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Rapid short-term poly-β-hydroxybutyrate production by *Thiosphaera pantotropha* in the presence of excess acetate

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During substrate-limited growth, some strains of Thiosphaera pantotropha *nitrify heterotrophically and denitrify aerobically. These processes are thought to be used as NADH overflow mechanisms, resulting from a restricted oxygen respiration capacity. In this study, the behavior of* T. pantotropha *regarding the NADH overflow in the presence of substantial concentrations of substrate was examined. Continuously grown (dilution rate 0.1 h^{-1}), acetate-limited cultures of* T. pantotropha *(30–285 mg dry weight l^{-1}) were exposed for short periods (10–90 min) to excess acetate in batch. After the transition from acetate limitation to acetate excess, the growth rate immediately increased from 0.1 to 0.2 h^{-1}. The acetate uptake rate was 1.1–1.2 μmol min^{-1} mg $protein^{-1}$, which would theoretically allow a growth rate of 0.35 h^{-1}. Acetate appeared to be converted mainly to poly-β-hydroxybutyrate (PHB) (57% wt/wt^{-1}). The initial PHB production rate was 27 μg PHB min^{-1} mg $protein^{-1}$. Respiration measurements showed that only 29% of the total acetate taken up was oxidized. The remainder (14%) was used for biomass synthesis. After acetate (10 mM) was completely taken up, the cellular PHB content was 42% of the dry weight. During PHB synthesis no heterotrophic nitrification or aerobic dentrification could be detected. The results indicate that in acetate-limited cells when exposed to excess acetate, PHB formation serves as an NADH overflow metabolism.*

Keywords: *Thiosphaera pantotropha;* poly-β-hydroxybutyrate; batch; excess acetate; overflow electron transport chain

Introduction

Poly-β-hydroxybutyrate (PHB) is a common storage product in both Gram-positive and Gram-negative bacteria. It is generally observed when batch-grown cells enter the stationary phase, and is synthesized under conditions of nutrient imbalance, such as when growth is limited by any essential nutrient except the carbon source.[1] Any excess organic carbon substrate may then be taken up by the cells and converted into reserve material. PHB synthesis by carbon- and energy-limited continuous cultures during alternating 4-h periods of acetate and thiosulphate supply in the medium feed has been reported for *Thiobacillus* S LMD 81.10.[2] *Thiobacillus* S was able to synthesize sufficient PHB during the acetate phases to meet its requirements for organic carbon when thiosulphate was the only substrate available. The production of reserve material may be caused by a bottleneck in the respiratory metabolism or by a restricted assimilatory capacity.[3]

Thiosphaera pantotropha is capable of denitrification, whether or not oxygen is present,[4,5] although some strains appear to have lost this property.[6] This organism is also able to nitrify heterotrophically.[7] It has been suggested that both processes are mechanisms for overcoming redox problems in the cytochrome chain caused by a rate-limiting step in electron transport to oxygen.[5] Both processes allow a faster reoxidation of NAD(P)H to meet the $NAD(P)^+$ demand of the cells. Under oxygen or nitrogen limitation, or during growth with hydroxylamine, PHB was synthesized by

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chemostat cultures growing on acetate.[8] PHB formation also uses NAD(P)H, and was proposed as a process of last resort,[5] when both aerobic denitrification and heterotrophic nitrification were not possible. It seems likely that a sudden surge of electrons into the cytochrome chain from a pulse of acetate might result in a rapid drop in the redox state of the cytochromes, triggering PHB formation, rather than an increase in either the nitrification or denitrification rates. With the electrons being channeled into PHB synthesis, the cytochromes would become less reduced, and therefore, no nitrification or denitrification will occur.

This article describes the rapid formation of PHB by acetate-limited *T. pantotropha* when suddenly provided with excess amounts of acetate. This rapid PHB formation is hypothesized to be due to the combination of an overcapacity for acetate uptake and a bottleneck in the respiration metabolism.

Materials and methods

Organism

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide-oxidizing wastewater treatment system.[9] Although its 16S rRNA is virtually identical to that of *Paracoccus denitrificans*,[10] *T. pantotropha* differs significantly as regards physiology and antibody reaction. *Acinetobacter calcoaceticus* LMD 79.41, a species known not to nitrify and denitrify, was obtained from Dr. B. J. van Schie.

