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Microbiology of Thiobacilli and Other Sulphur-Oxidizing Autotrophs, Mixotrophs and Heterotrophs [and Discussion]

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Microbiology of thiobacilli and other sulphur-oxidizing autotrophs, mixotrophs and heterotrophs

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Recent studies on the ecophysiology of the obligate chemolithotroph *Thiobacillus neapolitanus* have given better insight into its specialization for an autotrophic mode of life. This appears not only from its high constitutive levels of autotrophic enzymes, but also from its possession of carboxysomes, which seem to be specialized organelles for CO_2 fixation and concentrating reducing power. At the same time, these organisms are metabolically versatile with respect to nitrogen assimilation pathways, and during starvation are able to utilize endogenous resources such as polyglucose for carbon and energy.

Studies on the facultative chemolithotrophs such as *Thiobacillus novellus* and *Thiobacillus* A2 have shown that they can grow mixotrophically on mixtures of inorganic and organic substrates, i.e. they can utilize these compounds simultaneously provided that they are growth limiting. *Thiobacillus* A2 displays a remarkable flexibility not only with respect to the organic substrates that it can utilize but, for example, also in the choice of various pathways for glucose metabolism. Competition experiments carried out between specialized and versatile thiobacilli strongly indicate that the ecological advantage of the versatile thiobacilli may lie not so much in their short-term flexibility, but rather in their ability to grow mixotrophically.

Studies on most heterotrophic chemolithotrophs are still in their infancy. Promising progress has been made in the study of the physiology of *Beggiatoa* species.

Renewed interest in the sulphur-oxidizing bacteria stems from recent findings about their role in food chains, and their possible application in industry.

Introduction

Reduced inorganic sulphur compounds produced during mineralization processes in the environment are subject to biological oxidation in the absence and presence of light under aerobic and anaerobic conditions. This paper deals with the *omnium gatherum* of colourless bacteria involved in the oxidation of reduced inorganic sulphur compounds. This includes organisms with widely different types of physiology and morphology, ranging from specialist obligate chemolithotrophs, via facultative chemolithotrophs which may grow mixotrophically, to specialist heterotrophs, some of which may benefit from the oxidation of reduced sulphur compounds (table 1). In this review we shall refer to the sulphur-oxidizing chemolithotrophs simply as chemolithotrophs.

The obligate chemolithotrophs belong to the genera *Thiobacillus* and *Thiomicrospira*. These organisms are able to generate energy only from the oxidation of reduced inorganic sulphur compounds such as sulphide, thiosulphate and elemental sulphur and not from the oxidation of organic compounds. Organic carbon is synthesized by these bacteria from CO_2 via the Calvin cycle, but exogenous organic carbon compounds may contribute maximally to 20–30% of the total cell carbon.

Facultative chemolithotrophs are not only able to grow autotrophically with reduced inorganic sulphur compounds as energy source, but are also capable of heterotrophic growth. Such bacteria belong to the genera *Thiobacillus*, *Sulfolobus*, *Thermothrix* and *Paracoccus* (table 1). Several *Thiobacillus* species are able to utilize mixtures of inorganic and organic compounds simultaneously (often referred to as 'mixotrophic' growth). Depending on the ratio of inorganic and organic substrates, CO_2 may serve as an additional carbon source.

TABLE 1. COLOURLESS SULPHUR BACTERIA OXIDIZING INORGANIC SULPHUR COMPOUNDS

obligate chemolithotrophs		facultative chemolithotrophs		chemolitho-heterotrophs	heterotrophs	unclassified
aerobes	fac. anaerobes	aerobes	fac. anaerobes			
T. neapolitanus	*T. denitrificans*	*T. intermedius*	*Thiobacillus* A2 (on organic substrates)	*T. perometabolis*	*Beggiatoa* spp.	*Thiovulum*
T. thiooxidans	*T. thioparus* (to NO_2^-)	*T. novellus*	*Thermothrix thiopara*‡	*Pseudomonas* sp.	*Pseudomonas* spp.	*Thiophysa*
T. ferrooxidans	*Tms. denitrificans*	*T. acidophilus*	*P. denitrificans*§ (on H_2)			*Thiothrix*
T. kabobis†		*T. organoparus*				*Thiospira*
Tms. pelophila		*Sulfolobus acidocaldarius*				*Thioploca*¶
		Sulfolobus brierleyi				

† Recently isolated from soil adjacent to a sulphur stockpile. It differs from *T. thiooxidans* only in its ability to oxidize tetrathionate (Reynolds *et al.* 1981).
‡ This organism was isolated from hot sulphur springs (72 °C) (Brannan & Caldwell 1980).
§ Friedrich & Mitrenga (1981).
¶ Gallardo (1977).

Heterotrophic bacteria able to oxidize reduced inorganic sulphur compounds may be divided into two groups, those that obtain energy from the oxidation process and those that at first sight do not measurably benefit from it. The former group contains members of the genera *Thiobacillus* and *Pseudomonas*. For organisms that do not obtain energy from oxidation of sulphide, it may still be advantageous to oxidize this compound because of its toxicity. In the *Beggiatoa* species that lack catalase, the oxidation of sulphide may protect the cells from reactive harmful oxygen compounds such as hydrogen peroxide. At the same time, the oxidation of sulphide produces intracellular sulphur, which can serve as an electron acceptor for the oxidation of organic compounds under anaerobic conditions.

The physiological characteristics of the different types of bacteria and their interactions with each other, with the abiotic environment and with eukaryotes will be considered in this paper. The central theme is the question of how the physiology of these organisms can account for their distribution and role in Nature, and how differences in their physiology can explain their coexistence in a given environment. For a detailed discussion of the biochemistry of energy metabolism of the thiobacilli the reader is referred to a paper in this symposium by Kelly.

PHYSIOLOGY OF OBLIGATE CHEMOLITHOTROPHS

The obligately chemolithotrophic way of life has been a puzzling phenomenon ever since the foundation of the concept by Winogradsky (1888). Extensive reviews have appeared dealing with the highly specialized obligate chemolithotrophs (Rittenberg 1969; Kelly 1971; Rittenberg 1972; Schlegel 1975; Smith & Hoare 1977; Matin 1978). Attempts based on physiological data

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to explain why these organisms synthesize their organic carbon from CO_2 by the energy-consuming Calvin cycle and do not, or only to a limited extent, assimilate and dissimilate external organic carbon compounds, are still far from satisfying. It seems as if a complicated combination of metabolic lesions, a low transport capacity for organic compounds and a lack of induction of relevant enzymes, if present, in response to the appearance of potential organic substrates is responsible for the obdurate obligately chemolithotrophic way of life. At the same time, these metabolic features may be the consequence of the specialist way of life, thus providing optimal survival value. The necessity of these metabolic features for the survival of obligate chemolithotrophs in Nature is, however, not understood.

Since the review of Matin (1978) on this matter, research on obligate chemolithotrophs has been continued in only a few laboratories. We have studied *Thiobacillus neapolitanus* as a model organism for this group of bacteria. Attempts were undertaken to describe the ecological niche for this type of organism, which in Nature must compete for reduced inorganic sulphur compounds with the types of bacteria listed in table 1. *T. neapolitanus* was considered to be metabolically rigid owing to its lack of response to the appearance of organic substrates in the growth medium, the enzymes required for the metabolism of these compounds never being induced. Since the natural environment changes continuously, a rigid metabolism may be considered to be disadvantageous. Studies on the effects of fluctuating inorganic parameters on the organism showed, however, that *T. neapolitanus* was rather flexible with respect to the assimilation of CO_2 and nitrogen. The activity of the CO_2-fixing enzyme of the Calvin cycle, D-ribulose 1,5-bisphosphate carboxylase (RuBPCase), increased as the CO_2 concentration in the growth medium decreased. Maximal activity was detected in CO_2-limited cells (Beudeker *et al.* 1980). The increase in RuBPCase activity was due to an increase in RuBPCase protein levels. During CO_2 limitation, RuBPCase accounted for up to 17% of total cell protein in *T. neapolitanus*, whereas under thiosulphate limitation in the presence of 5% CO_2 (by volume) in the gas phase, the enzyme contributed only 4–5% of total cell protein (Beudeker *et al.* 1981*b*). The increase in abundance of RuBPCase relative to total cell protein paralleled the increase in the number of carboxysomes present in this organism.

