Metabolic Flexibility of *Thiobacillus* A 2 During Substrate Transitions in the Chemostat

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Abstract. During autotrophic growth, cells of *Thiobacillus* A 2 retained a considerable capacity to oxidize various organic energy sources. Heterotrophically grown cultures, on the other hand, were completely devoid of the capacity to fix CO₂ via the Calvin cycle and to generate energy from thiosulfate. During transitions from organic media to inorganic thiosulfate-containing media in the chemostat, a long lag-phase was observed before energy generation, CO₂ fixation and, consequently, measurable growth occurred. This lag-phase was practically abolished if substrates were present at very low concentrations in the thiosulfate mineral medium which could be used as an energy source. The same result was obtained when the cells contained reserve material at the moment of the transition. During transitions from thiosulfate-limited growth to starvation, the Q_{O₂}^{max}-thiosulfate and the capacity to fix CO₂ decreased very slowly, after an initial short (± 4 h) increase of both enzyme systems. In contrast, these two metabolic functions were inactivated relatively rapidly in the presence of an oxidizable organic carbon and energy source. This process of inactivation was instantaneously stopped and reversed into rapid enzyme synthesis upon replacement of the organic substrate by thiosulfate.

Key words: *Thiobacillus* A 2 — Mixotrophy — Enzyme-induction — Enzyme-inactivation — Metabolic flexibility

In this publication experiments are described which were aimed at investigating whether *T. A 2* indeed possesses a degree of physiological flexibility and reactivity which permits rapid change-over from heterotrophic to autotrophic growth and back again. Since in recent years it has become generally accepted (Goldberg and St. John 1976; Switzer 1977) that enzyme activity in microbial cells is not only the result of induction and repression but can also be subject to selective inactivation under conditions in which the presence of the enzyme is of no use or even particularly harmful, we have studied both appearance and disappearance of some enzyme activities during substrate transitions in the chemostat. Attention was focused mainly on the enzyme system responsible for the oxidation of thiosulfate and on the key enzyme of the autotrophic CO₂-fixation pathway in *T. A 2* (ribulose-1,5-bisphosphate carboxylase). In a previous publication both these enzyme activities have been shown to be subject to very accurate regulation mechanisms (Gottschal and Kuenen, 1980b).

The results presented below demonstrate the physiological flexibility and reactivity of *T. A 2* towards changing environmental conditions.

Materials and Methods

Organism. The organism used in this study, the facultatively chemolithotrophic *Thiobacillus* A 2, has been described previously (Taylor and Hoare 1969; Gottschal et al. 1979).

Media and Growth Conditions. The composition of the basal medium used in batch and continuous cultivation is the same as described in an earlier publication (Gottschal et al. 1979). To this basal medium different amounts of Na-thiosulfate, Na-acetate or fructose were added according to the description in the results section. The medium was sterilized by autoclaving for 30 min at 118°C. Magnesium sulfate plus trace elements and the different growth substrates were sterilized separately, each in 5% of the total volume of the medium. The continuous culture equipment used has been described by Harder et al. (1974) and Kuenen and Veldkamp (1973). The oxygen concentration in the culture was automatically controlled at 50% air saturation. Cultures were maintained at 28°C, at a pH 7.5 by automatic addition of 1 M Na₂CO₃ or 1 M HCl, depending on the substrate used.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (= SDS-PAGE). Slabgel electrophoresis was performed with 150 µg protein portions of crude cell-free extracts of cultures of *Thiobacillus* A 2 during a change-over from autotrophic to heterotrophic growth conditions or vice versa. The method used has been described by Laemmli (1970). A 3% stack-

Previous experiments (Gottschal et al. 1979; Smith and Kelly 1979; Gottschal and Kuenen 1980a; Gottschal and Kuenen 1980b) clearly pointed to an ecological niche for facultatively chemolithotrophic thiobacilli under conditions of a continuous supply of mixtures of inorganic and organic substrates. Furthermore, it was suggested (Gottschal et al. 1979) that an additional advantage of the facultative chemolithotrophs over the specialized obligate chemolithotrophs could be their metabolic flexibility. This might allow the versatile chemolithotrophs to thrive comparably well under conditions of a fluctuating or alternating supply of carbon and energy sources.