Growth

The cells used as inoculum for the short-term batch experiments were grown in acetate-limited chemostat cultures at a dilution rate of 0.1 h^{-1} (μ_{max} = 0.35 h^{-1})[4] and a dissolved oxygen concentration of 80% air saturation. The pH was maintained at 8.0 with 1N NaOH and 1N H_2SO_4, and the temperature at 37°C. The medium contained (g l^{-1}): K_2HPO_4, 0.4; KH_2PO_4, 0.15; $(NH_4)_2SO_4$, 1.32; $MgSO_4 \cdot 7H_2O$, 0.4; KNO_3, 0.51; $CH_3COONa \cdot 3H_2O$, 2.72 (20 mM); and 2 ml of trace element solution.[11] When grown mixotrophically, 0.79 g l^{-1} $Na_2S_2O_3$ was added to the medium. During heterotrophic growth on 20 mM acetate the yield was 220 mg dry weight l^{-1}.

Short-term batch experiments

One liter of a steady-state culture (acetate concentration was below the detection limit at <5 μM) was aseptically washed with 50 mM potassium phosphate buffer, pH 8.0, before use. We suspended 15–100 mg cells in 1-l Kluyver flasks[12] at 37°C, with a dissolved oxygen concentration above 80% air saturation. The medium contained (g l^{-1}): Na_2HPO_4, 4.19; KH_2PO_4, 1.5; NH_4Cl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; KNO_3, 0.51; and 2 ml of a trace element solution.[11] The cultures were provided with 10 mM sodium acetate at the start of the experiment. The batches were stopped at the times given in the text.

Analytical techniques

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer, Mannheim, Germany), or as total organic carbon. PHB was extracted from dried cells (150–200 mg) with 50 ml hot chloroform in a Soxhlet apparatus for 8 h.[13] The chloroform was then evaporated and the dried PHB was then depolymerized to crotonic acid with concentrated sulphuric acid at 100°C. The crotonic acid concentration was determined at 235 nm according to Law and Slepecky.[14] Protein was measured by means of the microbiuret method.[15] The total organic carbon (TOC) of washed cells was determined with a Beckmann TOCA master 915-B. Carbon, hydrogen, oxygen, and nitrogen (CHON) were measured with a Perkin Elmer 240 C. Electron microscopy with a Philips EM 400 was used to detect significant amounts of storage products. Before sectioning, the cells were fixed in glutaraldehyde and stained with osmium tetroxide and ruthenium red.

Respiration measurements

Respiration rates of whole cells were measured polarographically with a Clark-type oxygen electrode (Biological Oxygen Monitor; Yellow Springs Instruments, Yellow Springs, OH, USA) at 37°C. The oxygen consumption rate was measured during the first 2–4 min. The acetate-dependent oxygen uptake rate was corrected for endogenous respiration. The biomass concentration was 200 mg dry weight l^{-1}.

Estimation of the amount of storage compound

The chemostate-grown inoculum did not contain significant concentrations of storage compounds, and the CHON ratio from these cells was in good agreement with previous determinations from similar cultures.[5] The ratio $CH_{1.81}O_{0.54}N_{0.26}$ was therefore assumed to be representative of *T. pantotropha* biomass without storage products. Previous experiments revealed that the storage material shown on electron micrographs was PHB,[5] which has a CHO ratio of $CH_{1.5}O_{0.5}$. Thus, the total biomass composition is the sum of biomass plus the fraction (x) contributed by PHB, and is given by the equation:

$$CH_{1.81}O_{0.54}N_{0.26} + x\ CH_{1.5}O_{0.5} = C_AH_BO_CN_{0.26} \qquad (1)$$

$C_AH_BO_CN_{0.26}$ represents the element analysis found for the total biomass. Equation (1) is valid if PHB can be determined. After the calculation of x the amount of PHB in the biomass can then be estimated by:

$$\text{amount of PHB} = x/(1 + x) \times DW \qquad \text{mg } l^{-1} \qquad (2)$$

where DW represents the total biomass.

