Arch Microbiol (1988) 150:117–125

Archives of
Microbiology

Thiobacillus strain Q, a chemolithoheterotrophic sulphur bacterium

P. J. F. Gommers and J. G. Kuenen

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, NL-2628 BC Delft, The Netherlands

Abstract. The physiological properties of an organism isolated from a selective chemostat enrichment using acetate and thiosulphate as the limiting substrates, provisionally called *Thiobacillus* Q, were investigated. Although the organism made up 85% of the community in the enrichment culture, its expected chemolithotrophic nature was not apparent in batch experiments. The growth yield was not enhanced by the addition of thiosulphate to an acetate containing mineral medium, even though up to 50% of the thiosulphate was oxidized. Under acetate limitation in the chemostat, there was a linear increase in yield with thiosulphate addition up to a concentration of 7 mM. Higher thiosulphate concentrations resulted in loss of thiosulphate oxidizing capacity and a decrease in the biomass to the level obtained with acetate alone. This loss may be due to the presence of inhibitory (50–100 μM) levels of sulphite which is probably produced as an intermediate of the biological thiosulphate oxidation. Experiments with sulphide showed that *Thiobacillus* Q could also use it as an additional energy source. The complete lack of autotrophic growth, both in batch and chemostat experiments, together with the absence of even very low amounts of the key enzymes of the Calvin cycle demonstrated that this organism is a typical chemolithoheterotroph. Although this organism has provisionally been placed in the genus *Thiobacillus*, standard taxonomic procedures showed a close relationship with *Pseudomonas alcaligenes*. This study stresses the importance of quantitative chemostat studies in establishing the role of inorganic oxidations in energy metabolism and in the understanding of the role of heterotrophic sulphur oxidation in natural environments.

Key words: Sulphur bacterium – Sulphide/thiosulphate oxidation – Chemolithotroph – Mixed substrate – Sulphite inhibition – Non-autotrophic/heterotroph – *Thiobacillus*

Reduced sulphur compounds can be oxidized by many bacteria, some fungi and a few yeasts (Vitolins and Swaby 1969). Most of the known sulphur oxidizing bacteria are members of the so-called 'colourless sulphur bacteria' which include unrelated genera such as *Thiobacillus*, *Thiothrix*, *Thiomicrospira*, *Beggiatoa* and *Achromatium* (Kuenen 1988). It has been shown that in most of these non-phototrophic sulphur bacteria, the oxidation of sulphur compounds yields metabolically useful energy. However, in others, such as *Achromatium*, the physiological role of sulphur metabolism remains unknown. In some phototrophic bacteria, such as *Chromatium*, the reduced sulphur compounds may also serve as an energy source during microaerophilic, autotrophic growth in the dark (Kämpf and Pfennig 1980), although the primary role of reduced inorganic sulphur compounds in the phototrophs is to provide reducing power for CO_2 assimilation during anaerobic photosynthesis.

A number of bacteria which can also oxidize reduced sulphur compounds have not been included in "the colourless sulphur bacteria". With the exception of *Paracoccus denitrificans*, which can grow autotrophically on thiosulphate (Friedrich and Mitrenga 1981), most of the other examples are heterotrophic bacteria belonging to the genera *Pseudomonas*, *Streptomyces*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Micromonas*, *Alcaligenes*, *Brevibacterium*, *Achromobacter*, *Mycobacterium*, *Aquaspirillum*, *Xanthobacter* and *Escherichia coli* (Starkey 1935; Sijderius 1946; Vitolins and Swaby 1969; Mizoguchi et al. 1976; Schook and Berk 1978). The few species among the heterotrophs able to generate metabolically useful energy from the oxidation of reduced sulphur compounds are classified as chemolithoheterotrophs (Rittenberg 1969; Kelly and Kuenen 1984), but in most organisms the physiological role, if any, of the oxidation process is not known. A lack of well characterized strains of chemolithoheterotrophs has hampered the physiological study of this type of metabolism. The one named example, *Thiobacillus perometabolis*, has recently been shown to be able to grow autotrophically and therefore is a facultative chemolithoautotroph (London and Rittenberg 1967; Harrison 1983; Katayama-Fujimura and Kuraishi 1983). Tuttle and collaborators have described a number of marine heterotrophic sulphur oxidizers, one of which could be shown to obtain energy from thiosulphate oxidation (Tuttle et al. 1974; Tuttle and Jannasch 1977; Tuttle 1980). In view of the important role often ascribed to heterotrophic sulphur oxidizers both in soils (Guittonneau and Keilling 1932; Sijderius 1946; Swaby and Vitolins 1969; Skiba and Wainwright 1984; Wainwright 1984), and in marine environments (Tuttle and Jannasch 1972, 1973; Ruby et

Offprint requests to: J. G. Kuenen

al. 1981) it became desirable to try and isolate chemolithoheterotrophs by using selective chemostat enrichments (Gottschal and Kuenen 1980a). For this purpose the acetate/thiosulphate ratio chosen was high enough that acetate could serve as the only carbon source. Successful enrichments yielded two heterotrophic strains able to oxidize sulphur compounds. One organism, provisionally called *Thiobacillus* Q, was the dominant (86%) organism in the enrichment, while in a second experiment the chemolithoheterotroph made up 25% of the population.

However, it turned out to be extremely difficult to demonstrate their chemolithotrophic nature. This paper describes the results of this study, which indicates that, in the one species studied in detail, the oxidation of sulphur compounds yields metabolically useful energy only under substrate limiting conditions.

