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ACETATE METABOLISME IN THIOBACILLUS VERSUTUS

Proefschrift

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Stellingen behorend bij het proefschrift "ACETATE METABOLISM IN THIOBACILLUS VERSUTUS" van ir. P.A.M. Claassen

1. Aan de opmerking van Anthony welke luidt: "It is worth noting that the field of metabolic regulation is one in which complex and imaginative theories are relatively easier to produce than facts", wordt te weinig aandacht geschonken.
C. Anthony (1982) The Biochemistry of Methylotrophs, Academic Press London, p. 134

2. De bewering dat Thiobacillus versutus glutaraat niet als koolstofbron kan gebruiken is onjuist.
B.F. Taylor and D.F. Hoare (1969). J. Bact. 100:487-497.
Dit proefschrift.

3. Het is betreurenswaardig dat Gottschal en Kuenen zich beperkt hebben tot een methode voor de bepaling van 2-ketoglutaraat dehydrogenase activiteit in Thiobacillus versutus.
J.C. Gottschal en J.G. Kuenen (1980). Arch. Microbiol. 126:33-42

4. Door Kargi en Robinson wordt onterecht gesuggereerd dat tijdens "bioleaching" de samenhang tussen deeltjesgrootte en oxidatiesnelheid alleen door de afmeting van het beschikbare oppervlak bepaald wordt.
F. Kargi and J.M. Robinson (1984). Biotechn. and Bioengineering, 16: 41-49.

5. Van de Dijk en Troelstra leveren met hun methode geen enkel bewijs voor het optreden van heterotrofe nitrificatie in een zure heidebodem.
F.J. van de Dijk en F.R. Troelstra (1980). Plant and Soil 57: 11-21

6. De tendens wetenschappelijk onderzoek "maatschappelijk relevant" te maken dient vaak noch de maatschappij noch de wetenschap.

7. De functionaliteit van gebouwen zou in belangrijke mate toenemen indien de ontwerpers ervan verplicht zouden worden intern te verkeren.

8. De bepaling van lipide-gebonden-N-acetylneuraminezuur door oxydatie met perjodaat gevolgd door derivatisering met thiobarbituurzuur vormt geen betrouwbare methode om in serummonsters de aanwezigheid van een tumor vast te stellen.
L. Warren (1959). J. Biol. Chem. 234:1971-1975

9. De term grensverleggend onderzoek wordt te vaak gebruikt om probleemverschuivend onderzoek te beschrijven.

Voorwoord

Het op schrift stellen van al deze wetenschappelijke wetenswaardigheden en hersenspinsels heeft mijn fantasie uitgeput. Gelukkig resteert alleen nog een concrete zaak: het Voorwoord.
Allereerst wil ik alle leden van de Vakgroep Microbiologie bedanken voor het aangenaam verpozen in hun midden gedurende de afgelopen jaren.
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- Gerard Kortstee, zonder jouw ideeën was dit werk nooit geëntameerd
- Wilma van Vliet, misschien was ik wel harde en zachte sporen gaan kweken zonder jouw assistentie
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- Wim Harder en Hans van Dijken, bedankt voor jullie kritische opmerkingen tijdens onze ellenlange bijeenkomsten
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- Marcel van den Heuvel, die ELISA was natuurlijk wel een gepriegel, maar ook daarin liep onze samenwerking gesmeerd
- Ans Broersma, bedankt voor je eindeloze typewerk en voor het samenstellen van dit proefschrift

Kortom, zo'n proefschrift als dit is vaak niet het werk van een persoon en wil alleen lukken als de juiste voedingsbodem ervoor aanwezig is. Nou dat was zo; ik heb een fijne tijd gehad, veel geleerd en wil daarvoor iedereen bedanken.

Pieternel

Abstract

Thiobacillus versutus was chosen as a modelorganism to study the metabolism of acetate in isocitrate lyase negative organisms.
The potential operation of the tricarboxylic acid cycle was established after investigation of the oxidation of acetate by chemolithoautotrophically and heterotrophically grown cells and by measuring the enzyme activities involved in this oxidative cycle. Isocitrate lyase activity, the first enzyme of the glyoxylate cycle, was never observed in cells grown under aerobic conditions whereas malate synthase, catalyzing the second step in this assimilative cycle, was always highest in acetate-grown cells.
However, when T.versutus was grown on acetate under denitrifying conditions, isocitrate lyase activity was induced after a certain lag phase during which denitrifying growth took place without the presence of this enzyme. This isocitrate lyase activity was directly linked to the metabolism of acetate during denitrification. Following transition to aerobic growth on this substrate, the activity was actively repressed.
Malate synthase activity during denitrifying growth was again highest in acetate-grown cells and the amount was independent of the terminal electron acceptor present, indicating a physiological role of glyoxylate during denitrifying as well as during aerobic growth.
The authenticity of the observed isocitrate lyase activity was investigated and appeared to be comparable to isocitrate lyases from other organisms.
Although it was found during this study that T.versutus possesses the capacity to synthesize an authentic isocitrate lyase, the reason why this is linked to denitrifying growth and the way acetate is assimilated during aerobic growth remain to be solved.

C O N T E N T S

C H A P T E R 1

Introduction

Introduction

The title of this thesis "Acetate metabolism in Thiobacillus versutus" may appear to announce old news. However, in previous studies (Peeters et al. 1970; Gottschal and Kuenen, 1980) it has been demonstrated that during heterotrophic growth on acetate T.versutus lacks two crucial enzyme activities, namely 2-oxoglutarate dehydrogenase and isocitrate lyase. The former enzyme is required for the operation of the tricarboxylic acid cycle during heterotrophic growth (Fig. 1). Isocitrate lyase, observed only in plants and microorganisms (Lehninger, 1975), is the first enzyme of the glyoxylate cycle. This cycle is used as an anaplerotic route to the tricarboxylic acid cycle during growth on substrates metabolized directly via acetyl-CoA such as acetate, ethanol or 3-hydroxybutyrate. Despite the reported absence of 2-oxoglutarate dehydrogenase and isocitrate lyase, T.versutus grows well on acetate or other organic substrates. In view of the apparent nonfunctionality of the tricarboxylic acid and glyoxylate cycles, Gottschal and Kuenen (1980) suggested the existence of an alternative metabolic route possibly related to another, still obscure, isocitrate lyase negative pathway (see below). Based on this suggestion, this study has been undertaken in order to elucidate the metabolism of organic substrates in T.versutus with special emphasis on the intermediary metabolism of acetate.
Since control of culture conditions is of prime importance in physiological research, all studies were performed with cultures derived from a chemostat. However, as described in Chapter 2, prolonged continuous cultivation in a chemostat may disturb the uniformity of the culture.
In Chapter 3 the enzymatic profiles of T.versutus after chemolithoautotrophic and heterotrophic growth are presented. For comparative purposes a common heterotrophic organism, Pseudomonas fluorescens, has been included in this study. Subsequently the metabolism of acetate in T.versutus was studied in vivo using ^{13}C NMR spectroscopy (Chapter 4). In order to circumvent technical problems resulting from aeration in the NMR tube, cells were adapted to denitrification assuming that under denitrifying conditions the specific features of aerobic metabolism would be preserved. This assumption appeared to be incorrect as during denitrifying growth on acetate isocitrate lyase activity was observed in T.versutus (Chapter 5 and 6). Chapter 7 gives a summary of the most relevant data produced during this study.

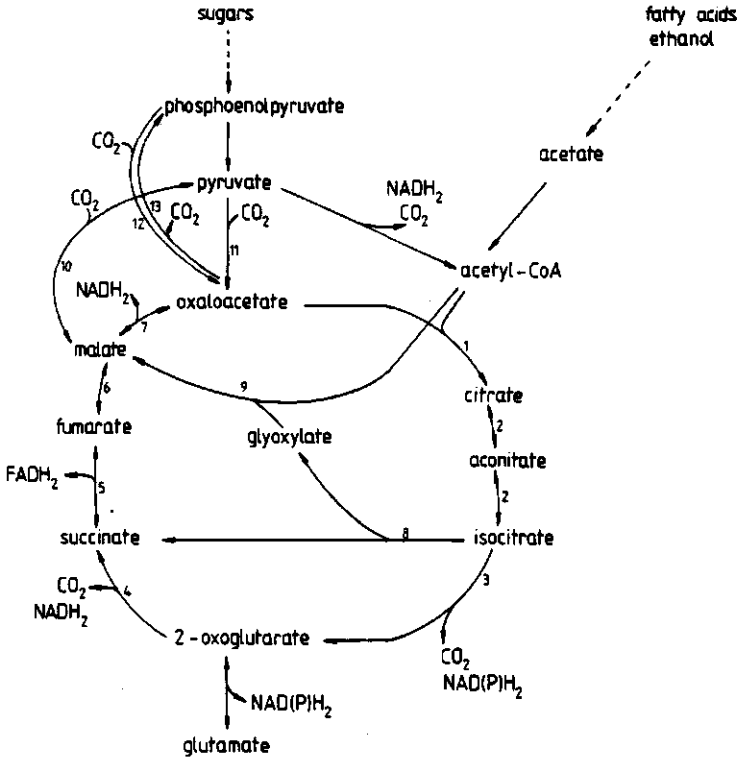

Fig. 1. Tricarboxylic acid and glyoxylate cycles. 1: citrate synthase, 2: aconitase, 3: isocitrate dehydrogenase, 4: 2-oxoglutarate dehydrogenase, 5: succinate dehydrogenase, 6: fumarase, 7: malate dehydrogenase. Anaplerotic enzymes; 8: isocitrate lyase, 9: malate synthase. 10: malic enzyme, 11: pyruvate carboxylase, 12: phosphoenolpyruvate carboxylase, carboxykinase, 13: phosphoenolpyruvate carboxytransphosphorylase.

Thiobacillus versutus

Organism

Thiobacillus versutus, formerly called Thiobacillus A2 (ATCC 25364) (Harrison, 1983) has been isolated by Taylor and Hoare (1969) during enrichment for anaerobic thiobacilli. The organism is gram-negative, coccobacillary in shape, non-sporulating and mesophilic. Data concerning the motility of T.versutus are contradictory. Taylor and Hoare (1969) describe the organism as being nonmotile while by Korhonen et al. (1978) flagella have been observed.
T.versutus is referred to as a facultative chemolithoautotroph because of its capacity to obtain metabolic energy from thiosulphate while using CO_2 as carbon source, or to grow methylotrophicallyl) or heterotrophically on a great variety of organic compounds (Taylor and Hoare, 1969; Wood and Kelly, 1977). Mixotrophic growth occurs when thiosulphate is added to acetate- or glucose-limited chemostat cultures (Gottschal and Kuenen, 1980; Gottschal et al., 1981; Smith et al., 1980). Under anaerobic conditions T.versutus can grow by nitrate respiration at the expense of heterotrophic or methylotrophic substrates (Taylor and Hoare, 1969; Peeters et al. 1970; Wood and Kelly, 1983). Robertson and Kuenen (1983) report chemolithoautotrophic growth under denitrifying conditions. In contrast, Taylor and Hoare (1969) and Wood and Kelly (1983b) have been unable to grow T.versutus on thiosulphate using nitrate as terminal electron acceptor.

Chemolithoautotrophic and methylotrophic metabolism

Thiosulphate is the only sulphur compound to support chemolithoautotrophic growth of T.versutus, even though cells grown on thiosulphate oxidize sulphur, sulphite and sulphide. Tetrathionate and thiocyanate are not oxidized (Taylor and Hoare, 1969). Methylotrophic growth has been observed on formate (Taylor and Hoare, 1969; Kelly et al. 1979), methylamine (Van Dijken and Kuenen, 1981; Haywood et al. 1982) but not on dimethyl- and trimethylamine (Van Dijken and Kuenen, 1981). Reports on growth on methanol are contradictory. In the original description of this organism (Taylor and Hoare, 1969) fast growth on methanol is reported. Subsequently it has been found impossible to grow T.versutus on methanol (Van Dijken and Kuenen, 1981) while Kelly and Wood (1982) observe slow growth (doubling times between 80 and 200 h).
In T.versutus the difference between chemolithoautotrophic and methylo-trophic metabolism is determined by the identity of the energy source. During chemolithoautotrophic growth, metabolic energy is derived from the oxidation of thiosulphate to sulphuric acid while during methylotrophic growth a C_1 substrate serves for this purpose. In both cases energy conservation is effected by electron transport phosphorylation. Oxidation of thiosulphate is mediated by a multi-enzyme complex and all generated electrons are transferred to cytochrome c (Lu et al., 1985). During methylotrophic growth, electrons may be donated to the electron transport chain employing NADH during growth on formate (Taylor and Hoare, 1969; Kelly et al., 1979) or a blue copper protein amicyanin as suggested for growth of T.versutus on methylamine (Haywood et al., 1982; Van Houwelingen et al., 1985). During chemolithoautotrophic as well as during methylotrophic

[1] The classification of Anthony (1982) defining growth on C_1 compounds as methylotrophic irrespective of the way C_1 units are assimilated, has been followed in this thesis.

Table 1. Specific activities of ribulose 1,5-biphosphate carboxylase in Thiobacillus versutus.

Growth substrate(s)	Specific activity	References
Thiosulphate + CO_2	75 nmol/min.mg protein	Gottschal and Kuenen (1980)
Thiosulphate + CO_2	50-70 " " "	Kelly et al. (1979)
Formate	60-115 " " "	" " "
Methanol	3.6 ± 0.6 nmol/min.mg dry wt	Kelly and Wood (1982)
Acetate	0.2 nmol/min.mg protein	Gottschal and Kuenen (1980)
Acetate	1 " " "	Kelly et al. (1979)
Fructose	1 " " "	" " "

growth, carbon is fixed as CO_2 by the Calvin cycle as evident from the increased specific activity of ribulose 1,5-biphosphate carboxylase (Table 1).
Data on the intermediary metabolism of T.versutus are less equivocal. It is now generally assumed that in many organisms the tricarboxylic acid cycle merely serves to supply biosynthetic precursors during autotrophic growth (Smith and Hoare, 1977; Kelly, 1971; Whittenbury and Kelly, 1977). Therefore, the specific activities of the tricarboxylic acid cycle enzymes are low and the cycle is often incomplete by lack of 2-oxoglutarate dehydrogenase. Carbon from the Calvin cycle is converted by the C_6 branch of this noncyclic pathway to glutamate and around the C_4 branch to succinate and their respective derivatives. The data on chemolithoautotrophic growth of T.versutus presented by Peeters et al. (1970) (Table 2) fit this noncyclic horse shoe pathway. Taylor and Hoare (1971) on the other hand report a low but considerable activity of 2-oxoglutarate dehydrogenase (Table 2). The presence of this enzyme is supported by the observation that autotrophically grown cells of T.versutus possess the capacity to oxidize organic substrates (Gottschal et al., 1981; Taylor and Hoare, 1969). Moreover, citrate synthase of T.versutus is inhibited by NADH instead of 2-oxoglutarate (Taylor, 1970). The reverse is usually found in organisms having an incomplete tricarboxylic acid cycle (Doelle, 1975). The operation of a cyclic pathway is further indicated by the observed incorporation of ^{14}C in aspartate and leucine after incubation of autotrophically grown cells with [^{14}C]-acetate (Kelly et al., 1979). However, a cyclic route can be formed not only via 2-oxoglutarate dehydrogenase but also via isocitrate lyase (Fig. 1). Unfortunately, analogous to the ambivalent reports on 2-oxoglutarate dehydrogenase, data on isocitrate lyase activity are also contradictory. Peeters et al. (1970) have shown both isocitrate lyase and malate synthase activity in autotrophically grown cells of T.versutus. The lack of isocitrate lyase in acetate-grown cells, reported by Gottschal and Kuenen (1980), renders the operation of the glyoxylate cycle however very unlikely.

Table 2. Specific enzyme activities in cell-free extracts of Thiobacillus versutus after chemolithoautotrophic growth.

Enzyme	Specific activity in nmol/min.mg protein		
	Peeters et al.(1970)	Taylor and Hoare(1971)	Gottschal and Kuenen(19
citrate synthase	23.2	35	N.D.[1)]
aconitase	35.4	29	N.D.
isocitrate dehydrogenase	34.8	177	325
2-oxoglutarate dehydrogenase	0	18	N.D.
succinyl-CoA synthase	0	N.D.	N.D.
succinate dehydrogenase	7.5	1670[2)]	N.D.
fumarase	195.0	807	N.D.
malate dehydrogenase	213.0	2590	239
isocitrate lyase	16.2	N.D.	1
malate synthase	10.3	N.D.	N.D.

1) N.D. is not determined
2) Determined in the particulate fraction.

Heterotrophic metabolism

Among the facultative thiobacilli, T.versutus possesses the largest heterotrophic growth potential. Heterotrophic growth is supported by amino acids, alcohols, organic acids and sugars (Taylor and Hoare, 1969; Peeters et al. 1970; Wood and Kelly, 1977, 1979, 1980, 1983a, 1983b; Wood et al., 1977; Gottschal and Kuenen, 1980; Smith et al. 1980). The metabolism during growth on sugars and organic acids has been most extensively studied. A striking feature of T.versutus during growth on glucose is the simultaneous utilization of the Embden-Meyerhof, the Entner-Doudoroff and the oxidative pentose phosphate routes. The relative ratio of the three pathways can vary with different growth conditions (Wood et al., 1977; Wood and Kelly, 1979; Smith and al. 1980), but gives growth yields indistinguishable within reasonable limits of experimental error (Wood and Kelly, 1979).
Peeters et al. (1970) and Gottschal and Kuenen (1980) have investigated the specific enzyme activities of the intermediary metabolism in T.versutus (Table 3). An important discrepancy in their data concerns the specific activities of 2-oxoglutarate dehydrogenase and isocitrate lyase. Gottschal and Kuenen (1980) report the absence of 2-oxoglutarate dehydrogenase and isocitrate lyase activity during growth on acetate. Accordingly, acetate cannot be metabolized via the tricarboxylic acid and glyoxylate cycles. However, 2-oxoglutarate dehydrogenase activity is observed by Peeters et al. (1970) in glutamate- and succinate-grown cells and seems sufficient to support the operation of the tricarboxylic acid cycle. A cyclic pathway during heterotrophic growth is also indicated by the observation that after incubation of acetate-grown cells with [^{14}C]-acetate, label is recovered in all amino acids investigated without a preferential labelling of glutamate and its derivatives as found in autotrophically grown cells (Kelly et al. 1979). However, when the same experiment is repeated with succinate-grown cells the distribution of label is similar to that observed in autotrophically grown cells (Kelly et al., 1979). With the exception of aspartate, the same is true of sucrose-grown cells (Whittenbury and Kelly,

The occurrence of isocitrate lyase activity in glutamate- and succinate-grown cells is very remarkable since in contrast to acetate, these substrates are not metabolized via the glyoxylate cycle. The observed activities are rather low and may have been misinterpreted because the spectrophotometric assay used was rather unspecific, giving positive results with any oxoacid e.g. 2-oxoglutarate which could be formed from isocitrate by isocitrate dehydrogenase in the presence of NAD(P)H (Quayle, 1975; Attwood and Harder, 1977).

Table 3. Specific enzyme activities in cell-free extract of Thiobacillus versutus after heterotrophic growth.

	Specific activity in nmol/min.mg protein Gottschal and Kuenen (1980) Peeters et al. (1970)		
	Growth substrate		
	acetate	succinate	glutamate
Enzyme			
citrate synthase	N.D.[1)]	6.0	10.2
aconitase	N.D.	21.5	N.D.
isocitrate dehydrogenase	751	258	140.8
2-oxoglutarate dehydrogenase	0.26	10.6	95.1
succinyl-CoA synthase	N.D.	267	N.D.
succinate dehydrogenase	N.D.	8.7[2)]	6.1[2)]
fumarase	N.D.	211	174
malate dehydrogenase	481	196.3	85.5
isocitrate lyase	1	9.7	17.3
malate synthase	N.D.	13.2	N.D.

1) N.D. is not determined
2) Determined in the particulate fraction

Mixotrophic metabolism

Addition of thiosulphate to chemostat cultures limited by glucose (Smith et al.,1979) or by acetate (Gottschal and Kuenen, 1980) results in mixotrophic growth, i.e. simultaneous utilization of inorganic and organic substrates as carbon and energy source. Additive growth yields (Smith et al., 1979) or growth yields up to 30% higher than expected as the sum of the separate yields (Gottschal and Kuenen, 1980) have been obtained during dual limitations. The thiosulphate oxidizing capacity, fully repressed during heterotrophic growth, is derepressed by inclusion of thiosulphate in the medium to an extent depending on the ratio acetate versus thiosulphate. In the same way an increase in the maximum acetate oxidation potential with an increase in acetate concentration in the medium was observed. During chemolithoautotrophic growth, acetate could however still be respired (Gottschal and Kuenen, 1980). Gottschal and Kuenen (1980) have determined a number of relevant enzyme activities of T.versutus grown in chemostat cultures on either thiosulphate, on acetate or on varying ratios of both substrates (Table 4).
They claim that the tricarboxylic acid and glyoxylate cycles cannot be operative in acetate-grown T.versutus since 2-oxoglutarate dehydrogenase and isocitrate lyase are never observed and since the other investigated tricarboxylic acid

cycle enzymes do not increase significantly in switching from chemolithoautotrophic to heterotrophic metabolism.
In conclusion: At this stage the data concerning the intermediary metabolism of T.versutus during heterotrophic growth are highly confusing. The operation of the tricarboxylic acid cycle is denied by Gottschal and Kuenen (1980) but conceivable when the results of Peeters et al. (1970) are taken into account. Isocitrate lyase activity is not observed in acetate-grown cells (Gottschal and Kuenen, 1980) even though Peeters et al. (1970) detect specific activities of 10 and 17 nmol/min.mg protein after growth on succinate and glutamate, being substrates not metabolized via the glyoxylate cycle.

Table 4. Specific enzyme activities in cell-free extract of Thiobacillus versutus after growth on varying ratios of thiosulphate and acetate. Data are from Gottschal and Kuenen (1980).

	Specific enzyme activity in nmol/min.mg protein				
Growth substrates					
Acetate (mM)	0	2	10	15	20
Thiosulphate (mM)	40	36	20	10	0
Enzyme					
isocitrate dehydrogenase	325	279	318	286	751
malate dehydrogenase	239	389	310	243	481
2-oxoglutarate dehydrogenase	N.D. [1)]	0	N.D.	0	0.26
isocitrate lyase	1	1	1	1	1
sulphite oxidase	298	290	237	312	245
ribulose biphosphate carboxylase	75	63.2	3.1	0.2	0.2

1) N.D. is not determined

Isocitrate lyase negative growth

The long standing problem of isocitrate lyase negative growth has been first encountered in organisms using the serine pathway during methylotrophic growth. Data concerning this phenomenon are excellently reviewed in the recent book by Anthony (1982) of which a short anthology is presented below.

