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SELECTIVE ENRICHMENT OF FACULTATIVELY CHEMOLITHOTROPHIC THIOBACILLI AND RELATED ORGANISMS IN CONTINUOUS CULTURE

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1. Introduction

The current opinion is that facultatively chemolithotrophic thiobacilli, sometimes referred to as "versatile" thiobacilli [1], often may have a significant ecological advantage over their more specialized counterparts, the obligately chemolithotrophic thiobacilli. It has been suggested that the presence of both inorganic and organic substrates in the natural environment would particularly favour growth of facultatively chemolithotrophic thiobacilli [2,3]. There exists, however, still too little direct experimental evidence to substantiate this point of view. Moreover only a very limited number of facultatively chemolithotrophic thiobacilli has been isolated in the past. This might be explained either by the fact that their occurrence in nature is very limited or by the lack of selective enrichment techniques, or by a combination of both.

In a study on the ecological niche of facultatively chemolithotropic thiobacilli, we investigated *Thiobacillus* A2 during growth in pure and in mixed cultures under dual substrate limitation in the chemostat [4,5]. Under these conditions *Thiobacillus* A2 was found to be able to utilize inorganic and organic compounds simultaneously. The outcome of competition between *Thiobacillus* A2 and both a specialized *Thiobacillus* sp. and a specialized heterotroph was shown to depend on the concentration-ratio of the organic and inorganic substrate (acetate and thiosulfate, respectively) in the medium-reservoir of the chemostat [4]. *Thiobacillus* A2 dominated the mixed population over a large range of different mixtures of thiosulfate and acetate. At relatively high thiosulfate-acetate ratios coexistence of *Thiobacillus* A2 and the specialized thiobacillus occurred, whereas at relatively low thiosulfate-acetate ratios *Thiobacillus* A2 coexisted with the specialized heterotroph. However, when the inflowing medium contained exclusively thiosulfate or acetate the specialized autotroph or heterotroph respectively, became dominant.

These results clearly pointed to a possible ecological niche for facultatively chemolithotrophic thiobacilli, since in many environments growth-limiting concentrations of inorganic and organic energy sources may be available simultaneously [6,7]. The outcome of the competition experiments also implied that it should be possible to enrich for facultatively chemolithotrophic thiobacilli in continuous cultures in which growth is limited simultaneously by organic and reduced sulfur containing inorganic substrates.

This paper reports the successful application of this method to isolate selectively these organisms from different fresh-water samples.

2. Materials and Methods

Samples of surface mud from marine and fresh-water habitats were filtered through membrane filters (Schleicher und Schüll, Selectron ST69) with a pore size of 1.2 micron to remove protozoa and 15–20 ml was used as inoculum for the chemostat enrichments.

2.1. Media

The basal medium used in the chemostat cultures contained: (% w/v) K_2HPO_4, 0.08; KH_2PO_4, 0.03; $MgSO_4 \cdot 7H_2O$, 0.04; NH_4Cl, 0.04; NaCl, 2.0 (only in media used for enrichment of marine organisms); in deionized water, plus 2 ml per liter of a trace elements solution [8]. This solution contained 2.2 g instead of the originally reported 22 g $ZnSO_4 \cdot 7H_2O$ per liter. Vitamin B12 and biotin were added to a final concentration of 15 μg and 10 μg per liter, respectively. The pH of the medium was 7.2. Different amounts of Na-acetate and Na-thiosulfate were added to the medium according to the description in the experimental section. The medium was sterilized by autoclaving for 30 min at 118°C unless otherwise stated. The solidified media used in platecounting were prepared as described above, the only differences being that yeast extract (0.005%) and agar (1.5%) were added. The pH of these solidified media was 7.5.

2.2. Cultivation

Enrichments were carried out at 28°C, at 50% air saturation and at a pH of 7.5 in chemostat equipment as described previously [4]. Batch cultures in which acid was produced from thiosulfate were neutralized manually with a 1 M Na_2CO_3 solution.

2.3. Miscellaneous methods

Oxygen consumption rates, thiosulfate, tetrathionate and acetate concentrations, cell density (as organic cell-carbon) and viability of the cultures were determined as described previously [4]. Carbon dioxide-fixation capacity in whole cells and ribulose-1,5-bisphosphate-dependent CO_2-fixation in cell-free extracts was measured as described earlier [5], using radioactive [^{14}C]bicarbonate. Sulfite and sulfide were determined according to the methods described in [9].