Materials and methods

Strain. The isolate used in this study, provisionally called *Thiobacillus* Q came from an enrichment inoculated with ditch water (originally designated as strain V, Gottschal and Kuenen 1980a). The enrichment procedure involved an energy limited chemostat enrichment supplied with 15 mM acetate and 10 mM thiosulphate). *Thiobacillus* Q is deposited as LMD 81.11 in the Delft culture collection.

Media. The basal medium used in batch experiments contained (g/l): K_2HPO_4, 8; KH_2PO_4, 3; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4. Only the two phosphate salts were changed for use in the chemostat (g/l): K_2HPO_4, 0.08; KH_2PO_4, 0.03 (unless stated otherwise). The pH was adjusted to 7.5 with KOH. All media also contained 2 ml/l of a trace element solution (Vishniac and Santer 1957). This solution contained 2.2 g rather than the originally reported 22 g $ZnSO_4 \cdot 7H_2O$ per liter. Vitamin B12 and biotin were added to a final concentration of 20 µg and 10 µg per liter. Media were sterilized by autoclaving for 20 min at 120° C. The trace element solution, the $MgSO_4$ and the vitamins were autoclaved separately. Acetate, thiosulphate and other substrates were supplied at the concentrations described in the Results section. Anaerobic growth tests were performed in the presence and absence of 20 mM KNO_3. Solid media were prepared by the addition of 2% Difco Bacto Agar to the appropriate liquid medium. Autotrophic growth was checked in this medium and in a ferric ammonium citrate medium as described by Schlegel et al. (1961). Whenever possible the growth tests were checked with the results from the standard taxonomic test arrays API NE 50 and API 10 S.

Cultivation. Batch cultures were incubated on rotary shakers. For substrate screening, cultures were transferred three times in the same medium to confirm positive results. Continuous culture experiments were performed in a 1.0–1.5 l vessel equipped with pH control, temperature control and dissolved oxygen measurement as described by Harder et al. (1974). A wash out experiment involved a change from a growth supporting medium (10 mM acetate + 5 mM thiosulphate) to a medium that would not support growth (5 mM thiosulphate) of *Thiobacillus* Q. The dilution rate was kept constant and therefore the biomass-concentration in the culture vessel was expected to decrease exponentially, according to the chemostat theory [$x = x_0 \cdot \exp(-D \cdot t)$, in which x is the biomass concentration, x_0 is the biomass concentration at zero time, t is time and D is the dilution rate].

Biomass determination. Four methods were used to determine steady state biomass concentration in the chemostat.

Optical density (OD_{430}) was measured with a Hitachi spectrophotometer, Model 100-60, at 430 nm against water as a blank. The OD_{430} showed a linear relationship with dry weight biomass content, except when polyphosphate had accumulated.

Dry weights were measured by centrifuging 20–40 ml of cell suspension at $6{,}500 \times g$ and then drying the pellet at 80° C for 24 h. Experiments were performed in triplicate and controls for salts in the remaining medium were included.

Protein determinations were performed according to Lowry et al. (1951) and by the Microbiuret method (Goa 1953) on cells washed twice in phosphate buffer. The first method gave slightly higher results, corresponding better with the generally accepted 60% protein content of bacteria, but since sulphur compounds interfere with this method, the second, less sensitive method was always used as a check.

Cell carbon was determined with a Beckman Tocamaster Model 15-B. The cultures were only considered to have reached a steady state when all parameters were stable for at least 2 volume changes.

Oxidation rates. Oxygen consumption rates of cell suspensions were measured polarographically using a Biological Oxygen Monitor (Yellow Springs Instruments). The endogenous activity of steady state cells was always below 20 nmol O_2/min · mg protein.

Preparation of cell free extracts. Bacteria were harvested from steady state chemostat cultures and centrifuged at 4° C and $11{,}000 \times g$ for 15 min. Pellets were washed in TRIS-HCl buffer (pH 8.2, 20 mM $MgCl_2$, 5 mM $NaHCO_3$ and 0.5 mM dithiothreitol) and resuspended in the same buffer. Cell densities were 5–20 mg protein per ml. The washed cells were sonified in melting ice with an ultrasonic probe (MSE ltd, 150 W), five times for 30 s with 30 s cooling intervals in between. Glass beads (Ballotini, diameter 0.10 mm) were added to the cell suspension prior to sonification (1 g beads per 2 ml suspension). After centrifuging at $11{,}000 \times g$ for 15 min the supernatant was used for enzyme assays.

Enzyme assays. Ribulose-bisphosphate carboxylase/oxygenase (RubP-case) activity in cell free extracts was determined as described by Beudeker et al. (1980) using ^{14}C-labeled $NaHCO_3$. Ribulose-monophosphate kinase activity was determined by the coupled assay with RubP-case as described by Siebert et al. (1981). The spinach RubP-case was obtained from Sigma.

CO_2 fixation. The fixation of CO_2 by whole cells was determined with the ratio-labeled tracer method described by Beudeker et al. (1980).

Crossed immunoelectrophoresis. Cell free extracts were examined with crossed immunoelectrophoresis with antibodies

prepared against RubP-case of *Thiobacillus neapolitanus* as described by Holthuijzen et al. (1986).