The serine patwhay is the third of the known pathways operating in bacteria during methylotrophic growth. It differs from the ribulose biphosphate and ribulose monophosphate patwhays in the nature of its intermediates which are amino and carboxylic acids instead of carbohydrates. There are two known variants of the serine pathway, i.e. an isocitrate lyase positive (Fig. 2) and an isocitrate lyase negative variant. In bacteria having the isocitrate lyase negative variant the reactions involved in the oxidation of acetyl-CoA to glyoxylate are obscure since all lack isocitrate lyase activity. In most of these organisms also no malate thiokinase and hence no "ATP malate lyase" activity can be measured. A relation between the function of these enzymes has been suggested as a mutant of Pseudomonas MS (wild type is isocitrate lyase positive) which is unable to grow on methylamine and acetate had lost both "ATP malate lyase" and isocitrate lyase activity. For all other reactions of this pathway leading to the formation of oxaloacetate from glyoxylate, formaldehyde and CO_2, extensive evidence based on radioactive-labelling, mutant studies and enzyme analysis has been provided. In facultative methylotrophs able to express isocitrate lyase activity, the conversion of isocitrate to glyoxylate and succinate is essential as well during methylotrophic as during heterotrophic growth on C_2 substrates. It has been suggested that during these different growth modi one and the same enzyme is involved. In Pseudomonas MA, on the other hand , two different isocitrate lyases have been observed (Bellion and Woodson, 1975). However, among the facultative methylotrophs using the isocitrate lyase negative serine pathway all except Organism 5B1 (Colby and Zatman, 1972) fail to induce isocitrate lyase activity during growth on substrates assimilated exclusively by way of acetyl-CoA, e.g. acetate, ethanol or 3-hydroxybutyrate. Therefore such organisms cannot utilize the glyoxylate cycle during heterotrophic growth on C_2 compounds. There are strong indications based on mutant studies by Dunstan et al. (1972b) that some reactions involved in isocitrate lyase negative methylotrophic growth are common to the pathway used during growth on C_2 substrates. In a mutant of Pseudomonas AM1 (an isocitrate lyase negative organism) loss of the ability to grow on methanol was accompanied by failure to grow on ethanol or 3-hydroxybutyrate. Growth on succinate was not affected. Addition of supplements of glyoxylate or glycollate were sufficient to restore growth of this mutant on C_1 or C_2 compounds. After isotope studies with the wild type organism, Dunstan et al. (1972a) proposed a route involving hydroxylation of acetate (or a derivative) to glycollate, followed by oxidation to glyoxylate.

Another alternative route for the oxidation of acetly-CoA to glyoxylate in Pseudomonas AM1 or Pseudomonas 80 has been proposed by Kortstee (1980, 1981). In this pathway, the oxidation of acetyl-CoA is effected via a homoisocitrate cycle homologous to the isocitrate lyase route. Even though the evidence is not complete, this route or a modified version thereof, seems to offer an elegant solution to the problem of isocitrate lyase negative metabolism.

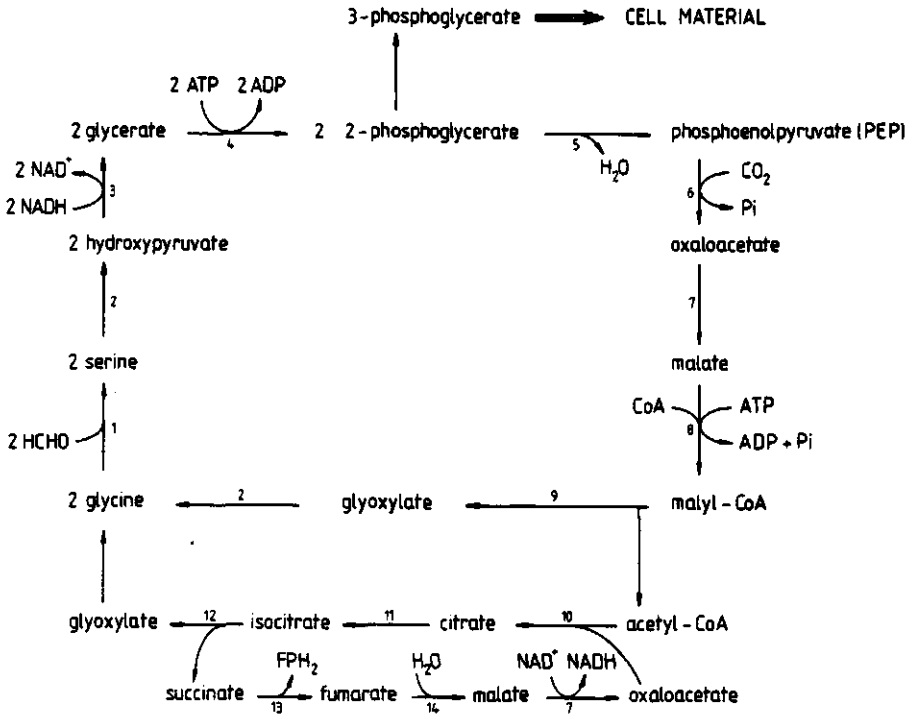

Fig. 2. The serine pathway of formaldehyde assimilation (ICL^+ variant). The ICL^- variant differs in lacking measurable malate thiokinase and isocitrate lyase. Precursors for biosynthesis can be removed at level of oxaloacetate or succinate. 1: serine transhydroxymethylase; 2: serine-glyoxylate aminotransferase; 3: hydroxypyruvate reductase; 4: glycerate kinase; 5: enolase; 6: PEP carboxylase; 7: malate dehydrogenase; 8: malate thiokinase; 9: malyl-CoA lyase; 10: citrate synthase; 11: aconitase; 12: isocitrate lyase; 13: succinate dehydrogenase; 14: fumarase (Anthony, 1982).

Aerobiosis versus denitrification

The capacity to utilize nitrate or other nitrogenous oxides as the terminal electron acceptor under anaerobic growth conditions is relatively wide-spread among prokaryotic organisms (Payne, 1973; Jeter and Ingraham, 1981; Whatley, 1982; Boogerd, 1984). This process is called denitrification, nitrate respiration or dissimilatory nitrate reduction. Bacteria reducing nitrogenous compounds to gaseous nitrogen compounds are classified as denitrifiers. In the complete denitrification process nitrate is reduced to dinitrogen; nitrite and nitrous oxide have been observed as intermediates but the information on nitric oxide is controversial. The same way as during reduction of oxygen, electrons donated by physiological electron donors such as NADH or succinate are transported by electron carriers located in the cell membrane. However, the identity and the localization of the redox carriers can differ dependent upon the electron acceptor present. It goes without say that among different species different redox carriers may be involved in the electron transport chain (Matsubara and Zumft, 1982). Even growth conditions can alter the content and the nature of some cytochromes in one and the same organism utilizing the same electron acceptor (Meyer et al., 1977).

Conservation of energy during nitrate respiration is effected by oxidative phosphorylation in a similar fashion as during reduction of oxygen. The efficiency of energy conversion during denitrification is generally lower, however, because of the involvement and orientation of different redox carriers. As usually observed, molar growth yields are lower too during denitrification as compared to aerobiosis. In this respect it should be noted that the lower energy conversion during denitrification cannot be applied to estimate differences in molar growth yields since only part of the substrate is used for energy production. The other part is used for biomass synthesis and for maintenance purposes. In chemostat cultures of Paracoccus denitrificans on succinate the molar growth yield during denitrification is 80% of that obtained under aerobic respiration. (Boogerd et al., 1984). This relatively high yield can tentatively be explained by a shift in the ratio of substrate dissimilated versus assimilated, favoring the amount used for dissimilation.

It is generally assumed that organisms lacking the capacity for fermentation maintain the specific aerobic features of metabolism during denitrification. The operation of the tricarboxylic acid cycle as an oxidative pathway has been shown by Forget and Pichinoty (1965) in Micrococcus denitrificans after aerobic and denitrifying growth. Pseudomonas denitrificans, on the other hand, cannot utilize ammonium ions as nitrogen source during denitrification, whereas fast growth is observed when the organism is incubated under air. Koike and Hattori (1975) suggest a block in amino acid biosynthesis in the absence of oxygen as the cause of lack of growth under nitrate respiration.

Less information is available on denitrifying growth of T.versutus. Taylor and Hoare (1969) report lack of chemolithoautotrophic growth on thiosulphate under denitrifying conditions. This is confirmed by Wood and Kelly (1983b) who have studied the capacity of T.versutus to oxidize thiosulphate under nitrate respiration in single substrate and mixotrophic cultures. The inability to oxidize thiosulphate anaerobically in the presence of nitrate is possibly due to the fact that electrons derived from thiosulphate enter the electron transport chain at the level of cytochrome c (Lu and Kelly, 1983 a and b; Lu et al., 1985) rendering the reduction of nitrate, presumably at a cytochrome b, impossible. However, contrasting results have been obtained by Robertson and Kuenen (1983). The controversy in the data from Taylor and Hoare (1969) and Wood and Kelly (1983b) on the one hand and Robertson and Kuenen (1983) on the other may be the result of strain differences at the level of the organization of the electron transport chain or even involving substrate-level phosphorylation dependent on adenylyl sulphate reductase.

Methylotrophic growth on formate under denitrifying conditions occurs concomitantly with a disappearance of nitrate. Molar growth yields on formate determined with nitrate or nitrous oxide are equal suggesting energetic equivalence of these electron acceptors as in P.denitrificans (Wood and Kelly, 1983b).

Denitrifying heterotrophic growth of T.versutus has been studied by Taylor and Hoare (1969), Peeters et al (1970), Robertson and Kuenen (1983) and most extensively by Wood and Kelly (1983b). Again equivalence of the electron acceptors nitrate and nitrous oxide during denitrifying growth on glucose has been observed (Wood and Kelly, 1983b). Molar growth yield on glucose under nitrate respiration has been compared to the yield obtained under aerobic respiration. A decrease to 60% of the aerobic yield was attributed to the difference in energy conservation during denitrification being about two thirds of the value obtained during aerobiosis. This explanation is incorrect since as mentioned above molar growth yield cannot be estimated from the energy yield. Wood and Kelly (1983b) observe a shift in the ratio of the utilization of the Embden Meyerhof (EM), Entner Doudoroff (ED) and pentose phosphate pathways during denitrifying growth on glucose as compared to aerobic growth. Substitution of oxygen by nitrate results in a shift in the relative contribution of these pathways to a predominance of the ED route (Wood and Kelly, 1983b). However, a similar predominance of the ED route has been observed in aerobic glucose-limited chemostat cultures of T.versutus by Smith et al. (1980). Furthermore in earlier papers Wood and Kelly (1979, 1980) discuss the difficulties in interpreting these complex radiorespirometric data and the fact that no clear pattern of response to environmental conditions could be determined. Taking these considerations into account the data concerning the shift in relative contributions of the metabolic pathways during denitrification lose ground. Peeters et al. (1970) have examined the levels of various enzymes involved in the intermediary metabolism of succinate during aerobic and denitrifying growth. In both cases a complete set of tricarboxylic acid and glyoxylate cycle enzymes has been found. The significant increase in specific activity of aconitase, succinate dehydrogenase, fumarase and malate synthase has been left undiscussed.

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C H A P T E R 2

Colonial heterogeneity of *Thiobacillus versutus*

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Abstract.

In acetate-limited chemostat cultures started with single colony cultures of Thiobacillus versutus, the appearance of a mutant was observed after approximately 85 volume changes. The inhomogeneity of the culture was detected by the development of two different types of colonies on agar plates. When a pure culture of the mutant was grown in a chemostat, parent colonies appeared after almost the same period of time. Electron micrographs of the mutant, grown on butyrate, showed the presence of fibrils surrounding the cells. The cells of the parent strain were bald when grown under the same conditions.The growth-kinetics of parent and mutant were investigated in batch cultures with a variety of substrates and were found to be identical. Major differences between the two strains were observed during growth on mannitol when the mutant attained a lower yield and excreted large amounts of extracellular polysaccharides.

Introduction

The loss of homogeneity of a bacterial culture during cultivation in a rigidly controlled environment is not an unkwnown phenomenon. Calcott (3) has recently summarized a number of studies in which parent-mutant transitions in chemostat cultures have been observed. In continuous cultures, at nutrient concentrations much lower than K_s, the outcome of the competition between parent and mutant depends on the $\mu max/K_s$ ratio. At higher substrate concentrations, the competition is always won by the organism with the lowest K_s, except for the situation in which the Monod curves cross over. In this situation, at substrate concentrations below the crossover point, K_s is decisive, but at higher concentrations the organism with the highest growth rate is selected (17). Physiological causes of parent-mutant systems have been investigated in a number of cases (3). Although some of these studies have led to an understanding of the observed phenomenon, in some transitions take-over by a mutant without obvious reasons has been noticed.

There are two possible ways to cope with the problem of the appearance of mutants in a pure culture. The first one is to maintain a chemostat culture only for a short period of time as suggested by Tempest (14), the second one is to wait until the most adapted organism has established itself (8).

Here we report the appearance of a mutant with a different colonial morphology in a pure chemostat culture of Thiobacillus versutus. To identify the nature of this parent-mutant transition, some growth-kinetic and physiological parameters of both strains have been investigated in chemostat and batch cultures.

Materials and methods

Organism, media and cultivation. Thiobacillus versutus, formerly called strain A2 (= ATCC 25364) (5) was a gift from Dr. J.G. Kuenen (Delft, The Netherlands). In the following this strain is called WS. The organism was maintained on thiosulphate agar slants, stored at 4° C and subcultured every two months. The basal medium for growth in batch culture was according to Taylor and Hoare (13), containing 2 ml instead of 5 ml trace element solution as described by Gottschal and Kuenen (4). For growth on methanol, formate, methylamine, ethanol, glutamate, 5-aminovalerate and organic acids, substrates were added at a concentration of 2.5 g/l of the sodium salt (except for methanol and ethanol). In the medium with methylamine, NH_4Cl was omitted. For growth in batch culture on mannitol, NH_4Cl was substituted by 1.1 g/l sodium glutamate. Mannitol, sterilized separately, was added to a final concentration of 5.0 g/l. Growth on

mannitol was also studied in a medium described by Zevenhuizen and van Neerven (20). Solid media were prepared by adding 15 g/l agar (Gibco, Europe Ltd, Paisley Scotland). The medium for chemostat cultures was according to Gottschal and Kuenen (4). Sodium acetate was added at a final concentration of 5.0 g/l. Cultures were grown at a dilution rate of 0.1 hr^{-1}. The pH was kept at 8.0 through automatic addition of 1 N HCl. Oxygen was supplied by sparging air through the culture at a rate of 1 litre air per litre culture per min. All cultures were grown at 30° C.

Colony transformation. Samples from the chemostat culture were regularly streaked onto agar plates with batch culture medium. Photographs were taken with a Wild M5 microscope with the enlargement set at 12 x. The mutant proportion was estimated by spreading 0.1 ml of a dilution series onto agar plates with the same substrate as used in the chemostat.

Electronmicroscopy. Butyrate grown cells were washed with 0.1 M cacodylate buffer, pH 7.2. Pellets were suspended in 1% (^{wt}/vol) purified agar (Gibco, Europe Ltd, Paisley Scotland) in cacodylate buffer at 45° C, followed by fixation in 2% (^{wt}/vol) paraformaldehyde and 2.5% (^{wt}/vol) glutaraldehyde in the same buffer. After fixation in 1% (^{wt}/vol) OsO_4, the specimens were dehydrated in graded ethanol and placed in propylene oxide. Dehydrated samples were embedded in Epon 812. Thin sections were stained with uranyl acetate (30 min at 40° C) and lead citrate (40 s at 20° C) using a LKB Bromma 2168 Ultrostainer.

Analytical methods. Growth in batch culture was followed by measuring the optical density of the bacterial suspension at 623 nm with a Vitatron Universal Photometer. Specific growth rates were calculated from the slope of the linear interval of the logarithmic growth curve. Dry weight of samples from the cultures was determined by washing the cell pellet from 50 ml culture twice with distilled water. The washed cell pellet was dried overnight at 105° C in predried and preweighed vials. Dissolved acetate in the chemostat culture was analyzed with a Varian gaschromatograph, equipped with a chromosorb 101, 80-100 mesh, column. The total carbohydrate content of batch grown cells, harvested through centrifugation, was determined with the anthrone-sulphuric acid reagent using glucose as a standard (15). The amount of poly-3-hydroxybutyric acid (PHB) was measured in 96% H_2SO_4 at 235 nm after extraction of acid hydrolyzed cells with chloroform (19). Extracellular sugars in the supernatant of centrifuged cultures were analyzed with the anthrone-sulphuric acid reagent (15) and the method of Somogyi-Nelson (10) for the determination of reducing end groups. Mannitol concentrations were spectrophotometrically quantified after oxidation with periodate followed by the chromotropic acid-formaldehyde reaction (2). For a qualitative determination mannitol was acetylated with acetic anhydride using 1-methylimidazole as the catalyst (1). The alditol acetate was extracted into dichloromethane and subsequently identified by gaschromatography using a Kipp Analytica 8200 gaschromatograph equipped with a WCOT fused Silica OV 225 capillary column, length 25 m, at 210° C. Identification was done by comparison with the derivative of a pure solution of mannitol.

Results

The original Thiobacillus versutus strain, in the following called WS, forms colonies which are best described as having a rather dense central region surrounded by a more or less irregularly shaped, spreading fringe (Fig. 1A). These colonies fit the description by Taylor and Hoare (13). After continuous cultivation of this strain in a chemostat for approximately three weeks (approx. 85 volume replacements) a novel type of colonies having smooth edges appeared besides the original when samples were streaked onto agar plates (Fig. 1B and 1C). Bacteria which form this type of colony have subsequently been called Thiobacillus versutus WH. Continuous cultivation of a pure culture of WH led to the appearance of WS resembling colonies. When observed through a light microscope the cells of each strain are indistinguishable: WS as well as WH cells are coccobacillary in shape. Electron micrographs of T.versutus WS and WH on the other hand demonstrate an important difference in cell surface (Fig. 2): WS cells are bald and WH cells are surrounded by fibrils.
The yield of T.versutus WS and WH in a chemostat culture on acetate was respectively 0.36 and 0.40 g/g acetate. Thus only a small difference could be observed. The ratio of the WH mutants in a WS culture or vice versa varied between 1 and 10 per 1000 cells after approx. 85 volume changes on acetate.

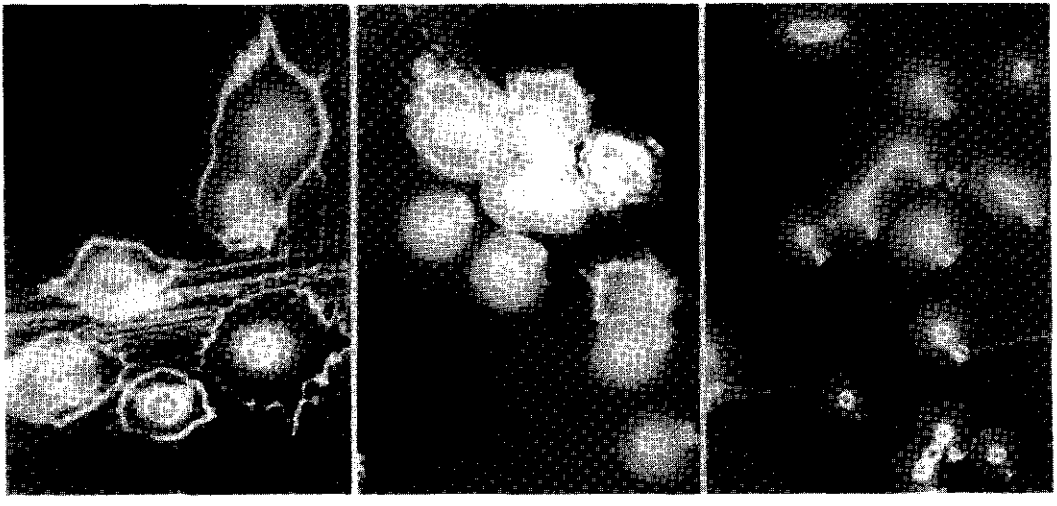

Fig. 1: Growth of T.versutus WS and WH on agar plates containing acetate as carbon and energy source. A: Sample from a pure batch culture of WS on acetate used to inoculate the chemostat. B: Sample from the chemostat culture on acetate after approx. 85 volume changes. Arrow: WH colonies. C: Sample from a pure batch culture of WH on acetate.

After six weeks in the chemostat (approx. 170 volume changes) this ratio did not change. Hence the observed mutant proportion seemed to have reached an equilibrium and was definitely higher than the ratio observed as a result of spontaneous mutations.
The versatility of T.versutus WS and WH with respect to growth potential was studied with a variety of substrates. Both strains grew in mineral media supplemented with the following substrates: thiosulphate, formate, methylamine, acetate, ethanol, glycolate, lactate, butyrate, 4-hydroxybutyrate, succinate, malate, 2-oxoglutarate,glutamate, glutaconate, adipate, 5-aminovalerate and mannitol. During growth on formate both strains formed clumps. Neither strain grew on methanol, not even after two weeks of incubation at 30° C. Growth on 4-hydroxybutyrate was characterized by an extremely long lag phase of 9 days. A selected number of maximum specific growth rates (μ max) of T.versutus WS and WH in batch culture were determined in media supplemented with the following

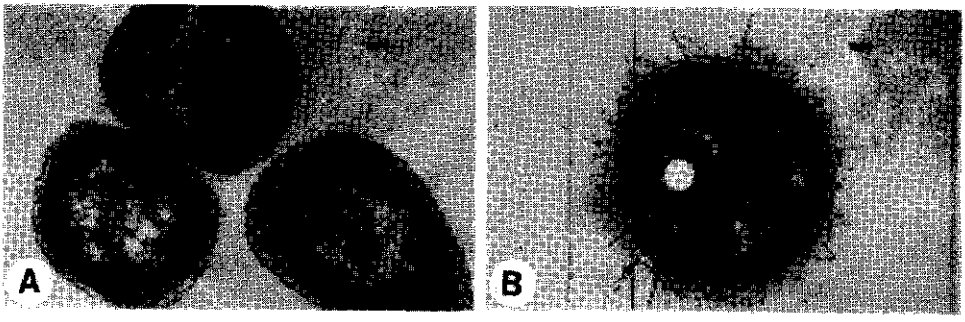

Fig. 2: Ultrathin sections of T.versutus grown on butyrate A: Strain WS with a bald cell surface. B: Strain WH with fibrils surrounding the cell wall. Bar indicates 0.1 μm.

substrates: thiosulphate, acetate, succinate, glutarate, glutamate and mannitol. The values for the μ max were virtually the same and ranged from 0.043 hr^{-1} (WS) and 0.046 hr^{-1} (WH) for growth on thiosulphate to 0.45 hr^{-1} (WS) and 0.44 hr^{-1} (WH) on succinate. Growth in basal medium supplemented with mannitol as carbon and energy source and glutamate as nitrogen source yielded a remarkable difference between the strains. The optical density at 623 nm of the stationary phase of WS cells was about 1.8 times higher than of WH cells. The values for μmax were, on the contrary, almost identical, 0.30 hr^{-1} (WS) and 0.32 hr^{-1} (WH). When T.versutus WS and WH were grown on mannitol and glutamate in a different medium, composed as described by Zevenhuizen and van Neerven (20), an even higher optical density was obtained by both strains. The prior observed difference in maximal optical density of WS and WH cultures was again noticed, together with the similarity in μmax: 0.29 hr^{-1} for WS and WH. The medium according to Zevenhuizen and van Neerven (20) contained less phosphate, more Ca and Zn but fewer Mn, Mo, Cu, Co and Fe ions than the medium used before. Furthermore, this medium was supplemented with H_3BO_3, biotin and thiamine. As omission

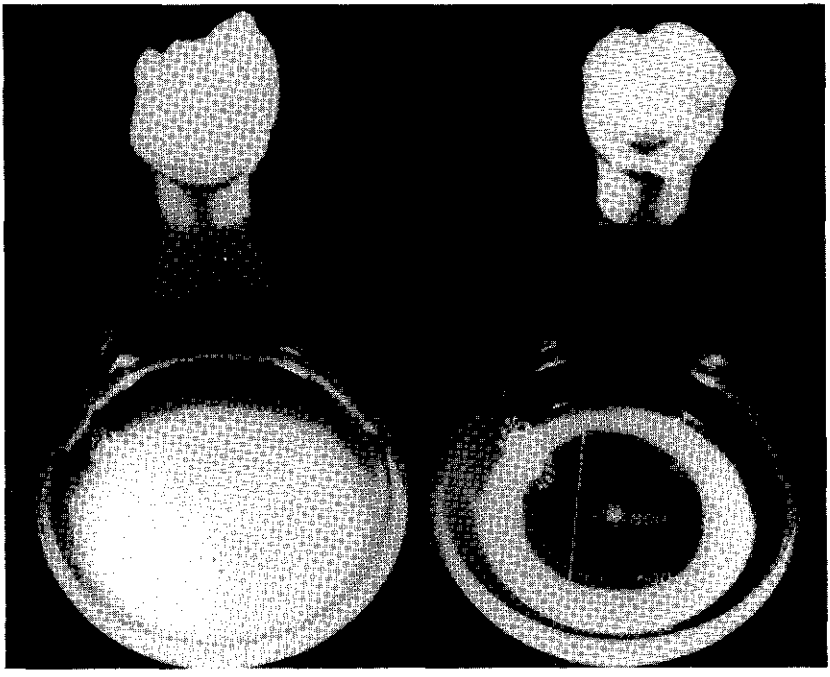

Fig.3: Settled batch cultures of WS(right) and WH (left) after growth on mannitol. Medium composition was according to Zevenhuizen and van Neerven (20). Note the diffuse layer on top of the WH pellet.