2.4. Enrichment technique

Samples from different habitats were used as inoculum for enrichment cultures in the chemostat. At first unrestricted growth was allowed to occur on a mixture of thiosulfate (5 mM) and acetate (2 mM), until both substrates had been utilized completely. At the end of this period (1–2 days), medium containing a mixture of acetate and thiosulfate was fed to the cultures, initially at a very low rate (D = approx. 0.01 h^{-1}). This rate was gradually increased until a dilution rate of 0.05 h^{-1} was established. During this initial period, great care was taken to avoid that more substrate was fed to the culture than actually could be metabolized. After 15–20 volume changes not only the total density (as organic cell-carbon) but also the densities of the different bacterial species in the culture appeared to be constant. This was concluded from colony plate-counts on 3 different media: acetate (5 mM) + yeast extract (0.05%); acetate (5 mM) + thiosulfate (10 mM); and thiosulfate (10 mM) alone. The culture viability (on a medium containing thiosulfate + acetate + 0.05% yeast extract) was always more than 92%.

3. Results

The results of the enrichments (Table 1) show that the freshwater enrichments contained high numbers of facultative chemolithotrophs or, in one case, of a chemolithotrophic heterotroph (Table 1: V). In contrast no such organisms could be enriched from marine samples. The details will be discussed below. The dominant isolates from enrichments I–VII will be referred to as strains I–VII, respectively.

3.1. Enrichments from freshwater environments

All dominant populations were able to grow heterotrophically and to oxidize thiosulfate (Table 1). The organisms present as secondary populations were mostly heterotrophs, some of which were able to oxidize thiosulfate. The secondary population in enrichment culture III was composed of a mixture of obligately and facultatively chemolithotrophic thiobacilli.

Pure cultures of most isolates required the presence of growth factors. This requirement could be satisfied by the addition of 0.01% yeast extract to the medium.

Whereas in batch culture the different isolates grew very well heterotrophically, the demonstration of autotrophic growth was more difficult. Only little

TABLE 1

Results of enrichment cultures (I–VII) after 15–20 volume changes in the chemostat under dual substrate limitation (thiosulfate and acetate) at a dilution rate of 0.05 h^{-1}

The total cell number in the cultures was $2–3 \cdot 10^9$, as determined by plate counts. Autotrophic growth was tested in batch cultures in basal medium supplemented with thiosulfate (20 mM) and yeast extract (0.01%), or in thiosulfate-limited chemostat cultures (III and V). Heterotrophic growth was tested in batch cultures in basal medium supplemented with acetate (10 mM) and yeast extract (0.01%). The thiosulfate-oxidizing potential was determined polarographically after growth in batch cultures in the presence of both acetate (5 mM) and thiosulfate (10 mM).

Enrichment	Sample	Substrate concentrations in the inflowing medium (mM)	Total cell density mg cell carbon/l	Dominant population				Secondary population			
				Percent of total cell number	Autotrophic growth	Heterotrophic growth	Thiosulfate oxidation	Percent of total cell number	Autotrophic growth	Heterotrophic growth	Thiosulfate oxidation
I	Pekeler Hoofddiep (= fresh water)	thiosulfate 30 + acetate 5	90	82	+	+	+	8	–	+	+
								10	–	+	+
II	Pekeler Hoofddiep (= fresh water)	thiosulfate 10 + acetate 15	146	75	+	+	+	25	–	+	+
III	Small ditch (= fresh water)	thiosulfate 30 + acetate 5	94	85	+	+	+	1	+	–	+
								7	+	+	+
								7	+	+	+
IV	Small ditch (= fresh water)	thiosulfate 20 + acetate 10	135	50	+	+	+	5	–	+	ND
								45	–	+	–
V	Small ditch (= fresh water)	thiosulfate 10 + acetate 15	139	86	–	+	+	14	–	+	–
VI	Tidal mud flat (= marine)	thiosulfate 30 + acetate 5	87	63	+	–	+	37	–	+	–
VII	Tidal mud flat (= marine)	thiosulfate 10 + acetate 15	140	81	–	+	–	19	+	–	+

ND, not determined.

growth occurred in the course of 10–14 days at 28°C in a thiosulfate (20 mM) and yeast extract (0.01%) containing medium in batch culture. Initially all strains I–V grew rapidly during the first 24 h at the expense of carbon derived from the yeast extract, as judged from organic carbon analysis. After this short period the growth rate drastically decreased. Carbon analysis clearly demonstrated that the total organic carbon content of the cultures, with the exception of the culture of strain V, increased slowly during the next 10–14 days. During this period the thiosulfate concentration dropped slowly as a result of acid production. These results strongly suggested that the secondary slow increase in cell density was due to autotrophic growth. Direct demonstration of short-term $^{14}CO_2$-uptake was only possible for strain I, which grew relatively fast under these conditions.