Intracellular phosphorous. Cells were harvested from steady state chemostat cultures and washed twice with TRIS-HCl buffer pH 7.5. For the assay of phosphate content the cells were dissolved as described by Ames and Dubin (1960). Phosphate was measured with the method described by Chen et al. (1956).

Miscellaneous. Thiosulphate was determined according to Sörbo (1957), sulphite according to Trüper and Schlegel (1964) with fuchsin except when thiosulphate was present. High (> 100 μM) concentrations of thiosulphate interfere with the sulphite determination and low concentrations of sulphite (< 100 μM) could only be determined qualitatively in its presence. Sulphate was measured with a Waters HPLC using a Chrompack ion-exchange column (Ionosphere TMA, 250 × 4.6 mm) and detection by refraction index measurement. Sulphide was determined after conversion to methylene blue as described by Trüper and Schlegel (1964) or as thiocyanate according to Sörbo (1957). In the latter case copper sulfate was omitted. Sulphur was determined by extraction of the pellet with 2 ml acetone, followed by cyanolysis in the acetone solution according to Sörbo (1957) (W. Hazeu, unpublished). Acetate was measured as organic carbon with the carbon analyser (see cell carbon). Solutions of sulphide and sulphite were made in anoxic demineralized water and kept under N_2 atmosphere when not used immediately. Thiosulphate solutions were made in demineralized water with a pH higher than 6.

Unless otherwise stated all chemicals were of analytical grade.

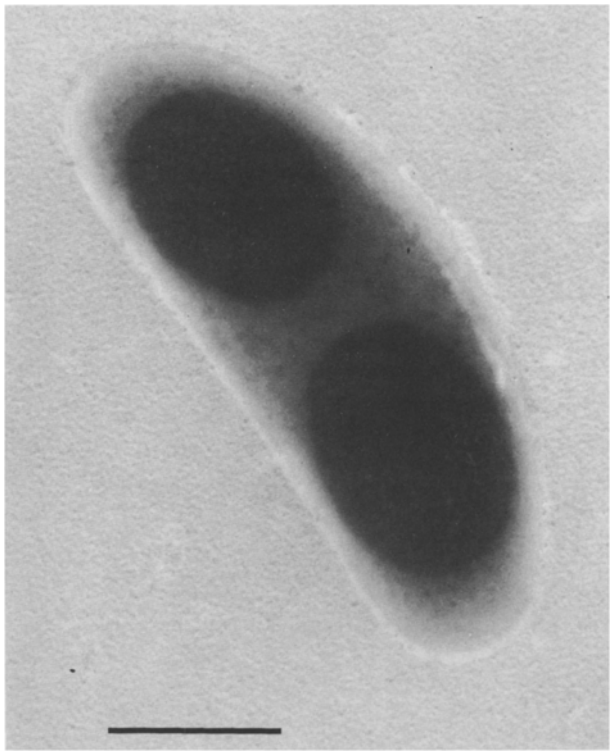

Fig. 1. Electron micrograph of *Thiobacillus* Q showing phosphate accumulation (the bar indicates 0.5 μm). The organism had been grown in an acetate limited chemostat at a D of 0.09 h^{-1}, in a medium containing 6.8 mM K_2HPO_4. When grown in 0.68 mM phosphate the organism does not show phosphate accumulation

Results and discussion

General description

Thiobacillus Q was a Gram-negative, vibrio shaped, motile organism with a tendency to form spirals when grown in rich media. Strain Q was capable of aerobic growth on the following carboxylic acids in mineral medium: acetate, glyoxylate, glycerate, propionate, lactate, malate, pyruvate, butyrate, succinate and fumarate. The organism had a vitamin requirement for biotin and vitamin B12. It could not grow on fructose, glucose, arabinose, mannose, galactose, maltose, lactose, xylose, mannitol, dulcitol, gluconate, glycogen, adipate, citrate, oxaloacetate, glycerol, dihydroxyacetone, ethanol, oxalate, formaldehyde, benzoate, methylamine, dimethylamine, urea, methionine and serine. Thus, in contrast to well known chemolithotrophic species (*Thiobacillus novellus*, *Thiobacillus versutus*) *Thiobacillus* Q is unable to grow on sugars and (poly)alcohols (Kelly and Harrison 1988). The organism was unable to grow anaerobically (in the presence or absence of nitrate) on any of the substrates tested. All attempts to grow strain Q autotrophically failed: it could not grow in biotin and vitamin-B12 supplemented mineral medium with thiosulphate, sulphide, formate or H_2. Supplementation of the media with low concentrations of yeast extract (1 – 50 mg/l) or a vitamin mixture (Bruinenberg et al. 1983) did not allow for autotrophic growth, which is in contrast to observations made for a number of *Thiobacillus novellus* strains (Matin 1978; Matin et al. 1980). Attempts to show growth in a ferric nitrate medium, which is often used for autotrophic growth on H_2 also failed. Optimum temperature for growth was between 30 and 35° C. The pH for growth ranged from 6.5 to 8.5 with an optimum of 7.5. On solid media containing acetate and thiosulphate, strain Q formed small (1 mm), creamy-coloured colonies with raised centers and flat, veil-like fringes. Growth of strain Q on thiosulphate agar produced the barely visible, pinpoint colonies typical of background growth on organic impurities in the agar. These were white and translucent, and contained some sulphur deposits.