Table 1. Dry weight, polysaccharide and poly-3-hydroxybutyrate analyses of cultures of T.versutus WS and WH after batch growth on mannitol [a]. Cells were harvested after 48 or 72 hrs. of incubation at 30° C.

	WS		WH	
	48 hrs.	72 hrs.	48 hrs.	72 hrs.
Pellet:				
dry weight (μg/ml)	3006	3006	1919	2025
% carbohydrate	30	12	11	16
% poly-3-hydroxybutyrate (PHB)	40	58	40	38
Supernatant				
mannitol (μg/ml)	0	0	937	957
extracell. polysaccharides (μg/ml)	343	289	1914	1829
% reducing sugars	9	10	74	86

a) Substrate concentration: mannitol 5.0 g/l. Medium composition was according to Zevenhuizen and van Neerven (20).

of the vitamins was without effect, the high optical densities of the cultures after growth in the Zevenhuizen medium were attributed to differences in mineral composition of the medium used. When cultures of T.versutus WS and WH, grown on mannitol in the medium according to Zevenhuizen, were left to settle, and interesting phenomenon was observed. T.versutus WS developed a compact pellet with a clear supernatant, whereas WH developed a pellet on top of which a thick layer of a diffuse substance was noticed. The supernatant was again very clear (Fig. 3). The cells of both cultures had the normal coccobacillary shape of T.versutus and appeared to be stuffed with poly-3-hydroxybutyrate (PHB). Lysed cells were not visible. The macroscopic differences between WS and WH cells after growth on mannitol and glutamate were investigated by chemical analysis of the cultures (Table 1). The composition of the cells of WS and WH was roughly the same. In the extracellular fluid, however, distinct differences were found. The yield of strain WS was 0.5 g/g substrate whereas WH reached only 0.4 g/g. The amount of mannitol left in the supernatant of the WH culture, quantified spectrophotometrically and identified by gaschromatographic analysis, was considerable. Furthermore, strain WH had produced a large amount of extracellular polysaccharides with a composition different to the polysaccharides excreted by T.versutus WS. The production of the extracellular material had apparently proceeded at the cost of biomass production.

Discussion

The appearance of a novel colony type in a culture which was homogeneous with respect to colony shape is called dissociation (11). The most well-known change is the transformation of smooth into rough colonies by Brucella abortis. At first this seemed to be a reversible mutation but later it became clear that the new smooth cells differed from the original in a distinct physiological aspect. Replacement of a parent organism by a variant with a different colony shape has also been observed in chemostat cultures of Enterobacter aerogenes. This transition was accompanied by changes in biochemical and immunological composition, in polysaccharide content, in survival properties and in substrate-accelerated death (12). Despite the physiological differences between parent and mutant in these studies, morphological transitions without any effect on growth have also been reported (11). T.versutus WS and WH seem to belong in this last category as the growth-kinetics of both strains were identical (see text). However, a marked difference was observed in the ultrastructure of WS and WH cells after growth on butyrate (Fig. 2) and in the composition of excreted polysaccharides during growth on mannitol (Table 1). At this moment we are not sure whether these strain differences are intrinsic to the strain or to the substrate. However, the observed differences illustrate the phenomenon that mutants appearing during prolonged continuous cultivation on acetate can differ in growth on other substrates too. Even though we were able to determine two major strain differences on arbitrarily chosen substrates, we are still confused about the real nature of the transition as apparently (a) during growth of T.versutus on acetate mutants arise easily, (b) the mutation is reversible and (c) no selective advantage as far as growth on acetate is concerned can be detected. Previous reports on growth of T.versutus on methanol (6,13,16), its capacity to oxidize thiosulphate under denitrifying conditions (9,13,18) and its motility (7,13) are controversial. In view of the here reported ease of mutation we feel that the occurrence of strain differences should be taken into account.

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C H A P T E R 3

Tricarboxylic acid and glyoxylate cycle enzyme activities in Thiobacillus versutus, an isocitrate lyase negative organism

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Abstract

An analysis was made of the specific enzyme activities of the TCA and glyoxylate cycle in Thiobacillus versutus cells grown in a thiosulphate-, or acetate-limited chemostat. Activities of all enzymes of the TCA cycle were detected, irrespective of the growth substrate and they were invariably lower in the thiosulphate-grown cells. Of the glyoxylate cycle enzymes, isocitrate lyase was absent but malate synthase activity was increased from 15 nmol.min^{-1}.mg^{-1} protein in thiosulphate-grown cells to 58 nmol.min^{-1}.mg^{-1} protein in acetate-grown cells. Suspensions of cells grown on thiosulphate were able to oxidize acetate, although the rate was 3 times lower than that observed with acetate-grown cells. The respiration of acetate was completely inhibited by 10 mM fluoroacetate or 5 mM arsenite. Partially purified citrate synthase from both thiosulphate- and acetate-grown cells was completely inhibited by 0.5 mM NADH and was insensitive to inhibition by 1 mM 2-oxoglutarate or 1 mM ATP. The specific enzyme activities of the TCA and glyoxylate cycle in T.versutus were compared with those of Pseudomonas fluorescens, an isocitrate lyase positive organism, after growth in a chemostat limited by acetate, glutarate, succinate or glutamate. The response of the various enzyme activities to a change in substrate was similar in both organisms, with the exception of isocitrate lyase.

Key words: TCA and glyoxylate cycle enzymes - acetate metabolism - isocitrate lyase negative - Thiobacillus versutus.

Introduction

The specific enzyme activities of the tricarboxylic acid (TCA) and glyoxylate cycle in Thiobacillus versutus, a facultative chemolithoautotrophic organism, have been investigated previously (Gottschal and Kuenen, 1980; Peeters et al., 1970; Taylor and Hoare, 1969). The reported data are, however, contradictory especially with respect to the activities of 2-oxoglutarate dehydrogenase and isocitrate lyase. Peeters et al. (1970) detected 2-oxoglutarate dehydrogenase activity in cell-free extracts of succinate- and glutamate-grown cells but not in thiosulphate-grown cells. In addition they reported isocitrate lyase activities in these cell-free extracts. In contrast Taylor and Hoare (1971) did observe 2-oxoglutarate dehydrogenase activity in cells grown on thiosulphate, while Gottschal and Kuenen (1980) detected hardly any activity in either thiosulphate-, acetate- or mixotrophically-grown cells of T.versutus. Gottschal and Kuenen (1980) also reported the absence of isocitrate lyase activity in these cells.

It is generally assumed that during chemolithoautotrophic growth of organisms, the TCA cycle only supplies biosynthetic precursors and therefore this cycle is usually considered to be incomplete (Kelly, 1971; Smith and Hoare, 1977; Whittenbury and Kelly, 1977). During heterotrophic growth, however, the TCA cycle generally fulfills two functions, namely the generation of energy and the supply of biosynthetic precursors. Because of this amphibolic role, there is a need for replenishment of TCA cycle intermediates which, during growth on acetate or comparable substrates, occurs by way of the glyoxylate cycle (Kornberg, 1966). These cycles apparently do not function in T.versutus during growth on acetate as neither 2-oxoglutarate dehydrogenase nor isocitrate lyase activity is observed (Gottschal and Kuenen, 1980). It is therefore possible that an alternative, 2-oxoglutarate dehydrogenase and isocitrate lyase negative, route may be involved in the metabolism of acetate in T.versutus.

Isocitrate lyase negative growth of bacteria on acetate or comparable substrates

is not a novel phenomenon. Several of the facultative methylotrophs which assimilate C_1 compounds by way of the serine pathway have been found to be isocitrate lyase negative, not only during methylotrophic growth, but also during growth on acetate, 3-hydroxybutyrate or ethanol (Anthony, 1982; Meiberg, 1979; Quayle, 1975). A partially common metabolic route in the metabolism of C_1 and C_2 compounds not involving isocitrate lyase was proposed in a mutant of Pseudomonas AM 1 because loss of the ability to grow on methanol was concomitant with loss of the ability to grow on ethanol. Growth on both substrates was restored by addition of glyoxylate or glycolate (Dunstan et al., 1972). In this paper we present a comparative study of the enzyme profiles of T.versutus after chemolithoautotrophic and heterotrophic growth. This study was performed to obtain more information about the possible operation of the TCA cycle during heterotrophic growth. The metabolism of acetate, glutarate, succinate or glutamate in T.versutus was also compared to that in an isocitrate lyase positive organism, Pseudomonas fluorescens.

Materials and methods.

Organisms. Thiobacillus versutus, formerly called Thiobacillus strain A2 (ATCC 25364) (Harrison Jr., 1983), was a kind gift of Dr. J.G. Kuenen (Delft, the Netherlands). This organism was maintained on thiosulphate agar slants. Pseudomonas fluorescens (ATCC 11250) was purchased from ATCC, Rockville, Maryland 20852, USA. This organism was maintained on yeast extract/glucose/$CaCO_3$ agar slants. Both organisms were subcultured every two months. The agar slants were stored at 4°C.

Growth of bacteria. The basal medium for batch cultures contained, in distilled water, 29.5 mM Na_2HPO_4, 11.0 mM KH_2PO_4, 0.4 mM $MgSO_4$ and 2 ml/l trace element solution (Gottschal and Kuenen, 1980). For autotrophic growth on solid media 40.3 mM $Na_2S_2O_3$, 5.6 mM NH_4Cl and 15 g/l agar were added. The composition of the yeast extract/glucose/$CaCO_3$ medium was, in distilled water, 5.7 mM K_2HPO_4, 10.0 mM $CaCO_3$, 4.1 mM $MgSO_4$, 5.1 mM glucose, 3 g/l yeast extract, 2 g/l nutrient broth and 15 g/l agar. Yeast extract, nutrient broth and agar were purchased from Gibco Europe Ltd, Paisley, Scotland. The basal medium for continuous cultivation contained, in distilled water, 8.5 mM KH_2PO_4, 1.6 mM $MgSO_4$, 7.5 mM NH_4Cl and 2 ml/l trace element solution (Gottschal and Kuenen, 1980). For autotrophic growth 40.3 mM $Na_2S_2O_3$ was added whereas for heterotrophic growth 25.0 mM acetate, 11.4 mM glutarate, 13.0 mM succinate, or 10.2 mM glutamate was added to the medium. The pH was adjusted to 5.0 before autoclaving. During autotrophic growth the pH was maintained at 8.0 by the automatic addition of 1 M Na_2CO_3, and during heterotrophic growth by addition of 0.5 M H_2SO_4. Oxygen was supplied by sparging air through the culture at a rate of 1 l air per l culture per min. The dilution rate was set at 0.05 or 0.1 h^{-1}. All cultures were grown at 30°C. Substrate limitation was determined by measuring the dissolved organic carbon (DOC) concentration in the supernatant of centrifuged cultures with a Total Organic Carbon Analyzer, Beckman model 915 A. Since the DOC concentrations were always very low ($\leq$ 0.13 g/l) and the optical densities of the cultures increased proportionally to an increase in substrate concentration in the medium reservoir, no further attempts were undertaken to identify the remaining carbon source.

Preparation of cell-free extracts. Bacteria were harvested from steady state chemostat cultures in quantities of A_{623} x vol (in ml) = 100, and centrifuged at 9 000 x g for 15 min. Pellets were washed with 30 mM potassium phosphate buffer, pH 7.2 and resuspended in 2 ml of the same buffer. The cells were broken by sonification (4 times during 30 s with 30 s intervals) using an ultrasonic disintegrator (MSE, London, England). After centrifugation at 20 000 x g for 20 min the supernatant was used for enzyme assays without further treatment unless otherwise stated. All steps were carried out at 4°C. The protein concentration in cell-free extracts was determined with bovine serum albumin as a standard, according to Lowry et al.(1951).

Enzyme assays. All enzyme activities were continuously measured in 1 ml reaction mixtures (1 cm cuvettes) in a Hitachi 101 Spectrophotometer at 20°C. The observed rates were proportional to the amount of extract added and linear with time for at least 2.5 min. The composition of the assay mixtures is given below. The buffer used in all assays was 50 mM Tris-HCl, pH 8.0. Citrate synthase, EC 4.1.3.7 (Reeves et al., 1971): 0.1 mM DNTB, 0.15 mM acetyl-CoA and 0.2 mM oxaloacetate; Aconitase, EC 4.2.1.3 (Reeves et al., 1971): 20 mM isocitrate; Isocitrate dehydrogenase, EC 1.1.1.42 (Gottschal and Kuenen, 1980): 1 mM $NADP^+$, 1 mM $MgSO_4$ and 1 mM isocitrate; 2-Oxoglutarate dehydrogenase, EC 1.2.4.2 (Amarasingham and Davis, 1965): 2mM cysteine-HCl, 0.1 mM HSCoA, 2 mM APAD+, 10 mM 2-oxoglutarate; Succinate dehydrogenase, EC 1.3.99.1 (Veeger et al., 1969): 1 mM KCN, 0.2 mM PMS, 0.025 mM DCPIP and 10 mM succinate; Fumarase, EC 4.2.1.2 (Reeves et al., 1971): 10 mM fumarate; Malate dehydrogenase (NAD dependent) EC 1.1.1.37 (Sottocasa et al., 1967): 0.1 mM NADH and 1 mM oxaloacetate; Malate dehydrogenase (PMS/DCPIP dependent), (Kornberg and Phizackerley, 1961): 1 mM KCN, 0.2 mM PMS, 0.025 mM DCPIP and 5 mM malate; Isocitrate lyase, EC 4.1.3.1 (Dixon and Kornberg, 1959): 50 mM $MgCl_2$, 5 mM phenylhydrazine-HCl, 2 mM cysteine-HCl and 5 mM isocitrate; Malate synthase, EC 4.1.3.2 (Reeves et al., 1971): 10 mM $MgCl_2$, 0.1 mM acetyl-CoA and 2 mM glyoxylate; The amount of protein added to the reaction mixtures ranged from 5 to 100 μg. All reactions were started by addition of the substrate.

O_2consumption. Cells from chemostat cultures were suspended in air saturated 30 mM potassium phosphate buffer, pH 7.2. The final concentration was approximately 0.25 mg dry weight/ml in the reaction vessel of a Biological Oxygen Monitor Model 53 (Yellow Springs Instrument Co., Yellow Springs, Ohio 45387, USA). The O_2 consumption was monitored at 30°C and the endogeneous respiration rate was substracted from the O_2 consumption rate observed after the addition of 5 mM acetate.

Partial purification of citrate synthase. Approximately 1 ml of crude cell-free extract was applied to a DEAE-Sephadex A-50 (1x5cm) column which was equilibrated with 50 mM Tris-HCl buffer, pH 7.4. Malate dehydrogenase activity was eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 M NaCl. After washing with the same buffer, citrate synthase was eluted by increasing the NaCl concentration to 0.6 M. The partially purified enzyme was dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.4. All steps were carried out at 4°C.

Biochemicals: Biochemicals were obtained from Sigma Chemical Company, St. Louis, U.S.A. All other chemicals were of analytical grade.

Results

All TCA cycle enzyme activities were detected in cell-free extract of Thiobacillus versutus after chemolithoautotrophic or heterotrophic growth (Table 1). The specific activity of these enzymes was 1.7 to 3.8 fold higher in cell-free extracts of acetate-grown cells as compared to thiosulphate-grown cells. Of the enzymes of the glyoxylate cycle, only malate synthase activity was detected. The specific activity of this enzyme increased about 4 fold during growth on acetate. The activity of 2-oxoglutarate dehydrogenase was investigated in more

Table 1. Specific enzyme activities[a] in cell-free extracts of T.versutus after growth in a thiosulphate- or acetate-limited chemostat at a dilution rate (D) of 0.05 h^{-1}.

Enzyme	Growth substrate	
	thiosulphate	acetate
citrate synthase	60	144
aconitase	148	395
isocitrate dehydrogenase ($NADP^+$)	212	372
2-oxoglutarate dehydrogenase ($APAD^+$)	7	18
succinate dehydrogenase (PMS/DCPIP)	4	15
fumarase	510	1286
malate dehydrogenase (NAD^+)	1864	3218
isocitrate lyase	2	1
malate synthase	15	58

a) specific activities expressed in nmol.min^{-1}.mg^{-1}protein.

detail. When 50 mM Tris-HCl, pH 8.0 was used instead of 30 mM phosphate buffer, pH 7.2 during the preparation of cell-free extracts, the specific activity was about 4 fold lower. Addition of 0.5 mM EDTA, 5 mM 2-mercaptoethanol and 5% ($^v/_v$) ethyleneglycol or 5 mM $MgCl_2$ and 5 mM dithiotreitol was without effect. The pH-activity curve of this enzyme demonstrated a gradual increase in specific activity with increasing pH of the assay mixture. The optimum was at pH 8.6. Succinate dehydrogenase activity was also measured in the particulate fraction of cell-free extracts. After centrifugation of the extract at 120 000 x g during 60 min the specific activities in the pellet and supernatant were respectively 51 and 3 nmol.min^{-1}.mg^{-1}protein. In the supernatant of the 20 000 x g spin succinate dehydrogenase activity was always much lower and never exceeded 15 nmol.min^{-1}.mg^{-1}protein. The maximum specific oxygen uptake rate, $Q_{O_2}^{max}$, for oxidation of acetate was measured in a Biological Oxygen Monitor (BOM) with thiosulphate- and acetate-grown cells of T.versutus. The $Q_{O_2}^{max}$ of thiosulphate-grown cells was 18 and of acetate-grown cells 60 nmol O_2.min^{-1}.mg^{-1}dry weight. The effect of two inhibitors of the TCA cycle on the oxidation of acetate by cell suspensions was also examined. Fluoroacetate, which impedes the operation of the TCA cycle through the formation of fluorocitrate (Lehninger, 1975), fully inhibited the oxidation of acetate at a concentration of 10 mM. Arsenite, which was found to diminish 2-oxoglutarate dehydrogenase activity in cell-free extracts to 50% of its original value at a concentration of 0.01 mM, stopped

respiration of whole cells at a concentration of 5 mM.
Table 2 summarizes some aspects of the regulation of citrate synthase. The presence of 2-oxoglutarate or ATP had no inhibitory effect on the activity of citrate synthase. However, the enzyme was very sensitive to NADH and was completely inhibited by the addition of 0.5 mM NADH to the reaction mixture. The activity was restored by increasing the ionic strength, indicating an allosteric type of inhibition (Taylor, 1970).
The specific activities of the TCA cycle enzymes detected in cell-free extracts of variously-grown T.versutus and P.fluorescens cells are given in Table 3 and

Table 2. Inhibition of partially purified citrate synthase[a)] of thiosulphate- or acetate-grown cells of Thiobacillus versutus, expressed in % of remaining specific activity.

	Growth substrate	
	thiosulphate	acetate
control (no additions)	100	100
+ 1 mM 2-oxoglutarate	115	134
+ 0.5 mM NADH	0	0
+ 0.5 mM NADH + 0.35 M KCl	136	74
+ 1 mM ATP	153	109

a) Citrate synthase was partially purified by ion exchange chromatography with DEAE-Sephadex A-50. Low molecular weight metabolites were removed by dialysis. The final preparation was free of malate dehydrogenase activity.

Table 3. Specific enzyme activities[a)] in cell-free extracts of T.versutus after growth in a carbon-limited chemostat in media containing acetate, glutarate, succinate or glutamate as the carbon and energy source. D = 0.1 h^{-1}.

Enzyme	Growth substrate			
	acetate	glutarate	succinate	glutamate
citrate synthase	225	109	82	79
aconitase	439	261	206	149
isocitrate dehydrogenase ($NADP^+$)	445	226	161	112
2-oxoglutarate dehydrogenase ($APAD^+$)	25	13	15	8
succinate dehydrogenase (PMS/DCPIP)	13	5	4	8
fumarase	1718	1185	797	825
malate dehydrogenase (NAD^+)	1109	673	736	968
isocitrate lyase	N.D.[b)]	N.D.	N.D.	N.D.
malate synthase	62	63	22	22

a) Specific activities expressed in $nmol.min^{-1}.mg^{-1}$protein.
b) N.D. = not detected.

