

Heterolactic fermentation of intracellular polyglucose by the obligate chemolithotroph *Thiobacillus neapolitanus* under anaerobic conditions

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Received 10 August 1981

Accepted 17 August 1981

1. INTRODUCTION

Thiobacilli thrive in environments where sulfide and oxygen co-exist, for example in the upper layers of sediments and in the so-termed chemoclines of water bodies [1]. In these layers sulfide diffuses upwards from the anoxic sulfide-bearing sediments and bottom waters and reacts with oxygen which diffuses downwards. When light is available sulfide may be used as electron donor in bacterial photosynthesis and, as a consequence of this light-dependent sulfide oxidation the zone of co-existing sulfide and oxygen may migrate up and downwards during a diurnal cycle [2,3]. The chemocline was found to be a few centimeters wide in, for example, the stratified Solar Lake (Sinai) [2] and even smaller in, for example, cyanobacteria mats [3]. A drifting O_2 - H_2S interface of a few centimeters imposes a selective pressure on thiobacilli to co-migrate and/or to be resistant to aerobic starvation periods as well as to anaerobic conditions with an abundance of sulfide but lack of oxygen as electron acceptor. Some *Thiobacillus* spp. are able to survive and grow in anaerobic environments using NO_3^- as electron acceptor, a

capability which is not shared by many other thiobacilli, however. *Thiobacillus neapolitanus*, for example, is not able to denitrify on reduced sulfur compounds.

Recently we detected the presence of polyglucose in this obligately chemolithotrophic organism. Under N-limited growth conditions *T. neapolitanus* accumulated polyglucose to approx. 8% of its dry weight. The polyglucose was shown to function as storage energy and storage carbon source under aerobic conditions [4]. This paper shows that under anaerobic conditions polyglucose was fermented by *T. neapolitanus* to ethanol, lactate and CO_2 via the heterolactic fermentation pathway. The key enzymes of this pathway showed activities in cell-free extracts derived from *T. neapolitanus* cells.

2. METHODS

2.1. Organism and growth conditions

T. neapolitanus strain X was cultivated in continuous culture as in [5]. Ammonium-limited growth was achieved as detailed previously [3]. The dilution rate was kept constant at 0.07/h; the dissolved oxygen concentration was 50% of air-saturated water.

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2.2. Anaerobic incubation

The aerobically grown cells were harvested by centrifugation in sterilized buckets for 40 min at $20\,000 \times g$ (28°C) and washed in a sterile medium containing (g/l): NH_4Cl , 0.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; cysteine-HCl, 0.032 (brought to pH 6.8) and a redox indicator resazurine. To one litre of medium, 2 ml of a trace element solution was added [6]. Cells were concentrated 20-fold in this medium, transferred into 35 ml flasks, and subsequently flushed with oxygen-free nitrogen gas. Nitrogen used was made oxygen-free by leading the gas from the cylinder through heated copper (Baker light copper turnings, Baker Chemicals, Deventer, The Netherlands). The change of colour from pink to colourless of the resazurine indicated that the solution was oxygen-free. As an extra safeguard for strictly anaerobic conditions these flasks were incubated at 28°C in a desiccator which was made anaerobic by flushing with oxygen-free nitrogen in combination with the Gaspak system. The efficacy of the catalyst was checked with a redox indicator which turned from blue to white on anaerobiosis (Becton-Dickinson, Amersfoort, The Netherlands).

2.3. Determination of the fermentation products

After 96 h of anaerobic incubation, *T. neapolitanus* cells were harvested by centrifugation for 40 min at $20\,000 \times g$ (4°C). The supernatant was filtered through a membrane filter of 0.2 μm pore size (Sartorius). The filtered supernatant was assayed for alcohols, volatile and non-volatile fatty acids by Gas Liquid Chromatography. Volatile fatty acids and methyl derivatives of organic acids were analysed using a Pye Unicam 104 gas chromatograph equipped with flame ionization detector. A glass column packed with 10% SP-1000 (w/w) on 100–120 mesh Chromosorb W-AW incorporated with 1% H_3PO_4 (w/w) was used as a stationary phase. The carrier gas was passed through the column at a flow rate of 30 ml/min; the temperatures of the injection port, column and detector were 175, 125 and 170°C , respectively. Flow rates of air and hydrogen were 250 ml/min and 15 ml/min. Volatile fatty acids were de-

termined by mixing 1 ml of supernatant with 0.4 g NaCl, 0.1 ml of 18 M formic acid and 1.0 ml of diethylether, and subsequent injection of 5 μl of the ether phase into the column. In order to quantify non-volatile fatty acids accurately, the supernatant was freeze-dried and the lyophilized residue was subsequently dissolved in distilled water to 5% (v/v) of the original volume. The organic acids were methylated overnight following standard procedures [7]. After methylation the mixture was equilibrated with chloroform; a 5 μl sample of the latter phase was injected into the column (see above). Malonic acid was used as an internal standard.