Using standard taxomomical routines, such as API NE 50 and 10 S, for heterotrophic bacteria, together with its general properties, strain Q would be classified as a *Pseudomonas alcaligenes* (Palleroni 1984). However, since the ability to utilize reduced sulphur compounds as an energy source is regarded as taxonomically significant (Harrison 1983), the isolate has been provisionally placed in the genus *Thiobacillus*. In view of the recent developments in biochemical taxonomy, for example 16 sRNA analysis (Fox et al. 1980; Lane et al. 1985), it was felt that naming of this organism should not be done before this analysis has been carried out and a possible taxonomic relation of strain Q with *Pseudomonas* or *Thiobacillus* has been established.

An unexpected property of *Thiobacillus* Q, not shown before for any known *Thiobacillus* sp., was its ability to accumulate polyphosphate under certain growth conditions. When grown in the chemostat under energy limitation (acetate or acetate + thiosulphate) in a phosphate buffer of 6.8 mM instead of the 0.68 mM, used throughout this study,

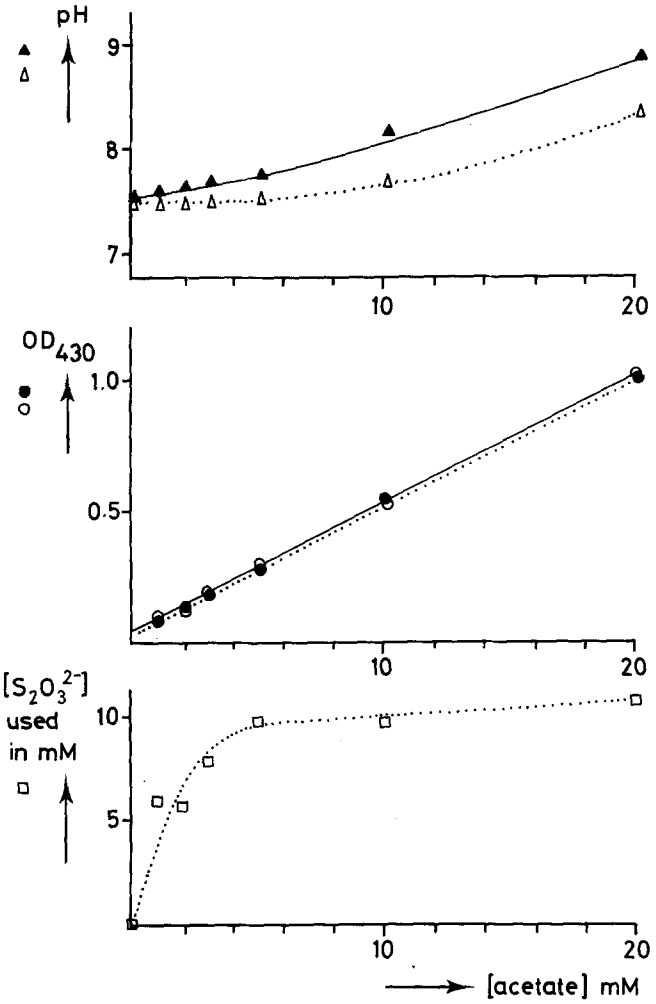

Fig. 2. Biomass concentration, pH at the end of the growth, and thiosulphate consumption of *Thiobacillus* strain Q in batch experiments as a function of the acetate concentration. *Solid line, closed symbols:* media with acetate only; *dotted line, open symbols:* media with acetate and 10 mM thiosulphate. The inoculum was taken from a chemostat culture growing under acetate and thiosulphate limitation

the accumulation of polyphosphates was visible as large, electron dense inclusion bodies (Fig. 1). Analysis showed that under these conditions up to 50% of the biomass could consist of polyphosphate (120 mg phosphate/240 mg dry weight). When the phosphate concentration in the medium was lowered from 6.8 to 0.68 mM the phosphorous content dropped drastically, ending at about 3% of the biomass. This is comparable with bacteria that do not accumulate phosphate. After this change a small increase of protein yield was observed and the large inclusion bodies completely disappeared.

Batch experiments

Batch growth experiments with different ratios of acetate and thiosulphate were performed to investigate the mixotrophic capabilities of *Thiobacillus* Q under non-limiting conditions. As judged from optical density and organic cell carbon measurements (Fig. 2), the addition of thiosulphate to the acetate medium did not result in higher biomass yields at any ratio of acetate to thiosulphate. Thiosulphate analyses and pH measurements showed that up to 50% of the thiosulphate added was oxidized to sulphate. Even when the batch cultures were inoculated with chemostat cultures of *Thiobacillus* Q that could effectively oxidize thiosulphate (see next paragraph), no cell yield increase was observed. Further experiments using the other 9 organic growth substrates, as mentioned above, with and without thiosulphate produced essentially similar results.

This failure of the yield to increase in spite of substantial thiosulphate oxidation has also been shown for batch cultures of the facultative autotroph, *Thiobacillus novellus* (Matin 1978; Perez and Matin 1980), and a number of marine heterotrophic thiosulphate oxidizers isolated by Tuttle et al. (1974). For *Thiobacillus novellus* it had been suggested that thiosulphate and/or sulphite toxicity caused general uncoupling of energy generation and growth (Perez and Matin 1980).

