 ml for aerobic incubations. Different substrates were added from oxygen free stock solutions. The final incubation concentration was 2 mM, 5 mM and 2 mM for acetate, thiosulphate and nitrate respectively. Approximately 2 μCi [^{14}C]-bicarbonate ([^{14}C]-$NaHCO_3$, NEN Life Science Products, USA) was added to each vial. Anaerobic vials were all evacuated and flushed with ultra pure nitrogen gas before substrates and a tracer were added from anaerobic stock solutions. The vials were shaken (150 r.p.m.) during incubation (4–6 h) at 22 ± 2°C. At the beginning and end of the incubation period, subsamples were collected by filtration onto 25 mm membrane filters (0.2 μm, Millipore). Excess radioactivity was removed by three washes (5 ml of tapwater each) followed by 5 ml of 0.05 N HCl. Washing with the diluted HCl only removed insignificant amounts of radioactive bicarbonate, indicating that the amount of precipitated bicarbonate in the sludge was low. The radioactivity on the filter was added to in a scintillation liquid (Ultima Gold XR, Packard Instrument) and counted in a Packard model 1600 TR liquid scintillation counter. The fixation rate of [^{14}C]-bicarbonate was calculated from total CO_2, the initial amount of [^{14}C]-bicarbonate added, the amount [^{14}C]-bicarbonate fixed in the biomass and the incubation time. Samples without thiosulphate and pasteurized samples (70°C, 10 min) served as controls. Total CO_2 varied between 14 and 24 mM in the activated sludge during the 6 month investigation period.

Bacterial identification and bacterial count

The filamentous bacteria present in the Grindsted samples were morphologically identified using the Eikelboom classification system (Eikelboom and van Buijsen, 1983), which includes phase-contrast microscopy and Gram and Neisser staining. Furthermore, fluorescence *in situ* hybridization (FISH) with 16S and 23S rRNA-targeted nucleic acid probes specific for Type 021N and *Thiothrix* spp., and for the gamma and beta subclasses of *Proteobacteria*, were used (Amann *et al*., 1995). Oligonucleotides were labelled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) or with sulphoindocyanine dyes (Cy3 and Cy5) (Interactiva, Ulm, Germany). One or 2 ml samples collected in time intervals of 4, 12 and 24 h from the various incubation conditions, were fixed in fresh 4% paraformaldehyde, washed twice in distilled water, immobilized on cover glass, dehydrated in ethanol and hybridized (Lee *et al*., 1999). Preparations were mounted using Citifluor (UKC ChemLab, UK).

The total number of bacteria in the activated sludge was measured using epifluorescence microscopy after sample homogenization and staining with DAPI (4′,6-diamidino-2-phenylindole) according to Frølund *et al*. (1996). The standard deviation for replicate samples was 7–15%. The measurement of the biomass of *Thiothrix* spp. in the sludge was conducted by measuring the total filament length (Sezgin *et al*., 1978) and the size of individual cells in the filaments was measured using light microscopy.

Formation of intracellular sulphur globules

Formation of sulphur globules in *Thiothrix* filaments from thiosulphate was investigated using microscopy. Preliminary experiments with variations in suspended solids (0.1–3 g SS l^{-1}), thiosulphate (1–10 mM) and oxygen (1–100% air saturation) were tested, and sulphur globules were found under all conditions. The conditions chosen for the investigation were slightly diluted activated sludge (0.5–1 g SS l^{-1}) with 5 mM thiosulphate and 100% air saturation. The sludge was investigated using microscopy after 2, 4, 8 and 24 h. Clear, bright S globules within the cells could easily be detected by phase-contrast microscopy. To verify that sulphur was present, ethanol was added to test that it could remove the globules in less than 1 min (Nielsen, 1984). FISH was used to confirm that all filaments containing sulphur globules were *Thiothrix* spp. Because ethanol is used in the FISH procedure and thereby removes the sulphur globules, the same field on a glass slide was observed (and images recorded) before and after the ethanol dehydration step and after the hybridization (Fig. 1).