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Abbreviations: SDS, sodium dodecylsulfate; SDS-PAGE, sodium dodecylsulfate — polyacrylamide gel electrophoresis; RubPcase, ribulose-1,5-bisphosphate carboxylase; PHB, poly-β-hydroxybutyrate

gel and a 10% running gel was employed in the procedure. A very clear protein band, corresponding to a molecular weight of approximately 54,000 ($\pm 1,000$), could be recognized in extracts prepared from autotrophically grown cultures and was completely absent in slabgels after electrophoresis of extracts prepared from heterotrophically grown cells. As the molecular weight of the large subunit of RubPcase, isolated from *T. A 2*, has been reported to be 55,500 (Charles and White 1976), this protein band was assumed to correspond to the large subunit of RubPcase. Changes in the density of the protein band were determined by absorption measurements after staining of the protein with Coomassie-blue (Laemli 1970).

For the determination of the molecular weight of the large subunit of RubPcase the following proteins have been used: β -galactosidase (M.W. 130,000), bovine-serum-albumine (M.W. 68,000), ovalbumine (M.W. 45,000), aldolase (M.W. 39,500) and chymotrypsinogen (M.W. 27,500).

Miscellaneous Methods. O_2 consumption rates, thiosulfate, protein, cell-density (as organic cell-carbon) and viability of the cultures were determined as described previously (Gottschal et al. 1979). Acetate was determined according to the method described by Laanbroek et al. (1977). Fructose was determined as organic carbon with a Total Organic Carbon analyzer (Gottschal et al. 1979). Poly- β -hydroxybutyric acid (= PHB) was determined with the method described by Law and Slepecky (1961). Formate was determined following the method described by Lang and Lang (1972). Ribulose-1,5-bisphosphate carboxylase (= RubPcase; EC 4.1.1.39) was assayed according to the method described before (Gottschal and Kuenen 1980). In extracts, prepared from cultures of *T. A 2* in which inactivation of RubPcase was observed, the presence of inhibitory or stimulatory compounds was investigated. For this purpose RubPcase-activity was measured in extracts prepared before and after the inactivating process had started and also in mixtures of these extracts. When the RubPcase-activity detected in such mixed extracts was the arithmetical mean of the activity in the two extracts separately, inhibitors or activators were assumed to be absent.

Chemicals. All chemicals used were of analytical grade (Merck or BDH).

Results

Enzyme Synthesis Under "Autotrophic" Growth Conditions in the Chemostat

Thiobacillus A 2 is able to grow autotrophically on thiosulfate and heterotrophically on a wide range of organic substrates (Taylor and Hoare 1969). Though the potential to use organic compounds was found to be repressed in autotrophically grown cultures of *T. A 2*, some residual oxidative capacity for many different organic compounds was invariably detected (Table 1). In contrast, cells of *T. A 2* grown in batch- or continuous culture for a prolonged period of time on an organic substrate had lost not only their capacity to oxidize thiosulfate ($= Q_{O_2}^{\max}$ -thiosulfate) but their ability to fix carbon dioxide as well. This indicated that such cultures, when transferred to a thiosulfate/bicarbonate mineral medium, would require a period of adaptation before being able to resume growth. The following experiments demonstrated that an adaptation-period indeed was required before adequate chemolithotrophic energy generation and CO_2 -fixation took place.

An acetate-limited continuous culture of *T. A 2* was set up at a dilution rate ($= D$) of $0.05\ h^{-1}$. After a steady state had been reached the acetate medium was replaced, at zero time, by a thiosulfate (40 mM)-mineral medium, while the dilution rate was reduced to $0.02\ h^{-1}$ (Fig. 1). A very slow increase of the capacity to oxidize thiosulfate ($\bullet\text{---}\bullet$) and of the ribulose-1,5-bisphosphate carboxylase ($=$ RubPcase-activity ($\circ\text{---}\circ$)) was observed during the first 6.5 h. No growth occurred in this period, as the cell-density in the culture decreased with a rate equal to the dilution rate.