Romano et al. (1975) suggested that sulphite toxicity was due to inhibition of specific enzymes from the metabolism of glucose. However, such an explanation in this case seems unlikely, since the growth yields in the presence and absence of thiosulphate were identical at a number of acetate to thiosulphate ratios. The question remained why *Thiobacillus* Q had become the dominant species during a substrate limited chemostat enrichment from a natural source with 10 mM thiosulphate and 15 mM acetate in the mineral medium. It seemed clear that this organism in the competition with other heterotrophs should have had the advantage of energy generation from thiosulphate.

Therefore experiments in continuous culture were conducted to demonstrate the potential chemolithotrophic nature of *Thiobacillus* Q.

Chemostat experiments with thiosulphate

Thiobacillus Q was grown in the chemostat in mineral medium under acetate limitation at a dilution rate of 0.09 h^{-1}. After steady state conditions were reached (4–7 volume changes) as judged by biomass parameters (OD_{430}, mg protein/l, mg cell carbon/l, mg dry weight/l), thiosulphate was added to the medium reservoir at a concentration of 2 mM. By this procedure thiosulphate could be added to the growing culture very slowly. During the following transient state the thiosulphate concentration in the culture increased for 1 h. After 30 min the thiosulphate oxidation potential had been induced, and after the first hour the thiosulphate concentration had dropped below the detection level (< 20 μM). Once a steady state had been reached, the thiosulphate concentration in the medium was increased stepwise up to 7 mM. Thiosulphate, sulphite and acetate were not detectable in the steady state supernatants. Biomass concentration and oxidation potentials were measured and are shown in Fig. 3A, B respectively. The biomass yield increase on thiosulphate was within the range generally observed for Thiobacilli (5–13 mg dry wt./mmol thiosulphate, Kuenen and Beudeker 1982). Under these conditions, *Thiobacillus* Q possessed a clear overcapacity for thiosulphate oxidation (Fig. 3B) which was already fully induced at 1 mM thiosulphate in the influent. These experiments show that under energy limited conditions *Thiobacillus* Q was indeed capable of making use of the energy from the oxidation of thiosulphate. A similar observation was made for *Thiobacillus novellus* grown under glucose

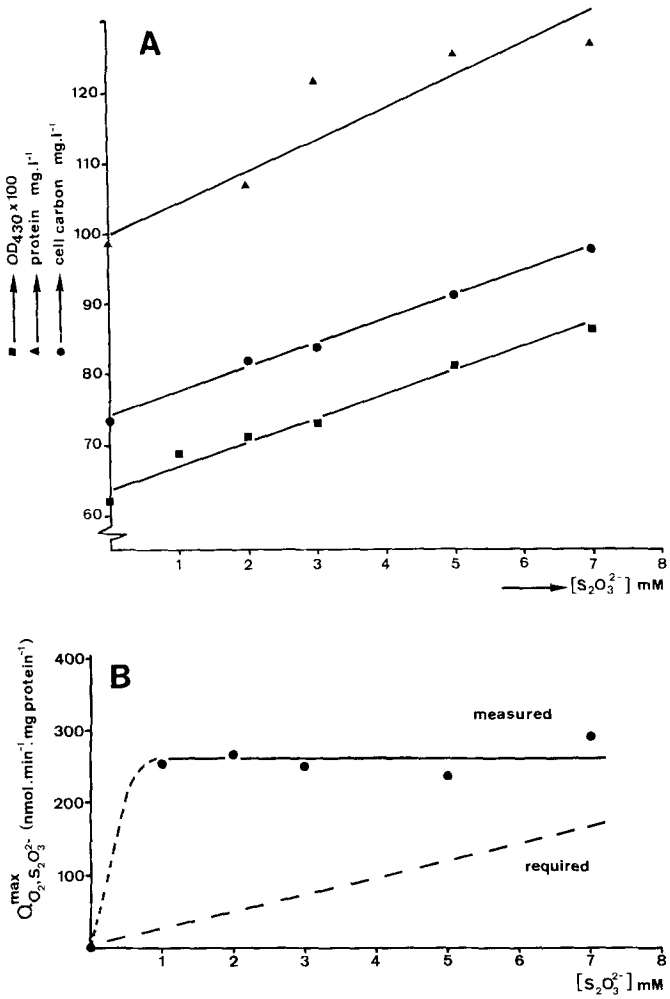

Fig. 3A, B. Biomass (as protein), concentration, OD_{430} and cell carbon (**A**) and thiosulphate oxidation potential (**B**) of steady state chemostat cultures grown under limitation by acetate (medium concentration 10 mM) and increasing amounts of thiosulphate in the medium (D = 0.09 h^{-1}, T = 37°C, pH = 7.5). *The dotted line* in **B** is the thiosulphate oxidation potential required to oxidize the thiosulphate present in the medium