Estimation of acetate metabolism during PHB formation

The data obtained from the short-term batch experiments were used to calculate to what extent the cells used the acetate to produce PHB. Acetate is used in three pathways: one is for PHB synthesis, the second is assimilation, and the third is dissimilation to provide adenosine triphosphate (ATP) and NAD(P)H. The acetate is taken up by means of active transport, and is converted to acetyl-CoA. Both of these processes require ATP equivalents to drive the reactions.[16,17] The production of β-hydroxybutyrate units (HB), the monomer of PHB, is given by the equation:

$$2 \text{ acetyl-Coa} + \text{NAD(P)H} \rightarrow (\text{HB}) + \text{NAD(P)} + + 2\ \text{CoA} \qquad (3)$$

Acetate dissimilation starts with acetyl-CoA and yields 2 NADH, 1 NADPH, 1 FADH, and 1 ATP per acetate.

Part of the reducing power is consumed by means of electron transport to oxygen to generate ATP. In view of the very low yields of *T. pantotropha*, a P:O ratio of 1:2 has been used in the calculations.[16] Most of the remaining reducing power is used in β-hydroxybutyrate synthesis (Reaction 3).

Results and discussion

Batch experiments

Short-term batch culture experiments with direct measurement of PHB showed that its formation could be detected in *T. pantotropha* after only 5 min *(Table 1)*. It can be seen that the proportion of protein in the total biomass decreased, and the ratio of C to N increased during the course of the experiment. Electron microscopy *(Figure 1a–c)* revealed the presence of the distinctive inclusions previously identified as PHB.[5] When the PHB content was estimated from the increase in carbon content and then subtracted from the total biomass of the cells, the protein content of the cells was found to remain constant at about 45% to 50% (not shown). This value is in good agreement with protein data previously obtained from steady-state cultures that were not accumulating PHB (protein content 50%).[5]

Two subsequent series (labeled 2 and 3 in *Table 1*) of short-term batch culture experiments (lasting from 5 to 35 min) were carried out during which the PHB content was measured directly. Acetate disappeared from the culture medium at a rate of about 1,100 to 1,200 nmol min^{-1} mg $protein^{-1}$. Using Equation (2) (see Materials and Methods), the PHB content was also calculated for series 1. In *Figure 2*, the percentages (wt/wt^{-1}) of PHB in the biomass are plotted against the time at which the sample was taken. It is clear that PHB production started immediately. A maximum PHB content of 42% (wt/wt^{-1}) of the biomass was found during the experiments with 10 mM acetate *(Figure 2)*.

Acetate-dependent oxygen uptake experiments

To examine the stoichiometry of substrate-dependent oxygen uptake by *T. pantotropha*, cells were taken from aerobic or anaerobic acetate-limited chemostat cultures. Pulses of acetate (10–40 μM) were given to *T. pantotropha* in an oxygen uptake chamber equipped with an oxygen electrode (biological oxygen monitor). Only 29% of the acetate was oxidized, as calculated from total oxygen uptake. Similar aerobic cultures of *A. calcoaceticus* gave 80% of the expected values derived from the stoichiometry of acetate oxidation to carbon dioxide and water *(Figure 3)*. The addition of dinitrophenol to the *T. pantotropha* cells to uncouple energy generation and biosynthesis did not result in increased oxygen uptake. It therefore appeared that not all of the added organic substrate was oxidized by this organism. That the problem was related only to organic carbon metabolism and not to the overall physiologic status of the cell was revealed using mixotrophically grown (simultaneously thiosulphate–acetate-limited growth in the chemostat) *T. pantotropha*. Although these mixotrophic cells showed the same amount of acetate oxidation as acetate-grown cells when provided with acetate, the oxidation of thiosulphate was nearly total (results not shown).

Similar observations were reported by Taylor and Hoare[18] with *Thiobacillus* A2 (now *Thiobacillus versutus*). This organism also took up 25–28% of the oxygen expected for the oxidation of acetate. It appeared that the acetate was not completely oxidized. Despite an apparent increase in the optical density, they observed that the protein content of the cultures was relatively unchanged. It was concluded that the levels of PHB in the cells were increasing as the cultures entered the stationary phase.