Table 1. Maximum oxidation potential ($= Q_{O_2}^{\max}$) in cultures of *Thiobacillus A 2* for several substrates during autotrophic growth under thiosulfate-limitation at a dilution rate of $0.05\ h^{-1}$

Substrate	$Q_{O_2}^{\max}$ ($\mu l\ O_2/mg$ cell carbon $\cdot h$)
Thiosulfate	1900
Acetate	70
Fructose	36
Formate	145
Lactate	72
Succinate	3
Butyrate	24
Glycollate	18
Malate	30

Fig. 1. Appearance of the thiosulfate-oxidation capacity and RubPcase in chemostat cultures of *Thiobacillus A 2* after a transition from acetate limited growth at a dilution rate of $0.05\ h^{-1}$ to growth in the presence of thiosulfate. Medium containing thiosulfate (40 mM) was supplied at a rate of $0.02\ h^{-1}$. In addition to the supply of thiosulfate, a medium containing acetate (10 mM) was fed to the culture at a rate of $0.03\ h^{-1}$, initiated at various times after the transition, as indicated by the solid arrows. $Q_{O_2}^{\max}$ -thiosulfate after addition of acetate at $t = 0$ ($\blacksquare\text{---}\blacksquare$), $t = 3$ ($\blacktriangle\text{---}\blacktriangle$) or $t = 6.5$ ($\bullet\text{---}\bullet$). RubPcase-activity ($\circ\text{---}\circ$) in the culture to which acetate was added at $t = 6.5$. In one of the experiments a pulse of acetate was added to a final concentration of 5 mM, 6 h after the transition as indicated by the dashed arrow.

Enzyme Synthesis Under "Mixotrophic" Growth Conditions in the Chemostat

The very low rate of appearance of the capacity to oxidize thiosulfate was possibly due to the unavailability to the cells of an energy- and/or carbon source which could be effectively metabolized. When at various times, 0, 3 or 6.5 h acetate was fed to the continuous culture at a rate of $0.03\ h^{-1}$ using a second pump, a very significant stimulation of the rate of appearance of the $Q_{O_2}^{\max}$ -thiosulfate was observed. Under these conditions the acetate concentration remained undetectably

low. Acetate did not significantly stimulate the rate of induction of RubPcase. This was to be expected since at the ratio of acetate-thiosulfate present in the feed, RubPcase was largely repressed in *T. A2* (Gottschal and Kuenen 1980b).

A similar stimulatory effect on the rate of induction was exerted by formate. Formate can serve as an organic energy source for autotrophic growth of *T. A2* (Kelly et al. 1979). After a transition of cells, grown under formate-limitation at a dilution rate of 0.05 h^{-1} , to a medium containing only thiosulfate no oxidation of thiosulfate occurred during the first 6 h. Within half an hour after the start of the supply of formate medium at a rate of 0.03 h^{-1} , thiosulfate oxidizing capacity was detected, followed by a rapid increase of this capacity. Since formate-grown cells contain high levels of RubPcase, the stimulation of the rate of enzyme-induction was apparently caused by the relief of a shortage of energy rather than that of organic carbon. Further experiments showed that any other substrate which could serve as a directly metabolizable auxiliary energy substrate was able to support a rapid induction of the "autotrophic" enzymes. This turned out to be true not only for the substrates mentioned in Table 1 (for which a residual oxidative capacity always was available) but also for intracellular poly- β -hydroxybutyrate. Likewise, cells of *T. A2*, when pregrown under dual limitation by acetate and thiosulfate under conditions in which no RubPcase was present were able to induce RubPcase rapidly when switched to a medium containing only thiosulfate.

Whereas acetate at very low concentrations ($< 100\text{ }\mu\text{M}$) stimulated the rate of induction of the thiosulfate oxidation capacity (Fig. 1), the presence of a higher concentration of acetate obviously repressed the synthesis of enzymes required for these capacities. When, for example, in the experiments described in Fig. 1 the growth limitation was suddenly relieved by adding a pulse of acetate to a final concentration of 5 mM, a strong repression of the enzyme-synthesis was observed (Fig. 1, $\blacktriangle$ — $\blacktriangle$). This repression was abolished not until the concentration of acetate had become very low. The RubPcase-activity remained undetectable throughout the course of this experiment.