4, respectively. The specific activities of these enzymes were highest in acetate-grown cells of T.versutus. Isocitrate lyase activity, the first enzyme of the glyoxylate cycle, was never detected in normal nor dialyzed extracts However, malate synthase activity in acetate- or glutarate-grown cells was 3 fold higher than in succinate- or glutamate-grown cells. The authenticity of the malate synthase reaction was investigated by substituting glyoxylate by 2-oxo-glutarate, pyruvate or aceto-acetate. No enzyme activity was observed with any of these compounds. Acetyl-CoA was not hydrolyzed when $MgCl_2$ was omitted from the assay mix. Since citrate synthase activity was detected irrespective of the presence of $MgCl_2$, malate synthase was assumed to be a different enzyme. The profile of the TCA cycle enzyme activities in P.fluorescens (Table 4) demonstrated only slight differences when different growth substrates were used. The specific activity of isocitrate dehydrogenase in acetate- or glutarate-grown cells was almost twice as high as in succinate- or glutamate-grown cells. Malate dehydrogenase, which in many organisms is a NAD dependent soluble enzyme,

Table 4. Specific enzyme activities[a] in cell-free extracts of P.fluorescens after growth in a carbon-limited chemostat in media containing acetate, glutarate, succinate or glutamate as the carbon and energy source. D = 0.1 h^{-1}.

Enzyme	Growth substrate			
	acetate	glutarate	succinate	glutamate
citrate synthase	342	222	301	281
aconitase	68	80	117	107
isocitrate dehydrogenase ($NADP^+$)	817	730	432	441
2-oxoglutarate dehydrogenase ($APAD^+$)	22	14	15	15
succinate dehydrogenase (PMS/DCPIP)	8	3	11	11
fumarase	N.D.[b]	829	483	764
malate dehydrogenase (PMS/DCPIP)	47	12	15	22
isocitrate lyase	257	170	37	54
malate synthase	293	224	144	147

a) Specific activities expressed in $nmol.min^{-1}.mg^{-1}$protein.
b) N.D. = not determined.

appeared to be membrane-bound in P.fluorescens since all activity was detected in the particulate fraction obtained after high speed centrifugation (see above). Activity of this enzyme was dependent on the combination of the artificial electron acceptors PMS and DCPIP, thus resembling the malate dehydrogenase enzyme of Pseudomonas ovalis Chester (Kornberg and Phizackerley, 1961). The increase in specific activity of the glyoxylate cycle enzymes in acetate- or glutarate-grown cells, as compared to succinate- or glutamate-grown cells, was drastic. Isocitrate lyase activity was also detected in cell-free extracts prepared from equal amounts of T.versutus and P.fluorescens cells grown on acetate. When acetate-grown cells of P.fluorescens were mixed with an equal

amount of acetate-grown cells of T.versutus prior to sonification, the specific activity of isocitrate lyase in the thus obtained cell-free extract was 90 $nmol.min^{-1}.mg^{-1}$ protein. In extract prepared from P.fluorescens cells alone, 174 $nmol.min^{-1}.mg^{-1}$ protein was found, demonstrating that no isocitrate lyase inhibitor was present in T.versutus cells.

Discussion

The data presented in this paper reveal that T.versutus possesses all TCA cycle enzyme activities during chemolithoautotrophic and heterotrophic growth. Because of the following three physiological phenomena, the operation of a regular TCA cycle for the oxidation of acetate in T.versutus seems very likely. Firstly, we found a significant increase in the specific activities of the TCA cycle enzymes when chemolithoautotrophic metabolism was changed to heterotrophic metabolism (Table 1). Secondly, two well-known inhibitors of the TCA cycle, i.e. fluoroacetate and arsenite, were shown to block completely the respiration of acetate, while the inhibition of citrate synthase by NADH, and not by 2-oxoglutarate (Table 2) as generally found in organisms with an incomplete TCA cycle (Doelle, 1975; Taylor, 1970), constituted the third important indication of a functional, complete, TCA cycle. In this respect T.versutus resembles another isocitrate lyase negative organism, Pseudomonas AM1, which was shown to lose the capacity to grow on 3-hydroxybutyrate, ethanol and other multi-carbon compounds if 2-oxoglutarate dehydrogenase activity was absent (Taylor and Anthony, 1976). Our conclusion is supported by the findings of Kelly et al. (1979), who demonstrated incorporation of label from [U-^{14}C]-acetate into the "glutamate"- and "aspartate"-family of amino acids by autotrophically grown cells of T.versutus. These investigators however, attributed the incorporation of label to the operation of the glyoxylate cycle instead of the TCA cycle and referred to the results of Peeters et al. (1970). As mentioned before, these authors observed isocitrate lyase activity, but failed to detect 2-oxoglutarate dehydrogenase activity in thiosulphate-grown cells. This inability to detect 2-oxoglutarate dehydrogenase activity might have been due to the use of 50 mM Tris-HCl buffer, pH 8.0, for the preparation of cell extracts. This as we have shown, significantly decreases the specific activity of this enzyme. The low activity of isocitrate lyase found by Peeters et al. (1970) is at variance with the data from Gottschal and Kuenen (1980) and might have been caused by the nonspecific enzyme assay used. Formation of a phenylhydrazone from phenylhydrazine occurs with any 2-oxo-acid, e.g. glyoxylate but also 2-oxoglutarate. This latter metabolite can be formed from isocitrate by the action of isocitrate dehydrogenase (Attwood and Harder, 1977; Quayle, 1975), an enzyme whose activity was detected (Tables 1, 3).

Comparison of the enzyme profile of an isocitrate lyase positive organism with that of T.versutus yielded interesting results (Tables 3 and 4). Although a clear difference must exist with respect to the assimilation of acetate in the two organisms, the overall appearance of the enzyme profiles was very similar, of course with the exception of isocitrate lyase activity. The significant increase in isocitrate dehydrogenase activity observed in both organisms after growth on acetate and glutarate, which in P.fluorescens is metabolized via acetyl-CoA (Numa et al., 1964), has been noticed in acetate-grown organisms before (Reeves et al., 1983). One reason could be to ensure sufficient supply of NADPH since malic enzyme is not functional during the metabolism of acetate (Reeves et al., 1983).

Our findings have led us to the conclusion that T.versutus possesses a complete set of TCA cycle enzymes and thus the capacity to dissimilate acetate in a

conventional manner. T.versutus is not able to express isocitrate lyase activity under the conditions tested by us but in view of the fact that malate synthase activity was increased during growth on acetate, a physiological role of glyoxylate in the assimilation of acetate seems conceivable. A possible route would be the conversion of acetate to glycolate followed by oxidation to glyoxylate as proposed by Dunstan et al. (1972) for the metabolism of acetate by Pseudomonas AM 1.

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CHAPTER 4

In vivo ^{13}C NMR analysis of acetate metabolism in *Thiobacillus versutus* under denitrifying conditions

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Abstract

The disappearance of 2-^{13}C-acetate and the subsequent incorporation of label into cellular metabolites were followed in denitrifying cells of Thiobacillus versutus by ^{13}C NMR spectroscopy. In cells grown under acetate-limitation, the specific rate of consumption was independent of the density of the cell suspension. An isotopic steady state was reached within 30 min if sufficient substrate was added to the cell suspension. In cells grown under nitrate-limitation, the consumption of 2-^{13}C-acetate proceeded at a significantly lower rate. The decrease and final disappearance of 2-^{13}C-acetate were accompanied by incorporation of ^{13}C into glutamate, glutamine, and by the release of labeled HCO_3^- and CO_2. The appearance of a broad resonance being the methyl endgroup of poly-3-hydroxybutyrate (PHB) was indicative for PHB mobilization during the incubation. The sequence of label incorporation and the distribution among the various carbon nuclei were consistent with the operation of the tricarboxylic acid cycle.

Keywords: Thiobacillus versutus - TCA cycle - ^{13}C incorporation - PHB metabolism.

Introduction

The application of ^{13}C NMR spectroscopy to elucidate metabolic pathways is now well-established. The sequence of metabolic reactions has been followed after the addition of ^{13}C enriched substrates to suspensions of intact cells of bacteria (Nicolay et al., 1983), yeasts (Den Hollander et al. 1979, 1981), fungi (Dijkema et al., 1985), and intact cells and whole organs of rats (Cohen et al., 1979a,b, 1981). The distribution of ^{13}C label among intermediates or endproducts has provided detailed information about the nature of certain metabolic routes. The most important advantage of ^{13}C NMR spectroscopy is the possibility to study the incorporation of the label without disrupting the cells. The uncertainty encountered in the extraction of metabolites with rapid turnover rates is thus avoided. Because of the broad spectral range of 300 ppm, inherent to ^{13}C NMR, the resonances of various C atoms within a molecule can be observed in the NMR spectrum. In combination with the analysis of NMR line splittings arising from adjacent ^{13}C nuclei, a well-resolved NMR spectrum is an irrefutable reflection of the metabolic route followed in the substrate utilization. The major drawback of this technique, however, remains its low intrinsic sensitivity which necessitates the use of very dense cell suspensions for time-resolved experiments. Consequently, the study of aerobic metabolism can be hampered by an insufficient supply of oxygen to the cells. Although some elegant devices for aeration of dense suspensions in a NMR tube have been designed (Dickinson et al., 1983; Ugurbil et al., 1978), it is still difficult to retain adequate homogeneity as the sample is heavily agitated in order to provide sufficient aeration. To circumvent this technical problem, we chose to investigate the metabolism of acetate by Thiobacillus versutus under denitrifying conditions. By doing so the problems accompanying the homogeneous and sufficient distribution of a gaseous substrate in a dense cell suspension can be avoided while the intrinsic features of aerobic metabolism are preserved.

Materials and Methods

Organism. Thiobacillus versutus, formerly called Thiobacillus strain A2 (ATCC 25364) (Harrison, 1983) was a kind gift of Dr. J.G. Kuenen (Delft, The Netherlands). The organism was maintained on thiosulfate agar slants, stored at 4'C and subcultured every two months (Taylor and Hoare, 1969).

Growth Conditions. The basal medium for the chemostat cultures contained per 1000 ml distilled water, 1.15 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.4 g NH_4Cl and 2 ml trace element solution (Gottschal and Kuenen, 1980). For acetate-limited growth 3.4 g CH_3COONa and 5.0 g KNO_3 were added per 1000 ml basal medium and for nitrate-limited growth 3.4 g CH_3COONa and 2.0 g KNO_3. The pH was adjusted to 5.0 before autoclaving. Cultures were grown at 30'C, pH 8.0 and at a dilution rate of 0.1 h^{-1}. The pH was kept at 8.0 by automatic addition of 0.5 M H_2SO_4. N_2 (3.0 technical grade) was sparged through the culture at a rate of 0.3 l per 1 culture per min.

Analytical methods. Dry weight of steady state cultures was estimated from a previously determined $O.D._{623}$ versus dry weight calibration curve. Bacterial cell counts were performed by plating samples of a dilution series onto agar plates containing basal medium (Taylor and Hoare, 1969) supplemented with either $Na_2S_2O_3.5H_2O$, 10 g/l, and incubated aerobically, or CH_3COONa, 2.5 g/l plus KNO_3, 2.0 g/l, and incubated anaerobically in a GasPak 150 system (BBL, Cockeysville USA). The acetate concentration in the medium was determined with a Varian gas-chromatograph equipped with a Chromosorb 101 (80/100 mesh) column and a flame ionization detector. Nitrogenous compounds were quantified with a Technicon autoanalyzer; NH_4^+ after reaction with salicylic acid, NO_2^- and NO_3^- (after reduction to NO_2^-) in the presence of sulfanilic acid and 1-naphtylamine.

NMR spectroscopy. Cells from steady state cultures were harvested by centrifugation and washed with cold NMR medium which contained per 1000 ml distilled water, 35 g K_2HPO_4, 13.5 g KH_2PO_4, 1.6 g NH_4Cl, 0.4 g $MgSO_4.7H_2O$ and 8 ml trace element solution (Gottschal and Kuenen, 1980). The pH of this medium was adjusted to 7.1. The washed pellets were resuspended in cold NMR medium to a final volume of 3 ml (concentrations of samples are specified in figure legends) and stored on ice prior to the NMR experiment. The pH of the samples, checked before and after the NMR experiment, remained the same. NMR experiments were started by adding 100 or 400 mol 2-^{13}C-acetate (90 atom %, purchased from Stohler Isotope Chemicals, Waltham USA and used without further purification), 160 resp. 640 μmol KNO_3, and 10% (v/v) ^2H_2O to the sample in the NMR tube. After addition of these substrates the samples were immediately transferred into the NMR probe. ^{13}C spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer equipped with a 10 mm probe operating in the Fourier-transform mode. Proton broadband decoupling was applied throughout all experiments. The free induction decays covering a spectral range of 20,000 Hz in the frequency domain, were recorded by using a 60' pulse (pulse time 12 μs) and 8,000 data points, accumulated in consecutive blocks of 5 min (600 transients) and sequentially stored on disk. The temperature in the probe during all experiments was maintained at approximately 15'C. The assignment of the resonances was done by incubating the suspension with a 5 fold isotopically diluted sample of 2-^{13}C-acetate. Under these conditions the resonances of the metabolites occur as singlets. The chemical shifts of the resonances were estimated using the value of the glutamate C-4 resonance (34.4 ppm) as an internal reference, derived from the mannitol values used by Dijkema et al. (1985). Amplitudes were determined by measuring the peak heights, and plotted on the time axis at the intermediate values between the endpoints of the consecutive blocks stored. The peak height of the acetate resonance in the first spectrum was used for calibration.

Results

Denitrifying growth of Thiobacillus versutus. To adapt aerobically grown cells to denitrification, aeration and substrate addition to an aerobic acetate-limited chemostat culture of T. versutus were stopped. Then 1.85 g acetate and 2.9 g KNO_3 were added and the culture was thoroughly flushed with N_2. Cells started to grow within approximately 7 h. Following this period of adaptation the substrate flow was started at a dilution rate of 0.1 h^{-1}. After ± 7 volume changes cells were harvested for further experiments. The steady state con-

Table 1. Steady state parameters of Thiobacillus versutus during denitrifying growth in chemostat cultures with acetate as carbon and energy source. The dilution rate is 0.1 h^{-1}.

		Limiting substrate	
		acetate	nitrate
Reservoir concentration of acetate	in g/l	2.5	2.5
Reservoir concentration of KNO_3	"	5.0	2.0
[Acetate] in supernatant	"	0	0.7
$[NO_3^-]$	"	0.6	0
$[NO_2^-]$	"	0	0
$[NH_4^+]$	"	0.032	0.047
Dry weight of the culture	"	0.68	0.49
Bacterial cell count	in bact./ml		
- thiosulfate, aerobic	"	6.2×10^9	4.0×10^9
- acetate plus KNO_3, anaerobic	"	7.1×10^9	3.6×10^9

ditions of the studied denitrifying cultures of T. versutus are presented in Table 1. The acetate-limited culture, grown on 2.5 g/l acetate and 5.0 g/l KNO_3 was carbon-limited as no acetate could be detected in the medium and an increase in acetate to 3.0 g/l resulted in a proportional increase in dry weight of the culture. The nitrate-limited culture was grown on 2.5 g/l acetate and 2.0 g/l KNO_3. This limitation was obvious from the absence of nitrate and the presence of acetate in the culture (Table 1).

Rates of incorporation of 2-^{13}C-acetate as observed by ^{13}C NMR. The effect of the density of the cell suspension from an acetate-limited culture on the rate of acetate consumption and incorporation into cellular metabolites is presented in Fig. 1A and 1B. In the low density suspension (15 mg dry wt./ml) the concentration of acetate was reduced to 50% after 22 min of incubation. In the high density suspension (50 mg dry wt./ml) this process took only 6.5 min. Thus, the rate increased proportionally to the dry weight of the NMR samples. It is interesting to note that when all 2-^{13}C-acetate had been consumed the ratio of the $H^{13}CO_3^-$ concentration to the concentration of labeled glutamate was significantly higher in the low density suspension. In the low density suspension (Fig. 1A) the glutamate resonances did not change any further after 30 min of incubation though the production rate of $H^{13}CO_3^-$ remained constant, indicating that an isotopic steady state had been reached. In the high density cell sus-

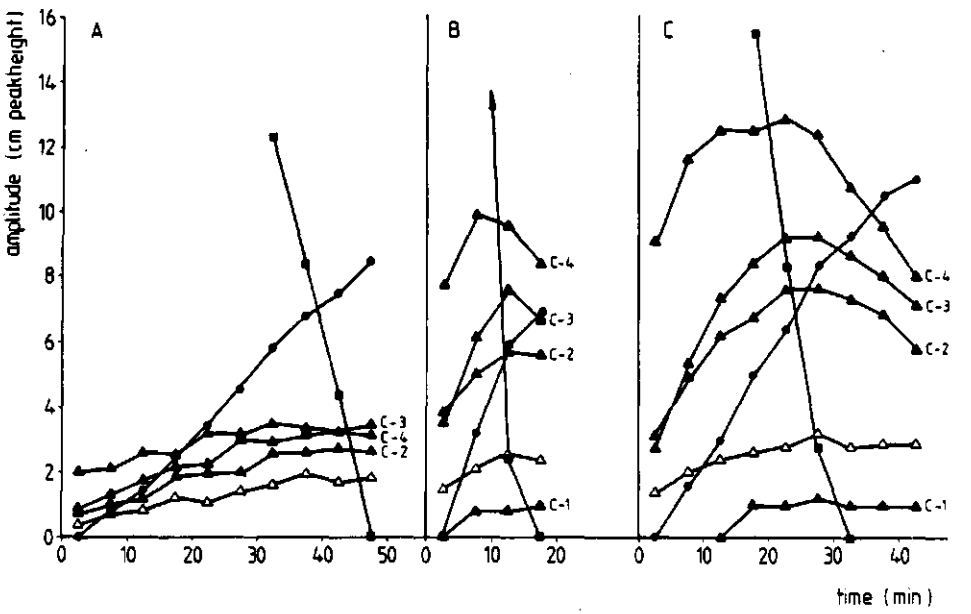

Fig. 1. Time course of labeling of various metabolites as observed by ^{13}C NMR spectroscopy in denitrifying cell suspensions of Thiobacillus versutus after the addition of 100 μmol 2-^{13}C-acetate and 160 μmol KNO_3. The cell suspensions were collected from an acetate-limited chemostat culture (acetate, 2.5 g/l and KNO_3, 5.0 g/l; A and B) or a nitrate-limited chemostat culture (acetate, 2.5 g/l and KNO_3, 2.0 g/l; C). All cultures were grown at D = 0.1 h-1. (■): 2-13C-acetate, (●): $H13CO_3$-, (▲): glutamate C-4, C-3, C-2 and C-1 and (△): glutamine C-4. A = Low density suspension; dry weight is 15 mg/ml. B = High density suspension; 50 mg/ml. C = High density suspension; 50 mg/ml

pension (Fig. 1B) the amount of 2-^{13}C-acetate supplied was not sufficient to attain a steady state. Due to the larger amount of cells present, the peak heights of the glutamate resonances were considerably higher. When 4 fold higher quantities of 2-^{13}C-acetate and nitrate were added to a high density suspension, isotopic steady state was also reached within 30 min (data not shown).
In Fig. 1B and 1C a cell suspension collected from an acetate-limited culture was compared with an equally dense suspension from a nitrate-limited culture. In cells grown under nitrate-limitation (Fig. 1C) the concentration of acetate was reduced to 50% after 12.5 min, instead of 6.5 min as observed in the sample from an acetate-limited culture (Fig. 1B).

Incorporation of ^{13}C from 2-^{13}C-acetate into cellular metabolites. A series of ^{13}C NMR spectra was recorded after addition of 100 μmol 2-^{13}C-acetate and 160 μmol KNO_3 (no isotopic steady state) to a high density cell suspension of T. versutus from an acetate-limited chemostat culture (Fig. 2). The natural abundance spectrum taken before the addition of the substrates revealed no resonances. The introduction of 2-^{13}C-acetate, resonating at 24.6 ppm, was followed by the appearance of a number of well-resolved resonances originating from small metabolites, and one very broad resonance. All resonances were assigned to known metabolites: the carbon atoms in glutamate, GLU C-3, C-4, C-2 and C-1 resonated at 28.5, 34.4, 55.7 and 175.4 ppm respectively, the carbon atoms in glutamine, GLN C-4 at 31.9 ppm, GLN C-3 and C-2 at 27.7 and 55.3 ppm, located on the flanks of the major glutamate resonances, and $H^{13}CO_3^-$ at 161.3 ppm. The broad resonance around 20 ppm, initially hardly visible, belongs to the CH_3-carbon of poly-3-hydroxybutyric acid (PHB). The series of spectra in Fig. 2 clearly demonstrates a time- dependent incorporation of label into glutamate, glutamine and HCO_3^-. From the sequence of label incorporation into glutamate a route for the metabolism of acetate could be derived. During the incubation first a singlet arises at the GLU C-4 position at 34.4 ppm, still visible in Fig. 2A, which originates from glutamate labeled only at the C-4 position. Already within the first 5 min of incubation a doublet grows in the spectrum around this C-4 singlet which amplitude increases simultaneously with the amplitude of the C-3 and C-2 resonances of glutamate (Fig. 2A-D). The doublet is due to C-4 – C-3 coupling within one molecule, demonstrating multiple incorporation of label originating from 2-^{13}C-acetate. Multiple incorporation is also reflected by the increase in the C-3 and C-2 resonances of glutamate during the incubation. This incorporation sequence at the C-4 and subsequently at the C-3 and C-2 positions in glutamate is consistent with the operation of the tricarboxylic acid (TCA) cycle (Lehninger, 1975). Of both carboxyl carbon atoms of glutamate only C-1 is observed. The amplitude of this resonance remained relatively low due to removal of label in going through the TCA cycle, the long relaxation time (T_1) of the C-1 nucleus and the Nuclear Overhauser Effect (NOE). This effect enhances signal intensity due to the presence of protons surrounding the ^{13}C nucleus. Since no protons are attached to the C-1 of glutamate, this carbon lacks the NOE (Wehrli and Wirthlin, 1976). Spectrum 2E has been recorded approx. 4 h after spectrum 2D. The decrease of the amplitudes clearly demonstrates continuation of metabolism after the exhaustion of substrate. The lower amplitude for GLU C-4 as compared to GLU C-3 and C-2 (in contrast to spectrum 2D) suggests replacement of ^{13}C by endogeneous ^{12}C, becoming available at that time. The viability of the cell suspension and the reproducibility of the observed phenomena were checked by adding another 100 μmol 2-^{13}C-acetate and 160 μmol KNO_3. The renewed addition of these substrates resulted in exactly the same process as depicted in Fig. 2.