Estimations of acetate metabolism during rapid PHB formation

The cells were pregrown at 0.1 h^{-1} in the chemostat. From the series 1 and 3 data *(Table 1)*, it was calculated that the immediate growth rate in the batches was 0.2 h^{-1}. Hence, the growth rate shifted two-fold in the presence of excess acetate. During acetate-limiting growth at 0.1 h^{-1}, a minimum acetate uptake rate of 303 nmol min^{-1} mg $protein^{-1}$ was necessary. This value should be at least twice as high in the batch cultures because of the two-fold increase of the growth rate. However, a maximum acetate uptake rate of 1,100 to 1,200 nmol min^{-1} mg $protein^{-1}$ was found. The total acetate flow within the cells is the sum of three processes: oxidation with oxygen, synthesis of hydroxybutyrate, and assimilation. The flow of acetate to hydroxybutyrate synthesis is divided into substrate and NAD(P)H pro-

Table 1 Poly-β-hydroxybutyrate (PHB) formation by *Thiosphaera pantotropha* during the early stages of batch growth

Series	Time (min)	Protein (mg l^{-1})	PHB (mg l^{-1})	Total biomass (mg l^{-1})	Protein %(wt/wt^{-1})	C	H	O	N
1	0	14.9	(0.0)	29.1	51	1.0	1.81	0.54	0.26
	30	15.6	(14.5)	41.8	37	1.5	2.66	0.84	0.26
	60	19.6	(22.2)	60.5	32	1.6	2.65		0.26
	90	23.9	(33.1)	79.1	30	1.7	2.91	0.87	0.26
2	0	125	9.0	285	45				
	5	118	14.2	297	40				
	10	133	37.9	334	40				
3	0	131	0.1	262	50				
	10	123	32.8	281	44				
	15	151	74.5	377	40				
	25	147	98.1	392	38				
	35	143	103.2	389	37				

The total biomass was calculated from total organic carbon measurements and element analyses. The PHB was determined directly, except those given between brackets. Protein was calculated as the percentage of the total biomass