Loss of the "Autotrophic Potential" During Starvation

In the next experiments attention was focused on the rate of disappearance of the $Q_{O_2}^{\text{max}}$ -thiosulfate and of RubPcase from autotrophically-grown cells of *T. A2*. Both capacities together will be referred to as the "autotrophic potential". As a first approach the change in the autotrophic potential was followed during starvation in cells of *T. A2*, grown autotrophically in a thiosulfate limited continuous culture at $D = 0.05\text{ h}^{-1}$. Starvation conditions were obtained by stopping the supply of thiosulfate medium to the chemostat. The results of such a starvation experiment are shown in Fig. 2. Most remarkable was the slight increase in the activity of the $Q_{O_2}^{\text{max}}$ -thiosulfate and/or RubPcase immediately following the start of the starvation period. The observed rise in enzyme activity was invariably found in autotrophically-grown cultures of *T. A2* entering a period of starvation, though the extent of it showed some variation. No significant change in protein and organic carbon content of the culture was detected in the experiment, yet protein synthesis was likely involved since chloramphenicol prevented the increase in enzyme activity in a parallel experiment (result not shown). Three to four hours after the start of the starvation period, the increase in enzyme activity came to a halt and was followed by a decrease of the $Q_{O_2}^{\text{max}}$ -thiosulfate and

Fig. 2. $Q_{O_2}^{\text{max}}$ -thiosulfate and RubPcase as a function of time during starvation of a culture of *Thiobacillus A2* grown autotrophically in the chemostat under thiosulfate limitation at a dilution rate of 0.05 h^{-1} . $Q_{O_2}^{\text{max}}$ -thiosulfate $\bullet$ — $\bullet$, RubPcase $\circ$ — $\circ$

the RubPcase-activity. After 24 h still 90–95% of the initial enzyme-activities remained. Longer periods of starvation caused a further gradual decrease of the enzyme-activities but still 40–50% of the initial $Q_{O_2}^{\text{max}}$ -thiosulfate and RubPcase-activity was found after five days. This process of enzyme-inactivation was not the reflection of the death-rate of the cells since the viability of a starving culture of *T. A2* (grown in a thiosulfate-limited chemostat) was still 85% after 4–5 days.

Loss of the "Autotrophic Potential" During Heterotrophic Growth

The loss of the autotrophic potential after changing over to acetate-limited growth was studied in cells of *T. A2*, initially grown under thiosulfate-limitation in the chemostat. The medium containing thiosulfate (40 mM) which had been supplied to the culture at a rate of 0.02 h^{-1} was replaced by a medium containing acetate (10 mM), while leaving the dilution rate unchanged. As became evident from measurements of protein and carbon, the transition from autotrophic to heterotrophic growth neither caused a change in growth-rate, nor resulted in a temporary accumulation of acetate (results not shown). In Fig. 3 the $Q_{O_2}^{\text{max}}$ -thiosulfate and the RubPcase-activity have been plotted versus the time following the substrate-transition. The RubPcase-activity dropped rapidly, in an exponential manner, immediately after the substrate-transition, whereas the $Q_{O_2}^{\text{max}}$ -thiosulfate decreased more slowly in the beginning. In order to decide whether the disappearance of enzyme-activity was due either to some kind of inactivation or to the mere cessation of enzyme synthesis, the units in which the parameters have been expressed were rendered independent of changes in the protein concentration of the culture. For that purpose the $Q_{O_2}^{\text{max}}$ -thiosulfate has been expressed as $\mu\text{l O}_2/\text{ml} \cdot \text{h}$, whereas the correction on the RubPcase-activity was made by multiplying the specific RubPcase-activity with the factor by which the protein content of the culture changed. To enable a comparison of the rate of loss of autotrophic potential and the rate of washout, the dilution rate (0.02 h^{-1}) has been plotted in Fig. 3 as dashed lines.

Comparison of the rate of loss of RubPcase-activity (0.075 h^{-1}) with the washout-rate clearly indicated that the

Fig. 3. Loss of $QO_2^{\max}$ -thiosulfate and RubPcase during acetate limitation ($D = 0.02 \text{ h}^{-1}$) from cells of *Thiobacillus* A2 initially grown under thiosulfate limitation at a dilution rate of 0.02 h^{-1} . $QO_2^{\max}$ -thiosulfate $\bullet$ — $\bullet$, RubPcase $\circ$ — $\circ$. The rate of washout (= dilution rate) is represented by the dashed lines

Table 2. Rate of inactivation of $QO_2^{\max}$ -thiosulfate and RubPcase during acetate and fructose limitation at various dilution rates