Carboxysomes (polyhedral bodies with an average diameter of 100 nm) occur in almost all obligately chemolithotrophic bacteria and in cyanobacteria (Codd *et al.* 1982) and have been shown to contain RuBPCase (Shively *et al.* 1973) and DNA (Westphal *et al.* 1979). The significance of the occurrence of carboxysomes in these bacteria has been an intriguing problem ever since their discovery. Recent experiments showed that a protein storage function of these bodies for *T. neapolitanus* was unlikely (Beudeker *et al.* 1981*b*). Evidence for a protective role against the oxygenase activity of RuBPCase resulting in the wasteful production of glycollate (Beudeker *et al.* 1981*d*) was not obtained either (Beudeker *et al.* 1981*b*). The possibility that CO_2 fixation through PEP carboxylase might be the primary CO_2 fixation mechanism was also considered. Although *T. neapolitanus* did possess the enzymes, short-term labelling experiments with $H^{14}CO_3^-$ showed the operation of the Calvin cycle to be the most important route for CO_2 fixation in *T. neapolitanus*. This also meant that the hypothesis of a role for carboxysomes analogous to the function of bundle sheath cells in C_4 plants had to be abandoned (Beudeker *et al.* 1981*b*). Further characterization of the organelles isolated from *T. neapolitanus* grown under CO_2 limitation showed the presence not only of RuBPCase but of all Calvin cycle enzymes in the carboxysomes. Additionally, high activities of malate dehydrogenase, aspartate aminotransferase and adenylate kinase were observed in pure carboxysome preparations

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(Beudeker & Kuenen 1981). The activity of one of the Calvin cycle enzymes, fructose 1,6-bisphosphatase (FBPase), was also demonstrated cytochemically (Beudeker *et al.* 1981*e*). Activities of the majority of the enzymes were absent in purified preparations that had not been ultrasonically disrupted, indicating that the preparation contained little or no cytosol contamination. It is important to note that carboxysomes from thiosulphate limited cells showed a tendency to disintegrate during purification (Y. Holthuis & J. G. Kuenen, unpublished observations) and were therefore, unsuitable for further study. The effects of several metabolites on RuBP-dependent CO_2 fixation by particulate fraction of a cell-free extract containing carboxysomes derived from *T. neapolitanus* pointed to an important role for malate in introducing reducing power to the Calvin cycle in the carboxysomes. A hypothetical model suggesting that carboxysomes function as 'Calvinosomes' is shown in figure 1. (see also Beudeker & Kuenen

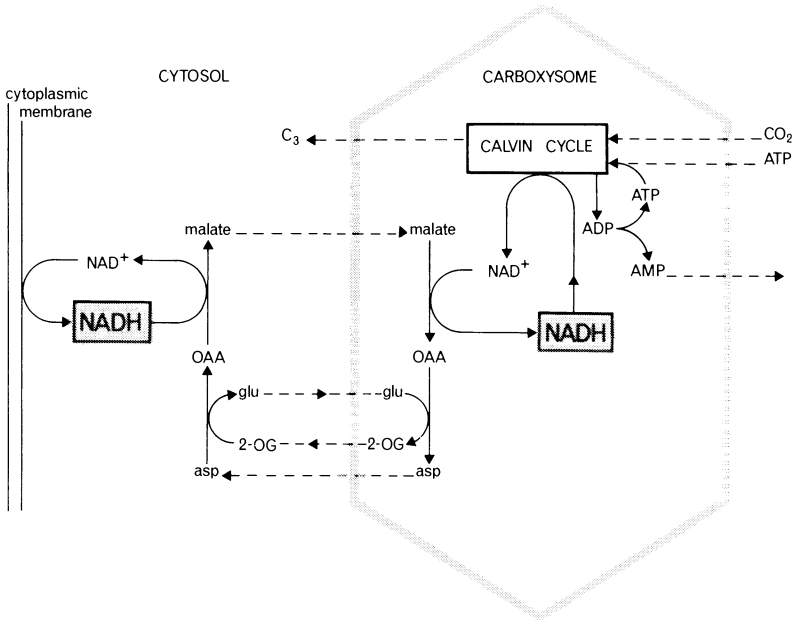

FIGURE 1. Hypothetical functioning of carboxysomes as 'Calvinosomes' in *T. neapolitanus* based on the presence of enzyme activities in purified carboxysomes. Activities of all relevant Calvin cycle cells enzymes have been detected in the carboxysomes, and, in addition, malate dehydrogenase, aspartate aminotransferase and adenylate kinase. Malate would have a function as the carrier of reducing power (NADH) to the carboxysomes. In the carboxysome malate would be dehydrogenated to produce oxaloacetate (OAA) and NADH. Subsequently OAA would be pulled from the reaction by aspartate aminotransferase. The reverse reaction would occur at the cytoplasmic membrane, where NADH is produced by energy-dependent reversed electron transport. Abbreviations: glu, glutamate; asp, aspartate, 2-OG, 2-oxoglutarate. For further details see text. (Reproduced with permission from Beudeker & Kuenen (1981).)

1981). NADH is used in the Calvin cycle by these organisms. The main problem of chemolithotrophy is the production of reducing power (NAD(P)H) since, for thermodynamic reasons, the oxidation of energy sources such as sulphide, ammonia or nitrite do not directly result in NADH production. Reducing power has to be formed by these organisms by energy-consuming reversed electron transport, whereby NADH is formed preferentially to NADPH by *T. neapolitanus* (Roth *et al.* 1973). Since cell-free extracts derived from all obligate chemolithotrophs hitherto examined exhibit NADH-oxidizing activity (albeit rather low), there is reason to

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assume that the existence of a free NADH pool in the cytosol would be energetically wasteful for these organisms. It is our hypothesis that to prevent the occurrence of free NADH in the cytosol, the reducing power is transferred to oxaloacetate (OAA), yielding malate. Malate serves as an intermediate carrier of reducing power from the cell membrane to the Calvin cycle in the carboxysomes. The activity of aspartate aminotransferase in the carboxysomes may prevent the accumulation of OAA, which otherwise would inhibit malate dehydrogenase activity. In addition, this reaction sequence might generate relatively high concentrations of NADH within the carboxysomes, allowing a higher rate of the reductive step in the Calvin cycle.

TABLE 2. ACTIVITIES OF GLUTAMINE SYNTHETASE, GLUTAMATE SYNTHASE, ALANINE DEHYDROGENASE AND GLUTAMATE DEHYDROGENASE IN DIALYSED CELL-FREE EXTRACTS OF *THIOBACILLUS NEAPOLITANUS* CELLS, GROWN AT VARIOUS LIMITATIONS IN THE CHEMOSTAT ($D = 0.07\ h^{-1}$)

(The presence or absence of NO_3^- reducing capacity is also indicated. Activities are expressed as nanomoles per minute per milligram of protein. After Beudeker *et al.* (1982 *b*).)

limitation	N source	glutamine synthetase	glutamate synthase	alanine dehydrogenase	glutamate dehydrogenase	capacity to reduce NO_3^- by whole cells
thiosulphate	NH_4^+	0	127	21	2	–
thiosulphate	NO_3^-	250	194	0	0	+
thiosulphate	urea	252	152	0	0	n.m.
nitrogen	NH_4^+	215	130	0	0	+
CO_2	NH_4^+	50	161	0	2	–

Notes: 0, not detectable; n.m., not measured.

The possession of these sophisticated organelles by this obligate chemolithotroph further indicates its extreme specialization to the autotrophic mode of life. It may be speculated that the carboxysomes in other chemolithotrophs would have a similar function. Interestingly, *T. denitrificans* is the only obligately chemolithotrophic bacterium to lack carboxysomes. This may be explained by the fact that, in this bacterium, electrons from sulphide or thiosulphate enter the electron transport chain at the flavoprotein level (Peeters & Aleem 1970; Timmer-ten Hoor 1977), whereas in all other thiobacilli cytochrome *c* accepts electrons from reduced inorganic sulphur compounds. Consequently, the formation of reducing power by *T. denitrificans* would consume less energy, compared with the other thiobacilli, which would make the functioning of carboxysomes less imperative.

With respect to the assimilation of nitrogen, *T. neapolitanus* appeared to be as flexible as many a heterotroph. The organism was able to use ammonia, nitrate and urea as the sole nitrogen source. *T. ferrooxidans* is at present the only *Thiobacillus* species for which fixation of N_2 has been reported (Mackintosh 1978). This may be linked with the occurrence of this type of organisms in acid mine waters where ammonia precipitates to jarosite (see review Lundgren & Silver 1980), possibly imposing a selective pressure on these organisms to develop N_2-fixing capacity. The pathway of ammonia assimilation by *T. neapolitanus* was shown to depend on the growth conditions. During N- and CO_2-limited growth, glutamine synthetase (GS) activity was derepressed. This enzyme, according to the literature, exhibits a high affinity for ammonia but its action consumes more energy than other ammonia-assimilating enzymes such as alanine dehydrogenase and glutamate dehydrogenase (table 2). During energy-limited growth, with

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excess of ammonia, glutamine synthetase activity was repressed and alanine dehydrogenase activity was induced. Further experiment showed that short-term regulation of ammonia assimilation included feedback inhibition of GS by compounds of low molecular mass (probably amino acids) and a reversible adenylylation of the enzyme (Beudeker *et al.* 1982*b*). The ability to use NO_3^- as a nitrogen source was present in cells cultivated under N-limiting conditions but not under energy-limiting conditions with excess ammonia as the source of nitrogen (table 2).