For an estimation of the isotopomers of glutamate present the splitting patterns resulting from multiple incorporation were studied after artificial line-narrowing, illustrated in Fig. 3 for the case of label distribution under conditions of isotopic steady state. The predominance of the doublet structure (34 Hz) around the C-4 singlet resonance at 34.4 ppm shows that almost all glutamate labeled at C-4 is also labeled at C-3. The GLU C-3 resonance is a superposition of the contributions of at least three glutamate fractions. The main fraction consists of glutamate labeled at the C-2, C-3 and C-4 positions; this fraction gives rise to a triplet structure (due to equal C-2 – C-3 and C-3 – C-4 couplings of 34 Hz) centered around the singlet value of 28.5 ppm for GLU C-3 (Walsh and Koshland, 1984). The small doublet structure (34 Hz) within this triplet arises from two different glutamate fractions, one labeled at both the C-3 and C-4 position and the other labeled at the C-3 and C-2 positions. A possible fourth glutamate fraction labeled only at the C-3 position will be hidden under the central line of the triplet at 28.5 ppm. Most interesting for

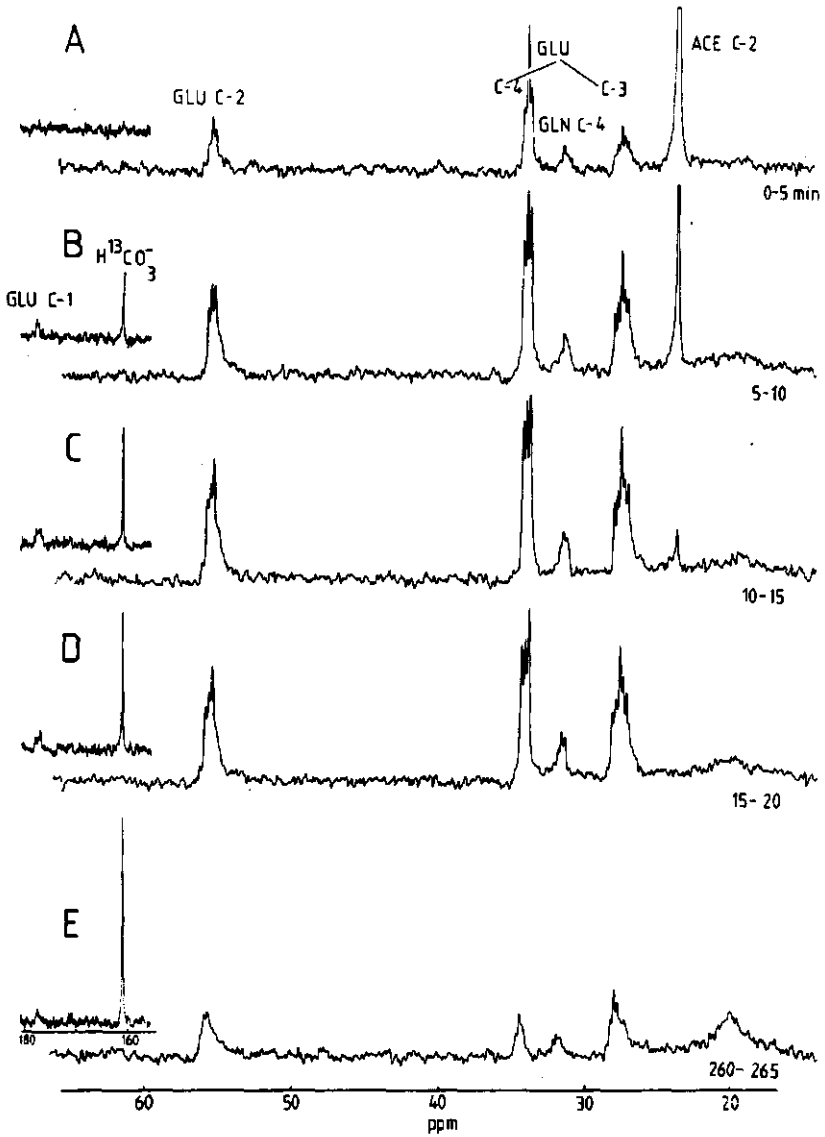

Fig. 2. ^{13}C NMR spectra of a denitrifying cell suspension of Thiobacillus versutus collected from an acetate-limited chemostat culture. Dry weight of the cell suspension was 50 mg/ml. The experiment was started by the addition of 100 μmol 2-^{13}C-acetate and 160 μmol KNO_3. Each spectrum represents the accumulation of 600 free induction decays at 0.5 s pulse intervals. A line broadening of 5 Hz is applied. Spectra A-D were recorded sequentially. Prior to the recording spectrum E the cell suspension had been kept on ice for 2 h and at room temperature for another 2 h. ACE: acetate, GLU: glutamate, GLN: glutamine.

our study is the GLU C-2 resonance composed of two major doublet lines with a splitting of 34 Hz due to C-2 – C-3 coupling, centered around the C-2 singlet resonance at 55.7 ppm. Both lines are split into a second doublet with a coupling of 54 Hz representing a second glutamate fraction labeled at the C-1, C-2 and C-3 positions. This fraction is also observed as a doublet around the singlet value of C-1 at 175.4 ppm. The occurrence of these two isotopomers differing in the labeling of the C-1 position in glutamate was also observed in Escherichia coli (Walsh and Koshland, 1984). Apart from this similarity our results demonstrate a very small contribution of singlet structures arising from single labeling at various glutamate carbons. This contribution is most probably the result of the use of 2-^{13}C-acetate enriched to 90 atom %, and dilution of ^{13}C acetate by endogeneous substrate, also observed in Fig. 2E. Other metabolites which have been observed in cell suspensions of T. versutus after the addition of 2-^{13}C-acetate and nitrate were CO_2, at 125.9 ppm, aspartate, C-3 at 38.1 and C-2 at 53.9 ppm, citrate, C-2 and C-4 at 46.7 and C-3 at 76.3 ppm, and succinate, C-2 and C-3 at 35.1 ppm (data not shown). Of these metabolites, CO_2 and aspartate appeared in small quantities in a high density suspension after the addition of 10 times as much 2-13C-acetate as used in the described experiments. Citrate was formed when cells were suspended in phosphate buffer with a much lower ionic strength than presently used. Succinate became detectable after the exhaustion of nitrate. The appearance of labeled succinate was always concomitant with the termination of $H^{13}CO_3^-$ production.

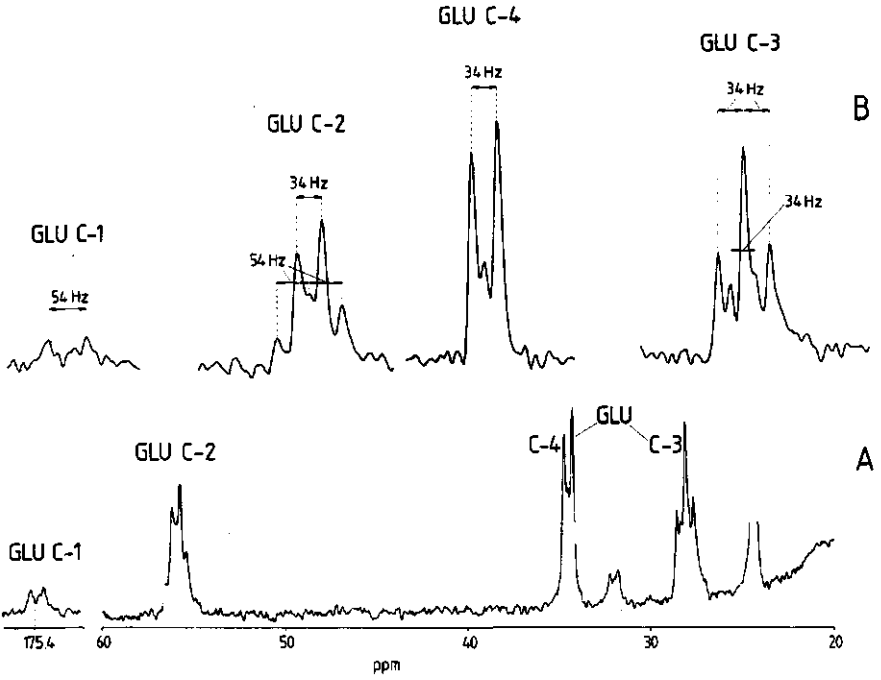

Fig. 3. A. ^{13}C NMR spectra of a denitrifying high density cell suspension of Thiobacillus versutus as in Fig. 2 under conditions of isotopic steady state, recorded between 45 and 50 min after addition of 400 μmol 2-^{13}C-acetate and 640 μmol KNO_3. B. The GLU C-1, C-2, C-3 and C-4 resonances are artificially narrowed applying Lorentz-Gauss multiplication; a negative line broadening of -5 Hz is used in combination with a Gaussian multiplication cuttoff factor of 0.15.

Discussion

During incubation in a NMR tube in the presence of 2-^{13}C-acetate and nitrate dense cell suspensions of denitrifying cells of Thiobacillus versutus consume acetate at a rate of 27-50 nmol/min mg dry wt. This rate is dependent upon the nature of the growth-limiting factor (Fig. 1B and 1C). Cells grown under acetate-limitation consume the substrate faster than cells grown under nitrate-limitation, perhaps due to a lower specific activity of the nitrogen oxide reductases in the latter case (Bryan, 1981). The specific rate of acetate consumption is independent of the density of the cell suspension (Fig. 1A and 1B) and can be varied by variation of the sample temperature. In order to obtain a reasonable time-resolution, we kept the temperature at 15°C. The observed rate of acetate consumption in the NMR probe at 15°C can stand comparison with the consumption rate in the chemostat where acetate is utilized at a rate of 103 nmol /min mg dry wt. (Table 1).

The sequence of label incorporation from 2-^{13}C-acetate into glutamate and the concomitant production of $H^{13}CO_3^-$ (Fig. 2) reflect the metabolism of acetate through the TCA cycle (Lehninger, 1975). The observation of TCA cycle activity in T. versutus is supported by a previous study in which all TCA cycle enzyme activities were observed after aerobic growth on acetate, glutarate, succinate and glutamate (Claassen et al., in press). Moreover, Peeters et al.(1970) demonstrated the presence of the complete set of TCA cycle enzymes in T. versutus after denitrifying growth on succinate.

The contribution of the TCA and glyoxylate cycles in the metabolism of acetate can be determined from the ratio of two isotopomers of glutamate. As described by Walsh and Koshland, (1984) TCA cycle activity alone results in 50% C-1, C-2, C-3 and C-4 isotopomer and 50% C-2, C-3 and C-4 isotopomer of glutamate. Glyoxylate cycle activity decreases the contribution of the isotopomer labeled at the C-1 position. Therefore the ratio of these isotopomers represents the flow of carbon through the respective cycles. However, ^{12}C nuclei are incorporated in glutamate emanating from unlabeled acetate and endogeneous substrate (Fig.2). The resulting singlet structure at GLU C-1 (Fig. 3) impairs a reliable quantitation of label at this position. Thus this experiment was not suitable for establishing a possible contribution of the glyoxylate cycle in the metabolism of acetate by T. versutus under these conditions.

The appearance of the CH_3 resonance of PHB is an interesting result. There are two different physiological processes which can lead to these data. The first one is biosynthesis of PHB from acetate, a reaction which usually occurs following the production of excess NADH. Accumulation of NADH can be due to a relatively slow rate of reduction or to a quantitative shortage of the final electron acceptor. When 2-^{13}C-acetate is used as substrate, the methyl carbon and the methylene carbon of PHB become labeled. As a consequence of the packed structure of the polymer, the signal of the methylene carbon will be broadened in ^{13}C NMR spectra beyond detection, whereas the more mobile methyl carbon will be visible as a narrow resonance (Nicolay et al., 1982). In the presented studies, stoichiometric additions of acetate and nitrate have been made to prevent NADH accumulation due to electron acceptor shortage. Therefore the production of PHB during this experiment seems unlikely. The second possibility leading to the same final result is mobilization of PHB followed by subsequent degradation into acetyl-CoA moieties. Several arguments favor this possibility; (1), a final increase in the singlet character of GLU C-4 (Fig. 2); (2), in spectrum 2E, the amplitude of GLU C-4 is decreased to a greater extent than the amplitudes of GLU C-3 and C-2, again indicating dilution of label; (3), after the exhaustion of labeled acetate, labeled glutamate is still oxidized; and (4), the increase in amplitude and decrease in line-width of the resonance around 20 ppm (Fig. 2). If no acetyl-CoA is provided, catabolism of glutamate through the TCA cycle leads to accumulation of oxaloacetate or aspartate (Den Hollander et

al., 1981). As these intermediates were not detected and as the $H^{13}CO_3^-$ peak kept increasing, labeled glutamate must be oxidized and/or substituted by unlabeled glutamate. Both ways the presence of unlabeled acetyl-CoA is obligatory. This mobilization hypothesis is further supported by the observation that anaerobically grown cells of T. versutus, whether cultured under acetate- or nitrate-limitation, contain a considerable amount of PHB granules clearly visible in a microscopic preparation.
In conclusion, the here described method, i.e. the use of denitrifying cells for in vivo ^{13}C NMR investigation, leads to reproducible incorporation of label into cellular metabolites which can be followed by well-resolved spectra resulting in an insight in the in vivo metabolism of acetate in T. versutus. We thus feel that the substitution of O_2 by nitrate constitutes a useful extension in the application of in vivo 13C NMR spectroscopy to study aerobic organisms, provided that they are able to denitrify.

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C H A P T E R 5

Isocitrate lyase activity in Thiobacillus versutus grown anaerobically on acetate and nitrate.

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Abstract

In cell-free extracts of Thiobacillus versutus, an organism which has been reported to be isocitrate lyase negative, an isocitrate lyase activity of 52 ± 18 nmol min^{-1} mg^{-1} protein was observed after anaerobic growth in a chemostat on acetate plus nitrate, i.e. during denitrification. Following growth on succinate plus nitrate, isocitrate lyase activity was only 1 ± 2 nmol min^{-1} mg^{-1} protein. In cell-free extracts derived from aerobic chemostat cultures this enzyme activity was always nil. The identity of the enzyme was analysed using a number of different methods, namely (a) three different enzyme assays, (b) ^{13}C NMR spectroscopy of the reaction products, (c) HPLC analysis of the reaction products, (d) mass spectrometry of derivatized glyoxylate enzymatically produced from isocitrate, and (e) radiography of derivatized glyoxylate enzymatically produced from [^{14}C]citrate. All methods used proved that the enzyme catalysed the conversion of isocitrate to glyoxylate and succinate.

Key words: Thiobacillus versutus - Isocitrate lyase - glyoxylate determination - Denitrifying growth on acetate.

Introduction

The metabolism of acetate or substrates which are converted to acetyl-CoA moieties without the intermediary generation of pyruvate proceeds through the tricarboxylic acid cycle (TCA cycle) and the glyoxylate cycle (Doelle, 1975; Lehninger, 1975; Schlegel, 1981). Dixon & Kornberg (1959) were the first to demonstrate the occurrence of the two key glyoxylate cycle enzymes, isocitrate lyase and malate synthase. However, bacteria have been isolated which are able to grow on acetate or substrates such as ethanol or 3-hydroxybutyrate without measurable isocitrate lyase activity. Most if not all of these organisms belong to the group of facultative methylotrophs using the isocitrate lyase negative serine pathway during methylotrophic growth (Anthony, 1982). As methylotrophic or heterotrophic growth on C_2 substrates apparently can occur without the presence of isocitrate lyase activity, these organisms were thought to possess an alternative metabolic route to generate glyoxylate. To obtain a better insight into the metabolism of acetate in an isocitrate lyase negative organism we chose Thiobacillus versutus, reported to lack isocitrate lyase (Gottschal & Kuenen, 1980), for our experiments. The intermediary metabolism of acetate was analysed in vivo by ^{13}C NMR spectroscopy. T.versutus was adapted to anaerobic growth on acetate in the presence of nitrate for the preparation of NMR samples. This was done in order to facilitate the NMR experiments. Moreover, we assumed that by substituting oxygen by nitrate as electron acceptor the metabolism of the carbon and energy source would not be altered. Very surprisingly, however, T.versutus was found to possess isocitrate lyase activity during denitrifying growth on acetate, whereas during aerobic growth on the same substrate no activity was detected. In view of this finding we felt obliged to confirm the nature of the observed enzyme activity by identifying the reaction products.

Methods

Organism, media and cultivation. Thiobacillus versutus, formerly called strain A2 (ATCC 25364, Harrison, 1983), was a gift from Dr J.G. Kuenen (Delft, The Netherlands). The organism was maintained on thiosulphate agar slants and sub-cultured every two months. The agar slants were stored at 4°C.

The basal medium for growth in chemostat culture contained, per litre distilled water, 1.15 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.4 g NH_4Cl and 2 ml trace element solution (Gottschal & Kuenen, 1980; Kuenen & Tuovinen, 1981). For aerobic growth, sodium acetate or sodium succinate was added at a final concentration of 3.4 g l^{-1}. For denitrification, the medium used for aerobic growth was supplemented with KNO_3 at a final concentration of 5.0 g l^{-1} for acetate-limited growth, and 2.0 g l-1 for nitrate-limited growth. The pH of the medium was adjusted to 5.0 before autoclaving. Cultures were grown at 30°C, pH 8.0 and a dilution rate of 0.1 h^{-1}. The pH was maintained at 8.0 by the automatic addition of 0.5 M-H_2SO_4. For aerobic growth, oxygen was supplied by sparging air through the culture at a rate of 1 litre air per litre culture per min. For anaerobic growth air was replaced by nitrogen (technical grade 3.0) at a rate of 0.25 litre per litre culture per min. Substrate limitations were determined from the proportional increase in optical density of the culture following an increase in substrate concentration in the medium reservoir.

For growth on thiosulphate or acetate (aerobic and denitrifying), the solid media described by Taylor & Hoare (1969) were used, except for the trace element solution which was according to Gottschal & Kuenen (1980). Sodium acetate was added at a final concentration of 2.5 g l^{-1} and agar (Gibco) at a final concentration of 15 g l^{-1}. Inoculated thiosulphate or acetate plates were incubated aerobically. When acetate plus KNO_3 plates were used incubation took place in a tightly closed jar in which oxygen was trapped after activation of a GasPak Disposable Hydrogen and Carbon Dioxide Generator Envelope (BBL) in the presence of a palladium catalyst. All incubations were done at 30°C. The purity of the chemostat cultures was regularly checked by streaking samples from a dilution series onto agar plates. Samples from aerobic cultures were incubated on acetate in the presence of air, samples from anaerobic cultures were tested on acetate plus KNO_3 plates under anaerobic conditions. The number of colonies obtained was compared to that found on aerobic thiosulphate plates inoculated with samples from the same dilution series.

Preparation of cell free extracts. Cells were harvested from steady state chemostat cultures by centrifugation at 9000 g for 15 min in quantities of OD_{623} x vol (in ml) = 100. Pellets were washed with 30 mM-potassium phosphate buffer, pH 7.2, and resuspended in 2 ml of the same buffer. The cells were broken by sonification (four times during 30 s with 30 s intervals) using an ultrasonic disintegrator (MSE). After centrifugation at 20 000 g for 20 min the supernatant was used for assays without further treatment except for dialysis if needed. In some experiments (see results section) the particulate fraction of cell-free extract was removed by centrifugation at 120 000 g for 2 h. All manipulations were done at 4°C. Protein concentrations were determined with bovine serum albumin as a standard according to Lowry.

Assays for determination of enzyme activity. Enzyme activities were measured in 1 ml reaction mixtures (1 cm cuvettes) in a Hitachi 101 Spectrophotometer at 20°C. The following assays were done in 50 mM- Tris/HCl buffer, pH 8.0: (i) isocitrate lyase, EC 4.1.3.1., according to Dixon & Kornberg (1959), in 50 mM-$MgCl_2$, 5 mM-phenylhydrazine/ HCl, 2 mM-cysteine/HCl and 5 mM-isocitrate; (ii) isocitrate lyase, EC 4.1.3.1., assayed according to Giachetti et al. (1984), in 2mM-cysteine/HCl, 0.1 mM-NADH, 10 µl lactate dehydrogenase (Boehringer) and 5 mM-isocitrate; and (iii) succinate dehydrogenase, EC 1.3.99.1. (Veeger et al.,

1969), assayed in 0.1 mM-PMS, 0.05 mM-DCPIP and 10 mM-succinate. For the determination of isocitrate lyase activity according to Dixon & Kornberg (1959), crude cell-free extract was used unless otherwise stated. For the assay according to Giachetti et al. (1984) only the soluble fraction of cell-free extract was added. The amount of protein used ranged from 40 to 300 μg. All reactions were started by adding substrate and followed for at least 2.5 min. The observed rates were proportional to the amount of extract added.
Isocitrate lyase activity was also discontinuously measured by following the production of glyoxylate. In this case cell-free extract, 5 to 9 mg protein, was incubated in 10 ml assay mix containing 50 mM- Tris/HCl, pH 8.0, 50 mM-$MgCl_2$, 2 mM-cysteine/HCl, 5 mM-phenylhydrazine/HCl and 5mM-isocitrate. At regular intervals 0.2 ml samples were removed. The reaction was stopped by adding 2.5 ml concentrated HCl. Any glyoxylate hydrazone formed was converted to 1,5-diphenylformazancarboxylic acid by adding 2.5 ml 1% (w/v) $KFe(CN)_6$ as described by Kramer et al. (1959). After removal of precipitated protein, the $\underline{A}$ 532 was measured. The concentration of glyoxylate was calculated from a calibration curve.

^{13}C NMR spectroscopy. Dialysed cell-free extract (approximately 50 mg protein) was incubated in a solution containing 30 mM-potassium phosphate buffer, pH 7.2, 2 mM-cysteine/HCl, 1 mM-$MgCl_2$ and 200 mM-isocitrate. The final volume was 30 ml. This mixture was slowly stirred during 6 h at 30°C. The reaction was terminated by boiling in a waterbath during 10 min. After removal of denatured protein the samples were concentrated by lyophilization and subsequently resuspended in 3 ml distilled water. ^{13}C NMR spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer equipped with a 10 mm probe operating in the Fourier-transform mode. Proton broadband decoupling was applied throughout all experiments. The free induction decays (FIDs) were recorded by using a 45° pulse (pulse time 8 μs) and 16 000 data points. Approximately 55 000 FIDs were accumulated. The temperature in the probe was kept at 270 K. The observed resonances were identified by comparison with solutions of the pure compounds.

Product analyses. For all product analyses, cell-free extracts were incubated in an assay mix according to Dixon & Kornberg (1959). The cell-free extract used was either crude extract, dialysed, or the soluble fraction alone. Incubation was done under air in open tubes or in a degassed assay mixture in closed tubes under nitrogen, always in a shaking waterbath at 30°C. The specific details of the incubations are described in the legends of the respective figures. Samples were deproteinized by addition of 7% (v/v) perchloric acid, followed by neutralization with $KOH/KHCO_3$ or by adding 0.65 M-H_2SO_4, followed by neutralization with KOH.

HPLC analyses. When samples were used for analysis of organic acids, phenylhydrazine was omitted in the assay mix. For cation exchange chromatography a Chrompack Organic Acids column (6.5 x 300 mm) in the hydrogen form was used. The column temperature was set at 65°C. Samples with a volume of 0.02 ml for qualitative and 0.20 ml for quantitative determinations were eluted with 0.005 M-H_2SO_4 at a flow rate of 0.8 ml min^{-1}. Detection was at 206 nm. For reverse phase chromatography a LKB Ultropac HPLC column (4 x 250 mm), LiChrosorb RB-8 (10 μm) was used at room temperature. Samples with a volume of 0.05 ml were eluted with 5% (v/v) acetonitril in 20 mM-potassium phosphate buffer, pH 5.8, at a flow rate of 1.5 ml min^{-1} as described by Bellion et al. (1981). Detection of phenylhydrazones was at 313 nm. All retention times ($\underline{R}_t$) were compared with retention times of pure solutions. Calibration curves were prepared by substituting cell-free extract by glyoxylate, succinate or fumarate.