Growth rate (h ⁻¹) before transition	Growth rate (h ⁻¹) after transition	Limiting substrate after transition	Rate of inactivation (h ⁻¹)	
			$QO_2^{\max}$ -thiosulfate	RubPcase
0.02	0.02	acetate	0.024	0.055
0.05	0.05	acetate	0.064	0.050
0.10	0.10	acetate	0.099	ND
0.02	0.02	fructose	0.032	0.053
0.10	0.02	acetate	0.016	0.086
0.02	0.10	acetate	0.116	0.053

enzyme disappeared more rapidly than expected when mere washout would have occurred. The difference between the rate of loss of enzyme activity and the rate of washout will be referred to as the rate of "inactivation", in this case equal to 0.055 h^{-1} . The $QO_2^{\max}$ -thiosulfate was also "inactivated", though at a lower rate, equal to 0.024 h^{-1} . The inactivation started only 12–16 h after the substrate-transition had taken place. Prior to the inactivation some synthesis actually had occurred, the extent of which was comparable to that observed in the initial period of starvation described above (Fig. 2). In a further series of experiments it was investigated whether the rates of inactivation of the autotrophic potential were dependent on the rate of growth (before and/or after the transitions), or on the nature of the organic substrate. The results of such experiments have been presented in Table 2. These data were obtained from graphs similar to that of Fig. 3. From the results it may be concluded that the rate of inactivation of RubPcase showed little variation in response to changes in the specific growth rate

Fig. 4. Loss of $QO_2^{\max}$ -thiosulfate and RubPcase from a culture of *Thiobacillus* A2 (pregrown autotrophically) during acetate limitation followed by induction of both enzyme activities after transition to inorganic thiosulfate containing medium. The dilution rate during growth on thiosulfate was 0.02 h^{-1} and it was 0.10 h^{-1} during growth on acetate. The dashed lines indicate the rate of washout (= dilution rate). $QO_2^{\max}$ -thiosulfate $\bullet$ — $\bullet$, RubPcase $\circ$ — $\circ$ and large subunits of RubPcase as judged from SDS-PAGE-data Δ — Δ

or the nature of the substrate. However, the rate at which the thiosulfate oxidizing capacity was inactivated appeared dependent on the specific growth rate and actually seemed numerically similar to the dilution rate after the transition.

The Reactivity of *T. A2* with Respect to its Autotrophic Potential

In Fig. 4 the results have been presented of an experiment aimed at investigating the rate of recovery of the autotrophic potential after a period of heterotrophic growth. At zero-time an autotrophically growing thiosulfate-limited chemostat culture of *T. A2* ($D = 0.02 \text{ h}^{-1}$) was made acetate-limited ($D = 0.10 \text{ h}^{-1}$) during 28 h. At the end of this period both RubPcase and thiosulfate oxidizing capacity had been inactivated to a large extent. In spite of the fact that the culture of *T. A2* was in the process of inactivating its autotrophic potential, an extremely rapid induction of this potential took place without a detectable lag when acetate was again replaced by thiosulfate.

In order to obtain information about the nature of the "inactivation"-process responsible for the decrease of RubPcase-activity during the change-over experiment, crude cell-free extracts were subjected to SDS-poly-acrylamide gel-electrophoresis (SDS-PAGE). The change in density of the protein-band, tentatively identified as the large subunit of RubPcase (methods section), was compared with the change in RubPcase activity during transitions from autotrophic growth to heterotrophic growth. The result of such an experiment, shown in Fig. 4, clearly demonstrated that the change in RubPcase activity ($\circ$ — $\circ$) ran parallel to that of the large

subunits of RubPcase (Δ — Δ). These results were confirmed in one of the experiments presented in Table 2 ($D = 0.10 \rightarrow 0.02 \text{ h}^{-1}$), in which again the rate of disappearance of the large subunit of RubPcase paralleled the rate of disappearance of activity of this enzyme. This result strongly indicated that the inactivation of RubPcase is a reflection of the breakdown of the enzyme. Consistent with this view was the absence of inhibitory metabolites in these crude cell-free extracts. This was inferred from results of RubPcase-activity measurements in mixtures of extracts prepared at various points of time following a transition from autotrophic to heterotrophic growth.