N-limited cells of *T. neapolitanus* contained an intracellular polymer that after isolation and characterization appeared to consist of glucose units joined by α-1,4 and α-1,6 linkages. Cells of *T. neapolitanus* that are unable to grow on exogenously supplied glucose appeared to be able to use intracellular reserves of polyglucose as a stored energy-source and storage carbon-source under aerobic conditions. This is clear evidence that an obligate chemolithotroph can derive energy from the oxidation of organic compounds. Polyglucose is very probably oxidized through the oxidative pentose phosphate cycle, producing NADPH (Beudeker *et al.* 1981*c*). Apparently NADPH rather than NADH is the source of energy in these organisms. Because of the low maintenance energy requirement during starvation periods, a difference in viability between cells with and without polyglucose was not observed during aerobic starvation periods (Beudeker *et al.* 1981*c*). During anaerobic starvation periods, however, cells containing polyglucose remained viable during a longer period than cells lacking this storage compound. Obviously this is due to the low energy yield of anaerobic metabolism. Polyglucose was fermented anaerobically to ethanol, lactate and CO_2 (1 : 1 : 1) via the heterolactic fermentation pathway by *T. neapolitanus* (Beudeker *et al.* 1981*a*). Cells without polyglucose fermented the ribose of their RNA. Nitrate could be used as an electron acceptor by cells containing polyglucose, yielding nitrite; CO_2 was the only product formed from polyglucose by these cells (Beudeker, de Boer & Kuenen, in preparation). Interestingly, some years ago it was observed that *T. thioparus* continued to produce nitrite from nitrate under anaerobic conditions even when the electron donor, thiocyanate, was not further oxidized (Woolley *et al.* 1962). This implies that an alternative electron donor, which might be polyglucose, was oxidized during this period.

From these results it may be concluded that the organism is flexible with respect to the assimilation of CO_2 and nitrogen, and even appeared to be able to accumulate and metabolize an organic storage compound. It seems as if, during evolution, only the flexibility with respect to the assimilation of exogenously supplied organic compounds has been lost by these bacteria. This might be due to a low transport capacity for organic compounds. Indeed, this seems to be the case for amino-acid transport in *T. neapolitanus*, which is mediated through an active transport system with low K_m but at the same time a very low V_{max} (Matin *et al.* 1974). Recent observations by Stark & Yankofsky (1981*a*, *b*) also indicate an active uptake system for amino acids in *T. thioparus* with a similarly low V_{max} but, in contrast to *T. neapolitanus* with a low affinity (high K_m) for amino acids. In a histidine-requiring mutant of *T. thioparus*, the V_{max} for histidine transport could be increased threefold during growth limitation by this amino acid. Even when induced the maximum transport capacity remained low and its K_m for histidine was not altered. The limited transport capacities of these obligate chemolithotrophs may be an adaption to minimize imbalance in amino acid supply from the autotrophic machinery, which is very sensitive to feedback inhibition (Matin 1978). Although this may be so, one might also argue that as a result of their extremely high respiratory capacity (4000 μl O_2 mg^{-1} d.m. h^{-1}) the cytoplasmic membranes of obligate chemolithotrophs contain so many respiratory enzymes that the presence of carriers for organic compounds is dramatically reduced because of lack of

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space. As a consequence of a low transport capacity for organic compounds, the intracellular concentration of potential substrates would never be high enough to allow the induction of relevant enzymes. Furthermore, even if induction was possible, the increase in transport capacity would be limited by the availability of space to insert carrier proteins. However, it is clear that the insensitivity of the metabolic machinery to exogenous organic compounds is not only due to low transport capacities. When the intracellular concentration of glycollate is high because of the oxygenase activity of RuBPCase, the low levels of those enzymes involved in the metabolism of this compound are not increased (Beudeker *et al.* 1981*d*). This lack of response may be particularly disadvantageous to the organism during CO_2 limitation, when 27% of all carbon fixed is excreted into the medium as glycollate. Chemolithotrophs occur, however, in environments where sulphide and oxygen coexist and consequently the $O_2:CO_2$ ratio will probably frequently be very low, preventing the formation of glycollate. Furthermore, one might speculate that an inducible glycollate metabolism would have a serious impact on the cellular physiology because glycollate is mainly metabolized to *malate* by *T. neapolitanus*. As indicated above, this compound is thought to play an important role as a carrier of reducing power in the operation of the Calvin cycle of this bacterium; tight control of malate levels might therefore be critical. If this were true, it would be more favourable for the organisms to excrete glycollate temporarily rather than to develop complicated regulatory mechanisms for its metabolism. It should be noted, however, that enzymes required for the aerobic and anaerobic metabolism of polyglucose clearly showed variable activities (Beudeker *et al.* 1981*a*; Beudeker, de Boer & Kuenen, in preparation), excluding the possibility that obligate chemolithotrophs possess inducible or modifiable enzymes only for the assimilation of inorganic compounds. The lack of enzyme induction on the appearance of exogenously supplied organic compounds may be rationalized because an inducible enzyme system for the metabolism of non-limiting concentrations of exogenously supplied organic compounds would imply a repression of the autotrophic potential (see next section), which may be of disadvantage during competition under an intermittent supply of sulphur compounds. The further implications of this specialized type of metabolism for its competitiveness and survival value will be discussed in a later section.

Physiology of facultative chemolithotrophs

The ability of facultatively chemolithotrophic bacteria to grow autotrophically on reduced sulphur compounds as well as heterotrophically on organic compounds is well known (Rittenberg 1969). The advantages and disadvantages of facultative chemolithotrophy for organisms during the struggle for life in which they must compete with organisms such as those listed in table 1 have been a subject of contradictory views. It has been postulated that a facultative chemolithotroph should either be a very good chemolithotroph or a very good heterotroph if it is to compete successfully with its counterparts (Whittenbury & Kelly 1977). Batch culture studies on physiology of facultative chemolithotrophs (for review, see Matin 1978) show many examples of the repression of both the autotrophic carbon assimilation and the generation of energy from the oxidation of sulphur compounds by the addition of organic substrates to the growth medium. For example, during cultivation of the facultative *Thiobacillus* A2 on acetate in batch culture, the autotrophic growth potential (i.e. thiosulphate-oxidizing capacity and RuBPCase activity) was completely repressed. Inclusion of thiosulphate in the growth medium partly relieved this repression. The thiosulphate-oxidizing activity increased with decreasing

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concentration of acetate in the growth medium, resulting in diauxic growth. It appeared that the degree of repression of the thiosulphate-oxidizing capacity was dependent on the acetate : thiosulphate ratio in the growth medium (Gottschal & Kuenen 1980*a*). Different results were obtained with *T. novellus* which consumed thiosulphate and glucose concurrently at various ratios of the substrates in batch culture (Perez & Matin 1980). *Thiobacillus* A2 appeared to be able to change over from autotrophic to heterotrophic growth without a detectable lag. Thiosulphate-grown cells always retained a residual capacity to oxidize organic substrates, enabling the organism to respond rapidly to the presence of these compounds. After prolonged heterotrophic growth, however, there was a lag phase of 7–10 h before the cells were able to grow autotrophically. This was due to a total repression of the autotrophic potential during heterotrophic growth. After the change over from thiosulphate limitation to acetate limitation, the $Q_{O_2^{max}}$ for thiosulphate was reduced and RuBPCase activity disappeared from the cells at a rate significantly faster than would be expected from mere wash-out. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of cell-free extracts derived from *Thiobacillus* A2 cells harvested during the period after the transition from thiosulphate limitation to acetate limitation showed that at least the large subunit of RuBPCase was subject to selective proteolysis (Gottschal *et al.* 1981*b*). This is not a general phenomenon, however, because after change over from thiosulphate limited *Thiobacillus* A2 cells to glucose limitation, RuBPCase activity disappeared at a rate slower than the dilution rate (Smith *et al.* 1980). The induction of the autotrophic potential after the change over from acetate-limited growth to growth on thiosulphate was markedly enhanced by the addition of low concentrations of organic substrates to the feed. The metabolism of these substrates provided the cells with energy for the synthesis of the 'autotrophic' enzymes during these conditions (Gottschal *et al.* 1981*b*).

Autotrophically batch-grown *T. novellus* cells were not able to oxidize sugars in the presence of thiosulphate, although the glucose transport system was still potentially active under these growth conditions (Matin *et al.* 1980). Utilization of glucose by these cells was prevented because thiosulphate inhibited both glucose uptake and also the activities of several key enzymes of the oxidative pentose phosphate pathway and TCA cycle. Moreover, autotrophically grown cells possessed far lower activities of these enzymes compared with heterotrophically grown cells, a situation which, although less pronounced in chemostat cultures of *Thiobacillus* A2 on acetate, is a general phenomenon among facultative chemolithotrophs (Gottschal & Kuenen 1980*a*; Smith *et al.* 1980; Matin *et al.* 1980; Wood & Kelly 1981). In *Thiobacillus* A2, mutants that grow more rapidly on the substrate provided are produced occasionally. Thus in Kelly's and our own laboratories, mutants that grew much faster on acetate than the parent strain were isolated. Similarly, Kelly & Wood have found mutants (strains GF) that grew much more quickly on glucose. This was particularly interesting because the parent strain grew rapidly on sucrose and fructose, suggesting that the isolate might be a transport mutant. Very recently Wood & Kelly (1982*a*, *b*) have reported that this was indeed so. The evidence presented, based mainly on inhibitor studies, indicated that the parent strain had a proton motive force (p.m.f.)-dependent glucose transport system, but the mutant had a high-energy phosphate-dependent glucose transport system (*not* a phosphoenolpyruvate transferase system), which allowed the mutant to grow faster on glucose than the parent strain.