Alcohols were determined using a Packard Becker 427 gas chromatograph equipped with flame ionization detector. A glass column packed with Porapak Type Q 100–120 mesh was used as a stationary phase (Waters Associates Inc., Milford, MA, USA). Flow rates of the carrier gas nitrogen and of hydrogen and air were as described above. The temperatures of the injection port, column and detector were 180, 140 and 150°C , respectively. CO_2 was determined in the inorganic channel of a Total Organic Carbon Analyzer (Beckman).

2.4. Preparation of cell-free extracts

Cultures were harvested by centrifugation for 40 min at $20\,000 \times g$ (4°C) and washed in the assay buffer containing 1 mM of dithiothreitol. Cell-free extracts were made by sonification for $5 \times 30\text{ s}$ at 0°C in the presence of 1:1 (w/v) ballotini beads (0.11 mm diameter). Beads and debris were removed by centrifugation for 20 min at $40\,000 \times g$ (4°C).

2.5. Enzyme assays

Alcohol dehydrogenase (alcohol:NADP oxidoreductase EC 1.1.1.2) reaction mixture contained 62.1 mM glycine-sodium pyrophosphate buffer adjusted to pH 9.0 with 1 M HCl; 72.8 mM semicarbazide-HCl, adjusted to pH 6.5 with NaOH; 1.8 mM NADP and cell-free extract (0.2 mg protein/ml of reaction mixture). The reaction was started by addition of ethanol (final con-

centration 57 mM) and the reduction of NADP was measured in a Perkin Elmer double beam spectrophotometer at 30°C.

Acetaldehyde dehydrogenase (aldehyde : NAD(P) oxidoreductase EC 1.2.1.5) reaction mixture contained 45 mM K-phosphate buffer pH 7.1; 20 mM MgSO₄; 0.1 mM coenzyme A; 1.8 mM NAD(P) and cell-free extract (0.2 mg protein/ml reaction mixture). The reaction was started by addition of acetaldehyde (final concentration 30 mM) and the reaction was followed as described above.

Phosphoketolase (D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate lyase (phosphate-acetylating) EC 4.1.2.9) was assayed at 37°C in a reaction mixture which contained 100 mM K-phosphate buffer pH 6.0; 3.3 mM MgCl₂; 1 mM thiamine pyrophosphate; 0.33 mM NADH; 1 U triosephosphate isomerase; 1 U α -glycerol-phosphate dehydrogenase (both purchased from Boehringer Mannheim, FRG). The reaction was started by addition of xylose-5-phosphate (Sigma Chemical Co., St. Louis, Missouri, USA) to a final concentration of 2.0 mM. The enzymic cleavage of xylulose-5-phosphate to glyceraldehyde-3-phosphate and acetyl phosphate was also checked by measuring the production of acetyl phosphate [8].

Lactate dehydrogenase (L-lactate: NAD(P) oxidoreductase) test mixture contained 62.1 mM glycine-sodium pyrophosphate buffer adjusted to pH 9.0 with 1 M HCl; 1.8 mM NAD(P) and cell-free extract (0.1 mg protein/ml of reaction mixture). DL-lactate (final concentration 29 mM) was added to start the reaction which was followed as described for alcohol dehydrogenase.

2.6. Protein

Protein in cell-free extracts was determined by the Coomassie blue method using bovine serum albumin as a standard [9].

2.7. Polyglucose

The total sugar content of the cells directly before and after anaerobic incubation was assayed with the anthrone reagent with glucose serving as a standard [10].

2.8. Contamination

Cultures were frequently checked for contaminants as in [5].