Mass spectrometry. Approximately 2 ml deproteinized sample obtained after incubation of cell-free extract of anaerobically grown cells in an assay mixture during 2 h was chromatographed on a reverse phase column (see HPLC analyses). In the reference sample cell-free extract was substituted by 0.5 mM-glyoxylate. Eluted fractions containing phenylhydrazone were collected, lyophilysed and resuspended in 1 ml distilled, acidified H_2O. The phenylhydrazone was extracted in 5 ml ethylacetate. After rotary evaporation, the samples were used for mass spectrometry. The spectrometer used was an AEI MS-9 mass spectrometer. The probe temperature was set at 30°C, source temperature at 300°C.

Radiography. To an assay mix in which isocitrate was substituted by 20 mM-[1,5-^{14}C]citrate (Amersham), diluted to a specific activity of 62.5 μCi mmol^{-1} (2.31 MBq mmol^{-1}), 2.6 mg protein of the soluble fraction of cell-free extract was added to make a final volume of 2 ml. No phenylhydrazine was added to the assay mix. The mixture was incubated in closed tubes during 45 min. A deproteinized sample of 0.05 ml was used for reverse phase HPLC (see HPLC analyses) after incubation with 10 mM-phenylhydrazine at 30°C during 30 min. Eluted fractions were collected at intervals of 12 s. After addition of 4 ml Aqualuma Plus (Lumac/3M b.v.) radioactivity in the collected fractions was counted using a LKB 1211 Rackbeta Liquid Scintillation Counter. The concentration of the glyoxylate phenylhydrazone in the sample was calculated from a calibration curve.

Results

Isocitrate lyase activity was determined by the method of Dixon & Kornberg (1959) in T.versutus after growth in five different chemostat cultures (Table 1). No isocitrate lyase activity was observed in aerobic cultures. In denitrifying cultures on acetate, however, the activity was found to be 52 and 111 nmol min^{-1} mg^{-1} in acetate-limited and nitrate-limited cultures, respectively. Cell-free extracts prepared from denitrifying succinate-limited cultures contained traces of, presumably, isocitrate lyase activity. Culture purity was confirmed from the agreement of the numbers of colonies on solid heterotrophic media with those obtained on solid media containing thiosulphate (Table 1). For the determination of isocitrate lyase activity according to Giachetti et al. (1984) in which the production of glyoxylate is followed by its reduction to glycollate in the presence of NADH and lactate dehydrogenase, NADH oxidase was removed by centrifugation at 120 000 g during 2 h. Again no isocitrate lyase activity was found in extracts from cells grown aerobically on acetate. The specific activity in the 120 000 g supernatant of cell-free extracts of cells grown on acetate under denitrifying conditions was 63 nmol min^{-1} mg^{-1} as determined by the method of Dixon & Kornberg (1959) and 21 nmol min^{-1} mg^{-1} as determined by the method of Giachetti et al. (1984). According to the method described by Kramer et al. (1959), the production of glyoxylate was quantified after its conversion to 1,5-diphenylformazancarboxylic acid. Using cell-free extract prepared from cells grown on acetate plus nitrate, 27 nmol product per min per mg protein was formed.

The specific activity was initially linear with time but decreased after approximately 60 min of incubation. No product was formed following incubation of cell-free extract from aerobically grown cells. For a qualitative identification of the reaction products formed from isocitrate after incubation of cell-free extracts of cells grown with O_2 or nitrate as electron acceptor, ^{13}C NMR spectroscopy was used. As no 13C enriched isocitrate was available, the assay

Table 1. Specific activity of isocitrate lyase (ICL) in cell-free extracts and viable cell counts of T. versutus from steady state chemostat cultures grown at a dilution rate of 0.1 h^{-1}, limited by different substrates.

Respiratory mode	Limiting nutrient	ICL ($nmol\ min^{-1}\ mg^{-1}$)	Viable cell count ($x10^8$ bacteria ml^{-1})			
			acetate	acetate $+KNO_3$	succinate $+KNO_3$	thiosulphat
aerobic	acetate	0±1(n=15)	94±16(n=5)			88±11(n=5)
denitrifying	acetate	52±18(n=16)		58±12(n=4)		52±10(n=4)
denitrifying	nitrate	111(n=1)		36(n=1)		40(n=1)
aerobic	succinate	1(n=1)				ND
denitrifying	succinate	1±2(n=8)			69±10(n=3)	68±14(n=3)

ND, not determined.

was dependent upon natural abundance of ^{13}C and thus large amounts of protein and substrate had to be used. The recorded ^{13}C NMR spectra are presented in Fig. 1. In spectrum B, the presence of isocitrate, citrate, aconitate, glyoxylate, fumarate, and malate, was observed. In spectrum A, only isocitrate, citrate and aconitate were found. Apparently the extremely large amount of isocitrate in the assay mixture had strongly favoured the synthesis of aconitate and citrate by aconitase and citrate synthase, respectively. The absence of succinate in spectrum B was presumably caused by conversion of succinate to fumarate and subsequently malate.

Cation exchange HPLC was used to determine the production of succinate and fumarate qualitatively and quantitatively after incubation of dialysed cell-free extracts for 2 h. When cell-free extracts of T. versutus grown anaerobically on acetate plus nitrate were incubated under air, only fumarate was found at a concentration of 0.076 mM. After incubation in a degassed, N_2 flushed mixture, 1.04 mM-succinate and 0.018 mM-fumarate were formed. When succinate dehydrogenase activity was reduced to about 7% of its original value by removal of the particulate fraction, 1.17 mM-succinate and 0.002 mM-fumarate were formed. In cell-free extracts of aerobically grown cells succinate and fumarate were never observed. The production of succinate, fumarate and glyoxylate (after derivatization according to Kramer et al .(1959)) was also determined as a function of time (Fig. 2). The amount of glyoxylate observed was not identical to the amount of succinate. This was due to the conversion of succinate to fumarate and malate.

For a qualitative and quantitative determination of glyoxylate or any other hydrazone forming compound, samples obtained after incubation during 2 h were chromatographed using a reverse phase HPLC column. By comparison with a chromatogram of phenylhydrazones of glyoxylate, 2-oxoglutarate and pyruvate, the product in the sample derived from anaerobically grown cells was identified as glyoxylate. No phenylhydrazone was observed in the sample prepared from an assay mixture containing cell-free extract of aerobically grown cells. For further identification of the hydrazone, chromatographed fractions were collected, extracted in ethylacetate and analysed by mass spectrometry. The theoretical accurate mass of the glyoxylate phenylhydrazone is 164.0586, the accurate mass of the chemically formed glyoxylate phenylhydrazone was found to be 164.0591 and that of the enzymatically produced hydrazone was 164.0587.

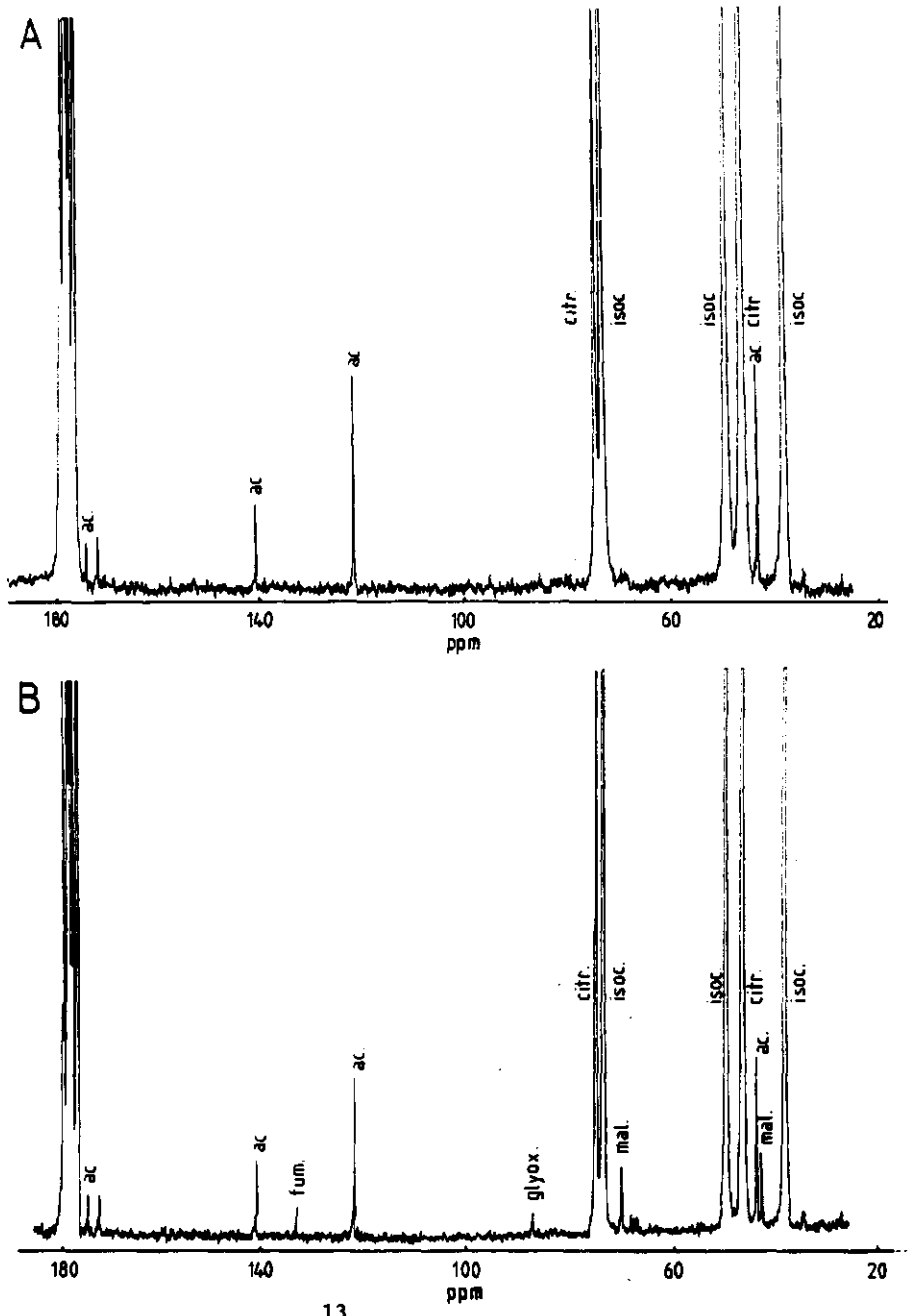

Fig. 1. Natural abundance ^{13}C NMR spectra of deproteinized samples obtained after incubation of dialysed cell-free extracts with isocitrate. (a): T.versutus from an aerobic acetate-limited chemostat culture. (b): T.versutus from a denitrifying acetate-limited chemostat culture.

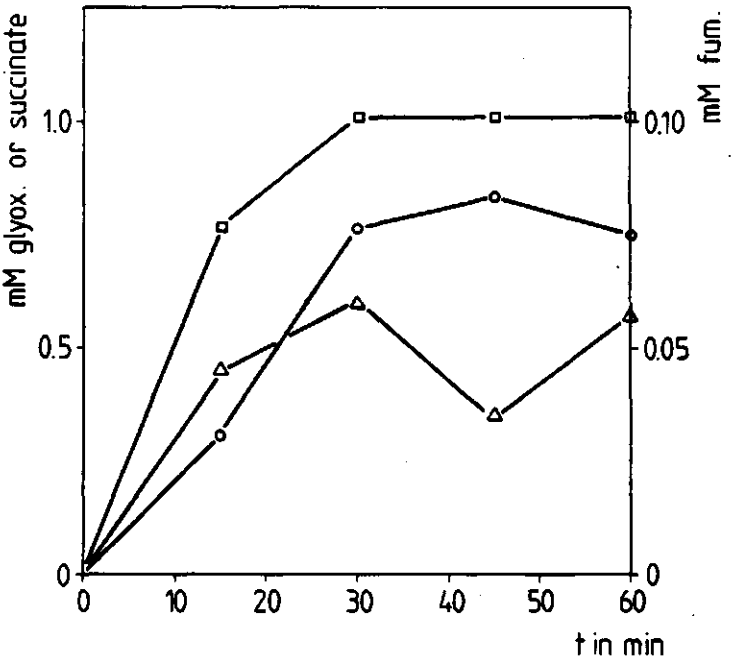

Fig. 2. Time course of succinate (Δ) and fumarate (O) formation as determined by cation exchange HPLC and of glyoxylate (□) formation as determined after its conversion to 1,5 diphenylformazancarboxylic acid. Incubation mixtures contained cell-free extract of T.versutus grown anaerobically on acetate and nitrate. Incubation conditions (5 ml volume) were as described in Methods with two modifications: the concentration of Tris/HCl, pH = 8.0 was lowered to 5 mM and $MgCl_2$ was omitted. Incubation was done in degassed, N_2 flushed mixture. To the mixture 5.0 mg protein was added. ICL sp. act. as determined from the glyoxylate production: 50 nmol min^{-1} mg^{-1}.

The origin of glyoxylate was determined by radiography. As no [^{14}C]isocitrate was commercially available [1,5-^{14}C]citrate was used as substrate. Deproteinized samples were, after incubation with phenylhydrazine, chromatographed on a reverse phase HPLC column and the radioactivity in collected fractions was counted by liquid scintillation. The chromatogram and the distribution of the label are presented in Fig. 3. The majority of the radioactivity migrated without retardation. The concentration of the glyoxylate hydrazone was 0.26 mM, calculated from a calibration curve. Therefore, as the specific activity of [1,5-^{14}C]citrate was diluted to 62.5 μCi $mmol^{-1}$ (2.31 MBq $mmol^{-1}$), the theoretical amount of d.p.m. of the glyoxylate hydrazone was 475. In the glyoxylate hydrazone fractions collected, a total of 492 d.p.m. was observed. An attempt was made to demonstrate the recovery of ^{14}C from [1,5-^{14}C]citrate in succinate, or a product derived from succinate, by cation exchange chromatography. Because of co-migration of succinate and aconitate (directly formed from citrate) and the always very low concentration of fumarate, this attempt was unsuccesful.

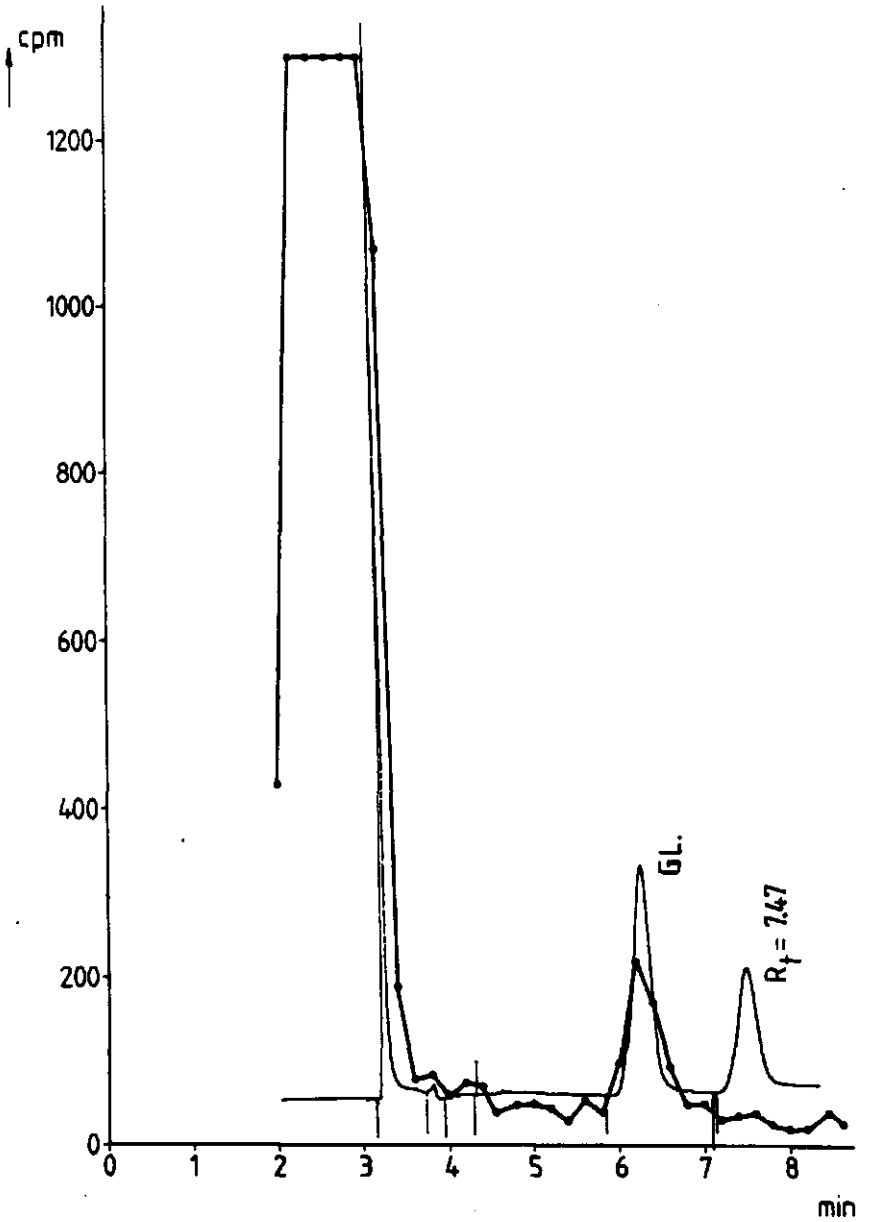

Fig. 3: Reverse phase HPLC chromatogram with ^{14}C label distribution (O) of a deproteinized sample obtained after incubation of cell-free extract of T.versutus, grown anaerobically on acetate and nitrate, with [1,5 - ^{14}C]citrate. Cell-free extract was prepared by centrifugation at 120 000 g for 2 h. Incubation conditions (2 ml volume) as described in Methods. To the mixture 2.6 mg protein was added. The reaction was ended after 45 min. The R_t of the glyoxylate phenylhydrazone was 6.22 min. The peak at 7.47 min was not identified.

Discussion

Our results on: (i) enzymatic formation of a glyoxylate phenylhydrazone or 1,5-diphenylformazancarboxylic acid, (ii) identification of glyoxylate and succinate as reaction products and (iii) recovery of ^{14}C from citrate in the glyoxylate fraction, leave no doubt as to the authenticity of the observed isocitrate lyase activity. Apparently T.versutus possesses the capacity to utilize the glyoxylate cycle when grown anaerobically on acetate in the presence of nitrate, i.e. during denitrifying growth on acetate. As in other micro-organisms (Dixon & Kornberg, 1959; Lehninger, 1975; Schlegel, 1981), this isocitrate lyase activity is associated with growth on a C_2 compound because hardly any activity is found after denitrifying growth on succinate. The observed specific activity of the enzyme in cells grown anaerobically on acetate and nitrate is not very high. However, the calculations by Quayle (1975) suggest that the specific activity is sufficient to maintain growth on acetate at a growth rate of 0.1 h^{-1}.

It is interesting to note that after incubation of cell-free extract obtained from anaerobically grown T.versutus with a large amount of isocitrate, not only glyoxylate and succinate can be observed, but also fumarate, malate, citrate and aconitate. The presence of these metabolites is most probably due to the activity of other tricarboxylic acid cycle enzymes, thus indicating the operation of the tricarboxylic acid and glyoxylate cycles during denitrifying growth on acetate. This assumption is supported by the results of Peeters et al. (1970) who demonstrated the presence of all tricarboxylic acid cycle enzyme activities during denitrifying growth of T.versutus on succinate.

Having shown that T.versutus metabolizes acetate via the glyoxylate cycle under denitrifying conditions, we are left with the question of how this substrate is metabolized during aerobic growth. The lack of isocitrate lyase activity during aerobic growth on acetate has been observed previously with this organism (Gottschal & Kuenen, 1980) and we never found any activity with any of the methods reported here in spite of the ability of T.versutus to grow well on acetate under aerobic conditions. A second major question arises from the observation that substitution of one electron acceptor by another seemingly confers a major effect on the route by which the growth substrate is metabolized. Although we have to leave these questions unanswered at the moment, we feel we have supplied extensive evidence on which further research concerning this interesting phenomenon can be based.

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C H A P T E R 6

Enzyme profiles of Thiobacillus versutus after aerobic and denitrifying growth: Regulation of isocitrate lyase

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Abstract

The specific activities of the tricarboxylic acid (TCA) cycle enzymes in Thiobacillus versutus were invariably lower after aerobic growth as compared to denitrifying growth in acetate- or succinate-limited chemostat cultures. Of the glyoxylate cycle enzymes, isocitrate lyase (ICL) activity was nil during aerobic and 76 $nmol.min^{-1}.mg^{-1}$ protein during denitrifying growth on acetate whereas malate synthase (MS) did not change. In succinate-grown cells ICL was always near nil. The change in ICL and MS was followed after pulse additions of acetate and nitrate to an aerobic acetate-limited chemostat culture made anaerobic prior to the first pulse. ICL remained nil during denitrifying growth after the first pulse but increased to 47 and 81 $nmol.min^{-1}.mg^{-1}$ protein after the second and third pulse respectively. MS remained unaltered. The appearance of ICL was dependent upon de novo protein synthesis. During transition in a steady state culture on acetate from oxygen to nitrate as terminal electron acceptor, denitrifying growth started after 0.6 volume replacements. The resumption of growth was concomitant with an increase in TCA cycle enzyme activities. ICL was observed only after 2 volume replacements. During the reverse transition, ICL disappeared at a rate twice the dilution rate. SDS polyacrylamide gelelectrophoresis of cell-free extracts containing ICL showed a major protein band with a R_f value identical to purified ICL and a mol.wt. of 60,000. ICL from T.versutus was inhibited by 1.5 mM itaconate but not by 10 mM phosphoenolpyruvate. Its activity was dependent upon the presence of Mg^{2+} and cysteine.

Key words: Thiobacillus versutus - TCA cycle - Glyoxylate cycle - Acetate assimilation - Aerobiosis - Denitrification.

Introduction

Growth of isocitrate lyase negative bacteria on certain C_1 and C_2 substrates has drawn the attention of many researchers for more than 10 years. During the investigation of the metabolism of facultative methylotrophs using the serine pathway for the assimilation of carbon, a problem arose when isocitrate lyase was found to be lacking in some organisms. This enzyme catalyzing the conversion of isocitrate to glyoxylate and succinate is a key enzyme in the oxidation of acetyl-CoA to glyoxylate. In the serine pathway, glyoxylate is the precursor of the acceptor molecule for the C_1 substrate. During growth on C_2 substrates , the de novo synthesis of C_4 intermediates is effected by condensation of glyoxylate and acetyl-CoA in the glyoxylate cycle. Among the facultative methylotrophs using the isocitrate lyase negative serine pathway, all, except Organism 5B1, do not show any isocitrate lyase activity when grown on C_2 substrates or other compounds assimilated exclusively via acetyl-CoA (Anthony 1982). Dunstan et al. (1972) isolated mutants of Pseudomonas AM1 which were unable to grow on C_1 and C_2 compounds. As growth on these substrates could be restored by adding glyoxylate to the medium, a partially common metabolic route for the metabolism of C_1 and C_2 substrates in this isocitrate lyase negative organism became conceivable.