As all strains had been isolated from thiosulfate-limited cultures, the very slow growth rate under "autotrophic" conditions in batch cultures might, at least in part, be explained by the presence of the relatively high thiosulfate concentration (20 mM). Therefore two strains (III and V) were selected for further experiments in continuous culture.

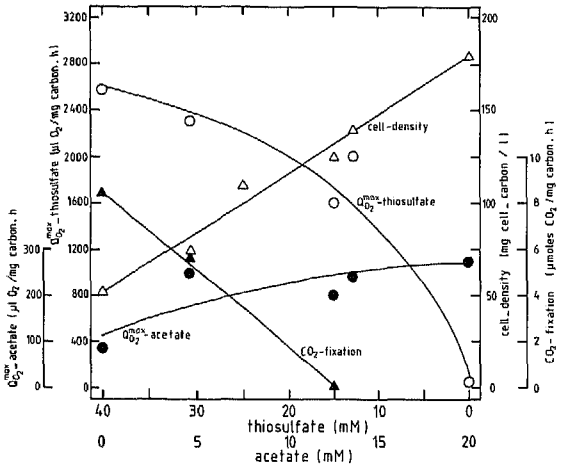

Fig. 1. Growth of strain III in pure culture in the chemostat at a dilution rate of 0.05 h^{-1}, limited by mixtures of acetate and thiosulfate. In steady-state cultures cell density as organic cell carbon (△——△), CO_2-fixation capacity (▲——▲), $Q_{O_2}^{max}$-thiosulfate (○——○) and $Q_{O_2}^{max}$-acetate (●——●) were determined as a function of different concentrations of acetate and thiosulfate in the reservoir medium.

3.2. Growth of strains III and V in continuous culture

Growth of strain III in a thiosulfate-limited chemostat (D = 0.05 h^{-1}) was excellent. Yeast extract (0.01%) was added to the thiosulfate (40 mM) containing medium, since the organism required a growth factor, which in later experiments was shown to be *p*-aminobenzoic acid. When the yeast extract was replaced by *p*-aminobenzoic acid the steady state cell density decreased by 15% from 52 mg cell-carbon/l (Fig. 1) to 44 mg. At a dilution rate of 0.05 h^{-1} steady states were also obtained with mixtures of thiosulfate and acetate as growth-limiting substrates. Cell density, substrate-oxidation-potential (= $Q_{O_2}^{max}$) and CO_2-fixation capacity were determined and the results are summarized in Fig. 1. Cell-density in the cultures increased linearly with the acetate concentration in the medium, indicating that the yield under these conditions was the sum of the yields on comparable amounts of both substrates separately.

The $Q_{O_2}^{max}$ values both for thiosulfate and for acetate appeared to change in parallel to the relative availability of these substrates.

The CO_2-fixation in this organism appeared to be ribulose-1,5-bisphosphate dependent indicating that the Calvin-cycle is operative in this bacterium. The activity of ribulose bisphosphate carboxylase (EC 4.1.1.39) was 105 nmol CO_2/mg protein · min^{-1} in cell-free extracts prepared from cells grown autotrophically in continuous culture at a dilution rate of 0.05 h^{-1}.

Rapid heterotrophic growth of strain III on acetate (5 mM) in batch cultures was observed with a μ_{max} of 0.24 h^{-1} at 28°C and at an initial pH of 7.5. Good growth also occurred on formate, ethanol, glucose, fructose, pyruvate, lactate, glycollate, succinate, and glutamate. No growth was observed on Na-citrate. Occasionally motile cells were observed. The organism was rod-shaped, 0.50–0.65 μm wide and 1.4–1.8 μm long and possessed a single flagellum as judged from electron microscopical observation.