Discussion

The ability to respond rapidly to changing environmental conditions might have great survival value to facultatively chemolithotrophic thiobacilli. In the present study we have demonstrated that *Thiobacillus* A2, grown autotrophically under thiosulfate-limitation in the chemostat, was able to continue to grow without a detectable lag when the thiosulfate was replaced by acetate or fructose. The absence of any appreciable lag after the transition could be explained by the fact that thiosulfate grown cultures always retained a residual, low, capacity to oxidize acetate, fructose and a large variety of other organic compounds. In contrast we have also shown that growth was resumed not until 7–10 h following a transition from "heterotrophic" to purely "autotrophic" growth conditions. This lag-phase was obviously caused by the complete absence of the capacity to generate energy from thiosulfate, to fix carbon-dioxide, and because the synthesis of the enzymes required for these metabolic capacities was very slow under the given conditions. The rate of enzyme synthesis during this period of adaptation was strongly enhanced by the presence of readily metabolizable substrates, provided these were supplied at very low concentrations (Fig. 1). These observations clearly demonstrated the importance to the organism of retaining enzyme-activities, particularly those involved in the generation of energy, in order to allow rapid adaptation to new growth conditions.

It appears that the observed complete loss of the "autotrophic potential" does not fit in a strategy based on metabolic flexibility, but one should take into account the rate at which the decrease of the enzyme-activities occurs. This rate of loss was shown to be remarkably low in *T. A2* during starvation. These properties seem to make *T. A2* fairly well adapted to survive periods of energy-starvation. In this context the facultatively chemolithotrophic *Nitrobacter agilis* should be mentioned which, with respect to its nitrite oxidizing capacity, behaved similarly during starvation (Steinmüller and Bock 1977).

While in the case of starvation, the $Q_{O_2}^{\max}$ -thiosulfate and the RubPcase-activity had decreased only slightly after 24 h following thiosulfate-limited autotrophic growth, a considerable part of the two enzyme-systems associated with these metabolic activities had been inactivated after one to two days of heterotrophic growth on acetate. Evidence was obtained to indicate that the inactivation of RubPcase was the result of proteolysis. The fact that proteolysis of this enzyme was apparently much slower during starvation than under conditions of active growth seems to contradict the common notion that overall proteolysis is strongly enhanced in microorganisms under substrate deficient conditions (Goldberg and St. John 1976). It should, however, be noted that in more recent reports strong evidence has been presented in favour of the view

that only a limited amount of the different classes of the microbial proteins respond in this way to starving conditions. In contrast a considerable amount of experimental evidence has accumulated demonstrating that general proteolysis is an energy requiring process (Goldberg and St. John 1976; Olden and Goldberg 1978; Murakami 1979) and thus does not occur during severe energy-starvation. In addition, various enzymes have been shown to become inactivated (in vivo) by unknown, though energy dependent, processes (Switzer 1977). Our observations that proteolysis of RubPcase hardly occurred during starvation, but took place rapidly during growth on acetate or fructose, strongly suggested that the (onset of) hydrolysis of RubPcase was energy dependent in *T. A2*. Energy-dependent inactivation of RubPcase probably occurred also in *Hydrogenomonas eutropha* (Kuehn and McFadden 1968) and in *Chromatium* strain D (Hurlbert and Lascelles 1963).

The inactivation of the enzyme-systems responsible for the oxidation of thiosulfate ($Q_{O_2}^{\max}$ -thiosulfate) turned out to be energy dependent too, since it took place much more rapidly in the presence of an oxidizable substrate than during starvation. The underlying mechanism of the inactivation of the $Q_{O_2}^{\max}$ -thiosulfate is unknown. A striking feature of this particular inactivating mechanism appeared to be its growth rate dependence. Not only did the rate of inactivation increase parallel to the growth rate on the organic substrate but both rates were similar as well. As far as we are aware, such a close coupling between the rate of enzyme inactivation and growth rate has not been reported so far. Investigations concerning the relation between growth rate and the rate of intracellular proteolysis invariably indicated an inverse relationship between both rates (Goldberg and St. John 1976; St. John et al. 1979). This makes it doubtful that specific proteolysis is the rate determining step in this enzyme-inactivating process.

From the foregoing we might conclude that *T. A2* does have a very flexible metabolism rendering the organism well suited to respond to changing environmental conditions. However, the important question whether these capacities enable the organism to compete indeed successfully with more specialized organisms like obligately chemolithotrophic thiobacilli and heterotrophs under such conditions, remains to be answered.

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