To study the possible reactions leading to the formation of glyoxylate in absence of isocitrate lyase we chose Thiobacillus versutus as a model organism. This bacterium is isocitrate lyase negative but is able to grow on C_2 compounds (Gottschal and Kuenen 1980). To tackle the problem of isocitrate lyase negative metabolism in T.versutus, in vivo ^{13}C NMR was applied. As initially no equipment was available for aeration in NMR tubes we decided to study the metabolism of acetate in denitrifying cells, assuming that substitution of the terminal

electron acceptor would not affect the oxidative metabolism in T.versutus during heterotrophic growth. However, this assumption appeared to be incorrect. In T.versutus grown in a denitrifying acetate-limited chemostat culture, the presence of isocitrate lyase activity was observed while in a similar aerobic culture no enzyme activity could be detected. In a previous study the authenticity of this isocitrate lyase reaction has been proven (Claassen and Zehnder 1986).
In this report data are presented concerning the regulation of the enzyme activities of the tricarboxylic acid and glyoxylate cycles in Thiobacillus versutus during growth under aerobic and denitrifying conditions.

Materials and methods

Organism. Thiobacillus versutus, formerly called Thiobacillus strain A2 (ATCC 25364, Harrison Jr. 1983) was a kind gift of Dr. J.G. Kuenen (Delft, The Netherlands). This organism was maintained on thiosulphate agar slants, subcultured every two months and stored at 4'C.
Growth. The basal medium contained, per 1000 ml distilled water, 1.15 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.4 g NH_4Cl and 2 ml trace element solution (Gottschal and Kuenen 1980). To this medium 3.4 g/l sodium acetate (42 mM) or sodium succinate (21 mM) was added prior to sterilization. The pH was adjusted to 5.0 before autoclaving. For aerobic growth oxygen was supplied by sparging air through the culture at a rate of 1 liter per liter culture per min. The oxygen concentration in the culture was monitored using an oxygen electrode and was kept at 50% (v/v) air saturation. For denitrifying growth, 5 g/l KNO_3 (50 mM) was added to the same medium as used for aerobic growth. After autoclaving, the medium was thoroughly flushed with N_2 (5.0 technical grade) and kept under N_2 during growth. To keep oxygen contamination to a minimum silicone tubing was replaced by butylrubber tubes. During growth, N_2 was sparged through the culture at a rate of 300 ml per liter culture per min. In all chemostat cultures the pH was maintained at 8.0 by the automatic addition of 0.5 M H_2SO_4. All cultures were grown at 30'C at a dilution rate of 0.1 h-1. Substrate limitation was checked by increasing the substrate concentration in the medium reservoir and determining the subsequent proportional increase in optical density of the culture. For pulse induction experiments 500 ml 3 fold concentrated basal medium containing various concentrations of acetate and nitrate was added to 1 liter chemostat culture after stopping medium inflow and culture outflow. The purity of the cultures was regularly checked by screening isolated colonies on atypical morphology and the ability to grow autotrophically on thiosulphate.

Preparation of cell free extracts. Cell-free extracts were prepared according to Claassen et al. (1986).

Enzyme assays. All enzyme activities, except for fumarase and malate synthase, were measured as described by Claassen et al. (1986). Fumarase, EC 4.2.1.2. (Hill and Bradshaw 1969) was measured in 50 mM Tris-HCl, pH 8.0, by adding 5 mM malate. The assay mix used for malate synthase, EC 4.1.3.2. (Reeves et al. 1971) contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 0.05 mM acetyl-CoA. The reaction was started by adding 2 mM sodium glyoxylate dissolved in distilled H_2O. Previously (Claassen et al. 1986) malate synthase was measured by adding 2 mM glyoxylic acid dissolved in 100 mM Tris-HCl, pH 8.0 . Under these conditions the specific activity of the enzyme was about 4 times lower. The amount of protein added to the reaction mixtures ranged from 5 to 100 μg. All activities were spectrophotometrically measured at 20'C.

O_2 consumption. Respiration of washed cells was determined according to Claassen et al. (1986).

Electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was done according to the method of Laemmli (1970) using for the separating gel a uniform acrylamide concentration of 10% (w/v). The gels were 1 mm thick. Per lane 0.015 to 0.15 mg extract protein was applied. Proteins were stained with Coomassie Brilliant Blue R. Electrophoresis was done overnight at 6 mA at 20°C. Molecular weight was determined using a high and a low molecular weight marker kit (Pharmacia, Uppsala, Sweden).

Enzyme purification. All manipulations except for fast protein liquid chromatography (FPLC) were done at 4°C. Cell-free extract was brought to 25% (w/v) saturation with respect to ammonium sulphate. The precipitate was removed by centrifugation at 8,000 x g during 15 min. The supernatant was subsequently brought to 40% (w/v) saturation. The precipitate was collected, redissolved in 30 mM potassium phosphate buffer, pH 7.4, and desalted by elution over a Sephadex G-50 column equilibrated in 50 mM Tris-HCl buffer, pH 8.0. The sample was further purified by ion exchange chromatography using a DEAE Sephadex A-50 column or a Mono Q FPLC column (Pharmacia, Uppsala, Sweden). Protein was eluted by a gradient of 0 to 1 M NaCl in 20 mM Tris-HCl buffer, pH 8.0. Fractions containing isocitrate lyase activity were obtained between 300 and 400 mM NaCl. These were again desalted in 20 mM Tris-HCl buffer, pH 8.0, and stored at -20°C.

Analytical methods. Protein content of bacterial suspensions was measured after washing and boiling (5 min), according to Lowry et al. (1951) using bovine serum albumin as a standard. Dry weight of washed pellets was determined after drying in predried vials overnight at 105°C. Acetate concentrations were determined in the supernatant of samples by gaschromatography using a Chromosorb 101, 80-100 mesh, column connected to a flame ionization detector. Nitrite concentrations were colorimetrically determined at 523 nm after adding 0.1% (w/v) sulphanilamide in 3 M HCl and 0.001% (w/v) N-(1-naphtyl) ethylenediamine di-HCl in H_2O to 200 fold diluted samples of supernatants. Nitrate concentrations were measured the same way after reduction to nitrite using a Technicon Autoanalyzer equipped with a Cd column.

Biochemicals. Biochemicals were obtained from Sigma Chemical Company, St.Louis, USA, unless otherwise stated. All other chemicals were of analytical grade.

Results

The specific enzyme activities of the tricarboxylic acid (TCA) cycle of *Thiobacillus versutus* after aerobic growth showed an overall increase when acetate was supplied as carbon and energy source instead of succinate, with the exception of 2-oxoglutarate dehydrogenase activity (Table 1). When cells were grown under denitrifying conditions on the same substrates and at the same dilution rate, again an increase in specific enzyme activities, except for 2-oxoglutarate dehydrogenase, was noted. The proportional difference in specific activities of the TCA cycle enzymes between acetate- and succinate-grown cells appeared to be independent of the electron acceptor used. Of the glyoxylate enzymes isocitrate lyase activity was never detected during aerobic growth but malate synthase increased from 79 during growth on succinate to 278 $nmol.min^{-1}.mg^{-1}$ protein during growth on acetate. After denitrifying growth on acetate or succinate the

Table 1. Specific enzyme activities in cell-free extracts and molar growth yields of Thiobacillus versutus after aerobic or denitrifying growth in acetate- or succinate-limited chemostat cultures. Dilution rate is 0.1 h^{-1}.

	aerobic growth on		denitrifying growth on	
	acetate	succinate	acetate	succinate
citrate synthase	299[a)]	119	462	241
aconitase	823	351	1617	640
isocitrate dehydrogenase	730	222	1621	640
2-oxoglutarate dehydrogenase	69	65	63	44
fumarase	2181	1154	3205	2201
malate dehydrogenase	5435	4478	6881	6490
isocitrate lyase	0	0	76	5
malate synthase	278	79	272	278
Yield g dry wt./mol substrate	21	43	17	36

a) Specific activities given in nmol.min^{-1}.mg^{-1} protein. The presented enzyme activities were determined in one experiment and are representative of other single determinations.

specific activity of isocitrate lyase was found to be 76 and 5 nmol.min^{-1}.mg^{-1} protein, respectively. Malate synthase activity was under these conditions independent of the growth substrate used and was equal to that found after aerobic growth on acetate.

The transition from aerobic to denitrifying growth was studied after three consecutive pulse additions of acetate and nitrate to an aerobic culture grown on acetate. Following the first addition of acetate and nitrate, the culture started to grow after a lag phase of approx. 4 h. The second and third pulses were not followed by a lag phase. However, as after the first addition, some nitrite accumulated in the medium even though the cells now assumedly possessed the complete set of nitrogen oxide reductases (Fig. 1A). During the transition from aerobic to denitrifying growth (after the first pulse), no isocitrate lyase activity could be detected. Following the second and third pulses, isocitrate lyase was induced to 47 and 81 nmol.min^{-1}.mg^{-1} protein, respectively. Malate synthase activity remained virtually the same throughout the entire experiment (Fig. 1B). The absence of isocitrate lyase activity during growth after the first pulse demonstrated that the previously observed coincidence of this enzyme activity and denitrification is not a conditio sine qua non. Even though different ways for the metabolism of the substrate were used, the yield expressed in mg protein per mg acetate was always 0.22. Denitrifying growth without isocitrate lyase activity proceeded however at a rate of 0.13 h^{-1} as compared to the faster rate of 0.20 h^{-1} after the second and third pulses.

The effect of changing the carbon and energy substrate in a denitrifying culture of T.versutus was studied after pulse addition of acetate and nitrate to a denitrifying steady state culture on succinate. Instantaneous cell growth was observed together with the utilization of acetate. After two hours of incubation isocitrate lyase activity had increased from 1 to 36 nmol.min^{-1}.mg^{-1} protein. The specific activity attained a value of 51 nmol.min^{-1}.mg^{-1} protein after 4 h and remained constant thereafter. Malate synthase activity increased slightly

during the experiment (Fig. 2A). When the experiment was repeated in the presence of chloramphenicol, isocitrate lyase activity was not observed and malate synthase activity did not change (Fig. 2B). After addition of basal medium containing only nitrate and no acetate, or after addition of acetate under aerobic conditions, no isocitrate lyase activity was observed. These findings demonstrated that under denitrifying conditions, induction of isocitrate lyase of T.versutus required de novo protein synthesis, probably triggered by

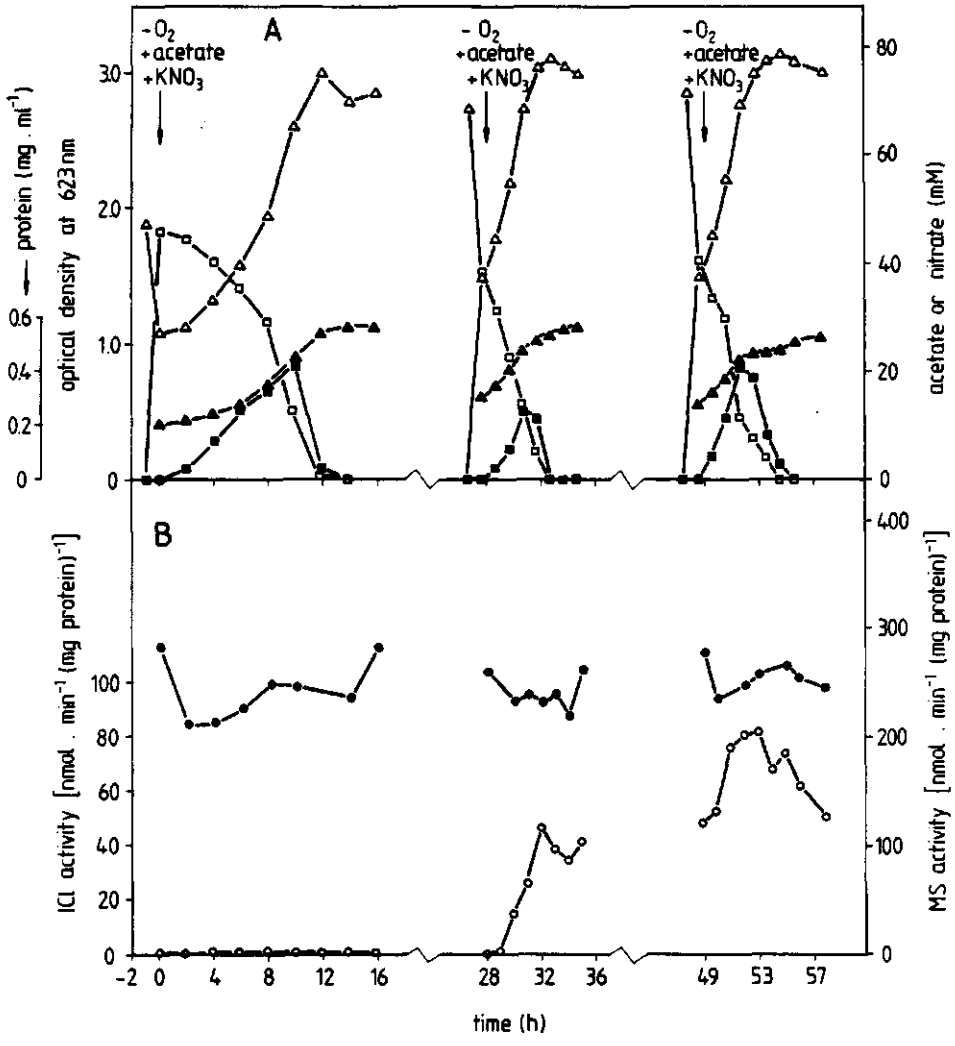

Fig. 1: Growth of Thiobacillus versutus after pulse additions of medium containing acetate and KNO_3 to a culture pregrown aerobically on acetate. At t = 0 h air was substituted by N_2 and acetate and KNO_3 (final concentrations 41 and 40 mM resp.) were added. At t = 27 and 48 h the same addition was done to the (now anaerobic) culture. A; optical density at 623 nm : △, [acetate] in supernatant: □, [nitrite] in supernatant: ■, protein content of culture: ▲. B; isocitrate lyase (ICL): ○, malate synthase (MS): ●.

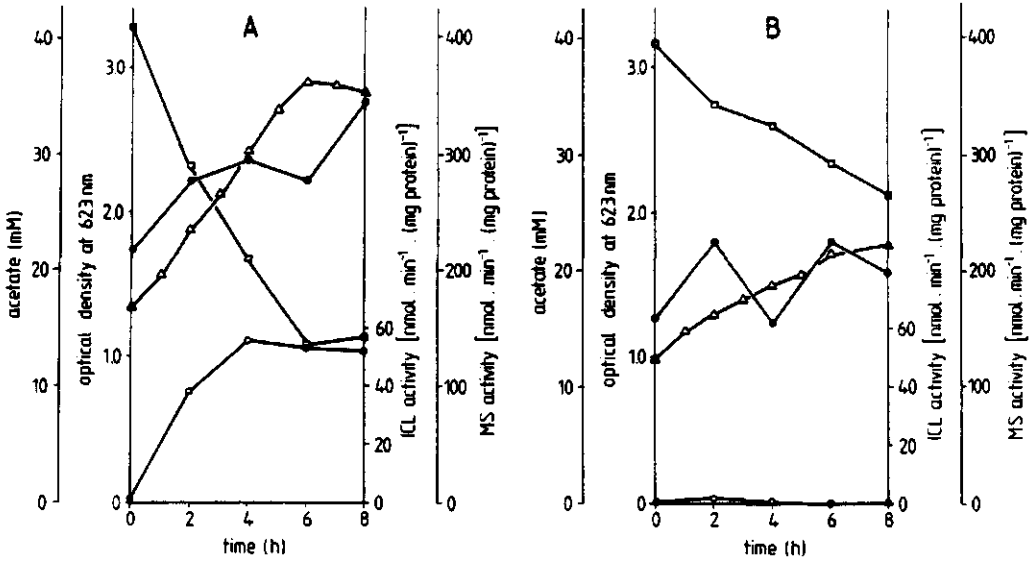

Fig. 2: Growth of Thiobacillus versutus after pulse addition of medium containing acetate and KNO_3 to a denitrifying culture on succinate. A; At t = 0 h acetate and KNO_3 (final concentrations 41 and 50 mM) were added. B; At t = 0 h acetate, KNO_3 and chloramphenicol (final concentrations: 41, 50 mM and 50 mg/l) were added. Optical density at 623 nm : Δ , [acetate] in supernatant: □ ,isocitrate lyase (ICL): ○, malate synthase (MS): ●.

acetate. As cell growth was prevented in the presence of chloramphenicol (Fig. 2B), the small increase in optical density was attributed to an increase in intracellularly stored poly-3-hydroxybutyric acid, always visible in denitrifying cells.

The effect of the substitution of one electron acceptor by another in acetate-limited chemostat cultures of T.versutus is shown in Fig. 3 and 4. When in an aerobic steady state culture, growing at a dilution rate of 0.1 h^{-1} on acetate, oxygen was replaced by nitrate, cell growth initially stopped as was evident from the decrease in optical density of the culture and the accumulation of acetate in the medium. At the same time nitrite was produced. Denitrifying growth, obvious from the halt in wash-out of the culture started approx. 6 h after the substitution of oxygen, resulting in a decrease and final disappearance of acetate and nitrite (Fig. 3A). Isocitrate lyase activity was not observed until after approx. 20 h and malate synthase remained unaltered (Fig. 3B). Prior to the appearance of isocitrate lyase and in fact concomitant with

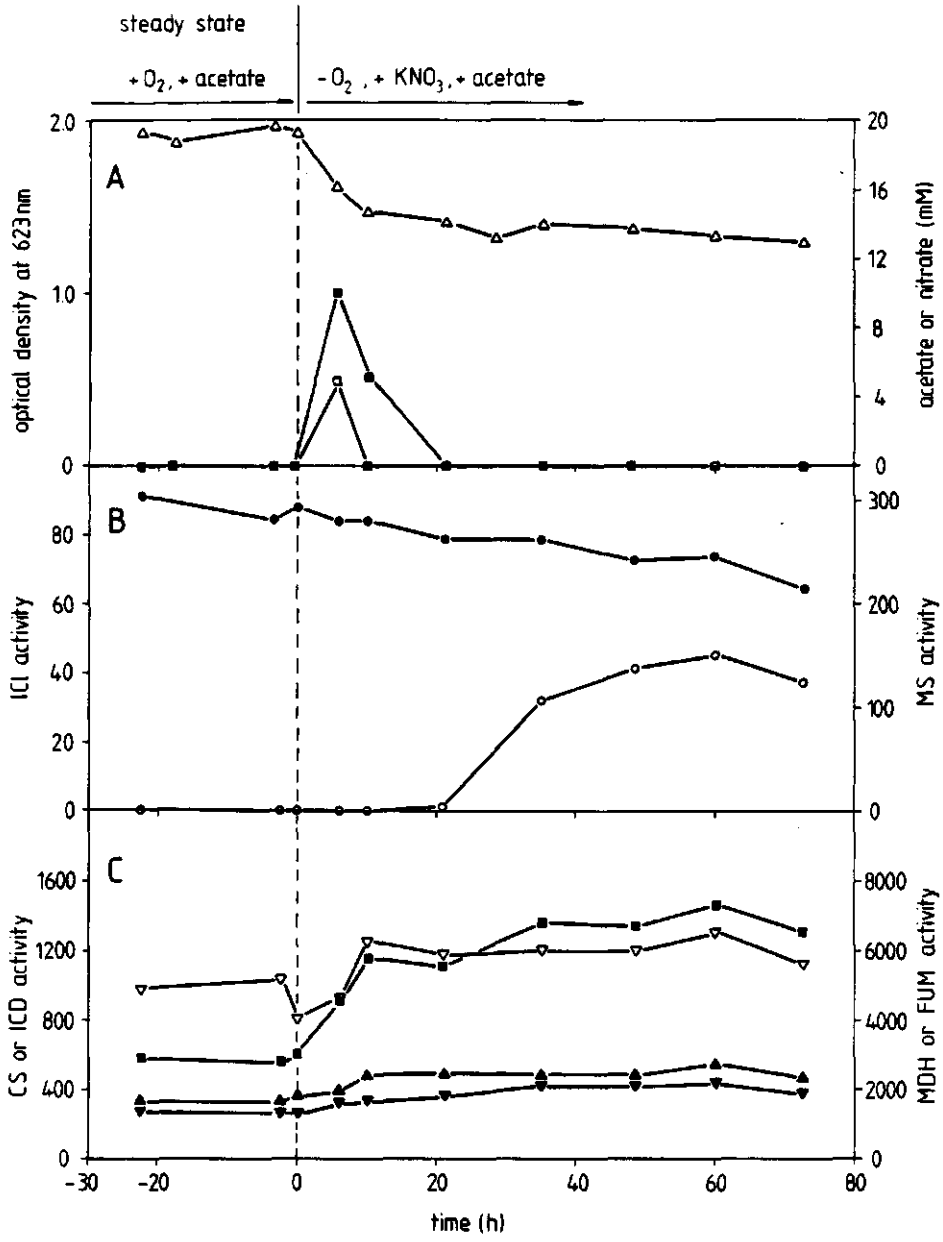

Fig. 3: Transition of Thiobacillus versutus from aerobic to denitrifying growth on acetate at a dilution rate of 0.1 h^{-1}. At t = 0 h acetate medium was replaced by acetate medium containing 50 mM NO_3 and air was substituted by N_2. A; optical density at 623 nm: △ , [acetate] in supernatant: □ , [nitrite] in supernatant: ■ . B; isocitrate lyase (ICL): ○ , malate synthase (MS): ● . C; citrate synthase (CS): ▼ , isocitrate dehydrogenase (ICD): ■ , fumarase (FUM): ▲, malate dehydrogenase (MDH): ▽ .

the resumption of growth, a great increase in isocitrate dehydrogenase activity was noticed. The increase in specific activity of the other TCA cycle enzymes investigated was less drastic but nevertheless occurred at an early stage during the transition before isocitrate lyase activity was observed (Fig. 3C). The reverse substitution of electron acceptor, i.e. substitution of nitrate by oxygen, was done in a subsequent experiment. This time the medium in the reservoir remained supplemented with nitrate. The cells switched immediately from denitrifying growth to aerobic growth as was evident from the accumulation of nitrate (Fig. 4A). Acetate was never detected in the culture. The decrease in specific activity of isocitrate lyase as a function of time was determined from a semilogarithmic graph and a value of 0.205 h^{-1} was found for the slope of ln sp.act. versus t (h). Since the dilution rate was set at 0.1 h^{-1}, isocitrate lyase activity was not washed out but actively repressed following the transition from denitrifying to aerobic growth on acetate (Fig. 4B).