Thiobacillus A2 is able to grow heterotrophically on mono-, di- and trisaccharides, amino acids, alcohols and organic acids (Taylor & Hoare 1969; Wood & Kelly 1977). Autotrophic growth of *Thiobacillus* A2 has been observed on formate (Kelly *et al.* 1979*b*), methylamine (van

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Dijken & Kuenen 1980), thiosulphate and also on sulphide (Beudeker *et al.* 1982*a*). Interesting observations have been made by Kelly and collaborators showing the existence of various overlapping pathways for the dissimilation of glucose in *Thiobacillus* A2. As judged from results of radiorespirometric experiments, it was concluded that batch cultures of *Thiobacillus* A2 (E.M.), Entner–Doudoroff (E.D.) and oxidative pentose phosphate pathways (Wood *et al.* 1977). The contribution of the E.M. pathway for glucose dissimilation in the wild-type *Thiobacillus* A2 during glucose-limited growth in continuous culture was called into question *Thiobacillus* A2 during glucose-limited growth in continuous culture was called into question by Wood & Kelly (1979). Subsequent enzyme studies showed that the key enzymes of the E.M. pathway, 6-phosphofructokinase, did not show any activity under such growth conditions (Smith *et al.* 1980; Wood & Kelly 1981). Thus only during growth in batch culture on glucose do the three pathways act simultaneously in *Thiobacillus* A2 (see also Wood & Kelly 1980). The relative significance of the various pathways depended on the growth conditions, but no apparent correlation was observed between growth conditions and the relative contribution of the various pathways to total glucose dissimilation (Wood & Kelly 1979). Results of similar radiorespirometric experiments carried out with *T. novellus* are most probably explained by the action of phosphoketolase, which cleaves xylulose 5-phosphate to yield equimolar amounts of acetyl phosphate and glyceraldehyde 3-phosphate. Phosphoketolase showed a relatively high activity (60 nmol min^{-1} mg^{-1} protein) in batch cultures of *T. novellus* on glucose plus yeast extract (Greenley & Smith 1979). Although these authors also detected a reasonably high activity of this enzyme in batch-grown *Thiobacillus* A2 (16 nmol min^{-1} mg^{-1} protein), a far lower activity was observed when this organism was cultivated in continuous culture (Smith *et al.* 1980). The latter authors do not attach an important role to phosphoketolase for the total dissimilation of glucose in *Thiobacillus* A2.

Glucose metabolism by cultures of *T. ferrooxidans* has received controversial attention over the years. Recently it was shown by Harrison *et al.* (1980) that cultures of the obligate chemolithotroph *T. ferrooxidans* were not only contaminated with the facultative *T. acidophilus* (Guay & Silver 1975), but also with various acidophilic heterotrophs. Batch cultures of *T. acidophilus* dissimilated glucose by the E.D. and pentose phosphate pathways (Wood *et al.* 1977). This cast some doubt on the validity of earlier report of glucose adaptation of *T. ferrooxidans* (see, for example, Tabita & Lundgren 1971) but in the light of a recent report (Billheimer & Blanchard 1981) this controversy has apparently not been resolved.

It was postulated originally by Rittenberg (1969, 1972) that the survival value of the facultatively chemolithotrophic way of life would lie in the capacity of these organisms to grow mixotrophically (see introduction for semantics). Using the powerful tool of the chemostat, true mixotrophy has been demonstrated in *Thiobacillus* A2 during growth limitation by mixtures of acetate plus thiosulphate (Gottschal *et al.* 1979; Gottschal & Kuenen 1980*a*), thiosulphate plus glucose (Smith *et al.* 1980) and formate plus glucose (Wood & Kelly 1981). The same phenomenon was observed in glucose-plus thiosulphate-limited cultures of *T. novellus* (Leefeldt & Matin 1980). The yields of *Thiobacillus* A2 on growth-limiting mixtures of acetate plus thiosulphate were about 30% higher than predicted from the sum of both yields on separate substrates. This may be explained by consideration of the energy-sparing effect of partially switching off the Calvin cycle, whose operation consumes more energy than the assimilation of acetate to organic cell carbon. However, dry masses of chemostat cultures of *Thiobacillus* A2 on formate plus glucose, and of *T. novellus* on glucose plus thiosulphate, indicated additive growth

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yields (Wood & Kelly 1981; Leefeldt & Matin 1980). Interestingly, the maximal autotrophic growth rate on thiosulphate (μ_{max} = 0.11 h^{-1}) of *Thiobacillus* A2 was exceeded during mixotrophic thiosulphate–acetate-limited growth up to a dilution (= growth) rate of 0.20 h^{-1}. During mixotrophic growth on limiting amounts of thiosulphate plus glucose, similar observations were made for *T. novellus* (Leefeldt & Matin 1980). Even at this high growth rate, thiosulphate could not be detected in the growth vessel indicating the organisms' potential to oxidize thiosulphate at a growth rate far above the maximal autotrophic rate (Gottschal & Kuenen 1980*a*).

Although most facultative chemolithotrophs fix CO_2 by the Calvin cycle during autotrophic growth, *Sulfolobus brierleyi* seems to be an exception to this rule. Short-term $^{14}CO_2$ labelling experiments were carried out with this organism grown autotrophically in batch culture on elemental sulphur. Aspartate, malate and glutamate were the most important products of CO_2 fixation after 5 s of labelling (Kandler & Stetter 1981). The results were interpreted as being indicative of the operation of a reductive TCA cycle for CO_2 fixation similar to that found in *Chlorobium limicola* (f. *thiosulfatophilum*) (Fuchs *et al.* 1980; Ivanovsky *et al.* 1980). The possible operation of a C_4 plant CO_2-fixation mechanism, which might give similar early labelling products, was not considered. Enzyme studies to be carried out with *S. brierleyi* should confirm whether the present interpretation of the $^{14}CO_2$ labelling experiments is correct.

As a follow-up to the early work of La Rivière on *Thiovulum*, Wirsen & Jannasch (1978) attempted to isolate and characterize this genus. It appeared to be extremely difficult to obtain pure cultures of *Thiovulum* because of its production of a matrix of slime threads and its requirement for an O_2–H_2S gradient. Enriched cultures remained contaminated with heterotrophs and autotrophs, making an unambigous interpretation of the data difficult. No clear uptake of organic compounds by *Thiovulum* was observed, whereas the presence of sulphide led to a significant increase in CO_2 fixation by the cells. Sulphur globules were stored intracellularly and disappeared after starvation periods. Thiosulphate was not oxidized by the cells and no growth on agar plates was obtained. At present, it seems clear that sulphide and sulphur play a role in the energy metabolism of *Thiovulum*. Short-term labelling experiments and enzyme studies should shed light upon the role of CO_2 as a carbon source for the growth of *Thiovulum*.

A particularly interesting organism is *Thermothrix thiopara*, a thermophilic, filamentous, facultatively anaerobic facultative chemolithotroph able to grow on a variety of organic compounds, on inorganic sulphur compounds using either oxygen or nitrate as the terminal electron acceptor. Like *Thiobacillus* A2, it can grow heterotrophically, but not autotrophically, under anaerobic conditions in the presence of nitrate. The reported μ_{max} of *Thermothrix thiopara* was very high, 0.3–0.4 h^{-1} (Brannan & Caldwell 1980). However, the reported CO_2-fixation rate of 340 nmol min^{-1} g^{-1} wet cells is between 50–100 times too low to account for this rate (cf. Beudeker *et al.* 1980). More detailed physiological studies on this organism should be rewarding.

Pure cultures of *Thiothrix* have recently become available (Larkin 1980). No significant progress has been made in the studies on the physiology of *Thiospira*, *Thiophysa* and *Achromatium*.

Physiology of sulphur compound-oxidizing heterotrophs

Though these bacteria appear to be the predominant sulphur-oxidizers in certain environments, for instance in soils or in the Black Sea (Trudinger 1967; Tuttle & Jannasch 1972), they

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have been paid relatively little attention. *T. perometabolis* is the best known representative, but information on the physiology of this bacterium still is rather scarce (London & Rittenberg 1967). *Thiobacillus* strain V formed the dominant population in chemostat enrichment cultures supplied with thiosulphate (10 mM) and acetate (15 mM) together as growth limiting substrates. At this ratio of thiosulphate to acetate, CO_2 fixation is not required (Gottschal & Kuenen 1980*b*). Apparently it is of competitive advantage for organisms to lack the genetic information for enzymes that are not needed during competition (see also Slater & Godwin 1980). Several heterotrophs have been described to be capable of oxidizing thiosulphate to tetrathionate (Trudinger 1967; Tuttle *et al.* 1974*b*). In some strains the ability to oxidize thiosulphate was induced during growth in the presence of thiosulphate (Tuttle *et al.* 1974*a*). Bacterial reduction of tetrathionate to thiosulphate under anaerobic conditions indicates the reversibility of the process (Tuttle & Jannasch 1973). The significance of these reactions for the organisms is puzzling because often no increase in yield or growth rate was observed (Trudinger 1967). Tuttle (1980), Tuttle & Jannasch (1977) and Tuttle *et al.* (1974) have produced cumulative evidence that the oxidation of thiosulphate to tetrathionate yields metabolically useful energy. In addition, unpublished results of Tuttle and also from our own laboratory (Kuenen, unpublished) show that the addition of thiosulphate to starved cells of such organisms leads to a rapid increase in ATP levels in the cells. However, the reported increases in yield from thiosulphate are difficult to quantify. A tenfold increase in yield in a glucose-limited chemostat (10 mg glucose l^{-1} (cf. Tuttle 1980)) is unrealistically high because the energy available from the oxidation of thiosulphate to tetrathionate is very low. For comparison, chemostat cultures of thiobacilli may produce maximally 5–13 mg dry mass per millimole of thiosulphate or sulphide, when complete oxidation to sulphate takes place (Timmer-ten Hoor 1976; Beudeker *et al.* 1982*a*). Contradictory views concerning possible energy generation from the oxidation of the reduced inorganic sulphur compounds have two likely explanations:

(1) the production of toxic amounts of sulphite in batch cultures of this type of organism; they should be treated very carefully and be pre-grown in carbon-limited chemostats to which increasing concentrations of thiosulphate are added (Gottschal & Kuenen 1980*b*; Kuenen, unpublished results);

(2) the existence of bacteria that do oxidize sulphur compounds but nevertheless do not directly gain energy from it.