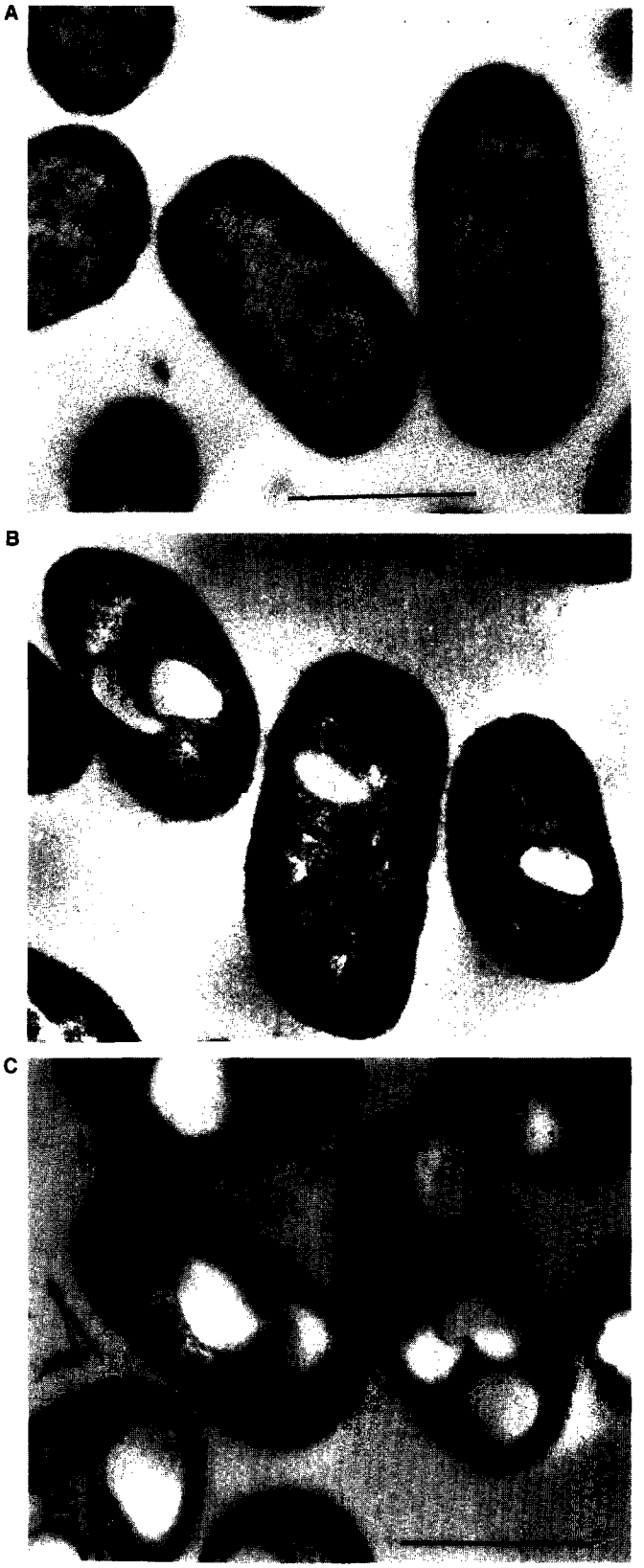

Figure 1 Thin sections of *Thiosphaera pantotropha* cells taken from batch cultures after: 1) 0 min; b) 10 min; c) 90 min. The white oval spheres indicate the presence of poly-β-hydroxybutyrate (bar represents 0.5 μm). Growth conditions: 10 mM acetate; 10 mM nitrate; 80% air saturation, pH 8.0

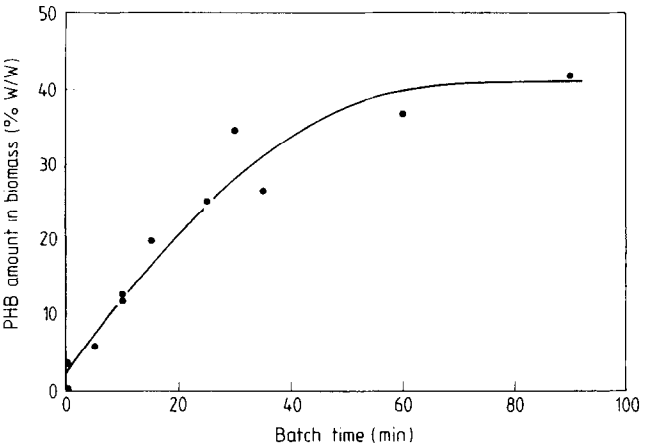

Figure 2 The amount of poly-β-hydroxybutyrate (PHB) formed by *Thiosphaera pantotropha* during the presence of excess acetate, expressed as a percentage of biomass (wt/wt^{-1}), plotted against time. The initial rate of PHB formation was calculated from the first 25 min. Batch growth conditions: 10 mM acetate; 10 mM nitrate; 80% air saturation, pH 8.0

duction (via citric acid cycle) flows. The maximum specific hydroxybutyrate production rate was 314 nmol min^{-1} mg protein^{-1} (628 nmol acetate min^{-1} mg protein^{-1}), as calculated from the data of series 2 and 3 during the first 25 min. The biomass production rate was about 57 mg l^{-1} h^{-1}. Of the total acetate uptake, 29% was oxidized using oxygen as the terminal electron acceptor *(Figure 3)*. From these figures, the relative flows of acetate to the different processes could be calculated *(Table 2)*. Thus, of the total acetate flow, 57% went into the PHB synthesis and 14% was used for biomass production. The sum of the total acetate flow within the cells as calculated from the figures in *Table 2* was 1,240 nmol min^{-1} mg protein^{-1}, which is comparable to the measured rate of acetate uptake by the cells (1,100–1,200 nmol min^{-1} mg protein^{-1}).