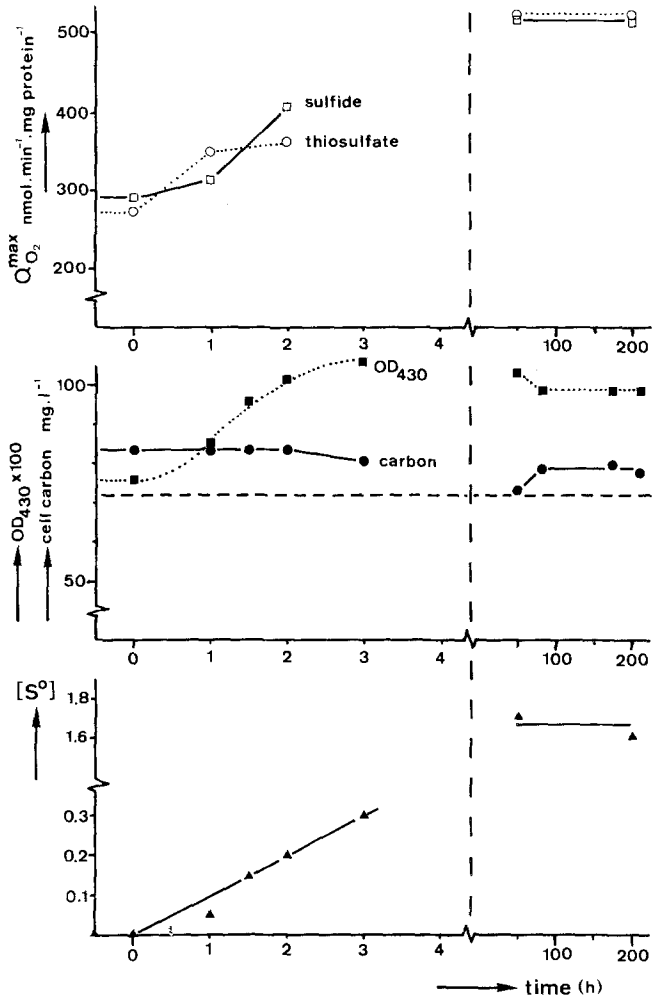

Fig. 4. Biomass concentrations as OD_{430} (■) and cell carbon (●), sulphur accumulation (▲) and oxidation potentials (sulfide, □; thiosulphate, ○) in chemostat cultures of *Thiobacillus* Q changing at zero time from 10 mM acetate plus 5 mM thiosulphate to 10 mM acetate plus 5 mM sulphide as growth limiting substrates (D = 0.09 h^{-1}, T = 30°C, pH = 7.5)

plus thiosulphate limitation (Leefeldt and Matin 1980). An analogous phenomenon occurs in cultures of *Acinetobacter calcoaceticus* grown on acetate supplemented with glucose, a substrate which this species only uses as an energy source (van Schie et al. 1984). No increase of the biomass yield was observed in batch experiments although in all experiments the glucose was completely converted to gluconic acid (B. J. van Schie, pers. comm.). However, in chemostat cultures the oxidation of glucose to gluconic acid resulted in an increase in the yield. While the explanation for this behaviour also remains unknown, the observations confirm that it is essential to use energy-limited chemostat cultures to study the role of auxiliary energy sources in metabolism. A detailed interpretation of the yield increase in terms of assimilatory and dissimilatory flows of carbon and energy sources will be presented elsewhere (Gommers et al. unpublished work).

Chemostat experiments with sulphide

Athough thiosulphate is a natural substrate in neutral environments, it usually originates from the oxidation of sulphide. For at least two other aerobic (facultatively), autotrophic *Thiobacillus* species, using substrate limited chemostat experiments, thiosulphate and sulphide were shown to be equivalent energy sources (Beudeker et al. 1982). The sulphide was oxidized under aerobic conditions without appreciable formation of elemental sulphur. However, the chemolithoheterotrophic *Hyphomicrobium* EG had a higher affinity for sulphide than for thiosulphate (Suylen et al. 1986). Therefore it was of interest whether *Thiobacillus* Q, would show similar or different behaviour towards sulphide. Addition of sulphide (1 mM) to the reservoir of an acetate limited culture resulted in a heavy sulphur deposit in the culture vessel even though the oxidation potential for sulphide had been induced at a level high enough to cope with the amount of sulphide added. Even after a high sulphide oxidizing potential was pre-induced with thiosulphate (5 mM), sulphur production started instantaneously when thiosulphate was replaced by sulphide (Fig. 4). The formation of sulphur explained the sudden increase in the optical density of the culture, while the amount of cell carbon remained fairly constant. After 5 to

Table 1. Sulphur balances of steady state chemostat cultures of *Thiobacillus* Q shown under acetate plus reduced sulphur compound limitation in a medium with 10 mM acetate plus 4.6 mM thiosulphate or 10 mM acetate plus 5.0 mM sulphide

Medium	10 μM acetate + 4.6 mM thiosulphate	10 mM acetate + 5.0 mM sulphide
In		
Thiosulphate	9.2[a]	–[c]
Sulphide	–	5.0
Sulphate in medium	0.4[b]	0.4
Sulphate acid titration	0.4[b]	2.5
Total	10.0	7.9
Out		
Thiosulphate	–	–
Sulphide	–	–
Sulphate	10.0	5.9
Sulphur	–	1.7
Total	10.0	7.6

[a] All numbers are expressed as mmol of sulphur in (spent) medium flowing in, or out

[b] The sulphur balance included sulphate in the medium and sulphate originating from sulphuric acid used for pH correction during cultivation in the chemostat

[c] (–) not detectable, that is < 0.5 μM for sulphide and < 10 μM for thiosulphate

20 volume changes (50–200 h) the sulphur balance for a 'semi steady state' (Table 1) showed that approximately 35% of the sulphide was converted to sulphur. The remaining 65% was oxidized completely to sulphate. The corresponding biomass yield was low compared to the steady state on 10 mM acetate plus 5 m thiosulphate, but still significantly higher than that obtained with an acetate grown culture. The observed yield increase by sulphide was 4.4 g dry wt. per mol which is also in the range generally observed for *Thiobacilli*. It can thus be concluded that *Thiobacillus* Q can use both thiosulphate and sulphide as metabolically useful energy sources.