This latter group of bacteria contains at least several *Beggiatoa* species. In carefully designed experiments it was shown that *Beggiatoa* cf. *leptomitoformis* (OH-75-B) oxidizes sulphide and thiosulphate to sulphur, which is subsequently stored intracellularly. No evidence for an increase in growth yields by the addition of sulphide was observed after correction for the mass of intracellularly stored sulphur. Convincing evidence was presented suggesting that intracellularly stored sulphur may function as an electron acceptor during anaerobic periods. Additionally, oxidation of sulphide by these cells, which lack catalase, may protect them from harmful reactive oxygen compounds such as hydrogen peroxide (Nelson & Castenholz 1981*a*). Acetate, lactate and ethanol could serve as sole organic carbon source for this *Beggiatoa* strain. TCA intermediates such as succinate, malate, oxaloacetate and fumarate had a strong synergistic effect on growth yields when added in combination with acetate to batch cultures (Nelson & Castenholz 1981*b*). In the same period a paper appeared claiming mixotrophic growth of *Beggiatoa alba* strain B 18 LD (Güde *et al.* 1981). The authors observed that the addition of

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sulphide markedly enhanced growth. In a subsequent paper it was demonstrated that the contribution of exogenous CO_2 to total cell carbon was very small and that CO_2-fixing enzymes had activities below the 1 nmol min^{-1} mg^{-1} protein level (Strohl *et al.* 1981). This clearly indicates that this would be a case of chemolithic heterotrophy. Whether one should call this mixotrophy is a matter of semantics (Rittenberg 1969). Some puzzling growth yields that, after some calculation, suggest the production of 139 mg dry mass from 1 mmol sulphide are presented. As mentioned earlier, *Thiobacillus* species form between 5–13 mg dry mass from 1 mmol sulphide under optimal growth conditions (Timmer-ten Hoor 1976; Beudeker *et al.* 1982*a*). It is known that the protein determination method (Lowry *et al.* 1951) employed by Güde *et al.* (1981) is severely interfered with by sulphur compounds, and therefore the data should be checked by other methods. Although mixotrophic growth of some *Beggiatoa* species cannot as yet be excluded, more convincing evidence is required. It should be realized that, as with the thiobacilli, the genus *Beggiatoa* may comprise a spectrum of bacteria with different types of metabolism.

Interactions between lithotrophic bacteria and their biotic and abiotic environment

In order to establish whether the mixotrophic way of life is of ecological importance, a series of competition experiments was set up by Gottschal *et al.* (1979). During competition between the facultative chemolithotroph *Thiobacillus* A2 and the obligate chemolithotroph *T. neapolitanus* for thiosulphate as the only growth-limiting substrate, cells of *T. neapolitanus* formed the dominant population (90 % of total cell numbers). *Thiobacillus* A2 maintained itself in the culture by growth on excretion products of *T. neapolitanus* such as glycollate (Cohen *et al.* 1979; Beudeker *et al.* 1981*d*). The addition of an organic substrate to the mixed culture clearly favoured *Thiobacillus* A2. With increasing input of organic compounds, cell numbers of *T. neapolitanus* declined and the organism was out-competed by *Thiobacillus* A2 at molecular ratios of acetate (or glycollate) to thiosulphate higher than 10 : 40. These results were virtually independent of the applied growth rate. The effects of increasing input of glucose to a thiosulphate-limited mixed culture of *Thiobacillus* A2 and *T. neapolitanus* were very similar, indicating that the observed phenomenon was not influenced by the kind of organic substrate applied (Smith & Kelly 1979). Since the competing organisms exhibit different pH optima, the outcome of the competition experiment depended on the pH (Smith & Kelly 1979). Nature harbours a spectrum of obligate and facultative chemolithotrophs, however, all with different pH optima; thus it seems reasonable to assume that another obligate chemolithotroph will take over the ecological niche of *T. neapolitanus* at higher pH values. Surprisingly, a freshwater obligate chemolithotroph with a pH optimum higher than 7.0 has hitherto not been described. *Thiobacillus* A2 also lost the competition for growth-limiting acetate when grown with a specialist heterotroph, *Spirillum* G7, thus agreeing with the predictions of Whittenbury & Kelly (1977). However, *Thiobacillus* A2 appeared to become the dominating population at intermediate ratios of thiosulphate and acetate during dual substrate-limited growth not only in two-membered mixed cultures, but also in cultures where the three physiologically distinct types of bacteria coexisted (figure 2) (Gottschal *et al.* 1979). From these results, it is concluded that the competitive strength of facultative chemolithotrophs lies in their capacity for mixotrophic growth. These results have been discussed in detail by Gottschal & Kuenen (1981). A selective enrich-

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ment procedure for facultative chemolithotrophs based on these observations has been developed. Various facultative chemolithotrophs were isolated from freshwater environments in a chemostat to which thiosulphate and acetate were both supplied as growth-limiting substrates (table 3) (Gottschal & Kuenen 1980*b*). For hitherto unexplained reasons, similar enrichment procedures for facultative chemolithotrophs from marine environments were never successful, mixed cultures of obligate chemolithotrophs and heterotrophs being obtained instead. Recently, *T. intermedius*, a facultative chemolithotroph was isolated from a salt-water environment by using a thiosulphate-limited chemostat maintained at pH 6.8 (Smith & Finazzo 1981). As

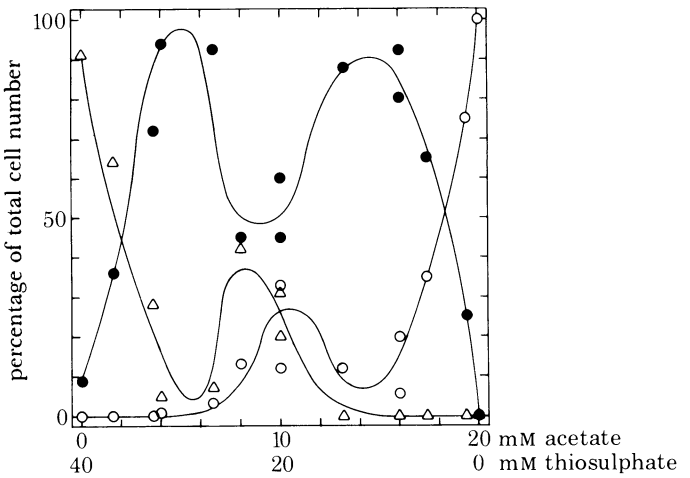

FIGURE 2. Results of competition experiments between the versatile *Thiobacillus* A2, the specialist chemolithotroph *T. neapolitanus* and a specialist acetate-utilizing heterotroph *Spirillum* G7. The three organisms were grown in the chemostat under simultaneous limitation by thiosulphate and acetate at a dilution rate of 0.075 h^{-1}. Concentrations of these substrates in the inflowing medium ranged from 0 to 20 mM for acetate and 40–0 mM for thiosulphate. After steady states with different ratios between acetate and thiosulphate concentrations in the feed had been established, relative cell numbers of *Thiobacillus* A2 (●), *T. neapolitanus* (△) and *Spirillum* G7 (○) were determined. Acetate and thiosulphate concentrations in the culture were below the detection level. (Reproduced with permission from Gottschal *et al.* (1979).)

TABLE 3. RESULTS OF ENRICHMENT CULTURES (I–VII) AFTER 15–20 VOLUME CHANGES IN THE CHEMOSTAT UNDER DUAL LIMITATION BY THIOSULPHATE AND ACETATE AT A DILUTION RATE OF 0.05 h^{-1}

(I–V were enrichments from freshwater samples; VI and VII had been inoculated with marine mud. Concentration of thiosulphate (T) and acetate (A) in the inflowing medium are given in millimoles per litre. After Gottschal & Kuenen (1980*b*).)

culture	substrate concentration	dominant physiological type	percentage of total number
freshwater			
I	30 T + 5 A	facultative chemolitho(auto)troph	82
II	10 T + 15 A	facultative chemolithoautotroph	75
III	30 T + 5 A	facultative chemolithoaurotroph	85
IV	20 T + 10 A	facultative chemolithoautotroph	50
V	10 T + 15 A	chemolithotrophic heterotroph	86
marine			
VI	30 T + 5 A	obligate chemolithoautotroph + heterotroph	67 + 37
VII	10 T + 15 A	obligate chemolithoautotroph + heterotroph	81 + 19

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shown above, *Thiobacillus* A2 was always out-competed under such growth conditions by *T. neapolitanus* unless pH values higher than 7.6 were applied (Gottschal *et al.* 1979; Smith & Kelly 1979). Against this background it seems hard to explain the successful enrichment of a facultative chemolithotroph from a saltwater environment, and it would appear that different parameters are of importance for the competition between lithotrophic bacteria in saltwater environments.