3. RESULTS

For the study of anaerobic breakdown of intracellular polyglucose by *T. neapolitanus* we used cells which had been grown aerobically under nitrogen limitation in the chemostat. The cells appeared to be able to degrade intracellular polyglucose under strictly anaerobic conditions. To measure the fermentation products quantitatively, polyglucose-containing cells had to be concentrated to a final density of about 2000 mg dry weight/l. The supernatant of *T. neapolitanus* cells incubated anaerobically for 96 h was assayed for volatile fatty acids, organic acids, alcohols and CO₂. Fermentation products appeared to be lactate, ethanol and CO₂ in a ratio of approx. 1:1:1. In a typical experiment, 0.35 mM (poly)glucose was fermented to 0.35 mM lactate, 0.31 mM ethanol and about 0.3 mM CO₂ (0.2–0.4 mM). This type of glucose fermentation pattern is known in the literature as the heterolactic fermentation pathway occurring for example in *Leuconostoc mesenteroides* [11]. Ethanol as fermentation product from *T. neapolitanus* polyglucose was detected only under strictly anaerobic conditions. The presence of traces of oxygen in the incubation medium resulted in the formation of other products such as acetate.

The key enzymes of the heterolactic fermentation pathway could be detected in cell-free extracts derived from aerobically grown ammonium-limited *T. neapolitanus* cells (Table 1). Activities of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase already had been determined [4]; these enzymes appeared to use NADP preferentially as electron acceptor. Phosphoketolase activity in cell-free extracts derived from *T. neapolitanus* was measured by the production of glyceraldehyde-3-phosphate from xylulose-5-phosphate (see METHODS). The detection of acetyl phosphate as second reaction product from the cleavage of xylose-5-phosphate confirmed the op-

eration of this enzyme in *T. neapolitanus* cells. According to the literature [11] acetyl phosphate can be reduced to ethanol via three steps: firstly acetyl phosphate is converted to acetyl-CoA by the action of phosphotransacetylase. The activity of this enzyme was not measured. Subsequently acetyl-CoA can be reduced to acetaldehyde by acetaldehyde dehydrogenase. This enzyme showed only activity in cell-free extracts derived from *T. neapolitanus* cells that had been incubated under anaerobic conditions. Cells from aerobically grown N-limited chemostat cultures of *T. neapolitanus* did not exhibit any acetaldehyde dehydrogenase activity. The enzyme, measured in the reverse direction, was active with NAD and also with NADP as an electron acceptor (Table 1). The reaction was completely dependent on the presence of coenzyme A for activity, indicating that the reaction product indeed is acetyl-CoA. Finally acetaldehyde can be reduced further to ethanol by ethanol dehydrogenase. This enzyme was equally active in aerobically grown cells as in those that had been incubated under anaerobic conditions. Interestingly, the enzyme, assayed in the reverse direction, showed only activity with NADP and not with NAD as an electron acceptor.

The C₃ cleavage product of xylulose-5-

phosphate, glyceraldehyde-3-phosphate, can be converted via a part of the Embden-Meyerhof pathway to pyruvate; glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase have been shown to be active in *T. neapolitanus* [12]. This pathway yields 1 mol of NADH and 2 mol of ATP by substrate-level phosphorylation per mol of glyceraldehyde-3-phosphate. The reducing power may be transferred to pyruvate yielding lactate, and indeed lactate dehydrogenase activity was detected in aerobically grown cells as well as in anaerobically incubated cells of *T. neapolitanus*. This enzyme, measured in the reverse direction, was equally active with NADP and NAD as electron acceptors.

Phosphofructokinase activity in extracts derived from the obligately phototrophic cyanobacterium *Anabaena variabilis* was revealed by variation of the ATP concentration in the assay mixture [13]. Similar experiments carried out with *T. neapolitanus* extracts did not result in detectable activity of this key enzyme of the Embden-Meyerhof pathway (not shown), consistent with results obtained by other authors [12,14].

4. DISCUSSION

As far as the authors are aware this is the first report on a heterolactic fermentation in an obligately aerobic bacterium (Scheme 1). Recently phosphoketolase activity was also detected in extracts derived from the facultative chemolithotrophs *Thiobacillus novellus* and *T. A2* [15] which indicates that one of the key enzymes of this pathway might be widespread among autotrophic bacteria.