Autotrophic potential

Even though *Thiobacillus* Q was capable of deriving metabolically useful energy from the oxidation of thiosulphate and sulphide, it could not be grown autotrophically on these substrates. An inability to fix CO_2 via the Calvin cycle might explain this. The key enzymes of this cycle, ribulose-1,5-biphosphate carboxylase (RubP-case) and ribulose monophosphate kinase (RumP-kinase) were measured in cell free extracts of *Thiobacillus* Q grown on 10 mM plus 5 mM thiosulphate. The results were negative, showing that under these growth conditions *Thiobacillus* Q lacks the ability to make these enzymes. Positive controls with *Thiobacillus neapolitanus*, *Thiobacillus versutus* and the addition of spinach RubP-case to the cell-free extract (not shown) confirmed the adequacy of the experimental procedures. Tests using crossed immunoelectrophoresis of cell-free extract with antibodies prepared against the RubP-case of *Thiobacillus neapolitanus* (Beudeker et al. 1981) showed no reaction. This is, of course, only negative evidence since the RubP-case from some species does not react with these antibodies (R. Beudeker, pers. comm.). However, a further indication was given by the absence of any detectable CO_2-uptake by whole cells. It could be argued that at the high acetate to thiosulphate ratio used in these experiments, autotrophic CO_2-fixation would not be induced, as was reported for *Thiobacillus versutus* (Gottschal and Kuenen 1980b). To circumvent this possibility and to check for autotrophic growth induced when large inocula are used, as reported for *Thiobacillus perometabolis* (Harrison 1983), a wash-out curve under autotrophic conditions (5 mM thiosulphate) of an actively growing, thiosulphate oxidizing culture of *Thiobacillus* Q was recorded. A steady state culture of *Thiobacillus* Q growing on 10 mM acetate and 5 mM thiosulphate was connected to a feed with 5 mM thiosulphate only. Any growth at a rate above 0.005 h^{-1} would result in a detectable deviation from the theoretically predicted wash-out curve. Figure 5 clearly shows an exact match between theoretical and experimental results for both the decrease in OD_{430} and cell carbon during the wash-out period, demonstrating the complete lack of autotrophic potential of *Thiobacillus* Q under these conditions. The appearance of thiosulphate in the culture supernatant only after 20 h from the start of the wash-out curve indicates that during the wash-out experiment the cells did not lose any thiosulphate oxidizing activity, demonstrating that non-growing cells remained metabolically active for at least 20–30 h.

Although the results of experiments to show the absence of an ability must be, by definition, negative, the weight of evidence presented here is such that it must be concluded that, under the experimental conditions tested, *Thiobacillus* Q should be regarded a true chemolithoheterotroph.

Thiosulphate oxidation and inhibition by sulphite

In mixotrophic steady state culture, *Thiobacillus* Q converted thiosulphate quantitatively to sulfate (Table 1). However, repeated experiments showed that such cultures could loose their thiosulphate oxidizing potential when the thiosulphate concentration in the influent was increased too fast. The same happened when the ratio of thiosulphate to acetate in the chemostat medium exceeded 0.7. It should be noted that although thiosulphate oxidation was abolished, the culture continued to grow in steady state on the acetate provided. The growth was identical to those of steady states in the complete absence of thiosulphate. It had been suggested that inhibition of thiosulphate oxidation may be due to sulphite formation (Gottschal and Kuenen 1980a). Indeed, in supernatants sulphite concentrations between 50 and 100 μM could be detected. To show that inhibition by sulphite was the possible cause of this phenomenon, oxygen uptake experiments were performed with thiosulphate and increasing amounts of sulphite. Cells from a steady state chemostat culture (10 mM acetate + 1 mM thiosulphate or 10 mM acetate + 1 mM sulphide) were used. Sulphite was not oxidized by *Thiobacillus* Q at all (neither were elemental sulphur or tetrathionate). Both with cells grown on acetate plus thiosulphate or acetate plus sulphide, complete inhibition of thiosulphate oxidation occurred at 400 μM sulphite (Fig. 6). Neither the acetate nor the sulphide oxidation were inhibited at this sulphite concentration. Even when excess

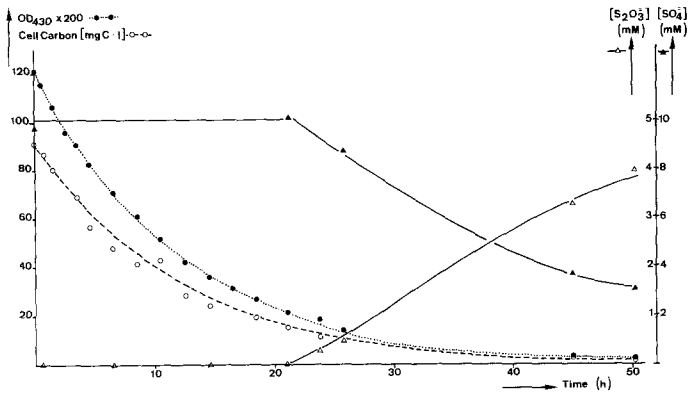

Fig. 5. Cell carbon, OD_{430} and thiosulphate concentration in the chemostat (D = 0.083 h^{-1}, T = 30°C, pH = 7.5) after a change in the medium composition from 10 mM acetate and 5 mM thiosulphate to 5 mM thiosulphate alone (autotrophic wash-out curve) at zero time. The increase of the thiosulphate concentration corresponded with the decrease of the sulphate concentration. The *broken lines* through the cell carbon and the OD_{430} data show the theoretical wash-out curves as expected if strain Q does not grow under these conditions