Aerobic incubations of activated sludge were performed in 50 ml serum vials with 20 ml of activated sludge and air in the gas phase. The anaerobic incubations were performed in serum vials where the gas phase consisted of ultra pure nitrogen gas after evacuation and flushing. Substrates were added from anaerobic stock solutions to a final concentration of 5 mM, 2 mM, and 1 mM for thiosulphate, nitrate, and nitrite respectively. For all anaerobic incubations, the substrates were added after 1 h preincubation under anaerobic conditions to ensure removal of any traces of oxygen, nitrate or nitrite. In some samples, acetate (final concentration 2 mM) was also added from anaerobic stock solution in the preincubation period. The samples were shaken at 150 r.p.m. at 22 ± 2°C.

Removal of sulphur globules under aerobic and anaerobic

conditions was also observed using microscopy after 2, 5 and 28 h. S globules in *Thiothrix* were produced by aeration of activated sludge with 5 mM thiosulphate for 5 h. Surplus thiosulphate was removed by washing the pelleted sludge three times with thiosulphate-free supernatant. The incubation conditions and tested substrates were as decribed above for sulphur formation. All studies concerning S globule formation or removal were conducted several times during a 6 month period, each performed in duplicate.

Autoradiographic incubations and procedures

The microautoradiographic experiments were performed using ^{3}H-labelled and ^{14}C-labelled acetate and glucose (Amersham-Pharmacia Biotech, UK) and ^{14}C-labelled bicarbonate. For uptake of organic substrates, 7 ml of diluted activated sludge (1 g SS l^{-1}) was transferred to glass serum vials (50 ml vials for aerobic incubations, 10 ml for anaerobic incubations) and preincubated for 1 h with unlabelled organic substrate under aerobic, anaerobic or anaerobic with nitrate conditions. The unlabelled acetate and glucose were added to a final concentration of 2 mM. After the preincubation period, 10 μCi [^{3}H]-acetate or [^{3}H]-glucose (specific activity of 100 and 10400 mCi $mmol^{-1}$ respectively) was added. Unlabelled organic substrates were added to a final concentration of 2.0 mM and the samples were incubated for 1–3 h. All vials for anaerobic incubation were closed with a gas tight rubber stopper and flushed with ultra pure nitrogen gas prior to the incubation. When thiosulphate was added, a final concentration of 2 mM was used. The preparation of labelled standards followed procedures described by Andreasen and Nielsen (1997) and Lee *et al*. (1999).

[^{14}C]-bicarbonate fixation in *Thiothrix* cells with intracellular S globules was investigated in some experiments. S globule formation took place when aerating activated sludge with 5 mM thiosulphate for 5 h. Surplus thiosulphate was removed by washing the pelleted sludge three times with thiosulphate-free supernatant. Other incubation conditions were as decribed above.

In the experiments where the uptake of bicarbonate and acetate were compared semiquantitatively based on MAR experiments, [^{14}C]-acetate was used. To keep the specific activity identical in the two incubations (1 μCi μmol^{-1} acetate or bicarbonate), 2 μCi [^{14}C]-acetate was added to vials with 2 mM acetate and 20 μCi [^{14}C]-bicarbonate was added to vials with 20 mM total CO_2. One millilitre of diluted (1 g SS l^{-1}) activated sludge was used in each of these experiments. The experiments were performed in duplicate.

Microscopy

A model LSM 510 scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633 nm) was used to record fluorescent signals from the gene probes and light microscopy for the silver grain from the MAR, as previously decribed (Lee *et al*., 1999). In some experiments the silver grain density was quantified by counting the number of silver grains on the top of the filaments and 5 μm to each side. At least 25 filaments were counted in each sample and the average grain number was expressed as number per μm filament. The background grain density was counted in areas >10 μm away from MAR-positive bacteria.

Analytical methods

Acetate was measured using a Dionex ion-chromatograph with a suppressed conductivity detector, 0.2 mM NaOH as mobile phase and an IonPac, AS11 column. Thiosulphate was determined using 21 mM NaOH as mobile phase and an IonPac, AS11 column. Total CO_2 was determined by adding 1 ml of activated sludge to a closed 50 ml serum vial, 1 ml 6 N HCl was then added and the released CO_2 measured in the gas phase after 30 min by gas chromatography. Total CO_2 was identical in both the supernatant and the full sludge, showing the absence of precipitated carbonates in the floc matrix that could disturb the use of [^{14}C]-bicarbonate by making precipitates. Suspended solids (SS) were determined by filtration and dry weight determination, while volatile suspended solids (VSS) were determined after ignition at 550°C, both according to Standard Methods (1995).

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