Neither anaerobically incubated nor aerobically grown *T. neapolitanus* cells exhibited any phosphofructokinase activity, ruling out the possibility of polyglucose fermentation via the Embden-Meyerhof pathway. This latter pathway should be operative in the facultatively anoxygenic cyanobacterium *Oscillatoria limnetica* under anaerobic conditions since polyglucose was fermented by this bacterium almost exclusively to lactate [16]. Another way of anaerobic polyglucose fermentation was reported for the obligately anaerobic

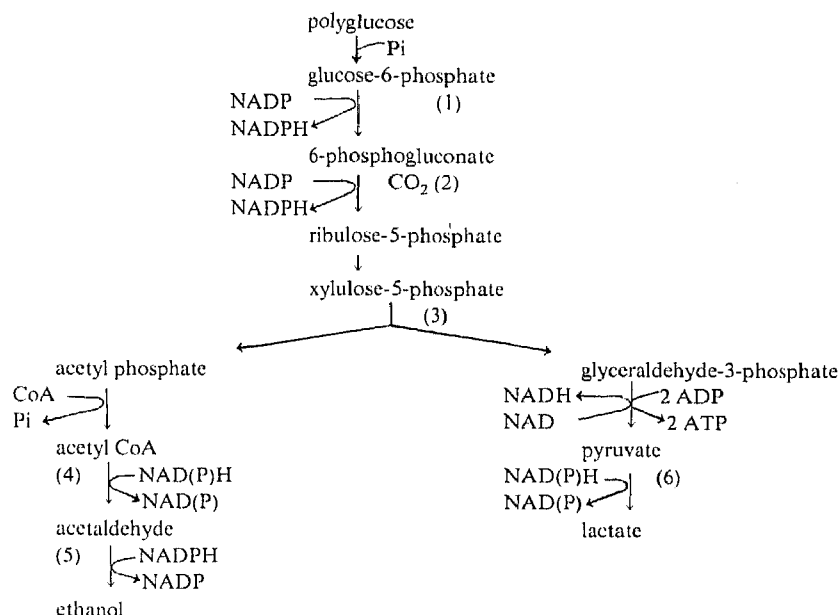
Table 1

Activities of the key enzymes of the heterolactic fermentation pathway in extracts derived from ammonium-limited *Thiobacillus neapolitanus* cells (D=0.07; dissolved oxygen tension=50% of the air saturation)

Enzyme	Activity (nmol/min/ mg protein)
(1) Glucose-6-phosphate dehydrogenase (NADP-dependent) ^a	15
(2) 6-Phosphogluconate dehydrogenase (NADP-dependent) ^a	24
(3) Phosphoketolase	11
(4) Acetaldehyde dehydrogenase ^b (NAD(P)H-dependent)	8
(5) Ethanol dehydrogenase (NADPH-dependent)	30
(6) Lactate dehydrogenase (NAD(P)H-dependent)	45

^a Data from Beudeker et al. [4].

^b Only active in anaerobically incubated cells.



Scheme 1. Proposed pathway of anaerobic polyglucose degradation by *Thiobacillus neapolitanus*. Numbers in parentheses refer to the enzymes listed in Table 1.

Chlorobium thiosulfatophilum which fermented its polyglucose to volatile fatty acids, among which acetate comprised the main part [17].

As pointed out in the introduction, thiobacilli are forced to co-migrate with the drifting O_2 - H_2S interface and/or should be able to stand aerobic and anaerobic starvation periods. The recent observations on chemotaxis by *T. thioparus* are of relevance in this respect. The observed positive attraction towards thiosulfate effected by rotation of polar flagella will enable these organisms to concentrate themselves in environments which contain reduced sulfur compounds [18]. Whether *T. neapolitanus* is also able to exhibit chemotaxis remains to be investigated. The present results clearly indicate, however, that the organism is able to survive under anaerobic conditions by fermentation of its intracellularly stored polyglucose, yielding two molecules of ATP from one mole of glucose-1-phosphate (Scheme 1). Since no ATP is required for the formation of glucose-1-phosphate from polyglucose, there is a net gain of 2 mol of ATP from 1 mole glucose equivalent of polyglucose. Preliminary experiments showed the viability

of the organisms to be over 90% after two days of anaerobic incubation. Polyglucose formation by *T. neapolitanus* cells occurs not only during N-limited growth [4], but also under thiosulfate limitation at a relatively low ammonium concentration (1 mM NH_4^+ ; $D = 0.03/h$; Beudeker and Riegman, unpublished). For this reason it seems likely that in their natural environment aerobically grown *T. neapolitanus* cells will possess some polyglucose as a rule rather than as an exception. This storage compound could sustain at least their maintenance requirement under aerobic and anaerobic starvation periods.

ACKNOWLEDGEMENTS

The investigations were supported by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). Prof. H. Veldkamp and Prof. W. Harder are gratefully acknowledged for valuable suggestions.

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