Another possible advantage of facultative chemolithotrophs over obligate chemolithotrophs was thought to be their metabolic flexibility (see the section on physiology). Although metabolic flexibility appears at first sight to be advantageous to an organism in an environment that is continuously changing, this clearly depends on the frequency at which a previous situation is

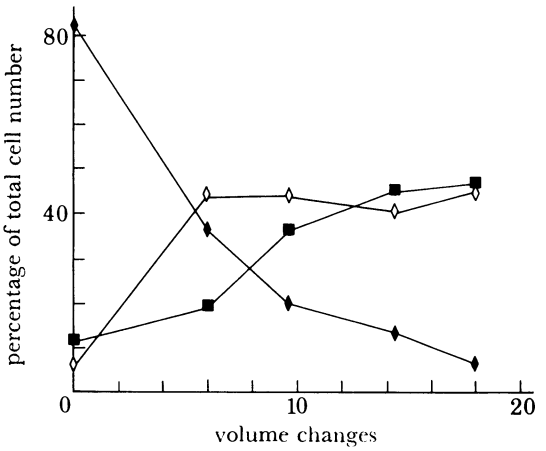

FIGURE 3. Competition in continuous culture between *Thiobacillus* A2, *T. neapolitanus* and *Spirillum* G7 for thiosulphate and acetate as growth limiting substrates, as a function of the number of volume changes (equivalent to time). The dilution rate was 0.05 h^{-1} with intermittent feeding of two media containing either thiosulphate (40 mM) or acetate (10 mM). The two media were supplied alternately to the culture, each during 4 h. *Thiobacillus* A2 (◆), *Spirillum* G7 (■) and *T. neapolitanus* (◇) are shown as a percentage of the total cell number present in the culture. (Adapted from Gottschal *et al.* (1981*a*).)

repeated. This may be illustrated by results obtained from studies on the effects of alternating substrate supply to chemostat cultures containing *Thiobacillus* A2 and *T. neapolitanus*. Mixed cultures were grown during alternating limitations of 4 h acetate and 4 h thiosulphate as sole substrate. Coexistence of the two organisms was observed, both contributing about 50 % of the population. As the concentrations of each substrate in the inflowing medium had been chosen such that yields on the separate substrates should be equal, the most likely explanation for these results is that *Thiobacillus* A2 was forced to grow as a heterotroph under such conditions, whereas *T. neapolitanus* used all the available thiosulphate. Not surprisingly, therefore, *Thiobacillus* A2 was out-competed when *Spirillum* G7 was added to a mixed culture of *Thiobacillus* A2 and *T. neapolitanus* under alternate limitation by acetate and thiosulphate (figure 3) (Gottschal *et al.* 1981*a*). Studies with pure cultures subsequently provided evidence to explain these observations (Beudeker *et al.* 1982*a*). To measure the affinity for the growth-limiting substrate at a fixed dilution rate, cells were grown on sulphide rather than on thiosulphate. Sulphide may be measured polarographically at concentrations even lower than 1 μM, whereas for the determination of thiosulphate one is committed to the use of a colorimetric assay with a far lower sensitivity (50 μM). Sulphide and thiosulphate appeared to be identical as energy sources

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for both organisms; yields and $Q_{O_2}^{max}$ on both substrates were virtually equal. *T. neapolitanus* cells were grown under alternating supplies of 4 h sulphide and 4 h sulphate (or acetate, which does not support growth either). During the latter period, washout of 20 % of the cells occurred. The concentration of sulphide was measured. At pH 6.8, the optimum for *T. neapolitanus*, the concentration of sulphide was always below 1 μM whereas at pH 7.5, the pH used during the competition experiments, the sulphide concentration maximally reached 4 μM. During prolonged starvation periods (up to 5 days), the autotrophic potential remained constant in *T. neapolitanus* cells. These properties permit the organisms to respond very quickly to the sudden appearance of a sulphur compound. Such a strategy results in maximal reactivity. In contrast, the metabolically flexible *Thiobacillus* A2 inactivated its autotrophic potential during the acetate period. Consequently the organism would not be able to respond as quickly as *T. neapolitanus* to the appearance of sulphide after the acetate period. Sulphide accumulated to up to 56 μM under such conditions. The lower affinity of *Thiobacillus* A2 for sulphide is due to its lower thiosulphate (sulphide) oxidizing capacity compared with *T. neapolitanus*, which possessed an overcapacity for the oxidation of reduced inorganic sulphur compounds under all growth conditions. The competitive position of facultative chemolithotrophs during such alternating growth conditions might improve if they had the ability to accumulate storage compounds during the heterotrophic period. Indeed, such an organism was found to be the dominant population in enrichment cultures in chemostats supplied with alternating growth-limiting concentrations of acetate and thiosulphate. This facultatively chemolithoautotrophic organism stored poly-β-hydroxybutyrate (PHB) up to 0.05 mg PHB per milligram protein during the acetate period. This was broken down in the first 2 h after the onset of the thiosulphate period. Our interpretation is that during the thiosulphate period, PHB served as ancillary carbon source, thereby saving energy for CO_2 fixation. This view is supported by the finding that the maximum CO_2 fixation capacity of this organism during the first $1\frac{1}{2}$ h of the 'autotrophic' period was too low to account for the observed growth rate (Kuenen & Spijkerman, unpublished). Competition experiments between this isolate and *T. neapolitanus* and *Spirillum* G7 during alternating growth limitation by acetate and thiosulphate have not yet been performed.

Our results allow the conclusion that both obligate chemolithotrophs and facultative chemolithotrophs occupy distinct ecological niches, at least in freshwater environments. During simultaneous limitation by reduced sulphur compounds and organic substrates (conditions that permit mixtrophic growth), the strategy of survival of *Thiobacillus* A2 will be most successful. Obligate chemolithotrophs such as *T. neapolitanus*, on the other hand, will thrive in environments where the turnover rate of reduced inorganic sulphur compounds is high relative to that of organic compounds. These organisms are also well adapted to a fluctuating supply of reduced sulphur compounds because they are very resistant to starvation periods. Chemolithotrophic heterotrophs will flourish under conditions of a high turnover rate of organic compounds relative to that of reduced sulphur compounds. Under such conditions these organisms were selectively enriched from freshwater environments (Gottschal & Kuenen 1980*b*). From these findings, we constructed a hypothetical model predicting the occurrence of chemolithotrophic sulphur bacteria in freshwater environments in relation to the relative turnover rates of inorganic sulphur compounds and organic substrates (figure 4).

In (eutrophic) environments where parameters other than the energy source may sometimes be growth-limiting, different principles will underlie the distribution of lithotrophic bacteria. Sulphur bacteria occur at the O_2–H_2S interface, which may migrate during a diurnal cycle as a

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consequence of sulphide oxidation and oxygen production by phototrophic organisms. (Jørgensen *et al.* 1979). For this reason it may well be that these bacteria will often have to compete for oxygen. Different affinities for oxygen among sulphur bacteria have not yet been looked for, though it seems reasonable to assume that organisms with a high thiosulphate or sulphide respiring capacity such as *T. neapolitanus* will exhibit a relatively high affinity for oxygen. Whether sulphur-oxidizing bacteria are able to compete successfully with the chemical oxidation of sulphide has been an open question. Since the chemical oxidation of two molecules of sulphide yields one molecule of thiosulphate it is obvious that the bacteria would more profitably oxidize the sulphide themselves. Difficulties very often arise when these bacteria are grown in batch culture on sulphide because this compound is toxic at high concentrations. We have grown *T. neapolitanus* and *Thiobacillus* A2 successfully in a sulphide-limited chemostat with aeration control. Growth yields of these bacteria cultivated at a dissolved oxygen tension

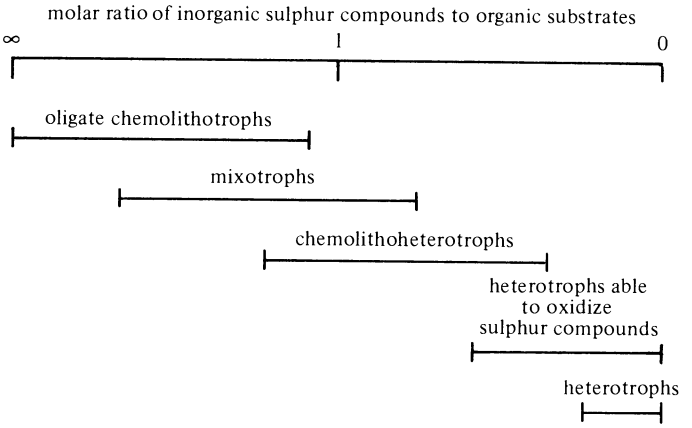

FIGURE 4. Model predicting the occurrence of sulphur-oxidizing bacteria as a function of the relative turnover rate of reduced inorganic sulphur compounds and organic substrates during energy-limiting growth conditions in fresh water environments.

of 5% of air saturation were almost equal of those found on thiosulphate. These results indicate that the chemical oxidation of sulphide at this oxygen tension does not play a significant role (Beudeker *et al.* 1982*a*). In chemostat cultures of *Thiobacillus* A2 grown at 50% air saturation on sulphide, sulphur accumulated and yields became very low, whereas on thiosulphate the oxygen tension had little influence on growth yields. It appeared that chemical oxidation of sulphide led to the formation of sulphur, which is not significantly metabolized by *Thiobacillus* A2. In contrast, similar cultures of *T. neapolitanus* showed only slightly (10%) reduced yields under similar conditions. As long as sulphide is growth-limiting it is apparently not toxic, but at higher concentration it may inhibit or retard growth. An observation relevant to the ecological niche of sulphide-oxidizing bacteria is that *Thiomicrospira pelophila* and another recently isolated *Thiomicrospira* sp. seem to have a higher tolerance to sulphide toxicity than thiobacilli such as *T. thioparus* or *T. neapolitanus* (Kuenen & Veldkamp 1972; Ruby & Jannasch 1982; Beudeker & Kuenen, unpublished).