Strain V, unable to grow autotrophically in batch culture with thiosulfate as the sole substrate, was nevertheless able to derive energy from thiosulfate since the steady-state cell-density of a continuous

culture increased from 83 mg cell-carbon/l to 123 when the medium containing only acetate (10 mM) was replaced by a medium containing a mixture of acetate (10 mM) and thiosulfate (20 mM). Cells of strain V, taken from such a dual-substrate-limited continuous culture, were unable to fix CO_2 with thiosulfate as a source of energy. Since the capacity to fix CO_2 might still be repressed by the relatively high concentration of acetate in the medium (compare strain III; Fig. 1) the medium-concentration of thiosulfate, relative to that of acetate, was increased to 32 mM thiosulfate and 4 mM acetate. Even under these conditions no thiosulfate dependent CO_2-fixation could be observed. When the ratio was changed to 36 mM thiosulfate and 2 mM acetate still no CO_2-fixation could be detected. At the same time a considerable portion of the thiosulfate supplied to the culture remained unused and the culture was slowly washed out.

3.3. Inhibition of thiosulfate oxidation in strain III

When grown in thiosulfate-limited continuous culture strain III oxidized the thiosulfate completely to sulfate as indicated by the extent of acid-production and by the absence of sulfur, tetrathionate, sulfide and sulfite in the supernatant of the culture. In contrast, when excess of thiosulfate (5–10 mM) was added to the chemostat-culture, the $Q_{O_2}^{max}$-thiosulfate decreased rapidly, and the culture was washed out. Analysis of the supernatant showed that sulfite had accumulated (Fig. 2B). Sulfide, tetrathionate or sulfur could not be detected. It should be noted here that the usually rapid auto-oxidation of sulfite is largely prevented in such cultures by the presence of excess of thiosulfate [10,11]. In a separate experiment, sulfite (0.5–2.0 mM) was shown to cause severe inhibition of the thiosulfate oxidation (Fig. 2A). At concentrations of 20–40 μM, sulfite already was highly toxic to strain III since growth stopped within an hour even in the presence of acetate. Both the production of sulfite and the striking inhibitory effect of it on thiosulfate oxidation observed in batch cultures of strain III were demonstrated earlier in cultures of *T. novellus* [10]. This has not been reported for cultures of *Thiobacillus* A2. The fact that actively thiosulfate-oxidizing cells of strain III poison their environment by the

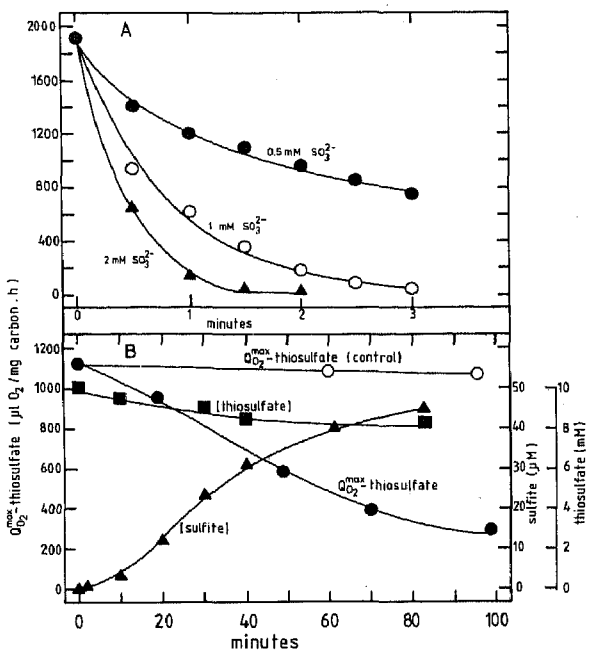

Fig. 2. The poisoning effect of sulfite on the thiosulfate-oxidizing capacity of strain III. (A) Effect of 0.5 mM (●——●), 1 mM (○——○) and 2 mM (▲——▲) sulfite on the $Q_{O_2}^{max}$-thiosulfate of cells pregrown under thiosulfate limitation in continuous culture. Measurements were done in an oxygen electrode chamber. (B) Formation of sulfite (▲——▲) after addition of excess of thiosulfate (■——■) to cells of strain III growing under thiosulfate limitation in continuous culture. At timely intervals the $Q_{O_2}^{max}$-thiosulfate was determined (●——●) in an oxygen electrode chamber. Part of the starting culture was kept without thiosulfate and its $Q_{O_2}^{max}$-thiosulfate (○——○) was measured for comparison.

production of sulfite, if high concentrations of thiosulfate are present, illustrates the adaptation of this bacterium to low substrate levels and the difficulty in handling this particular one, and comparable organisms, in batch culture.

3.4. Enrichments from a marine environment

At the end of the enrichment procedure, cultures VI and VII (Table 1) contained a mixture of a specialized (obligately chemolithotrophic) spirillum-shaped autotroph. This autotroph was very similar to *Thiomicrospira pelophila* [12] both in morphology, typical colony type and basic physiology.