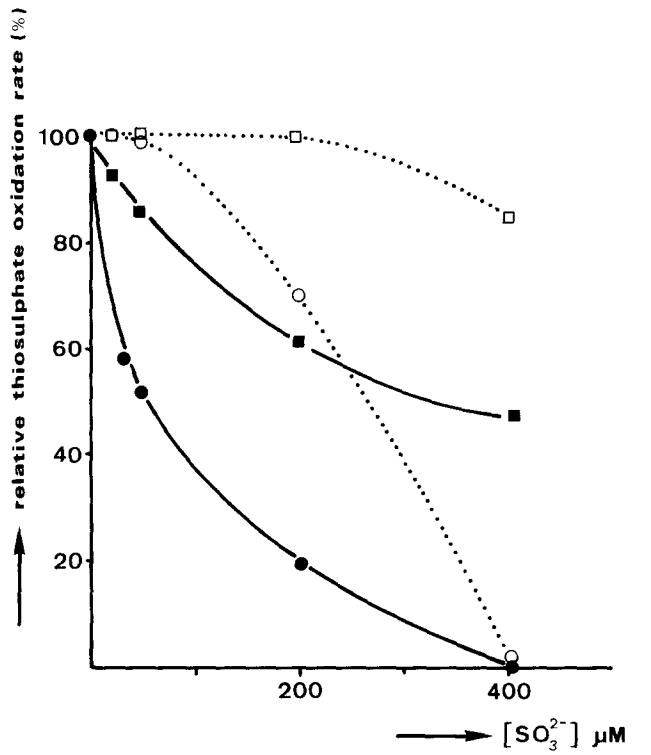

Fig. 6. Relative thiosulphate oxidation rate of *Thiobacillus* Q as a function of the sulphite concentration with (*solid lines*) or without (*dotted lines*) preincubation for 1 h in the presence of the corresponding sulphite concentration. Cells were pregrown in a chemostat with 10 mM acetate plus 1 mM thiosulphate. The oxidation potential was tested with 40 µM thiosulphate (*circles*) and with 1 mM thiosulphate

thiosulphate was added, the oxidation was still strongly inhibited by sulphite at 400 µM. Preincubation with sulphite for 1 h (or the addition of 0.1% Triton X) increased the inhibitory effect of the sulphite (Fig. 6). Under all of these conditions, acetate oxidation was not inhibited. This suggests that long 'contact' times with even the very low concentrations of sulphite which occurred in the chemostat vessel could cause the disappearance of the thiosulphate oxidation capacity during growth.

Ecological and practical significance

Thiobacillus Q became the dominant species in a chemostat enrichment with 15 mM acetate and 10 mM thiosulphate as the limiting substrates using an inoculum known to contain both heterotrophic and facultative chemoautotrophic organisms (Gottschal and Kuenen 1980a). This can only be explained if this organism had had a selective advantage under these conditions. The ability to obtain energy from low concentrations of reduced sulphur compounds in the presence of an organic substrate should confer an advantage on this type of organism during competition with other heterotrophs. When the relative concentration of the reduced sulphur compound was low, the energetically expensive CO_2 fixation would not be useful and thus the lack of autotrophic potential in *Thiobacillus* Q might be advantageous in competition with facultative chemolithoautotrophs.

The maximum specific thiosulphate or sulphide oxidation rates of strain Q are 10 to 20-fold lower than the maximum specific activities of autotrophic and mixotrophic sulphur oxidizers (Beudeker et al. 1982), but at the given ratio of acetate to thiosulphate or sulphide turnover, the rate apparently is sufficiently high to give *Thiobacillus* Q a competitive advantage. Given the fact that the ecological niche of *Thiobacillus* Q would lie in environments with low sulphur concentrations, the induction of its maximum oxidation potential at low concentrations of the sulphur compounds indicates an efficient adaptation to this type of environment. Also the toxicity of high concentrations of thiosulphate is indicative that the metabolism of strain Q is adapted to deal only with the relatively low concentrations of sulphur compounds found in many habitats. Whether the same may be found for other heterotrophic sulphur oxidizers remains to be seen. The role of chemolithoheterotrophic sulphur oxidation in waste water treatment is unknown. Based on the observations by Robertson and Kuenen (1983) it should be expected that where influents contain high ratios of organic to sulphur compounds, as is the case in domestic sewage, chemolithoheterotrophs may be selected. Such systems would have a relatively small resilience for sudden increases in sulphur supply, due to the relative low maximum thiosulphate or sulphide oxidation capacity of these organisms. It may be speculated that the well known odour (H_2S) problems in routine waste water treatments may in some cases be traced back to the limited capabilities of chemolithoheterotrophic sulphur oxidizers.

Acknowledgements. We thank L. A. Robertson for critical reading of the manuscript, B. Reitsma for help with some of the chemostat experiments, W. Batenburg for taking the E. M. pictures, J. v. d. Toorn for assistance with the bacteriological identification and Y. A. Holthuijzen for performing the crossed immunoelectrophoresis. The investigations were supported in part by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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Received December 9, 1987/Accepted February 29, 1988