Table 2 also shows the rate of ATP production necessary

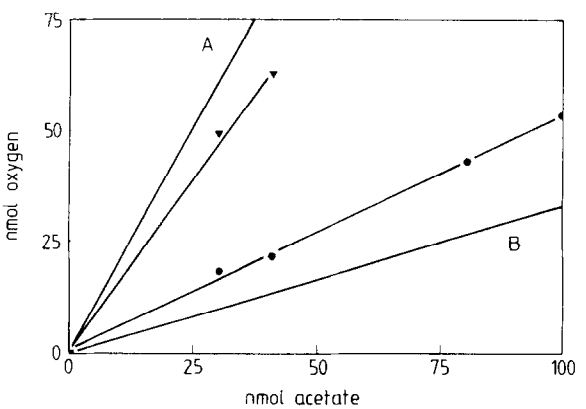

Figure 3 Total oxygen uptake by cell suspensions in the biological oxygen monitor by *Acinetobacter calcoaceticus* (closed triangles) and *Thiosphaera pantotropha* (closed circles). Line (A) represents the oxygen required for the complete conversion of acetate to CO_2 and H_2O. Line (B) represents the oxygen required for the maximum conversion of acetate to β-hydroxybutyrate

Table 2 Calculated values of the acetate flows and adenosine triphosphate (ATP) requirements in *Thiosphaera pantotropha* when provided with excess acetate

nmol Acetate min^{-1} $mg\ protein^{-1}$				nmol ATP min^{-1} $mg\ protein^{-1}$		
HB synthesis					Total ATP production rate	
HB	NAD(P)H	Assimilation for biomass	Oxidation by O_2	Total ATP required	P:O = 1	P:O = 2
628	79	172	360	3,050–3,170	2,240	3,320

HB, β-hydroxybutyrate. Total ATP required = the ATP necessary for active transport and activation of acetate plus the ATP for biomass formation

for transport and activation of the acetate that was used for PHB and biomass production. In bacteria, transport of one acetate molecule requires 0.33 to one ATP equivalent (H. W. van Veen, personal communication). Activation requires one ATP per acetate molecule.[16] The biomass production was calculated using a range of 50% to 100% of the theoretical value of Y_{ATP} on acetate (10 g biomass mol ATP^{-1}),[16] because the experimental Y_{ATP}^{max} is always smaller than the theoretical.[17] Indeed, with a P:O ratio of approximately 2, and with a maximum acetate oxidation rate for *T. pantotropha* of about 360 nmol min^{-1} mg $protein^{-1}$, the oxidation of acetate is sufficient to provide the cell with enough ATP to allow both the high PHB production rate and the biomass production rate *(Table 2)*.

The high value found for the acetate uptake rate is in the order required at maximum growth rate if it is assumed that the yield does not change ($\mu_{max} = 0.35\ h^{-1}$, acetate uptake rate 1,060 nmol min^{-1} mg $protein^{-1}$). This means that *T. pantotropha* has a high overcapacity for acetate uptake and metabolism at low growth rates during acetate-limiting growth. The maximum oxidation rate for acetate was 360 nmol min^{-1} mg $protein^{-1}$ *(Table 2)*, which allowed *T. pantotropha* to grow maximally at a growth rate of $0.2\ h^{-1}$. Thus, it can be suggested that the respiration rate was restricted, resulting in a rapid turnover of acetate into PHB. This supports the theory of the existence of a bottleneck in the cytochrome chain of *T. pantotropha*.[5]

Conclusion

From these results, it can be concluded that when confronted with a sudden excess of acetate, *T. pantotropha* is capable of rapid acetate uptake and immediate and rapid PHB synthesis. The rate of PHB formation (27 μg PHB min^{-1} mg $protein^{-1}$) is about five to 20 times higher than those found for organisms such as *Azotobacter beijerinckii*,[19,20] *Azotobacter vinelandii*,[1] *Hydrogenomonas* H16,[21] and *Thiobacillus* S.[2] This tendency to form PHB rapidly may explain why mass spectrometry experiments involving acetate pulses and $^{15}NO_3^-$ failed to demonstrate aerobic dentrification by *T. pantotropha* (E. W. J. van Niel, L. A. Robertson and R. P. Cox, unpublished results). Steady-state chemostat experiments using a mutant of *T. pantotropha* were more successful.[6]

It has already been suggested that aerobic denitrifiers may be favored in situations where the oxygen supply fluctuates. Rapid PHB formation as described here would be of similar value in an environment where the substrate supplies also fluctuate. The results reported here, when considered together with data already available on *T. pantotropha*,[5,8,22] provide a picture of a versatile organism well adapted to life in a dynamic environment.

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