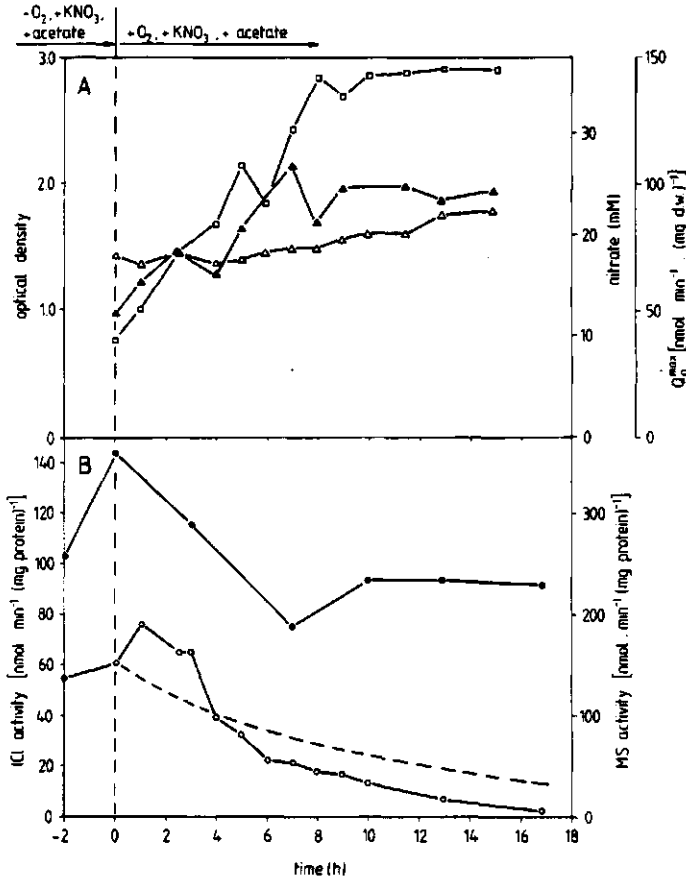

Fig. 4: Transition of Thiobacillus versutus from denitrifying to aerobic growth on acetate at a dilution rate of 0.1 h^{-1}. At t = 0 h the N_2 flow was substituted by flow of air. A; optical density at 623 nm: Δ, (nitrate) in supernatant: □, $Q_{O_2}^{max}$: ▲. B; isocitrate lyase (ICL): O, malate synthase (MS): ●, wash-out kinetics: --.

Using SDS polyacrylamide gel electrophoresis an almost identical protein pattern was found in cell-free extracts prepared from T.versutus after aerobic growth on acetate or succinate (Fig. 5). These patterns showed distinct differences with those obtained after denitrifying growth. The most important difference was the presence of a major protein band in cell-free extract after denitrifying growth on acetate, located at the same place as the purified isocitrate lyase. One band in cell-free extract from succinate-grown cells possessed approx. the same R_f value as isocitrate lyase. However, as the latter band was absent after removal of the particulate fraction, it could not be assigned to the soluble isocitrate lyase. A SDS-mol.wt. of 60,000 was determined for isocitrate lyase from T.versutus, a value which is either identical or close to the subunit mol.wt. of isocitrate lyase from various other organisms (McFadden et al. 1968; Johanson et al. 1974b; Chell et al. 1978; Takoa et al. 1984). Using purified fractions inhibition of enzyme activity by phosphoenolpyruvate and itaconate was investigated. Addition of up to 10 mM phosphoenolpyruvate had no effect on the enzyme activity. This was at variance with data from numerous other studies in which inhibition by phosphoenolpyruvate has been reported (Ashworth and Kornberg 1963; Rao and McFadden 1965; Johanson et al. 1974a; Sariaslani et al. 1975; Chell et al. 1978; Takao et al. 1984). Although in two separate cases (Syrett and John 1968; Roche et al. 1970) the inhibition by phosphoenolpyruvate was observed to be markedly dependent upon the pH, a fine control excerted by this metabolite on the enzyme from T.versutus seems unlikely. Itaconate inhibited the enzyme to 50% of its original activity at a concentration of 1.5 mM. This metabolite, presumably acting as a succinate analogue, was found to inhibit isocitrate lyase from Pseudomonas indigofera (Rao and McFadden 1965; Williams et al. 1971; Williams Rittenhouse and McFadden 1974) and from a Bacillus sp. (Chell et al. 1978). The activation of enzyme activity by Mg^{2+} was determined using a concentration series ranging from 0 to 50 mM $MgCl_2$. No activity was observed at 0 mM, then a sharp increase to 96 nmol.min-1.mg-1 protein at 6 mM, followed by a very slight gradual decrease to 72 nmol.min-1.mg-1 protein at 50 mM $MgCl_2$. The presence of a sulfhydryl protective reagent was also essential. No activity was detected when cysteine was omitted from the assay mixture. In this respect the enzyme from T.versutus is similar to many other isocitrate lyases (Smith and Gunsalus 1957; Shiio et al. 1965; Takao et al. 1984). Finally, the stability of the enzyme after storage at -20'C was determined. After 45 days at -20'C only 20% of the specific activity was lost.

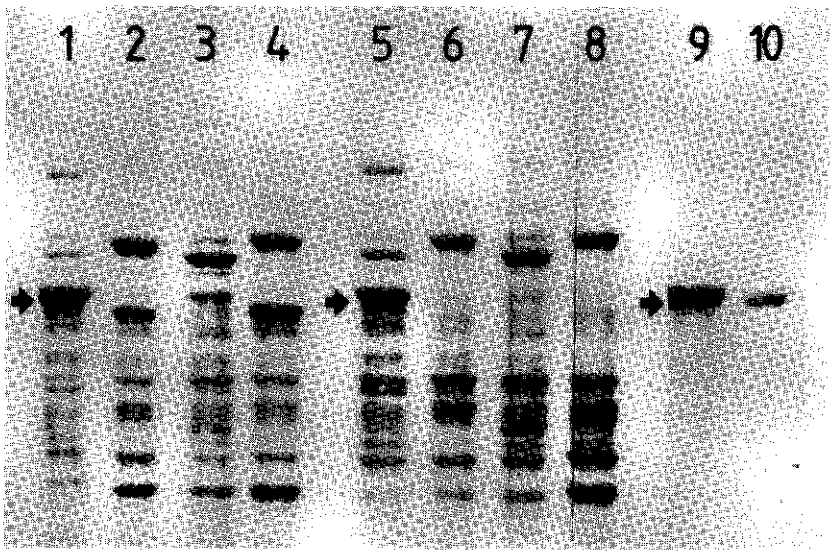

Fig. 5: Protein stains of SDS PAGE gels of cell-free extracts prepared from Thiobacillus versutus after aerobic or denitrifying growth on acetate or succinate. 1: denitrifying on acetate, 2: aerobic on acetate, 3: denitrifying on succinate, 4: aerobic on succinate. Lane 5 to 8 as lane 1 to 4 but after removal of the particulate fraction by centrifugation at 120,000xg during 2 h.
9: isocitrate lyase after DEAE Sephadex chromatography, 10: isocitrate lyase purified by Mono Q (FPLC) chromatography. Lane 1 to 8 : 0.10 mg, lane 9 and 10: 0.015 mg protein. Arrows indicate isocitrate lyase.

Discussion

The presented data describe a novel physiological phenomenon. It is shown that in Thiobacillus versutus during denitrifying growth acetate can be assimilated via the glyoxylate cycle whereas during aerobic growth this cycle is incomplete because of lack of isocitrate lyase activity. Apparently, denitrifying growth has a direct or indirect influence on the way acetate is metabolized in this organism. These results are in contrast with the general assumption that the specific oxidative features of metabolism are preserved upon transition from

aerobiosis to denitrification, with the exception of the metabolism of substrates requiring incorporation of molecular oxygen (Payne 1973; Young 1984). Notwithstanding this assumption, an obvious effect of denitrifying growth is a lower molar growth yield due to less efficient energy conservation during the reduction of nitrate as compared to oxygen (Boogerd et al. 1985). Since a lower molar growth yield is linked to an increase in the ratio of substrate dissimilated versus assimilated an increase in the specific activities of the TCA cycle enzymes as observed in T.versutus (Table 1, Fig. 3) is quite conceivable. These findings are corroborated by a previous report on the specific enzyme activities of the TCA cycle during denitrifying growth of T.versutus on succinate in batch culture (Peeters et al. 1970). Although not an overall increase was observed, the specific activities of aconitase, succinate dehydrogenase and fumarase were greatly increased and no enzyme activity was lower than observed during aerobic growth (Peeters et al. 1970). With respect to the assimilation enzymes, i.e. isocitrate lyase and malate synthase during growth on acetate, we are faced with a strange phenomenon (Table 1). No isocitrate lyase activity is observed during aerobic growth whereas during denitrification an activity of 76 $nmol.min^{-1}.mg^{-1}$ protein is found. The specific activity of isocitrate lyase observed in T.versutus is considerably lower than found in other organisms grown on acetate (Dijkhuizen and Harder 1979; Levering and Dijkhuizen 1985) but theoretically sufficient to sustain growth on acetate at a growth rate of 0.1 h^{-1} (Quayle 1975). Apparently T.versutus possesses the genetic information for two different acetate assimilation routes: one used during aerobic growth and another used during denitrification. The transition experiments demonstrate that the presence of isocitrate lyase is not a prerequisite for denitrifying growth on acetate (Fig. 1 and 3). As isocitrate lyase is rapidly induced following the substitution of succinate by acetate in a denitrifying culture, this route seems however to be preferred during denitrification (Fig. 2). The fast repression of enzyme activity after the transition from denitrifying to aerobic growth shows that the opposite is true during aerobiosis (Fig. 4). It is interesting to note that during denitrifying growth without isocitrate lyase, i.e. using the "aerobic" assimilation route, the molar growth yield is the same as in the presence of isocitrate lyase (Fig. 1A, B and C). As denitrification causes a change in the dissimilation versus assimilation ratio, one would expect the yield obtained using the "aerobic" route to be different especially since at least one different enzyme is involved in the assimilation of the substrate. The second enzyme of the glyoxylate cycle, malate synthase, behaves independently of isocitrate lyase. During aerobic growth on acetate malate synthase activity is high suggesting a physiological role of glyoxylate, the product of the reaction catalyzed by isocitrate lyase which is not present (Table 1). When isocitrate lyase activity in acetate-grown cells is induced after the transition to denitrifying growth, malate synthase activity remains at the same level (Figs. 1 and 2). After substitution of oxygen by nitrate in succinate-grown cells (no isocitrate lyase present) a large increase in malate synthase activity is observed (Table 1). An even more dramatic increase of this enzyme activity was reported by Peeters et al. (1970) who compared aerobic to denitrifying growth of T.versutus on succinate, a substrate not metabolized via the glyoxylate cycle.

Although the significance of the glyoxylate cycle during growth on acetate or metabolically comparable substrates, e.g. fatty acids, has been well established in many organisms, studies on the in vivo regulation of the glyoxylate cycle enzymes are less equivocal. Data obtained with Escherichia coli strains strongly suggest regulation of isocitrate lyase activity by a repression/derepression mechanism controlled by the intracellular concentration of phosphoenolpyruvate or a closely related metabolite (Kornberg 1966; Vanderwinkel and De Vlieghere 1968; Ornston and Ornston 1969). On the other hand, induction of isocitrate lyase synthesis by acetate or a related compound and catabolite repression by a

C_4 intermediate of the TCA cycle has been proposed for the regulation of the glyoxylate cycle in Acinetobacter sp. (Herman and Bell 1970) and in Nocardia salmonicolor (Sariaslani et al. 1975). Fewer data are available on the regulation of malate synthase involved in the metabolism of acetate. Vanderwinkel and De Vlieghere (1968) have demonstrated a close linkage between the structural genes governing the synthesis of malate synthase and isocitrate lyase in E.coli. Regulation of these loci, possibly part of a single operon, occurs by repressive control exerted by phosphoenolpyruvate or a related metabolite. At this moment our evidence is insufficient to decide on the nature of the regulation mechanism operative in T.versutus, even though preliminary results seem in favor of an induction/repression mechanism, at least during denitrifying growth.

As described above, an increase in TCA cycle activity as a result of less efficient energy conservation during nitrate respiration is quite conceivable. Alterations of pathways involved in the still oxidative metabolism of the electron donor seem less likely, with of course the exception of substrates which are metabolized by direct oxygenation (Payne 1973; Young 1984). However, T.versutus has been reported to lack the capacity to grow anaerobically on thiosulphate in the presence of nitrate (Taylor and Hoare 1969; Wood and Kelly 1983). Although this finding is in disagreement with data from Robertson and Kuenen (1983), possibly as the result of strain differences, inability to couple thiosulphate oxidation to nitrate reduction or repression of thiosulphate oxidation due to anaerobiosis seem reasonable suggestions (Wood and Kelly 1983). Moreover, in the same organism a difference in participation of the Embden-Meyerhof, Entner-Doudoroff and oxidative pentose phosphate pathways has been found when aerobic and denitrifying growth on glucose were compared (Wood and Kelly 1977; 1983). The greatest difference in aerobic and denitrifying physiology has been observed in Pseudomonas denitrificans (Koike and Hattori, 1975). This organism is incapable of denitrifying growth on glucose, mannitol, lactate and other organic acids, notwithstanding its capacity to grow on various amino acids using nitrate as electron acceptor. A block in amino acid biosynthesis as the result of anaerobiosis has been suggested to be the main cause of lack of growth.

In conclusion: Our results demonstrate that transition form aerobic to denitrifying growth has a major effect on the way acetate is metabolized in T.versutus. This organism offers two interesting opportunities, (i) the study of physiological phenomena related to oxygen versus nitrate respiration and (ii) the study of isocitrate lyase negative and positive metabolism in one and the same organism.

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C H A P T E R 7

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Samenvatting

Summary

In contrast to earlier observations Thiobacillus versutus was found to possess all tricarboxylic acid (TCA) cycle enzymes after aerobic growth in a chemostat on thiosulphate, acetate, glutarate, succinate or glutamate. The specific enzyme activities were invariably lower after chemolithoautotrophic growth as compared to heterotrophic growth. The capacity of thiosulphate-grown cells to oxidize acetate supported the presence of a complete TCA cycle. The overall increase in specific enzyme activities after transition from chemolithoautotrophic to heterotrophic growth indicated a dual role of the TCA cycle during heterotrophic growth, i.e. the supply of biosynthetic precursors and reducing power. However, isocitrate lyase activity was not observed in T.versutus after aerobic growth on acetate, rendering the replenishment of the TCA cycle via the glyoxylate cycle impossible. As isocitrate lyase of Pseudomonas fluorescens was not affected by inclusion of cell-free extract of T.versutus in the assay mixture, the presence of an isocitrate lyase inhibitor was excluded. In spite of the lack of isocitrate lyase activity, malate synthase activity was always highest in acetate-grown cells, suggesting a physiological role of glyoxylate in the metabolism of acetate in T.versutus.

In order to obtain a better insight in the in vivo metabolism of acetate, whole cells of T.versutus were studied using Nuclear Magnetic Resonance (NMR) spectroscopy. To circumvent technical problems resulting from aeration of a dense cell suspension, the metabolism was examined under denitrifying conditions. The in vivo operation of the TCA cycle was demonstrated by the sequence of incorporation of label from 2-^{13}C-acetate at the C-4 postition in glutamate, followed by simultaneous incorporation at the C-2 and C-3 positions. In a subsequent enzyme study, very surprisingly, isocitrate lyase activity was observed in T.versutus after denitrifying growth on acetate.

This enzyme catalyzed the conversion of isocitrate in glyoxylate and succinate and was therefore considered to be a true isocitrate lyase. Furthermore, the examined physico-chemical parameters were either identical or very close to those of isocitrate lyases of other organisms. Isocitrate lyase of T.versutus was inhibited by itaconate but not by phosphoenolpyruvate as generally observed.

To establish the physiological significance of the presence of isocitrate lyase an attempt was made to determine from the labelling pattern of glutamate, the relative flow of carbon through the TCA and glyoxylate cycles. Due to the use of 90% instead of 100% enriched 2-^{13}C-acetate and dilution by acetate emanating from intracellularly stored poly-3-hydroxybutyrate, this attempt was unsuccesful.

The regulation of the enzymes of the intermediary metabolism was studied during transition from aerobic to denitrifying growth on acetate. Of the specific activities of the TCA cycle enzymes an overall increase was observed simultaneously with the start of denitrification. Isocitrate lyase appearance occurred much later, approximately after two volume replacements in the chemostat culture. Malate synthase activity always remained the same. The retarded induction of isocitrate lyase was illustrative of the capacity of T.versutus to grow on acetate under denitrifying conditions with or without the presence of this enzyme. Assimilation of acetate via the glyoxylate cycle seemed however to be preferred as the enzyme was activily repressed after supplying air to denitrifying cells and because isocitrate lyase activity appeared within two hours after transition from succinate to acetate under denitrifying conditions. Since this induction was prevented by adding chloramphenicol, it was obvious that the regulation of isocitrate lyase was dependent upon de novo protein synthesis, at least when the carbon and energy substrate was replaced by acetate under denitrification.

Samenvatting

In tegenstelling tot eerdere waarnemingen werden alle enzymen van de citroenzuurcyclus aangetroffen in Thiobacillus versutus na aëroob kweken in een chemostaat op thiosulfaat, acetaat, glutaraat of glutamaat. De specifieke enzymactiviteiten waren na chemolithoautotrofe groei steeds lager dan na heterotrofe groei. Het vermogen van thiosulfaat-gekweekte cellen om acetaat te oxideren ondersteunde de aanwezigheid van een volledige citroenzuurcyclus. De algehele toename in specifieke enzymactiviteiten na overgang van chemolithoautotrofe naar heterotrofe groei was een indicatie voor de tweeledige rol van de citroenzuurcyclus, i.e. het leveren van precursors voor biosynthese en reducerend vermogen. Isocitraat lyase activiteit werd evenwel nooit gevonden in T.versutus na aërobe groei op acetaat. De aanvulling van de citroenzuurcyclus via de glyoxylaatcyclus is dientengevolge onmogelijk. Aangezien isocitraat lyase van Pseudomonas fluorescens niet beinvloed werd door toevoeging van celvrij extract van T.versutus aan het testmengsel, werd de aanwezigheid van een remmer van isocitraat lyase uitgesloten. Ondanks het gemis van isocitraat lyase activiteit was malaat synthase activiteit altijd hoger in acetaat-gekweekte cellen van T.versutus. Dit suggereerde een fysiologische rol van glyoxylaat in het metabolisme van acetaat. Teneinde meer inzicht te krijgen in het in vivo metabolisme van acetaat werden hele cellen van T.versutus bestudeerd met behulp van Nucleaire Magnetische Resonantie (NMR) spectroscopie. Om technische problemen die samenhangen met het aëreren van dikke celsuspensies te omzeilen werd het metabolisme onder denitrificerende omstandigheden onderzocht. Het in vivo functioneren van de citroenzuurcyclus bleek uit de volgorde van label incorporatie uit 2-^{13}C-acetaat op de C-4 positie in glutamaat gevolgd door gelijktijdige incorporatie op de C-2 en C-3 posities.

In een bijbehorende analyse van enzymactiviteiten werd, zeer verrassend, isocitraat lyase activiteit gevonden in T.versutus na denitrificerende groei op acetaat. Dit enzym katalyseerde de omzetting van isocitraat in glyoxylaat en succinaat en werd daarom beschouwd als een echt isocitraat lyase. Bovendien waren de fysisch-chemische parameters, voorzover onderzocht, hetzij identiek hetzij bijna identiek aan die van isocitraat lyases uit andere organismen. Isocitraat lyase van T.versutus werd geremd door itaconaat maar niet door fosfoenolpyruvaat zoals vaak gevonden.

Om de fysiologische betekenis van de aanwezigheid van isocitraat lyase vast te stellen werd een poging ondernomen om uit het labellingspatroon van glutamaat de relatieve flux van koolstof door de citroenzuur- en glyoxylaatcycli te bepalen. Tengevolge van het gebruik van 90% in plaats van 100% verrijkt 2-^{13}C-acetaat en de verdunning door acetaat afkomstig van intracellulair opgeslagen poly-3-hydroxybutyraat, was deze poging zonder succes.

De regulering van de enzymen van het intermediair metabolisme werd bestudeerd tijdens de overgang van aërobe naar denitrificerende groei op acetaat. Een algehele toename in specifieke activiteiten van de citroenzuurcyclus enzymen werd gelijktijdig met de aanvang van denitrificerende groei waargenomen. Het verschijnen van isocitraat lyase vond veel later plaats, ongeveer na 2 volume wisselingen in de chemostaat cultuur. Malaat synthase activiteit bleef altijd hetzelfde. De vertraagde inductie van isocitraat lyase was illustratief voor het vermogen van T.versutus om met of zonder de aanwezigheid van dit enzym op acetaat te groeien onder denitrificerende omstandigheden. De assimilatie via de glyoxylaatcyclus van acetaat leek echter de voorkeur te genieten aangezien het enzym actief gerepresseerd werd na het aanbieden van lucht aan denitrificerende cellen en omdat na overgang van succinaat naar acetaat onder denitrificerende omstandigheden, isocitraat lyase activiteit binnen een uur waarneembaar was. Omdat deze inductie verhinderd werd in aanwezigheid chlooramfenicol, was het duidelijk dat de regulering van isocitraat lyase afhing van de de novo eiwit synthese, tenminste wanneer de koolstof- en energiebron vervangen werd door acetaat tijdens denitrificatie.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 18 april, 1955 in Hapert, Noord Brabant. De Middelbare School opleiding vond plaats in Eindhoven aan het Lyceum Augustinianum en werd afgesloten met het diploma Gymnasium B in juni 1973. Het daaropvolgende jaar heb ik doorgebracht als student aan de University of Idaho in Moscow, USA, op een beurs van het International Institute of Education in Princeton. Het curriculum bestond uit: American Government, International Law and Organization, Sociology, Russian en Biology. In september 1974 begon ik met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen. Mijn doctoraalpakket bestond uit Toxicologie, Biochemie en Genetica. In het kader van mijn hoofdvak Biochemie was ik van april 1980 tot januari 1981 als research-assistant verbonden aan het Cancer Research Center van Purdue University in West-Lafayette, Indiana, USA. Teneinde een onderwijsbevoegdheid in Scheikunde te verkrijgen werden als aanvulling cursussen in anorganische Chemie, Didactiek en Pedagogie gevolgd. In juni 1982 werd het diploma van Landbouwkundig Ingenieur, cum laude, behaald.
Het onderzoek voor dit proefschrift vond plaats aan de Vakgroep Microbiologie van de Landbouwhogeschool, van oktober 1982 tot oktober 1985 gesubsidieerd door de Dutch Organization for Fundamental Research (ZWO) en van december 1985 tot juli 1986 als werknemer van de Landbouwhogeschool.
Met ingang van 4 augustus 1986 ben ik werkzaam als wetenschappelijk medewerker in de divisie "Fermentation and Microbiology" van het Shell Research Centre in Sittingbourne, UK.