The difference in sulphide tolerance may be due to the high affinity for iron of *Tms. pelophila* compared with *T. thioparus*. Iron will precipitate in the presence of sulphide and thus might become growth-limiting. Therefore the affinity of various sulphur bacteria for iron may be an important selective factor during growth on sulphide (Kuenen *et al.* 1977).

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Those species that are able to use nitrate as alternative electron acceptor may have an advantage over others unable to denitrify on reduced sulphur compounds. *T. denitrificans* and *Tms. denitrificans* are the only well established examples of facultatively anaerobic obligate chemolithotrophs. Their metabolic features were compared by Timmer-ten Hoor (1977). *Tms. denitrificans* synthesized its denitrifying capacity constitutively under anaerobic and aerobic conditions, whereas in *T. denitrificans* this capacity was repressed under aerobic conditions. Moreover *Tms. denitrificans*, in contrast to *T. denitrificans*, was microaerophilic, indicating that this organism is more adapted to anaerobic conditions. The μ_{max} of both organisms are virtually equal. Results of chemostat enrichments in an anaerobic, thiosulphate-limited chemostat that had been inoculated with marine mud showed that *Tms. denitrificans* became dominant. *Tms. denitrificans* did not appear as long as oxygen was not rigorously excluded from the system.

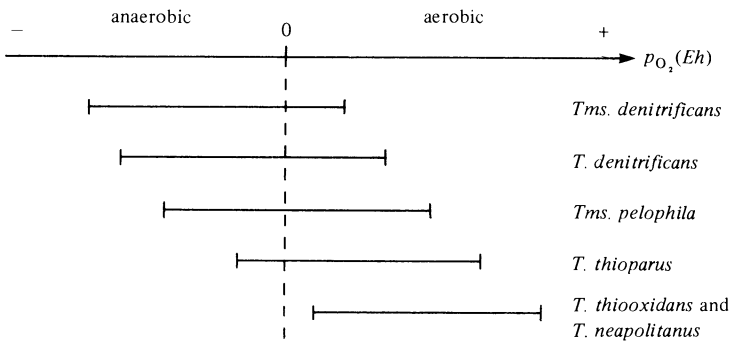

FIGURE 5. Hypothetical model showing the distribution of some colourless sulphur bacteria in relation to p_{O_2} or *Eh*. The model only serves to give trends. (After Timmer-ten Hoor (1977).)

Preliminary results showed that in the latter case, *T. denitrificans* might become dominant. This outcome seems to indicate that *Tms. denitrificans*, although it can grow under microaerophilic conditions, cannot compete successfully with other sulphide-oxidizing bacteria as long as a substantial turnover of oxygen is possible. Thus the ecological niche of *Tms. denitrificans* might lie in anaerobic environments with a high turnover rate of sulphide which may contain traces of oxygen. Based on the different behaviour of *Thiobacillus* and *Thiomicrospira* species towards oxygen, sulphide (iron), and redox potential (Sokolova & Karavaiko 1964), a model showing the respective ecological niches of several obligate chemolithotrophs as related to the redox potential or p_{O_2} as fluctuating parameter has been presented (Timmer-ten Hoor 1977) (figure 5).

Sulphur-oxidizing chemolithotrophs occur in widely different habitats (for review see Kuenen 1975). Their ecology is discussed by Jørgensen at this meeting. Dense populations of *Beggiatoa* sp. have been reported to occur within the upper few centimetres of oxic sediments of a brackish fjord in Denmark. Their occurrence was related to the sediment properties; they were absent in fine and medium grained sand but abundant in muds (Jørgensen 1977). It may be that their gliding motility (for review see Reichenbach 1981) enables them to thrive in muddy sediments where they resist temporarily anaerobic conditions by using intracellularly stored sulphur as an electron acceptor for the oxidation of organic substrates available in the anaerobic sediment.

Interactions between acidophilic lithotrophs, heterotrophs and iron-oxidizing bacteria are important for the dissolution of pyrite, but at present the kinds of interactions involved are still

unknown (Norris & Kelly 1978). One type of interaction is obvious, however, since at low pH values organic acids excreted by the bacteria are undissociated and may easily enter the cells, causing damage; the presence of heterotrophs will prevent accumulation of these organic acids (Kelly *et al.* 1979*a*).

Interactions between sulphur-oxidizing prokaryotes and eukaryotes

Of the known interactions between sulphur-oxidizing prokaryotes and eukaryotes, recycling of sulphide to sulphate by the former may seem trivial, but it is obviously of crucial importance to provide soluble sulphate as the sulphur source for growth of the eukaryotes. Recently new interactions between lithotrophic prokaryotes and eukaryotes have been discovered. It has long been realized that, in principle, autotrophic chemolithotrophs have the potential to be the only primary producers in ecosystems where photosynthesis is not possible, but where a supply of inorganic sulphur compounds would be available from geological deposits. During marine expeditions near the Galapagos rifts, hydrothermal vents were found to release geothermally produced reduced inorganic compounds into the surrounding waters. Sulphide was the most abundant chemical substance emitted from the vents. Near these vents, in complete darkness at a depth of 2500–2600 m, dense populations of worms, clams, mussels, crabs, anemones and fish were encountered, and it was postulated that these animals were dependent on chemolithotrophic bacteria for their food supply. Electron microscopic observations of the vent water indicated the presence of numerous bacteria, some of which show a characteristic morphology similar to *Beggiatoa*, *Leucothrix*, *Thiothrix*, *Homoeothrix* and stalked bacteria (Jannasch & Wirsen 1981). Enrichment cultures inoculated with vent water yielded several strains of the obligately chemolithotrophic genus *Thiomicrospira* (Ruby *et al.* 1981). The first species of this genus was isolated originally from the Dutch Waddenzee (Kuenen & Veldkamp 1972) and, as previously mentioned, was shown to be very resistant to high concentrations of sulphide. This tolerance would allow *Thiomicrospira* to thrive particularly well in the sulphide vents. It has been postulated that the organisms might subsequently be consumed by the numerous filter feeders arrayed around the vents, establishing *Thiomicrospira* as a primary producer for this ecosystem.

The occurrence of sulphur-oxidizing prokaryotes around the thermal sulphide vents was not confined to the abiotic environment. Bacteria have been found fluorishing inside a number of eukaryotic organisms surrounding the vents such as clams and worms. One of these worms, the 2 m long *Riftia pachyptila* (Jones), lacks both mouth and gut, its coelomic cavity being filled by an organ called the trophosome, which is richly surrounded by blood vessels. This trophosome has been found to contain a dense population of bacteria (3.7×10^9 per gram of wet tissue) together with sulphur inclusions (Cavanaugh *et al.* 1981). It was postulated that the bacteria would fix carbon dioxide and thus provide the organic carbon for the worm. The bacteria in the trophosome are provided with oxygen and carbon dioxide by the blood of the worm whose haemoglobin appears to be well suited for this task (Arp & Childress 1981). Significant activities of the key enzymes of autotrophic sulphur metabolism, RuBPCase, phosphoribulokinase, rhodanese, APS reductase and ATP sulphurylase have been detected in the trophosome tissue, as shown in table 4 (Felbeck 1981). The first two enzymes are clear evidence for the presence of a Calvin cycle in this tissue. The latter three enzymes are likely to play an important role in the energy generation of a number of thiobacilli (see paper by Kelly in this symposium). The

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combination of all these enzymes clearly indicates that the symbionts may be chemolithoautotrophs. The presence of symbiotic lithotrophs has not been confined to the rift worms, but has also been demonstrated in other worms occurring in more normal habitats (Southward *et al.* 1981), and in several bivalve molluscs such as *Solemya* sp., which have a greatly reduced gut (Fellbeck *et al.* 1981; Cavanaugh *et al.* 1981). In these animals, the enzyme activities (table 4) appear to be associated with the gill tissue in which the presumptive prokaryotic symbionts can be seen.