The failure to select for facultatively chemolithotrophic thiobacilli from the marine environment confirms results of several earlier analogous experiments. As facultatively chemolithotrophic thiobacilli, originally present in the sample might have been killed by the initial presence of excess of substrate, the chemostat was re-inoculated, after several volume changes, with a sample from the same environment; however, without an effect on the outcome of these experiments.

4. Discussion

From the results presented it can be concluded that facultatively chemolithotrophic thiobacilli were isolated from different fresh water environments.

The dominant organism from one of the enrichments showed a marked physiological resemblance with *Thiobacillus* A2 under mixotrophic growth conditions in the chemostat [5]. However, comparison of this organism with *Thiobacillus* A2 in respect to cell size, colony type, growth requirements, cell yield and growth on thiosulfate in batch culture indicated clear differences as well. Therefore this isolate is distinct from *Thiobacillus* A2 and will be referred to as *Thiobacillus* III.

The autotrophic growth yield of *Thiobacillus* III (2.2 g/mol thiosulfate) is rather low when compared to yields of other thiobacilli, such as *Thiobacillus* A2, *T. neapolitanus* and *T. novellus* which have yields of 5–6 g/mol thiosulfate at the same growth rate [13, 14]. The cell density of a continuous culture of *Thiobacillus* III during mixotrophic growth ($D = 0.05\ h^{-1}$) limited simultaneously by thiosulfate and acetate also was rather low: both in pure culture and in the mixed culture from which the organism was isolated (Table 1: III) the cell density was about 50% of that observed in *Thiobacillus* A2 cultures grown under the same conditions [5]. The fact that *Thiobacillus* III, under identical growth conditions, had become the dominant species in the chemostat enrichment, indicates that a low yield does not necessarily need to be a disadvantage during the competition for two growth-limiting substrates. It is of interest in this context that results of mathematical modelling indicate that it is the ratio of the yields on the two growth-limiting substrates which is a factor of importance for the outcome of the competition rather than their absolute value [15,16].

To our knowledge the results presented are the first example of a successful selective enrichment technique for facultatively chemolithotrophic thiobacilli. This result justifies earlier surmises concerning the selective advantage of the mixotrophic way of life [2,3]. Furthermore it confirms the more specific prediction that conditions in which thiosulfate and an organic substrate limit growth simultaneously should lead to selective enrichment of facultatively chemolithotrophic thiobacilli [4,15]. It should be expected that very similar results will be obtained with combinations of sulfide and organic compounds, since it has been observed that several thiobacilli and related organisms respire sulfide at rates identical to that of thiosulfate as has been shown for *Thiobacillus* A2 and *T. neapolitanus* (Gottschal and Kuenen, in preparation) and for *Thiobacillus* III and strain V (data not shown).

In enrichment V the dominant organism was not a facultatively chemolithotrophic *Thiobacillus*. From this culture a heterotrophic organism, able to derive energy from the oxidation of thiosulfate, was isolated instead and therefore is very similar to *T. perometabolis* [17]. The explanation for this somewhat unexpected result might be found in the composition of the medium. This contained a very high concentration of acetate compared to thiosulfate, which implies that the dominant organism, metabolizing both substrates, does not require the enzymic machinery to use CO_2 as the main carbon source. It might even be a selective disadvantage to an organism under the given conditions to carry the genetic information for such redundant enzymes.

The two enrichments from a marine environment (Table 1: VI and VII) led to mixed cultures composed of a heterotroph and a specialized chemolithotrophic *Thiobacillus* or *Thiomicrospira*-type organism. The explanation for the striking difference between enrichments from freshwater samples and from marine samples is at present only open for speculation.

The fact that we have obtained successful enrichments from all freshwater samples demonstrates that facultatively chemolithrophic thiobacilli and thiosulfate oxidizing heterotrophs must commonly occur in the natural environment. This implies that there are probably many habitats in which the simultaneous

occurrence of low concentrations of organic compounds and thiosulfate (or sulfide) provides an ecological niche for these metabolic types. More ecologically directed research will be needed to quantify the contribution of these organisms to the total turnover of sulfur compounds.

The general principle of this enrichment technique may also be applied to the specific enrichment of other facultative chemolithotrophs or, in general, to the enrichment of metabolically versatile bacteria. Their isolation from batch culture enrichments has always been a rather laborious and aspecific procedure, as these bacteria never become dominant in such enrichments.

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