If the free-living lithotrophic bacteria or the symbiotic prokaryotes were the primary producers of the ecosystem, one would expect that the $\delta^{13}C$ value would be highly negative relative to the ratio in the bicarbonate of the surrounding water (the $\delta^{13}C$ value is expressed as the relative difference (per mille) between the ratio of ^{13}C and ^{12}C in a given sample and the

TABLE 4. ENZYMES OF THE CALVIN CYCLE AND SULPHUR METABOLISM IN MARINE ANIMALS LIVING AT THE SULPHIDE–OXYGEN INTERFACE

(Activities expressed as micromoles per minute per gram wet mass of tissue. Adapted from Felbeck *et al.*(1981).)

species and habitat	RuBPCase	Ru-5Pkinase	ATP sulphurylase	APS reductase	rhodanese
rift vents					
Riftia pachyptila (worm)	0.22	19.0	74.0	23.3	7.6
Riftia sp. (worm)	1.13	—	133.0	30.1	5.2
sewage outfall					
Solemya panamensis (bivalve)	2.4	4.4	77.0	4.1	0.7

standard ratio in a geological sample, the PDB carbonate standard). It is known that organisms that fix CO_2 by the Calvin cycle exhibit a discrimination against ^{13}C of about -22 to $-40‰$ (for review see Troughton 1979). The $\delta^{13}C$ found in the tissue of the clams around the hydrothermal vents was about $-32‰$, compared with $-4‰$ in the available inorganic carbon of the surrounding water. In another worm occurring in the Bay of Biscay, *Pogonophora*, even lower $\delta^{13}C$ values were found (Southward *et al.* 1981). These low values support the view that RuBPCase is the primary CO_2-fixing enzyme in these organisms. A puzzling observation was that in *Riftia pachyptila* from the thermal sulphide vents, the $\delta^{13}C$ value was only $-10‰$. As the autotrophic nature of their metabolism now seems to be well established, the question of whether such organisms might have a C_4 plant CO_2-fixation mechanism, via phosphoenolpyruvate carboxylase, arises. This would not show the same discrimination against $H^{13}CO_3^-$. However, the enzyme could not be detected. Another possibility is that CO_2-limited conditions exist in the trophosome tissue, as a result of which little or no discrimination might occur (Rau 1981). Further work is apparently needed to solve this and many other interesting questions concerning symbiotic sulphide-dependent CO_2 fixation.

It appears to be of particular general importance that the symbiotic sulphur-oxidizing lithotrophs may occur in many animals occurring all over the world in habitats that contain sulphide and oxygen. Thus, the recycling by these symbionts of sulphide originating from the dissimilatory sulphate reduction may be a most important step in the food chain. Furthermore, if sulphide from hydrothermal vents contributed substantially to the total input of energy into the sea, it could be of great importance to our understanding of carbon and energy cycling in the oceans.

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Applied microbiology and sulphur-oxidizing bacteria

The economic importance of sulphur bacteria in general is discussed by Postgate (this symposium). We shall therefore only touch upon a few applications directly relevant to this paper.

Renewed interest in the industrial application of sulphide-oxidizing bacteria stems from the increasing importance of anaerobic waste water treatment. In these systems sulphate is reduced to sulphide, which must be removed. One option is to use aerobic or facultatively anaerobic sulphide oxidizers. Our better understanding of their physiology and ecology may help to improve the management of such plants. An unexpected application of sulphur bacteria in waste water treatment may lie in nitrate removal. When effluent from sewage treatment plants contained nitrate, methanol has often been supplied as an energy source for bacterial denitrification. With the increasing cost of methanol, it has been proposed that methanol be replaced by elemental sulphur, which is cheaply available (Batchelor & Lawrence 1978). The use of sulphur-oxidizing bacteria in the treatment of paper mill waste was described some years ago (Sivelä & Sundmann 1975). The waste contained high concentrations of malodorous methylated sulphides (CH_3SH, $(CH_3)_2S$, $(CH_3)_2S_2$), which were removed by bacterial oxidation in a biofilter. The organisms presumably responsible were obligately chemolithotrophic *Thiobacillus* species which can (co)metabolize $(CH_3)_2S$ during growth on sulphide.

The microbial leaching of ores with cultures of acidophilic thiobacilli in particular *T. ferrooxidans*, is the best known process by which sulphur-oxidizing bacteria are successfully used on an enormous physical and economic scale. For this highly specialized subject the reader is referred to a recent review by Kelly *et al.* (1979). The same organisms may be used for the microbiological desulphurization (depyritization) of coal (Dugan & Apel 1978).

A possible futuristic application of chemolithotrophic sulphur bacteria may be found in establishing sulphide-dependent food chains. An anaerobic waste treatment in the marine environment would lead to the production of sulphide, which could be used to feed symbiotic sulphide-oxidizing animals (for example clams) to produce useful biomass for protein production.

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Discussion

J. M. Shively (*Department of Biochemistry, Clemson University, U.S.A.*). Since I have been accomplishing carboxysome research for over ten years, I believe that a few comments are appropriate.

1. We have never been able to purify the carboxysomes by two sucrose gradients. We find that an electrophoresis step is necessary. Our criteria for purity includes electron microscopy and sucrose density gradient and analytical ultracentrifugation.

2. The polypeptide composition (sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis) of carboxysomes from *Nitrobacter* (E. Bock, Hamburg University), *Chloroglespsis* (G. A. Codd, Dundee University) and *Thiobacillus neapolitanus* are remarkably similar. From seven to ten polypeptides are obtained, not twenty-seven.

3. Carboxysomes obtained from carbon dioxide and thiosulphate-limited *T. neapolitanus* cells show the same SDS polyacrylamide gel electrophoresis pattern.

4. Our carboxysomes are remarkably stable, i.e. we would not expect a loss of other Calvin cycle enzymes if they were present.

5. Phosphoriboisomerase, phosphoribulokinase, fructose bisphosphate phosphatase and carbonic anhydrase *do not* copurify with the carboxysomes. Sonication has no effect.

J. G. Kuenen. We have recently become aware that Dr Shively has not been able to reproduce our results. So far our common discussions have not produced a reasonable explanation for the differences found in his and our laboratory. As stated in the paper, our main criterion for the association of the Calvin cycle enzymes and, for example, malate dehydrogenase with the carboxysomes is that in the purified carboxysome preparation *no* activity of the enzymes can be detected *unless* the carboxysomes have been sonicated in 0.1 M Tris buffer. We have noticed on several occasions that only chemostat-grown steady-state CO_2-limited cells ($D = 0.07\ h^{-1}$) of *T. neapolitanus* produce carboxysomes suitable for our purification procedure.

If the carboxysome preparation were contaminated with other cell fractions one would also expect activities of 'typical' other enzymes that can easily be detected in cell-free extracts, such as glucose 6 phosphate dehydrogenase and 6 phosphogluconate dehydrogenase. Although these activities in cell-free extracts are 52 and 77 nmol min^{-1} mg^{-1} protein respectively, no such activity was detectable in sonicated carboxysome preparations. The question of contamination cannot apply to our cytochemical staining of fructose bisphosphatase. Obviously the staining only takes place if the enzyme activity is present inside the carboxysomes.

When CO_2-limited cells are shifted to growth in CO_2 excess the carboxysomes are actively broken down. With the electron microscope we observed that 'holes' appear in the carboxysomes in the first hour after the shift. This means that *T. neapolitanus* is able to degrade actively (specifically ?) the contents of the carboxysome. The fact that these cells do possess this ability may mean that also during purification lytic enzymes can become active and may, in Dr Shively's purification procedure, destroy the activity of these enzymes. It should be stressed that if the carboxysomes contain all the Calvin cycle enzymes they must *also* be present in the cytosol, for example for the operation of the oxidative pentose phosphate cycle. Copurification will therefore not be observed until all 'outside' activity has been removed.

It is obvious that these suggestions are only speculations and that further work will be necessary to resolve the controversy.

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G. A. Codd (*Department of Biological Sciences, University of Dundee, U.K.*). As we have seen, *Thiobacillus neapolitanus* contains polyhedral bodies, which were renamed carboxysomes by Dr Shively, who found that the organelles contain ribulose bisphosphate carboxylase. Professor Kuenen has proposed a scheme whereby the carboxysomes may serve to direct reducing power, via malate, to a fully functional Calvin cycle inside the organelles. With these interesting possibilities in mind, are there any suggestions about why carboxysomes are absent from some species, including *Thiobacillus novellus*, *Thiobacillus* A2 and *Thiobacillus denitrificans*?

J. G. Kuenen. The facultatively chemolithotrophic thiobacilli such as *T. novellus* and *Thiobacillus* A2 seem to have evolved for optimal functioning under mixotrophic growth conditions (see our paper). Under such conditions NADH from an organic carbon and energy source would be available. As in our hypothesis the carboxysomes are important in producing high NADH concentrations for optimal CO_2 fixation, carboxysomes would only be functional in these organisms during autotrophic growth. It might be speculated that, in Nature, these organisms encounter autotrophic growth conditions too infrequently to carry the information for the possession of carboxysomes. The only facultatively chemolithotrophic *Thiobacillus* that does have carboxysomes is *T. intermedius*. In this organism the carboxysomes are induced under autotrophic conditions (Purohit *et al.*, *J. Bact.* **127**, 516–522 (1976)). In the paper we have also speculated why *T. denitrificans* might not possess carboxysomes. This would be due to the fact that in this organism NADH production probably requires less energy than in the other thiobacilli.

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